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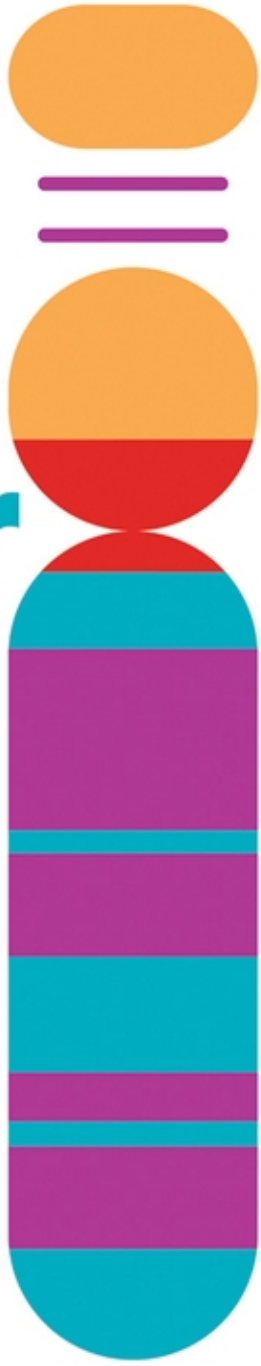
Second Edition

AN INTRODUCTION TO

Human Molecular Genetics

Mechanisms of Inherited Diseases



Jack J. Pasternak

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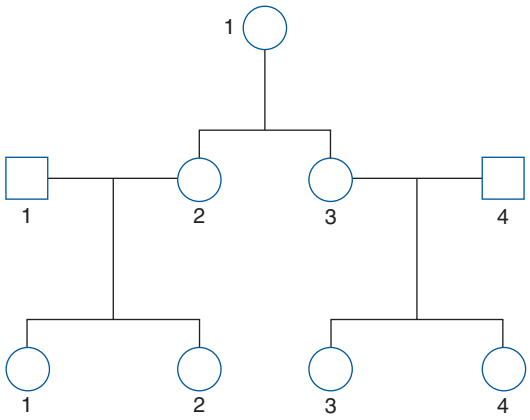
10 9 8 7 6 5 4 3 2 1

For my family

Lili (I-1)
Lisa (II-2)
Dahna (II-3)
Martin (II-1)
Mike (II-4)
Madelaine (III-1)
Miranda (III-2)
Nina (III-3)
Maya (III-4)



If we begin with certainties, we shall end in doubts; but if we begin with doubts, and are patient in them, we shall end in certainties.

FRANCIS BACON (1561–1626)

Our doubts are traitors,
And make us lose the good we oft might win,
By fearing to attempt.

WILLIAM SHAKESPEARE (1564–1616)

Contents

Preface

Any textbook is a work in progress. Consequently, a second edition provides a rare opportunity to amend, revise, update, elaborate, discard and insert new material. Moreover, specifically for *An Introduction to Human Molecular Genetics*, the first edition preceded completion of the human genome sequence, which is now essential for initiating studies of the molecular basis of human genetic disorders. The human genome is not only accessible online, but clones are available for any region of the human chromosome complement. Both of these resources have made the previous methods of identifying and isolating human disease-causing genes obsolete.

In this edition of *An Introduction to Human Molecular Genetics*, there are new chapters on complex genetic disorders, human population genetics, genomic imprinting, bioinformatics including proteomics, and clinical genetics. Many of the original chapters have been overhauled because of the advances in understanding the molecular genetics of various disorders. As well, the number of review questions for most chapters has been increased. However, the overall rationale for *An Introduction to Human Molecular Genetics*, as described in the previous preface, has not changed significantly.

The advice, comments, and criticisms of many of the anonymous reviewers of the first drafts of some of the new chapters were exceptionally helpful and greatly appreciated. I'm also thankful to the readers of the first edition who pointed out errors. It has been a pleasure working with everyone at J. Wiley and Sons. Finally, I'm exceedingly grateful to all my family for their encouragement and forbearance.

Preface to the First Edition

An Introduction to Human Molecular Genetics: Mechanisms of Inherited Diseases was written for advanced level undergraduate courses, introductory graduate level courses, and basic medical school courses on human genetics. The text examines how human genes are discovered and, once a gene is known, how the defective versions(s) causes a particular disorder. Humans are fascinated with everything to do with being human, and there is a keen interest in how the human genetic system functions and what causes inherited disorders. This book is derived from years of teaching Human Molecular Genetics at both the graduate and undergraduate levels at the University of Waterloo. These courses were initiated in the mid-1980s before any significant number of disease-causing genes had been mapped, isolated, or characterized. At that time, it seemed clear that the new gene technologies, based primarily on recombinant DNA technology, were going to make the direct study of human genes commonplace. To paraphrase one biologist, researchers will no longer need to rely on "breeding fruit flies and counting chiasmata" to appreciate the workings of human genes. Rather, a significant new phase in the study of human genetics was emerging. In the past decade, the developments in this research area have been phenomenal. In Star Trek parlance, human genetics is proceeding at "warp speed." Hardly a week now passes without a report in a major journal or newspaper stating that "The gene for disease X has been discovered!" And with the full flowering of the Human Genome Project, we are indeed in the midst of the genomic age in the life sciences.

This text has been structured to provide flexibility in the way topics might be covered within the time constraints of a traditional academic term. The first three chapters (Part One) review the fundamentals of genetics and focus on basic cytogenetics and Mendelian genetics. Some students, whose knowledge of these topics may be a bit rusty, would need this information as a brief refresher course. Obviously, these chapters can be omitted if an instructor feels that his/her students understand the material. Part Two covers the concepts of molecular genetics (chapter four); the tools, resources, and strategies for manipulating genes (chapter five); genetic an physical mapping of human chromosomes (chapter six); and isolating disease-causing genes (chapter seven). The chapters in Part Three of the book build on the information presented in the earlier chapters and focus on the molecular genetics of selected biological systems. Specifically, gene-based knowledge of inherited disorders of muscle,

the nervous system, and the eye are presented in detail. In these chapters and those that deal with inheritance of mitochondrial disorders and cancer, the biological basis of each particular system is described. Then, the discussion focuses on the mapping, identification, and characterization of genes that contribute to a system and how that system is affected by mutations in different genes. The goal is to familiarize the reader with the anatomical, physiological, and biochemical underpinnings that pertain to these genetic studies. The final chapter covers the exciting field of human gene therapy. Unfortunately, because human molecular genetics is such a broad-ranging scientific discipline, not all basic systems could be handled in this manner. In spite of these "omissions," there is plenty of challenging and interesting material in the book to occupy readers and provide a solid foundation for understanding the many facets of human molecular genetics.

In 1938, Herbert Walter (1867–1945), in the preface to the fourth edition of his book *Genetics* noted ruefully that "Any book concerning the growing subject of genetics is bound to be out of date as soon as it appears, just as every automobile on the road must be classified as a 'used car'." How true! Both Walter's pronouncement and his analogy are applicable today. There is fun and frustration in writing about human molecular genetics. On the one hand, conveying how a gene was discovered (often an arduous process) and explaining how it may function is exciting. On the other hand, by the time the book emerges, statements such as "It has not been established that gene X is responsible for disease Y," which are sprinkled throughout the book, may be passé. Clearly, some excellent candidate disease genes will have been confirmed and others shown not to be causative of the condition in question. Moreover, additional genes will have been found that cause disorders of the systems considered in the book. Undoubtedly, any text in human molecular genetics is "a work in progress" because of the dizzying pace of research in this field. This text is no different; however, the focus on key ideas and scientific principles, and the careful detail in which they are explained will allow students to acquire a solid foothold in human molecular genetics so that they can pursue additional topics in this area.

Because the field of human molecular genetics draws on information from so many scientific disciplines, and because it is based on a number of new technologies, there are many new technical terms in this text that might seem forbidding to students on their initial encounter. This trade language is not meant to mystify or be exclusive; rather, it is used for precision and to streamline communication. As Don DeLillo (B. 1936) warns us in his novel *Underworld*, "You didn't see the thing because you don't know how to look. And you don't know how to look because you don't know the names." For us to be able "to look" at human molecular genetics, we need to "know the names." Throughout this text I have tried to avoid technical jargon, but the use of some specialized nomenclature is inevitable. Each chapter ends with a list of Key Terms that are then discussed in a comprehensive Chapter Summary. Also, an extensive Glossary is included at the end of the text to help students with unfamiliar terminology.

The "from the Human Genetics files" feature in each chapter provides additional material dealing with experimental procedures or other aspects of human

molecular genetics that are not covered in the main body of the chapter. Some of the "files," for example, provide information about the important human genetics Internet site, On-line Mendelian Inheritance in Man (OMIM); spectral karyotyping; comparative genomic hybridization; DNA chips; and transgenic animal models. The Review Questions at the end of each chapter are usually of a general nature to ensure that the reader has grasped the various topics and that he/she is able to convey these concepts in writing without the aid of the book. The Reference sections contain most of the sources for the information presented in each chapter. Some of the references are highly technical research papers; others are review articles. Because human molecular genetics is such a rapidly changing discipline, it may be more helpful for the reader who wishes to pursue specialized items in the literature to conduct an up-to-date search of published articles. The complete author citations are presented for each reference not only to give full credit to all those who participated in a particular research project, but also to underscore the collaborative nature of modern genetics research.

Acknowledgments

An Introduction to Human Molecular Genetics would not exist if it weren't for the invaluable contributions of a large number of people. The entire manuscript was converted into computer files from less-than-legible long-hand drafts and then read for "sense" and commas by my wife, Lili. Critical reviews of sections and chapters by Edward Berger of Dartmouth College, Geoffrey M. Cooper of Boston University, Paula Gregory of Ohio State University, John Hardy of the Mayo Clinic, J. Fielding Hejtmancik of the National Eye Institute (NIH), Louis Kunkel of Harvard Medical School and the Howard Hughes Medical Institute, Marie Lott of Emory University, Donald Nash of Colorado State University, Hesed Padilla-Nash of the National Cancer Institute (NIH), Thomas R. Ried of the National Cancer Institute (NIH), Mark Sanders of the University of California, Davis, Alan Schecter of the National Institute for Digestive Diseases and Kidney Disorders (NIH) and Steven Wood of the University of British Columbia helped enormously to improve the presentation of the material. Most importantly, the reviewers—all of whom were exceptionally thorough and diligent—pointed out errors, misleading statements, and both minor and major problems pertaining to scientific matters. Needless to say, errors of any kind that remain are entirely my responsibility. K. Hesselmeyer-Haddad, H. Padilla-Nash, T. Ried, S. Stenke, H.-Ulli Weier, and D. Winkler kindly provided the magnificent fluorescent in situ hybridization (FISH) images found in the color insert; C. Lengauer furnished the FISH image on the cover. Michael Goldberg and Jeff Holtmeier of the American Society for Microbiology Press graciously allowed material from *Molecular Biotechnology: Principles and Applications of Recombinant DNA*, 2nd ed. by Bernard R. Glick and Jack J. Pasternak to be used here. Nancy Knight did a thorough and impressive job of converting "final" manuscript copies into publishable versions. Susan Graham coordinated the production process with efficiency, aplomb, and patience. The book was designed by Susan Schmidler with her usual deft touch. The team headed by Karen Hawk at Precision Graphics produced the book

and created its "look." Thanks to Hope Page, who is more adept at sending and opening attachments than her boss. Last, but not least (as the cliché goes), I am grateful to Patrick Fitzgerald, who nurtured the book from its conception, assembled the team to bring it to fruition, was an unflagging source of encouragement, and contributed ideas by the bushelful. If it can be believed, Patrick makes all phases of writing a book pleasurable!

chapter 1

Understanding Human Disease

Diseases crucify the soul of man, attenuate our bodies, dry them up like old apples, make them so many anatomies.

ROBERT BURTON (1577–1640)

All interest in disease and death is only another expression of interest in life.

THOMAS MANN (1875–1955)

OF ALL LIVING ORGANISMS, only humans can learn to write, read, and understand this sentence. Our ability to observe, reason, analyze, remember, and transmit acquired information to each other and from one generation to the next has enabled us to survive over the past 100,000 years and to be especially successful in the last 10,000 years. As accidental death and disease have dominated our continued struggle for existence, we have sought ways to prolong life, cure diseases, and alleviate suffering. These ends are not easily accomplished. Diseases can be caused by viruses, bacteria, fungi, parasites, toxic substances, malnutrition, or biologically based dysfunctions. The impact of pathological diseases depends, to a great extent, on the conditions of life. Twenty-five thousand years ago, when Stone Age people lived in small isolated bands, an infectious agent might be lethal to a localized group of individuals but would be unlikely to run rampant through all bands. In those times, malnutrition was a major cause of early death. By contrast, in the last 1000 years, infectious diseases were devastating to those living in densely populated centers, decimating vast numbers. During the worldwide influenza outbreak of 1918 and 1919, for example, as many as 20 million people died.

Humans have gone to great lengths and experienced considerable confusion in explaining the basis of human disease. Evil spirits, ill winds, and angry gods have been invoked as causes. Treatments often included charms, magic potions, chants, incantations, and prayers. Fortunately, practical knowledge, based on trial and error, has supplemented these supernatural strategies throughout the ages. Plants, especially, have provided a rich source of natural products used to alleviate pain and lessen the impact of certain diseases. Except in rare cases, however, the relationship between treatment of diseases and specific cures has been tenuous. Usually, diseases ran their courses regardless of treatment. In many instances, treatments accelerated patient deaths. During the 1800s, a more systematic and less anecdotal approach revealed the origins of many human dis-

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eases and provided a rational basis for developing effective treatments. Medical researchers turned to laboratory experimentation, doctors began to specialize, aseptic techniques were introduced, surgery became more effective with anesthesia, better instrumentation was developed, and research reports appeared in journals distributed internationally. On the basis of published case histories, sets of symptoms were assembled to describe more or less discrete diseases. These consensus-generated checklists led to more accurate and consistent diagnoses and supplanted a haphazard system in which every practitioner speculated differently on the specific actions of a given disease in each patient.

A monumental breakthrough in the understanding of human disease came in 1876, when, independently, Robert Koch (1843–1910) and Louis Pasteur (1822–1895) proved that a particular microorganism (*Bacillus anthracis*) caused a specific disease (anthrax). Clearly, as one historian noted, this discovery established that "man was indeed the victim of numberless, invisible assassins." By the early decades of the twentieth century, the causative agents for typhus, tuberculosis, syphilis, smallpox, bubonic plague, cholera, yellow fever, malaria, and other diseases had been established. With this knowledge, vaccines, drug treatments, and public health measures curtailed many of these former human scourges. Recent epidemiological studies have shown that many of these diseases were actually in decline before the widespread use of vaccines and drugs. This improvement is attributed to better nutrition, enhanced sewage disposal, improved working conditions, and clean water. Nonetheless, vaccines and drugs reduced mortality rates from infectious diseases to negligible levels, at least in the developed world. Some diseases continue to defy prevention (malaria and AIDS), and others flare up periodically, either because of growing resistance to specific antibiotics (tuberculosis) or as the result of significant lapses in sanitary conditions (cholera and pneumonic plague).

As knowledge about infectious diseases accumulated in the late nineteenth century, it became clear that many diseases were not caused by infectious agents. Noninfectious diseases leading to malignant, degenerative, and chronic progressive disorders were known but were considered less fearsome than those spread by contagion. By 1900, some researchers began to focus attention on illnesses that "ran in families" (familial) and on other conditions such as cancer and mental (psychological) disorders that tended to be sporadic. New emphasis on these kinds of diseases coincided with the rediscovery in 1900 by Hugo de Vries (1848–1935), Carl Correns (1864–1933), and Erich von Tschermak (1871–1962), each independently, of the laws of inheritance first formulated in 1865 by Johann Gregor Mendel (1822–1884).

Human Genetic Disease

Humans have a long-standing fascination with and interest in the continuities of form and structure that organisms exhibit from one generation to the next (inheritance, heredity). In less technical language, the basic premise is, for example, that when an elephant gives birth, the offspring, by the broadest visible criteria, can only be considered to be another elephant. Moreover, if we know that an elephant is pregnant, we expect a baby elephant at birth and not a kitten or puppy. Similarly, humans give birth to humans, rhinoceroses to rhi-

noceroses, and, after planting seeds from radish plants, we never anticipate that roses will grow.

Although the rule that "like begets like" seems straightforward, the problem is complicated by the occurrence of variation. Without much trouble, we can identify a human as a human or a dog as a dog. But we can also see that humans, with rare exceptions, are each obviously different from one another. Indeed, close inspection of all organisms reveals that considerable variation can occur within a species or strain. Underlying these commonplace observations are two fundamental problems. First, what determines the constancy, more or less, of form through successive generations? Second, how do members of the same species become different from one another? The resolution of these problems is part of the ongoing research agenda of molecular geneticists and other scientists. Considerable understanding of these issues, which form the foundation of this book, was achieved in the latter half of the twentieth century.

Even in prehistoric times, some humans used heredity for practical purposes. The development of agriculture, which provided a more secure but more arduous way of life than hunting and gathering, depended on an understanding of domestication. Both plant and animal breeding entail choosing organisms with specific characteristics (traits) and mating (crossing) them, either to perpetuate the original traits or to identify among the offspring representatives with superior features. These offspring, in turn, can be bred for the improved characteristics. Implicit in this apparently simple strategy of breeding and selection is the expectation that certain characteristics will be passed on from one generation to the next and that discriminating choices will create animals and crops with increased productivity that will, more or less, breed true. A successful breeding program requires patience and judgment. But it does not require knowledge of the principles of inheritance.

Historically, many cultures understood that certain human disorders tend to "run in families" and responded with medical and cultural traditions to accommodate this unexplained phenomenon. Judaism, for example, absolutely requires circumcision for boys that is performed eight days after birth. However, Talmudic law, established more than 1500 years ago, provides for specific exceptions. If two sisters have each lost a son as the result of excessive bleeding after circumcision, then neither they nor their other sisters are required to have subsequent sons circumcised. If one mother has lost two sons as a result of the operation, then subsequent sons, although not exempt, must be circumcised at an older age when, presumably, they are stronger. Although not completely precise, these religious regulations make the inheritance of hemophilia one of the earliest recorded genetic disorders.

Familial disorders, although observed and recorded for centuries, were not studied systematically. Recurrences of abnormalities in the histories of families were often noted as curiosities and attributed to evil spirits or rumored to be the consequences of matings between humans and animals. There were, however, exceptions to such bizarre explanations.

In 1644, Sir Kenelm Digby (1603–1665) cited a family in which at least eight members in five generations—a great-great-grandmother, a great-grandmother, a grandmother, four daughters, the grandmother's only granddaughter (but none of the grandmother's sons)—each had an extra thumb on

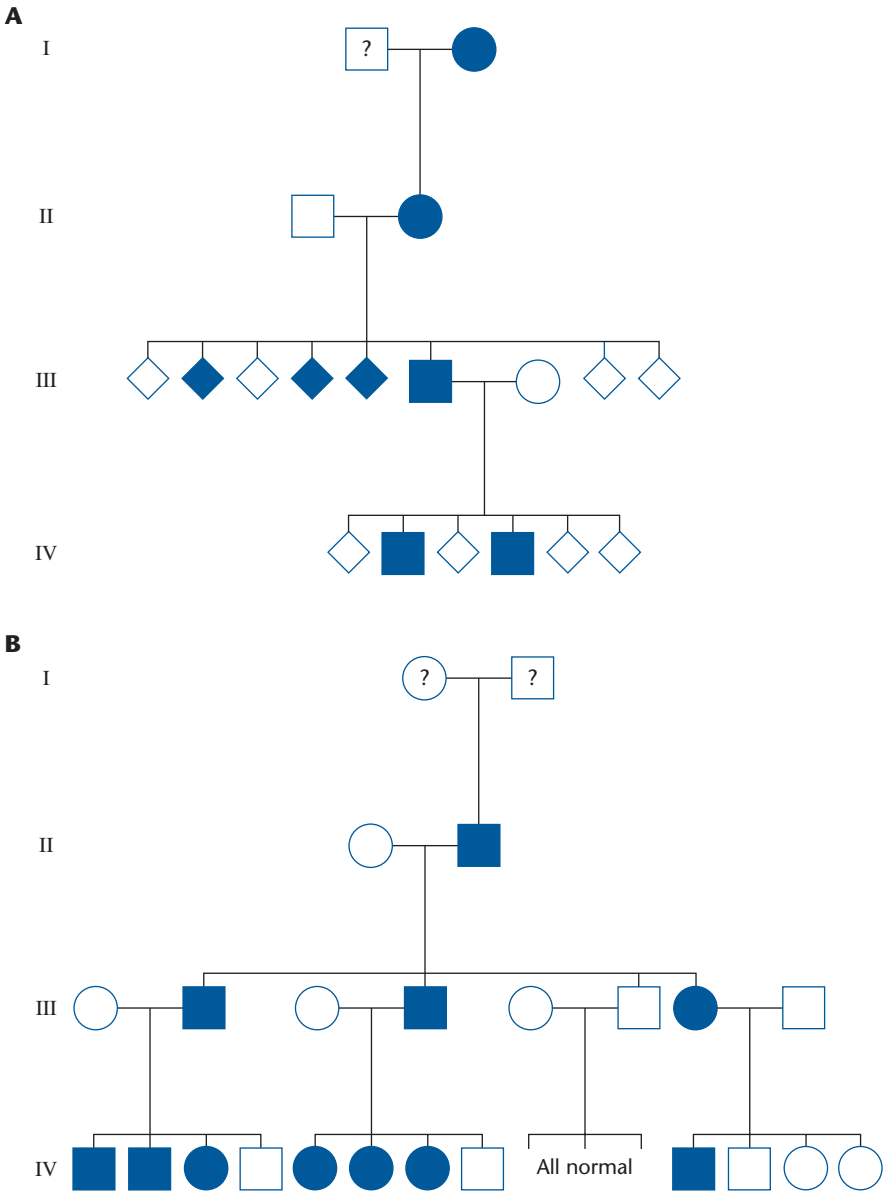


Figure 1.1 Representations of the descriptions of polydactyly in the Ruhe (A) and the Kellaia (B) families as recorded by Maupertuis and Réaumur, respectively. Squares are males, circles are females, open symbols are unaffected individuals, solid symbols are polydactylous individuals, diamonds indicate that the sex of the individual was not known, and question marks signify that the occurrence of polydactyly was not established. Roman numerals denote successive generations.

her left hand. Digby, who personally examined the grandmother, her daughters, and the granddaughter, used this example to support his view that the parts ("juices") of the body of an individual determine the nature of the offspring in the next generation. The occurrence of extra fingers, toes, or partially formed extra digits (polydactyly) in humans was known in Digby's time. In the Bible, for example, one of the Philistine champions had six fingers on each hand and six toes on each foot (2 Sam. 21:20). The Bible does not mention whether the parents or offspring of this unnamed individual had additional fingers or toes.

A century after Digby's description of familial polydactyly, Pierre Louis Moreau de Maupertuis (1698–1759) became, in 1745, the first person known to study comprehensively the pattern of inheritance of a human disorder within four generations of a single family (Figure 1.1A). The disorder happened to

be, again, polydactyly. Maupertuis was a brilliant scientist and theoretician, who articulated the fundamental principles of heredity and evolution. Unfortunately, his ideas on these subjects were largely ignored. Coincidentally, and apparently unknown to Maupertuis, René Antoine Ferchault de Réaumur (1683–1757) published a complete description of another polydactylous family about seven years later (Figure 1.1B). Although these data depict only two families with the same disorder, some distinctive features of inheritance are evident. Each polydactylous offspring had at least one parent with the condition, and, where the sex is known, more or less equal numbers of males and females were affected. In the language of human genetics, as explained later in this book, these pedigrees show the hallmarks of an autosomal dominant disorder.

Later in the eighteenth century and in the beginning of the nineteenth century, other researchers carried out similar analyses to establish patterns of inheritance of hemophilia and defects in color vision. In these cases, patterns of inheritance were similar to each other but differed from those observed by Maupertuis and Réaumur. Both hemophilia and color vision defects occur much more frequently in males than females. Often neither parent is affected, and an affected father never has an affected son. In today's parlance, these features are consistent with an X-linked inherited condition. Unfortunately, none of these studies contributed to the formulation of the basic laws of inheritance, nor did they stimulate others to follow this line of research in a thoughtful manner.

Human Genetics from 1900 to 1957

The rediscovery of Mendel's laws of inheritance and their confirmation in 1900 profoundly changed the study of human genetics (Table 1.1). Mendel showed that certain features of an organism were determined by units of inheritance, later called genes, that were transmitted from one generation to the next with mathematical precision. Mendelian genetics relies on the examination of large numbers of offspring from controlled matings to establish whether a condition is inherited and, if so, its mode of inheritance. Obviously, the requirement for controlled matings is impossible to meet when the genetics of human traits are being studied. The first human geneticists realized, however, without any knowledge of Maupertuis's study, that the Mendelian patterns of inheritance in humans could be deduced with a reasonable degree of reliability from either extensive multigeneration family trees (pedigrees) or large numbers of small pedigrees that each showed the same condition. By adopting this Mendelian strategy, an unofficial catalog of inherited conditions was initiated. By the 1960s, approximately 1500 different genes were recorded in the official catalog of human Mendelian traits. With the development of powerful non-Mendelian strategies for identifying human genes, the list has expanded dramatically. In the early 1990s, approximately 5500 genes had been described. By the late 1990s, estimates of the number of human genes ranged from 80,000 to 100,000. However, with the sequencing of the entire human genome by 2001, the number of human genes based on gene-prediction software was pegged, surprisingly, at only 25,000 to 35,000.

Inherited differences can affect any part or function of the human body. The resulting alternatives to normal features can have different degrees of severity.

Table 1.1 Milestones of human genetics.

1900	de Vries, Correns, and Tschermak, independently, rediscover Mendel's laws of inheritance
1901	Landsteiner discovers the human ABO blood groups
1905	Farabee deduces autosomal dominant inheritance based on a multigeneration pedigree with brachydactyly (short fingers)
1908	Garrod develops his theory of "inborn errors of metabolism," postulating that human biochemical disorders—albinism, alkaptonuria, pentosuria, and cystinuria—are inherited as Mendelian traits and due to defective enzymes
1908	Hardy and Weinberg, independently, show that gene and genotypic frequencies remain stable from one generation to the next under specific conditions in large, interbreeding populations
1909	Johanssen introduces the term "gene" for the unit of inheritance and creates the terms "genotype" and "phenotype" to distinguish between the genetic constitution and the physical appearance of an individual
1910	Weinberg develops methods for determining Mendelian inheritance from small pedigrees
1910	Dungern and Hirszfeld show that the ABO blood groups conform to Mendelian inheritance
1919	Haldane formulates the relationship between recombination frequency and linkage map distance and coins the term "centimorgan"
1925	Bernstein postulates that the ABO blood groups are due to multiple alleles
1927	Muller proves that gene mutations can be induced by X-rays
1930–32	Fisher, Haldane, and Wright establish the principles of population genetics
1935	Haldane determines the spontaneous mutation frequency of a human gene
1937	Newman and colleagues study the occurrence of various traits in monozygotic and dizygotic twins
1944	Avery, MacLeod, and McCarty show that DNA is the genetic material
1953	Watson and Crick determine the structure of DNA
1955	Morton devises a method for estimating human linkage using a likelihood of the odds of linkage (LOD) score
1956	Tjio and Levan show that the human diploid number is 46
1957	Ingram establishes that sickle cell anemia is due to a single amino acid change in the β-subunit of hemoglobin
1959	Lejeune and colleagues show that trisomy of chromosome 21 (21/21/21) is associated with Down syndrome
1964	Littlefield develops the HAT medium for selecting human-mouse somatic cell hybrids
1968	McKusick publishes his first edition of Mendelian Inheritance in Man (MIM)
1971	Q-banding patterns for all human autosomes is reported
1973	Boyer and Cohen lay the foundation for recombinant DNA technology
1975	Köhler and Milstein describe the production of monoclonal antibodies
1978	Human insulin is produced in the bacterium *Escherichia coli*
1980	Botstein, White, Skolnick, and Davis establish the principles of restriction fragment length polymorphism (RFLP) mapping
1983	Gusella and colleagues determine that the Huntington disease gene is located on the short arm (p) of chromosome 4
1986	Mullis and colleagues describe the polymerase chain reaction (PCR) method
1987	Kunkel and colleagues characterize the gene for Duchenne muscular dystrophy with positional cloning strategies
1990	The Human Genome Project (HGP) is launched in the US
1990	Approval is granted in the US for a trial of human somatic cell gene therapy to treat an inherited disease
1994–96	Formulation of high-resolution linkage maps for all human chromosomes
1998	Radiation hybrid map with 30,000 gene-based markers for all human chromosomes
2001	Draft sequence of the human genome (~85%) is published and made available to the public via the Internet
2003	Human genome is 99.9% finished
2004	Maps of interacting proteins (interactomes) of *Caenorhabditis elegans* and *Drosophila melanogaster* are published

Some conditions cause death, others are seriously debilitating, and a large number have mild effects. Some disorders occur more frequently and fall within the range of one affected individual per 2500 to 10,000 live births. Rare inherited disorders may affect as few as one in 100,000 newborns.

In 1908, Archibald Garrod (1858–1936), an English physician and biochemist, suggested that some human genetic diseases were caused by faulty "ferments" (enzymes). He designated this category of diseases as "inborn errors of metabolism." Today, there are dozens of examples of defective enzymes that cause genetic diseases. Alkaptonuria (alcaptonuria), a condition Garrod deduced in 1902 as an example of inborn error of metabolism, illustrates some of the fundamentals of this important type of genetic disorder. Alkaptonurics, in contrast to those with many other inherited disorders, have no serious maladies, although in later years they often develop a form of arthritis called ochronosis. The feature that attracted the attention of various researchers was that the urine of alkaptonurics turns black after a brief period of time outside the body. Garrod and others determined that an excess of homogentisic acid (alkapton) accumulated in the urine of alkaptonurics. On the basis of his own observations and those of others, Garrod noted that a large proportion of observed cases of alkaptonuria were from marriages between first cousins. And, although the condition was rare, often two or more sibling offspring from nonaffected parents had the condition. In Mendelian terminology, this pattern of inheritance was consistent with the interpretation that two recessive factors of a single gene pair were responsible for the condition. Garrod argued that alkaptonuria was inherited as a Mendelian trait and that the buildup of homogentisic acid was the result of a faulty enzyme that, if functioning normally, should have converted homogentisic acid to a different compound.

Generally, an enzyme catalyzes the conversion of one substance into another. A set of enzymatic conversions that produces a specific end product is called a biochemical pathway (Figure 1.2). Biological systems have a large number of such pathways for the production (anabolism) of essential compounds and the breakdown (catabolism) of scores of compounds. If an enzyme at one of the steps in a pathway is defective, then at least two consequences follow. The compound not converted by the faulty enzyme accumulates, and the last product (end product) of the pathway is not produced. The excess of an intermediate product may be toxic and cause a damaging disorder or, as in the case of alkaptonuria, may have no significant impact on the proper functioning of cellular processes. In addition, the end product of a pathway may be vital and, in its absence, an abnormality arises. Some biochemical pathways are branched, and others are interdependent. In these cases, a faulty enzyme can lead to complex biochemical changes.

Garrod's explanation for alkaptonuria was correct. Years would pass before additional research identified homogentisic acid as an intermediate compound in the catabolism of the amino acids phenylalanine and tyrosine to fumaric acid and acetoacetic acid (Figure 1.3). More than 50 years after Garrod's work, sci-

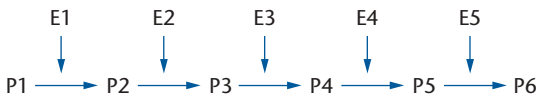


Figure 1.2 Schematic representation of a biochemical pathway. The notations P1–P5 represent compounds that are substrates for enzymes E1–E5, respectively. Compound P6 is the end product of the pathway.

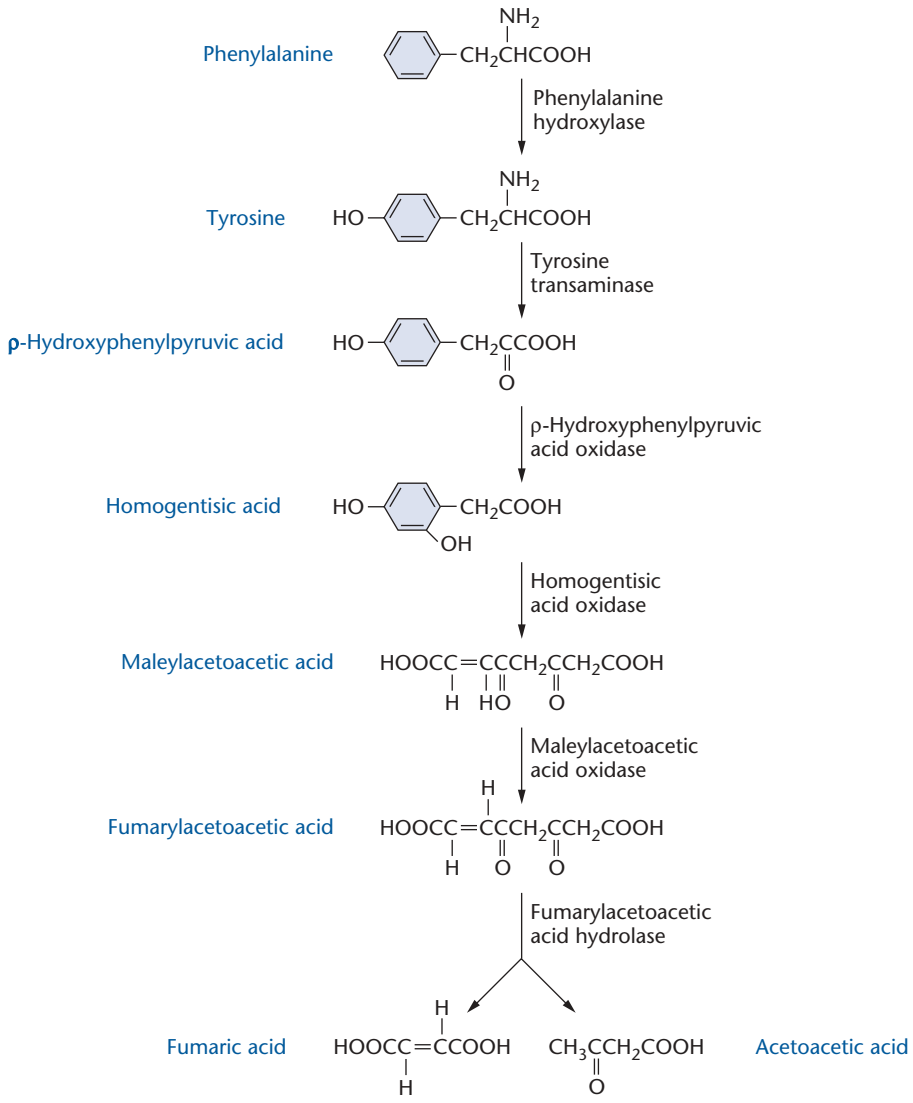


Figure 1.3 The catabolic pathway of phenylalanine and tyrosine to fumaric acid and acetoacetic acid.

entists demonstrated that the defective enzyme in alkaptonurics was homogentisic acid oxidase (Table 1.2). Different genetically determined metabolic disorders have different degrees of impact. Some, such as alkaptonuria, are benign, whereas others lead to serious complications. Phenylketonuria (PKU), for example, can cause severe mental retardation. Tay–Sachs disease leads to death, in most cases before the affected child reaches the age of 10. Other defective enzymes do not allow any form of development to occur. In a number of conditions early death is inevitable, and in others lifelong suffering is a prevalent feature of the disorder. For the most part, studies of human genetics are based on inherited disorders that enable some form of survival.

Garrod's concept of inborn errors of metabolism was essential both for the eventual understanding of the biochemical basis of many human genetic diseases and for stimulating research on biochemical pathways in living organisms. In addition, the demonstration of a relationship between genetic disorders and faulty enzymes, soon identified as proteins, focused attention on the pos-

Table 1.2 Activities of phenylalanine and tyrosine catabolic enzymes in liver samples from normal and alkaptonuric individuals.

Enzyme	Enzyme activity	
	Normal	Alkaptonuria
Tyrosine transaminase*	3.6	3.2
ρ-Hydroxyphenylpyruvic acid oxidase*	6.7	4.6
Homogentisic acid oxidase*	26.8	<0.0048
Maleylacetoacetic acid isomerase**	960	780
Fumarylacetoacetic acid hydrolase*	29	22

*Units are μmol/h/0.1 g wet weight.
**Units are Δlog optical density/h/0.1 g wet weight.
Adapted from La Du BN, Zannoni VG, Laster L, Seegmiller JE 1958. The nature of the defect in tyrosine metabolism in alkaptonuria. *J Biol Chem* **230**:251–261.

sibility that genetic information was responsible in some way for the production of proteins. Decades of research eventually established that a unit of genetic information (gene) did indeed determine the sequence of amino acids in a protein chain.

Eugenics: Genetics Misinterpreted

During the first quarter of the twentieth century, excessive public and scientific enthusiasm tended to designate any trait that "ran in families" as genetically determined. In many cases, the criteria of Mendelian inheritance were relaxed or ignored, and few observers bothered to determine whether a particular condition merely mimicked a Mendelian pattern of inheritance. Often, obvious social and environmental factors were disregarded. Pellagra, for example, is a persistent condition that causes skin lesions and severe nervous and mental disorders. Pellagra was common throughout the southern United States in the early 1900s. Although many researchers thought the disease was infectious, some geneticists argued forcefully, and apparently convincingly, that pellagra was an inherited disease, because in some cases both parents and offspring showed its effects.

Fortunately, a clinical researcher, Joseph Goldberger (1874–1929), showed in 1916 that this condition was caused neither by an infectious agent nor by a faulty gene, but by a nutritional deficiency. The absence of the vitamin niacin from the diet caused pellagra symptoms. However, the acceptance of a genetic cause for pellagra was so ingrained in the United States, and with it the notion that the disease was inevitable and incurable, that public policy did not begin mandating niacin as a dietary supplement until 1933. The object lesson of this case, which even today is not fully appreciated, is that the presence of a trait in successive generations of a family is not definitive proof that the characteristic is genetically determined. More stringent criteria must be used before a genetic cause is implicated. Such a cautious approach is especially important for complex traits with major behavioral components, such as intelligence and personality.

By 1920, unfounded genetic explanations for virtually all aspects of human conduct were rampant. Many complex human cultural features (entrepreneurial success, poverty, piety) and social behaviors (mental disorders, criminality,

intelligence) were considered to be genetically determined. Popular textbooks of the period freely made such generalizations as "the hereditary nature of criminal propensity is unquestionable" and "vagabondism, pauperism, and crime run in certain families for generations." These claims were often accepted without critical analysis. On the basis of this fallacious notion of genetics, concerns were raised that "civilization" (usually a code word for those who were well-to-do and descended from Northern Europeans) was in danger of genetic degeneration, because "bad" genes (ascribed to those who were poor or who were not descended from Northern Europeans) would soon outnumber "good" genes. To prevent this presumed catastrophe, organizations were formed to encourage couples with "good" genetic stock to breed and to advocate ways to prevent those with "bad" genes from having offspring.

The concept of improving human qualities by promoting and prohibiting specific marriages is called eugenics. In the United States, the eugenics movement quickly became popular and had significant effects on the formulation of federal immigration laws. The movement also pushed for and achieved the passage of widespread state legislation allowing court-ordered sterilization of individuals who, for various reasons, were considered unfit to have children. In 1927, the United States Supreme Court, by a vote of eight to one, upheld the right of states to implement sterilization for eugenic purposes. The viewpoint of the majority of the justices was encapsulated by Justice Oliver Wendell Holmes's terse declaration that "Three generations of imbeciles are enough."

By the late 1920s, scientists understood that the genetic contribution to physical health, intelligence, and moral character was exceptionally complex. Moreover, it was becoming apparent that the primary biological assumptions of the eugenics movement were based on faulty information and unsubstantiated claims. In addition, findings in the social sciences contradicted many of the beliefs promulgated by the more ardent eugenicists. Despite a mass of accumulating evidence that eugenics programs could never be effective, the enthusiasm for controlling the perceived negative impact of "bad" genes continued during the 1930s. Sterilization laws were passed in a number of countries. It was only after the horrendous consequences of Nazi attempts to wipe out whole groups of people in the 1940s that the eugenics movement lost all credibility.

Mainstream research in human genetics in the 1930s was devoted to analyzing the genetic structure of populations. Often theoretical, these studies emphasized the frequencies of genes and gene combinations (genotypes) in various groups. For example, the distributions of the A, AB, B, and O blood groups were commonly used for studies of historical relationships, migratory patterns, and other features of various human populations.

Also during this period, identical twins, who share the same genetic information, became the object of studies to ascertain which traits might have genetic components (nature) in contrast to those that might be caused by nongenetic means (nurture). The rationale for such studies, briefly, is to compare the frequency of occurrence of a trait in both members of sets of identical (monozygotic, MZ) twins and fraternal (dizygotic, DZ) twins. If a trait is present in both members of a twin set, it is called concordance. If a trait is entirely the result of genes, then MZ twins should show 100% concordance and DZ twins should show 50% concordance, because MZ and DZ twins share

100% and 50% of their genes, respectively. Various statistical approaches have been used over the years to determine when a significant relationship exists between concordance scores for MZ and DZ twins when the concordance for MZ twins is less than 100%. If a trait shows a much higher concordance value for MZ than for DZ twins, then it can be concluded that the trait is, to some extent, genetically determined. From a genetic perspective, these kinds of studies are not very informative. Little is learned about the nature of the inheritance of a trait, how many genes are involved, whether a major gene plays a role in determining a trait, or what gene products contribute to that trait. Twin studies do, however, point researchers in particular directions so that they can gain a better understanding of the causes of complex conditions.

By 1950, human genetic studies had made considerable contributions in identifying inherited diseases and complex conditions with genetic components. Mathematical strategies were developed to enable researchers to deduce patterns of inheritance by examining large numbers of sets of brothers and sisters (sibships) from a single generation. Five hundred inherited conditions had been recorded. Most of these were pathological, with approximately 100 disorders affecting the skin, 100 affecting the eyes, and 100 affecting the skeletal system. Approximately 20 abnormalities were identified for the blood system, 20 for the nervous system, and 20 for muscle tissue, as well as approximately 20 examples of metabolic and endocrine disorders. By contrast, very few nonpathological conditions, other than blood group antigens, color vision defects, hair whorl patterning, the ability to taste phenylthiocarbamide, and some others, had been described. Little was known about the biochemical consequences of most pathological diseases. Moreover, the actual relationship between a defective gene and a genetic disease was a complete enigma. No specific therapies for genetic diseases had been devised, and no procedures for diagnostic testing had been developed. Although possibly too harsh an assessment, the study of human genetics seemed to become intellectually stagnant after its first 50 years. There were no new methodologies to probe the basis of human genetic diseases, and novel ideas, in the absence of innovative techniques, were scarce.

The Molecularization of Genetics

The study of human genetic diseases was changed forever when the field became "molecularized" in the 1950s. "Molecularization" is scientific jargon for studies concerned with characterizing biological processes at the level of proteins and nucleic acids (ribonucleic acid, RNA; deoxyribonucleic acid, DNA) and with determining how alterations of these macromolecules disrupt basic biological processes. In 1953, James D. Watson (b. 1928) and Francis H. C. Crick (1916–2004) revolutionized all of the biological sciences with their discovery of the double helical structure of DNA. Previously, DNA had been shown to be the genetic material of living organisms. Researchers established that the units of genetic information (genes) somehow encoded information for the synthesis of enzymes, a theory designated at the time as the "one gene—one enzyme" hypothesis. But the question of how the DNA information of a gene generates a sequence of amino acids of an enzyme or any other protein chain remained a mystery. Nor was there any clear idea of the extent to which

a protein has to be changed to be nonfunctional or to have altered properties that, for example, could cause a human genetic disease.

The discovery of the molecular basis of an inherited disorder called sickle cell anemia provided some fundamental answers to these questions. Sickle cell anemia results from a breakdown of circulating red blood cells (hemolytic anemia). Affected individuals suffer from chronic ill health and long-term complications in the heart, brain, kidneys, and other organs and often die early in life. A microscopic comparison of the blood from patients with sickle cell anemia and healthy individuals shows differences in the shape of the red blood cells. Instead of being smooth and biconvex, the red blood cells are jagged and sickle-shaped in patients with the anemia. This observation gave the disease its name. The cause of the sickling of these red blood cells was unknown. Hemoglobin is the major protein of red blood cells and is responsible for transporting oxygen to organs within the body. In 1949, researchers found that the hemoglobin from the red blood cells of sickle cell anemics differed in its electrophoretic mobility from the hemoglobin of normal red blood cells (HbA). Accordingly, sickle cell anemia was characterized as a molecular disease with the genetic alteration giving rise to an altered hemoglobin molecule (HbS).

The normal HbA molecule is a protein multimer consisting of two α-chains and two β-chains. An α-chain has 146 amino acids, and a β-chain has 141 amino acids. In 1957, Vernon Ingram (b. 1924), using protein fingerprinting and sequencing techniques, determined that HbS had a single amino acid difference from HbA in the β-chain. The amino acid sequences of the α-chains of HbA and HbS were identical. However, on the β-chain of HbS the amino acid in the sixth position was valine, whereas in HbA this amino acid was glutamic acid. This difference is the result of a change in the DNA sequence that encodes the β-chain. This seemingly minor change of a protein alters the stacking properties of the HbS molecules, which form a viscous gel within the red blood cells, causing the shape of the cell to become distorted. Because of their jaggedness, sickled red blood cells do not flow smoothly through capillaries, small veins, and arteries. They become tangled and form blockages that prevent adequate distribution of oxygen to the major organ systems, leading to tissue damage and causing severe pain. Sickle cell anemia was the first example, of which there are now hundreds, of a single amino acid alteration of a protein chain that is responsible for a human genetic disease.

A quarter of a century would pass before researchers could sequence DNA routinely to determine the kinds of changes that occur at the level of the gene and cause many human genetic diseases. The ability to identify, isolate, and characterize genes that cause different genetic diseases is the major feature of current research in human genetics. This book is concerned with explaining how these studies are carried out, which genetic defects lead to disorders, and what biological consequences stem from these defects.

Genes and Phenotypes

To what extent do genes determine human nature? This question has been debated for centuries. More than 700 years ago, Thomas Aquinas (1225?–1274), a prominent Roman Catholic theologian, expressed the view that the intrinsic nature of a person may, in some instances, be determined by

heredity, noting "that even some defects of [the] soul are transmitted in consequence of a defect in the bodily habit, as in the case of idiots begetting idiots." To this day, we have little understanding of how the totality of the array of attributes, both biological and environmental, contributes to making each of us a unique personality with our own distinctive attitudes, tastes, behaviors, and abilities.

We do know that this process, whatever it involves, is a complex mixture of genetic and nongenetic factors. At one extreme, children raised without significant human contact during their early years seem unable to develop a full range of speech, their learning is limited, and their ability to interact socially with other humans is highly constrained. But we also have learned that conditions such as PKU, fragile X syndrome, myotonic dystrophy, and many other disorders with serious effects on behavior and mental competence are unquestionably the result in each instance of an alteration of a single gene.

By contrast, the role and extent of the genetic contribution to the formulation of complex characteristics such as intelligence (which is not synonymous with an intelligence quotient or IQ score), giftedness, personality, sexual orientation, social behaviors, and other attributes are largely unknown. Ignorance in these matters has not prevented speculation that, in a number of instances, has been accepted as fact. In the past, explanations that attempted to account for the origins of complex human characteristics tended to be polarized. One camp held the view that only environmental influences (nurture) determine human behavioral variability. The dictum that "all men are created equal" reflected this viewpoint. The other camp, at its extreme, argued that the most significant factor in determining human behavior is genes (nature). Historically, the latter school of thought was often inclined to accept, without sufficient proof, various attributes as genetically determined. Those with this viewpoint often have allowed both social and political ideology—not human genetics—to decide which human characteristics are inherited. And, for those characteristics that were considered to have a negative impact on society, the proposed remedies were driven by political convictions, not by medical or scientific reasoning.

Even today, the nature–nurture controversy is not entirely resolved. However, it is clear that the question can no longer be framed as an either/or issue. It seems probable that, for many complex traits, both genetic and environmental factors play roles in determining final outcomes. The problem for the unbiased researcher is to assess to what extent either genetic or environmental factors influence a specific characteristic.

A highly oversimplified formula, $P = G + E$, can be used to illustrate the nature–nurture problem, where P represents the characteristic (phenotype) and G and E represent genetic and environmental contributions, respectively. The sum of G and E equals 1 (unity). For example, a viral infection during early childhood may cause a severe mental disability. In this case, the phenotype results entirely from an environmental factor; hence, P equals E, with G equal to 0 ($P = E$; $G = 0$). On the other hand, the genetic disease PKU will cause severe mental disability if left untreated. Thus, for this form of mental impairment, P equals G and the contribution of E is 0 ($P = G$; $E = 0$). When treatment for PKU is initiated soon after birth and maintained, severe mental impairment does not necessarily occur, although, in some instances, complete

from the HUMAN GENETICS files

OMIM: An Important Online Source of Information About Human Genetic Disorders

Victor McKusick (b. 1921), a human geneticist at Johns Hopkins University with admirable foresight, established in 1966 a catalog of human genetic disorders with 1487 entries. Today, McKusick's brainchild, called Online Mendelian Inheritance in Man (OMIM), has grown to more than 15,000 entries and is fully accessible online at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM.

For each specific human genetic disorder, an OMIM entry provides a body of technical detail that is updated at regular intervals. The entries vary in size according to the amount of published information that is available for a particular genetic disorder. Each entry is assigned a six-digit OMIM number that may include different symbols. Details about this nomenclature and all other aspects of OMIM are available on its home page.

An entry has information, depending on the extent of current knowledge of a disorder, about clinical features, clinical management, molecular genetics, population genetics, animal models, and so on. The fields are not comprehensive essays, but rather they contain information culled from research articles. Initially, some readers may find the contents of a section arcane, overly technical, possibly disjointed, and too succinct. Moreover, because research is self-correcting, incremental, and produced at a rapid rate, some sections contain contradictory information. Notwithstanding, OMIM is very helpful for those who have some basic knowledge of human molecular genetics and a rudimentary understanding of the genetic disorder of interest, although these are not absolute requirements to consult this database. In this context, an extensive list of references accompanies each entry file, enabling users to delve deeper into the literature and learn more about a specific genetic disorder.

Overall, OMIM is a superb source of information. The CREATION DATE and EDIT HISTORY segments tell the user when the file became an OMIM entry and when it was last updated, respectively. If a disease-causing gene has been characterized, its OMIM entry contains a list of ALLELIC VARIANTS that briefly specifies the known mutational changes at various sites within the gene. For genes that have been studied thoroughly, such as the β-hemoglobin chain gene, there can be an extremely long list of allelic variants.

Entries are retrieved from the OMIM database by an effective search procedure that allows the user to include or exclude various fields, such as the title, text, references, allelic variants, etc. For example, a search of all fields with the term "alzheimer" produces a list of approximately 150 entries. Fortunately, the retrieved files are ranked in order of the number of "hits" for the search word. Reading entries on a computer screen is tedious. A user should consider saving entries of interest, reading them at a more leisurely pace offline, and, if required, printing the relevant entries.

OMIM has a number of other important features including links to a large number of databases of potential interest to OMIM users. In addition to OMIM, there are sites on the World Wide Web devoted to specific genetic disorders, such as Duchenne muscular dystrophy, that can be accessed by using addresses generated through a search engine. Anyone with more than a casual interest in human genetics should become familiar with OMIM. Try it! You'll like it!

Below is a sample of part of the OMIM entry for alkaptonuria (#203500):

Alternative titles; symbols
AKU
HOMOGENTISIC ACID OXIDASE DEFICIENCY

Gene map locus 3q21–q23

TEXT

A number sign (#) is used with this entry because alkaptonuria is caused by mutation in the homogentisate 1,2-dioxygenase gene (HGD; 607474).

CLINICAL FEATURES

Alkaptonuria enjoys the historic distinction of being one of the first conditions in which mendelian recessive inheritance was proposed (by Garrod, 1902, on the suggestion of Bateson) and of being 1 of the 4 conditions in the charter group of inborn errors of metabolism. The manifestations are urine that turns dark on standing and alkalinization, black ochronotic pigmentation of cartilage and collagenous tissues, and arthritis, especially characteristic in the spine. Cunningham et al. (1989) observed rapidly evolving osteoarthrosis of the right hip in a 65-year-old woman who had unusual stress after being forced to abandon a train as a result of a bomb threat and having to carry 2 heavy suitcases. Carrier and Harris (1990) reported the case of a 70-year-old man who underwent bilateral hip and knee total joint arthroplasties for the treatment of ochronotic arthropathy. Dereymaeker et al. (1990) described a patient in whom calcified aortic valve disease secondary to ochronosis necessitated urgent aortic valve replacement. There are reports of urolithiasis in AKU patients in middle and late adulthood who have already developed the full clinical picture of the disorder (e.g., Sener, 1992). Zibolen et al. (2000) emphasized the increased frequency of urolithiasis in AKU patients younger than 15 years. They reported 5 such patients, in one of whom the diagnosis of urolithiasis had been made at the age of 2 years. Phornphutkul et al. (2002) provided a review of the natural history of alkaptonuria. They based the review on an evaluation of 58 patients with the disorder ranging in age from 4 to 80 years. They found that joint replacement was performed at a mean age of 55 years and that renal stones developed at 64 years, cardiac-valve involvement at 54 years, and coronary artery calcification at 59 years. Linear regression analysis indicated that the radiographic score for the severity of disease began increasing after the age of 30 years, with a more rapid increase in men than in

women. In the 58 patients reviewed by Phornphutkul et al. (2002), kidney stones were documented in 13 male and 3 female patients. Of the 27 men who were 31 to 60 years old, 8 had prostate stones. The development of prostate stones was not associated with the development of kidney stones. Three patients, each over the age of 50 years, had undergone aortic valve replacement.

CLINICAL MANAGEMENT

Lustberg et al. (1970) presented evidence that ascorbic acid in high doses decreases binding of C(14)-homogentisic acid (HGA) in connective tissues of rats with experimental alkaptonuria. Long-term therapy in young patients with alkaptonuria is indicated. Wolff et al. (1989) treated 2 adults and 3 infants with high doses of ascorbic acid and studied the effect on the excretion of homogentisic acid and its toxic oxidation product, benzoquinone acetic acid (BQA). The purpose was to determine whether concentrations in body fluids of the latter substance, the putative toxic metabolite in alkaptonuria, would be reduced. Indeed, disappearance of BQA from the urine was observed in adults, whereas the level of excretion of homogentisic acid did not change. This could have relevance to the pathogenesis of ochronotic arthritis. In 2 of the infants studied (a 4- and a 5-month-old), ascorbic acid may have doubled the amount of homogentisic acid in the urine, presumably through an effect on the immature ρ-hydroxyphenylpyruvic acid oxidase. Dietary reduction of the intake of tyrosine and phenylalanine substantially reduced the excretion of homogentisic acid. In a 48-year-old man in whom alkaptonuria had been diagnosed during infancy, Mayatepek et al. (1998) studied the effects of 4 different therapeutic 1-month trials consisting of supplementation with ascorbic acid (1 g/day and 10 g/day) or a low-protein diet (0.3 g/kg/day and 1.3 g/kg/day). Administration of ascorbic acid in doses of 1 g/day or more resulted in a significant and constant decrease of urinary BQA, whereas the excretion of HGA could not be substantially reduced. Mild and severe dietary reduction of protein intake alone also substantially reduced the excretion of BQA, whereas the level of HGA excretion could not be drastically reduced. Such an extreme protein restriction would not, however, have been acceptable for a longer period of time. They concluded that supplementation of ascorbic acid in doses of 1 g/day represents a simple and rational treatment in patients with alkaptonuria. La Du (1998) examined the question "Are we ready to try to cure alkaptonuria?" He explored the possibilities and indicated that model animal systems, either those representing known spontaneous hereditary deficiencies of homogentisic acid or appropriate "knockout" animals with created deficiencies of the enzyme, need to be tested before human trials are undertaken. He said that it is to be hoped that some, perhaps all, of the adverse effects of alkaptonuria can be prevented by new molecular therapeutic approaches; however, trading alkaptonuric problems for even more serious metabolic disturbances because of the pronounced toxicity of the later tyrosine metabolites is not an acceptable alternative. Suzuki et al. (1999) used 2(-2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) as a therapeutic agent for alkaptonuria. NTBC is a potent inhibitor of ρ-hydroxyphenylpyruvate dioxygenase, which catalyzes the formation of homogentisic acid from ρ-hydroxyphenylpyruvic acid, and had been used in the treatment of type I tyrosinemia (276700). In a murine model of alkaptonuria that had been created with ethylnitrosurea by Montagutelli et al. (1994), they observed a dose-dependent reduction in urinary output of homogentisic acid with administration of NTBC. Phornphutkul et al. (2002) reported that, in a 51-year-old woman, urinary HGA excretion fell from 2.9 to 0.13 g per day after a 10-day course of nitisinone. In a 59-year-old woman, similar reductions were observed after 9 days of treatment. Plasma tyrosine levels in these patients rose, with no clinical signs or symptoms. They emphasized that the long-term safety and efficacy of this treatment requires further evaluation. Nitisinone is a triketone herbicide that inhibits 4-hydroxyphenylpyruvate dioxygenase by rapid, avid binding that is reversible. The agent had been approved by the FDA for the treatment of tyrosinemia type I.

MAPPING

In 6 Slovak pedigrees selected from a total of 191 alkaptonuria patients registered at the Institute of Clinical Genetics in Martin, Slovakia, Janocha et al. (1994) demonstrated linkage to microsatellite markers from proximal 3q. Markers on that chromosome were selected for study because of previously demonstrated homology of synteny with mouse chromosome 16. Independently, Pollak et al. (1993) used homozygosity mapping to locate the alkaptonuria gene to 3q2 in a 16-cM region. They studied 2 consanguineous families with 4 affected children and 2 nonconsanguineous families which supported the linkage. They pointed out that Garnica et al. (1981) described coinheritance of alkaptonuria and sucrase-isomaltase deficiency (222900), which maps to 3q25–q26. Furthermore, Steinmann et al. (1984) described coinheritance of neonatal severe hyperparathyroidism and alkaptonuria. The former condition is thought to be the recessive (i.e., homozygous) form of familial hypocalciuric hypercalcemia (145980), which maps to 3q21–q24. In the case reported by Steinmann et al. (1984) , both parents, who were related, had familial hypocalciuric hypercalcemia.

MOLECULAR GENETICS

In patients with alkaptonuria, Fernandez-Canon et al. (1996) identified missense mutations in the homogentisate 1,2-dioxygenase gene that cosegregated with the disease (607474.0001, 607474.0002), and provided biochemical evidence that at least one of these missense mutations is a loss-of-function mutation. Studying 4 alkaptonuria patients from Slovakia, where alkaptonuria has a notably high frequency, Gehrig et al. (1997) found 2 novel mutations in the HGD gene (607474.0003, 607474.0004). Other mutations in the HGD gene were identified in patients with alkaptonuria by Beltran-Valero de Bernabe et al. (1998, 1999), Muller et al. (1999), Rodriguez et al. (2000), Zatkova et al. (2000), and Phornphutkul et al. (2002). In Turkey, Elcioglu et al. (2003)

continued

described a 39-year-old male patient with typical features of alkaptonuria. In addition to the typical changes in the skin at many sites and in the pinnae and sclerae, there were grayish-blue longitudinal rigging of his fingernails and bluish-gray pigment deposition on the tympanic membrane. He was found to be compound heterozygous for 2 mutations in the HGD gene: gly270 to arg (G270R; 607474.0011) in exon 11 and 342delA (607474.0006) in exon 3 leading to a frameshift after arg58 and a subsequent premature stop codon.

POPULATION GENETICS

Alkaptonuria was found to be unusually frequent in the Dominican Republic (Milch, 1960) and in Slovakia (Cervenansky et al., 1959). According to O'Brien et al. (1963) , more cases (126) had been reported from Czechoslovakia than anywhere else. From Germany 108 had been reported, and from the United States 90.

ANIMAL MODEL

In the course of an ethylnitrosourea mutation study, Guenet (1990) and his group detected a mutation for alkaptonuria in the mouse by the finding of black wood shavings in the mouse boxes. Affected mice showed high levels of urinary homogentisic acid without signs of ochronosis or arthritis. Montagutelli et al. (1994) demonstrated that the locus, symbolized aku, maps to mouse chromosome 16 in a region of syntenic homology with human 3q.

HISTORY

Stenn et al. (1977) provided evidence that the Egyptian mummy Harwa, dating from 1500 B.C., had alkaptonuria. A phenocopy (a nongenetically produced phenotype resembling a gene-determined one) of alkaptonuria is provided by the ochronosis that develops after the prolonged use of carbolic acid dressings for chronic cutaneous ulcers (Beddard, 1910; Beddard and Plumtre, 1912). Fortunately this is a thing of the past. Quinacrine (atabrine), after prolonged administration, also causes an ochronotic change with pigmentation in many of the same sites as in alkaptonuria, and probably with a comparable arthrosis (Ludwig et al., 1963). Snider and Thiers (1993) described a case of exogenous ochronosis due to use of bleaching creams that contain hydroquinone by black women to lighten their complexion. Sandler et al. (1970) raised the question of whether parkinsonism occurs in increased frequency with alkaptonuria, either as a complication or as a syndromal entity separate from ordinary alkaptonuria. Phornphutkul et al. (2002) provided a review of the natural history of alkaptonuria in the year marking the one-hundredth anniversary of Garrod's description of the disease as the first disorder in humans found to conform to the principles of mendelian autosomal recessive inheritance (Garrod, 1902).

intellectual competency is not achieved. In these latter cases, the final phenotype is the result of both genetic and environmental factors (P = E + G). Most of the examples in this book will be of the P = G; E = 0 type. However, the reader should not get the impression that all human characteristics are completely genetically determined. Moreover, in the absence of definitive proof, differences among individuals or between groups of individuals should not automatically be attributed to genetic factors.

In the 1990s, the view that human behaviors are determined to a significant extent by genetic factors gained additional support. This hereditarian attitude was bolstered indirectly by significant successes in the discovery of genes responsible for a range of physical disorders, including Duchenne muscular dystrophy, cystic fibrosis, myotonic dystrophy, retinitis pigmentosa, fragile X syndrome, Huntington disease, and others. In these cases, knowledge of defective genes and their normal counterparts should promote a more complete understanding of the molecular basis of the diseases and stimulate the development of corrective therapies. In addition, both familial studies and comparisons of traits of twins who have been reared apart suggest that many human behavioral traits, including social attitudes, are associated with some genetic input. In many of these cases, the observed trait is the consequence of the activity of a number of different genes. How a set of multiple genes determines a complex characteristic remains a complete mystery. Even if most of our behavior was the result of our genes—and this is a big if—genetic disorders can be corrected and ameliorated. For complex traits, the long-term challenge will be to find humane strategies and treatments to override the negative consequences of genetically determined dispositions.

key terms

amino acids
chromosome
deoxyribonucleic acid
dizygotic twins
environment
eugenics
gene
genetics
genotype
heredity
infectious
inheritance
monozygotic twins
nature
nurture
phenotype
polydactyly
protein
ribonucleic acid
variation

summary

Humans have always been interested in the nature and function of the human organism. And, by necessity, we have sought ways to control the disastrous impact that disease can have on our lives. With the discovery in the late nineteenth century that many diseases were caused by microorganisms, both the treatment and management of these scourges became possible. The addition of vaccines and drugs in the twentieth century brought most of these diseases under total control. Consequently, biological disorders of the heart and circulatory system, cancers, and a variety of genetic disorders have now become major concerns.

At the beginning of the twentieth century, the rediscovery of Mendel's laws of inheritance stimulated researchers to develop strategies and methods for determining whether certain human disorders were inherited. By studying large, multigenerational families and large numbers of small families with the same condition, inherited human conditions were identified and classified. In addition, A. E. Garrod, first in 1902 and later in 1908, postulated that some inherited diseases were the result of defective enzymes. These "inborn errors of metabolism," as Garrod called them, showed that a unit of heredity (gene) was, in some way, responsible for the proper functioning of an enzyme. By the 1970s, the details of this relationship were worked out. Briefly, a gene encodes the information that ensures that a protein chain will have a specific sequence of amino acids.

By the 1920s, an excessive enthusiasm for theories of heredity attributed almost all aspects of human nature to genetic causes. For example, social evils—such as unemployment, crime, and pauperism—and social virtues—including financial success, piety, and resoluteness—were considered to be strictly genetically determined. It was believed that a person either possessed the genes for exemplary qualities or he/she did not. This view, which led to the formation of eugenic societies dedicated to preventing the so-called deterioration of the genetic makeup of future generations, was not based on any sound experimental evidence. A number of scientists pointed out the fallacies of these pronouncements, and, in time, the claims of the eugenics movement were discredited. In addition, it soon became scientifically clear that selective breeding of humans was unnecessary, not only because of the dubious genetic basis of the traits that were to be maintained or selected, but because complex genetic conditions would make such programs futile.

The "nature–nurture" debate, which stems from the human genetics of the 1920s, is, in modified form, a persistent controversy. In its earlier formulation, there were those who believed that environmental factors were the most important determinants in making humans human. In contrast, another faction argued that human patterns of behavior were completely genetically determined. The nature–nurture argument becomes most heated when it deals with complex traits, such as intelligence, that cannot be precisely defined genetically. Currently, many aspects of human nature seem to have genetic components. However, the characteristics that are most studied from this perspective have been found to be so complex and interwoven with contributions from both environmental and genetic factors that it is impossible to determine the specific role of genes in forming these behaviors.

Despite successes and controversies, by the 1950s the study of human genetics and genetic studies in general needed a scientific breakthrough to establish new momentum in the field. The fundamental problem was that little was known about the molecular relationship between the unit of inheritance (gene) and its product, a protein chain. Within a short period of time, the structure of DNA was determined, a single amino acid change in a protein chain was shown to be sufficient to cause an inherited human disease, and the process by which the encoded information within a gene is used to produce the specific sequence of amino acids of a protein was understood. This research momentum has continued. Espe-

continued

cially important in the 1990s was the development from recombinant DNA technology of a number of techniques and strategies for determining the chromosome location of a genetic disease and for identifying and characterizing the disease-causing gene. Finally, the most significant advance in human molecular genetics was the publication of the draft version of the human genome sequence in 2001. This resource, which is continually updated, has streamlined human gene discovery. In addition, the characterization of disease-causing genes leads to defective-gene detection assays and more effective treatments for genetic diseases.

References

Digby, K. 1644. *Two Treatises, in the One of which, the Nature of Bodies; in the other, the Nature of Mans Soule; is looked into: in way of discovery, of the Immortality of Reasonable Soules.* Gilles Blaizot, Paris. [Facsimile: Friedrich Frommann Verlag (Günther Holzboog), Stuttgard-Bad, Canstatt, 1970.]

Dunn, L. C. (ed.). 1951. *Genetics in the Twentieth Century: Essays on the Progress of Genetics during its First 50 years.* Macmillan, New York.

Garrod, A. E. 1902. The incidence of alkaptonuria: A study in chemical individuality. *Lancet* **ii**:1616–1620.

Glass, B., O. Temkin, and W. L. Strauss, Jr. (eds.). 1968. *Forerunners of Darwin: 1745–1859.* Johns Hopkins Press, Baltimore, MD.

Hubbard, R. and E. Wald. 1993. *Exploding the Genetic Myth: How Genetic Information is Produced and Manipulated by Scientists, Physicians, Employers, Insurance Companies, Educators, and Law Enforcers.* Beacon Press, Boston.

Kevles, D. J. 1985. *In the Name of Eugenics: Genetics and the Uses of Human Heredity.* A. A. Knopf, New York.

La Du, B. N. et al. 1958. The nature of the defect in tyrosine metabolism in alkaptonuria. *J Biol Chem* **230**:251–261.

Magner, L. N. 1992. *A History of Medicine.* Marcel Dekker, N.Y.

Mayr, E. 1982. *The Growth of Biological Thought: Diversity, Evolution and Inheritance.* Harvard University Press, Cambridge, MA.

McKeown, T. 1988. *The Origins of Human Disease.* Basil Blackwell Inc., Oxford.

Métraux, G. S. and F. Crouzet (eds.). 1963. *The Nineteenth Century World.* Mentor Books, Toronto, Canada.

Shannon, T. W. 1917. *Eugenics or the Laws of Sex Life and Heredity.* S. A. Mullikin, Marietta, OH.

review questions

1. What are the differences between infectious and noninfectious diseases? What are genetic diseases?
2. What is domestication? Without any knowledge of the principles of inheritance, how can plants and animals be domesticated?
3. Who were the first people to record an inherited defect within a family? Describe in detail the features of the pattern of inheritance they observed.
4. What are "inborn errors of metabolism"?
5. Outline and discuss critically the principal concerns of the supporters of the eugenics movement.
6. What is the rationale for studying genetic traits in twins?
7. Describe why a condition that appears to "run in a family" is not necessarily genetically determined.
8. What is sickle cell anemia? How did the study of the cause of this disease play a role in the understanding of the molecular basis of genetic diseases?
9. Discuss and describe the nature–nurture controversy.
10. Discuss the status of the study of human genetics in 1950.

chapter 2

The Genetic System: Chromosomes

Anyone who declines the labor of becoming familiar with the fundamental features of the genetic system and its method of operation cuts himself off from the possibility of understanding the nature of man and the origin of his peculiarities.

H. S. JENNINGS (1868–1947)

BIOLOGICAL CONNECTIVITY FROM ONE GENERATION of humans to the next is maintained by the fusion of a sperm from a male parent with an unfertilized egg from a female parent to produce a fertilized egg (zygote). After implantation, the zygote undergoes a series of cell divisions as the developmental program is implemented. After nine months, this results in the formation of an individual with many different cell types, tissues, and organs. This generative process is extremely complex and involves a vast array of biochemical, cellular, and tissue interactions.

The genetic system plays a decisive role in this multifaceted program and can be appreciated from three perspectives. First, it is important to understand how each cell and zygote receives a complete set of the carriers of the genetic information (chromosomes). Second, the conceptual framework for determining patterns of transmission of units of genetic information (genes) from one generation to the next, established by Mendel, forms the basis for studying inherited traits in humans and all other sexually reproducing organisms. Third, it is essential to know how the information encoded in the genetic material (DNA) can be decoded and how proteins are produced. In other words, the fundamental principles of human molecular genetics cannot be examined in any detail without some understanding of cytogenetics, Mendelian genetics, and the molecular biology of DNA.

Human Chromosomes

Maintaining the Chromosome Number

Under normal conditions there are 46 microscopic threadlike structures (23 pairs) called chromosomes in the nucleus of the fertilized human egg. After the first embryonic cell division, each nucleus of the two daughter cells has 23 pairs of chromosomes. And, after the 40th cell division, when there are about 1×10^{12} cells, each nucleus of each cell still has 23 pairs of chromosomes.

An Introduction to Human Molecular Genetics, Second Edition, by Jack J. Pasternak
ISBN 0-471-47426-6

Clearly an effective process, the cell division cycle (mitotic cycle) ensures that after each cell division, each daughter cell receives the same number of chromosomes.

However, this remarkably consistent pattern of chromosome distribution raises a provocative conundrum. Both sperm cells (spermatozoa) and unfertilized eggs (ova), which collectively are called gametes, are also the products of cell division. Accordingly, one might expect that a fertilized egg would have 92 chromosomes, that is, 46 chromosomes contributed by the spermatozoon and 46 by the ovum. Moreover, if this example held true, the number of chromosomes per nucleus would double with each successive generation. After 10 generations, each nucleus of each cell of an individual would have 47,104 chromosomes. Fortunately, a process called meiosis ensures that each gamete receives only 1 member of each pair of 23 chromosomes. Thus, after fertilization, a zygote will have 23 pairs of chromosomes.

The cell division cycle perpetuates cells with the same number of chromosomes, and meiosis creates gametes that have only one member of each pair of chromosomes. Cells that do not give rise to gametes are called somatic cells and normally have a diploid (2N) number of chromosomes. Gametes have a haploid (1N) chromosome number and arise from germ line cells.

Cell Division Cycle: The Mitotic Process

The interval that extends from a newly divided cell to the production of two cells is the cell division cycle and is divided into four successive phases, called gap 1 (G_1), synthesis phase (S), gap 2 (G_2), and mitosis (M) (Figure 2.1). During the G_1 phase, the chromosomes within the nucleus are highly elongated and, consequently, are not readily seen through a light microscope as discrete units after cytochemical staining. A G_1 chromosome is a single, complete double-stranded DNA molecule with associated proteins that are mostly histones. A number of different cellular proteins are synthesized during G_1, and

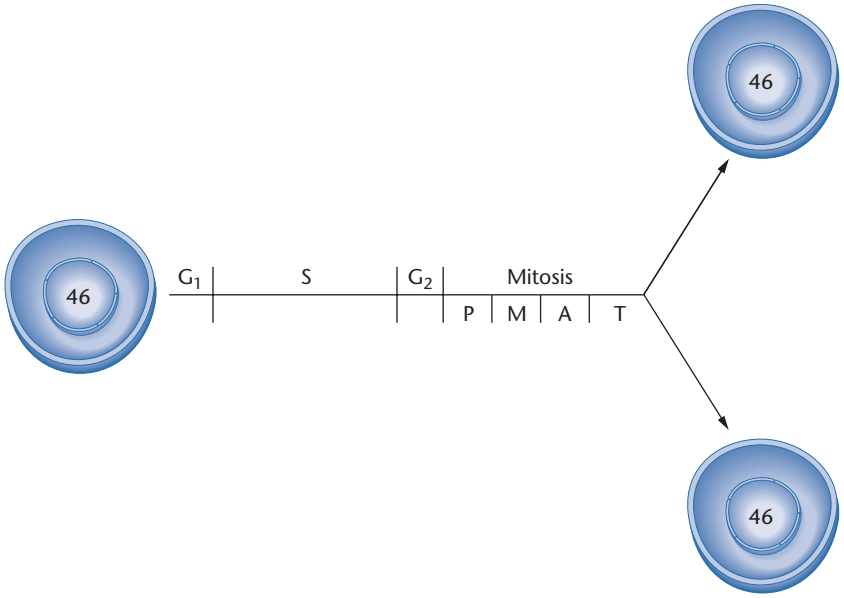


Figure 2.1 Cell division cycle. A dividing cell with 46 chromosomes passes through the G_1, DNA synthesis (S), and G_2 phases before entering mitosis, which consists, in succession, of prophase (P), metaphase (M), anaphase (A), and telophase (T). The end result of the cell division cycle is the formation of two daughter cells, each with 46 chromosomes.

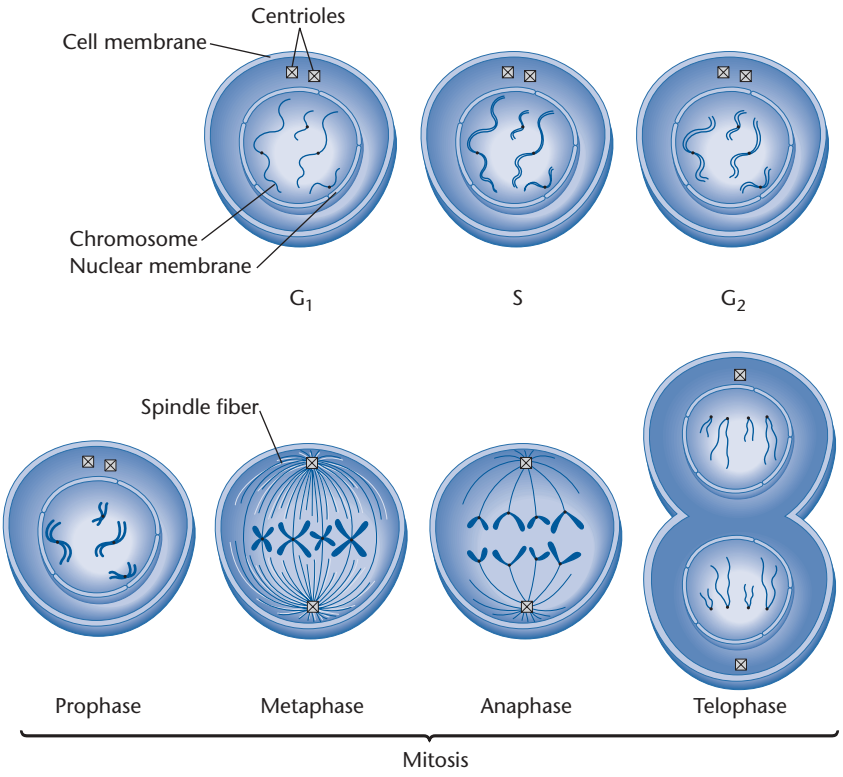


Figure 2.2 Essential features of the phases of the cell division cycle of a cell with two pairs of chromosomes.

many of these expedite entry into the S phase. Every chromosome is precisely and completely doubled (duplicated) during the S phase. At the end of the S phase, each chromosome is a joined duplicated structure consisting of two sister chromatids (Figure 2.2). During the G_2 phase, components are produced that facilitate the distribution of one chromatid of each duplicated chromosome to a daughter cell. The mitotic phase (mitosis) is a continuous series of events divided into substages: prophase, metaphase, anaphase, and telophase (Figure 2.2). A successful mitosis depends on the coordination of chromosome movement, the function of the subcellular organelles called centrioles, the activity of microtubules, the disassembly and reassembly of the nuclear membrane, and the cleavage of the original cell into two cells (cytokinesis).

The onset of prophase is marked by the condensation of the chromosomes. Outside the nuclear membrane, the two centrioles begin to separate from one another. By the end of prophase, the centrioles occupy a position 180° from each other at opposite poles (spindle poles), the nuclear membrane has disassembled, and the chromosomes are highly condensed. During early metaphase, chromosome condensation is nearly complete. Viewed with a light microscope after cytochemical staining, metaphase chromosomes often have a characteristic X-shaped structure (Figure 2.3). The joining point of a metaphase chromosome is the centromere (primary constriction). The centromere is a constant zone of each chromosome and is present at the same site in all mitotic cycles. In some chromosomes, the centromere is closer to one end and not necessarily in the middle. Specialized proteins bind to the outer faces of the two centromeres of each duplicated chromosome to form laminar structures called kinetochores.

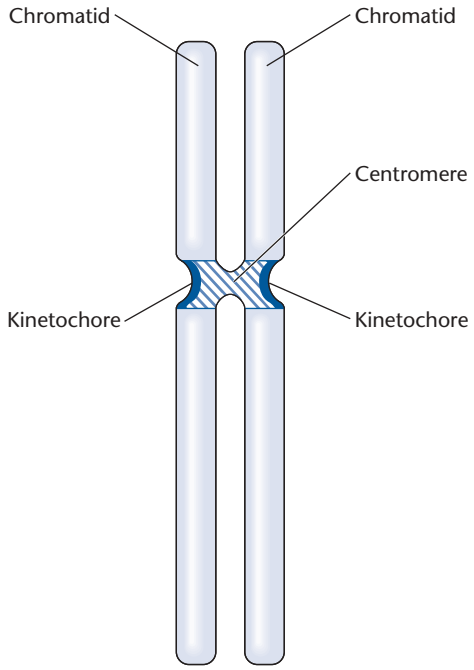


Figure 2.3 Metaphase chromosome. The centromere (primary constriction) is marked by the downward diagonal pattern. The kinetochore is the region of attachment of spindle fibers that forms on the outward portion of the centromere. At metaphase the chromosome is duplicated, and each complete chromosome is a chromatid.

The centrioles act as centers (spindle poles, centrosomes) for the assembly of thin, cylindrical protein tubes (microtubules). The microtubules, together with associated proteins, form spindle fibers that radiate out in all directions from each spindle pole. Some spindle fibers make contact with and bind to the kinetochores. After attaching to the kinetochores, the microtubules, by means of molecular motors, orient and position each duplicated chromosome so that, by the end of metaphase, all the chromosomes are aligned equidistant between the spindle poles (Figure 2.2). This midpoint is called the spindle equator (metaphase plate).

Anaphase is initiated with the separation (disjunction) of the chromosomes at the centromeres. Then, the single chromatids of each duplicated chromosome move to opposite spindle poles (Figure 2.2). As a complete set of single chromatids approaches a spindle pole, they become less compacted (decondense). During telophase, a nuclear membrane reassembles around each complete chromosome set, and the cell membrane grows (invaginates) between the two nuclei, cleaving the original cell into two daughter cells (Figure 2.2). Later, in the G_1 and S phases of the next cycle, the single centriole outside each nucleus directs the production of a second centriole. Overall, the cell division cycle produces two cells, each with a full diploid complement of chromosomes.

The Meiotic Process

The meiotic process is confined to the gonads (testes and ovaries) of sexually mature individuals and produces gametes that contain one member of each pair of chromosomes of the chromosomal complement. Two successive division cycles called meiosis I and meiosis II are required (Figure 2.4). During the first meiotic cycle, the G_1, S, and G_2 phases resemble those of a typical mitotic process. Even after G_2, the roles of the centrioles, microtubule assembly, and

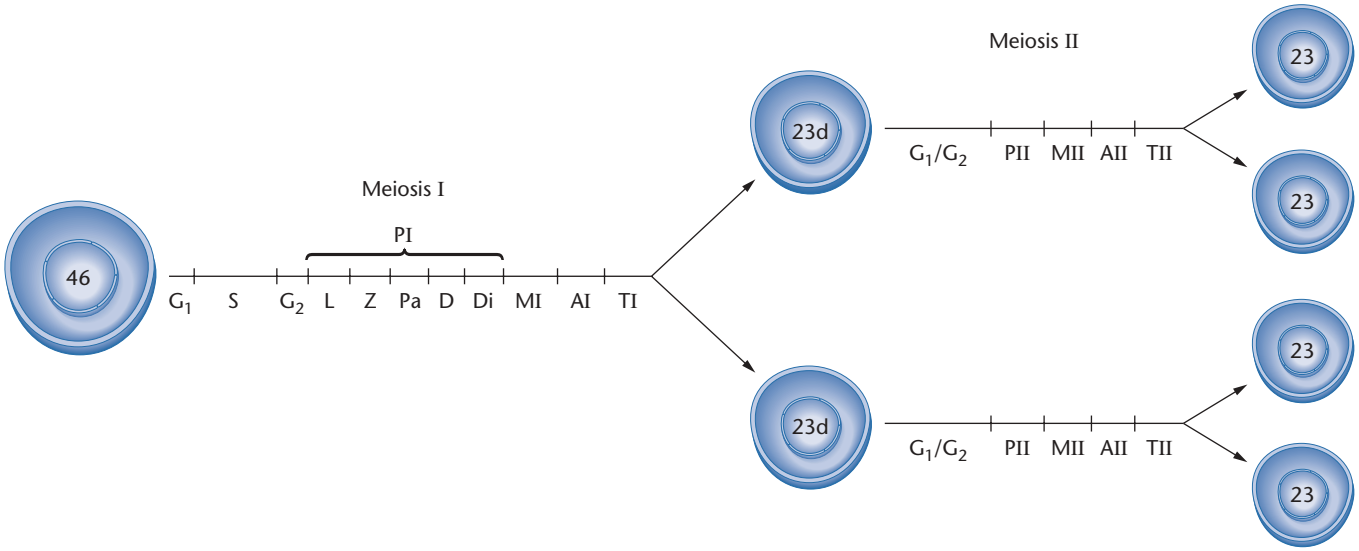


Figure 2.4 Meiotic process. Meiosis I consists of G_1, DNA synthesis (S), and G_2 phases; prophase I (PI), which includes leptotene (L), zygotene (Z), pachytene (Pa), diplotene (D), and diakinesis (Di); metaphase I (MI), anaphase I (AI), and telophase I (TI). The products of meiosis I carry 23 duplicated chromosomes (23d) from cells that entered meiosis I with 46 chromosomes. Meiosis II consists of a combined G_1/G_2 phase followed by prophase II (PII), metaphase II (MII), and telophase II (TII). The final products of the meiotic process are cells that contain 23 chromosomes that comprise one of each of the original set of 23 pairs of chromosomes. In human females, each meiotic process produces one functional gamete and three nonfunctional cells called polar bodies. In human males, all meiotic products are functional.

nuclear membrane disassembly are the same as during a mitotic cell division cycle. However, the behavior of the chromosomes of the prophase of meiosis I (prophase I) is not at all similar to that in the mitotic prophase.

Prophase I is divided into five substages called leptotene (leptonema), zygotene (zygonema), pachytene (pachynema), diplotene (diplonema), and diakinesis. During leptotene, the doubled chromosomes begin to condense. Zygotene is marked by lengthwise joining between members of each pair of chromosomes (homologous chromosomes) throughout their entire lengths (Figure 2.5). This pairwise coming together of doubled chromatids is called synapsis. Synapsis creates chromosomes that have four chromatids, two from each duplicated chromosome. Synapsed duplicated chromosomes traditionally are referred to as bivalents.

At this stage in humans, there are 23 bivalents. During pachytene, the bivalents continue to condense, and a reciprocal exchange of chromosomal material (crossing over) can occur between two nonsister chromatids of the bivalents (Figure 2.5). Crossing over leads to new combinations of genes along a chromosome. The onset of diplotene is marked by a recession of synapsis, with the pairing remaining at the few locations where crossing over has occurred (chiasma, sing., and chiasmata, pl.). The diplotene bivalents continue to condense, which leads into diakinesis. During diakinesis, the chiasmata recede from the centromeres of the bivalents and are released at the ends (telomeres) of condensed chromosomes. The centrioles become positioned at opposite poles, and the nuclear membrane disassembles.

The metaphase of meiosis I (metaphase I) is the same, in principle, as the metaphase of a cell division cycle. However, as a result of synapsis, bivalents are aligned at the spindle equator (Figure 2.5). During anaphase I, duplicated chromosomes (dyads) of each bivalent move to opposite poles. Telophase I entails the reassembly of a nuclear membrane around each group of chromosomes at each spindle pole, and cytokinesis creates two daughter cells (Figure 2.5). In humans, the nuclei of cells produced by meiosis I contain 23 duplicated chromosomes, one duplicated chromosome for each chromosome pair.

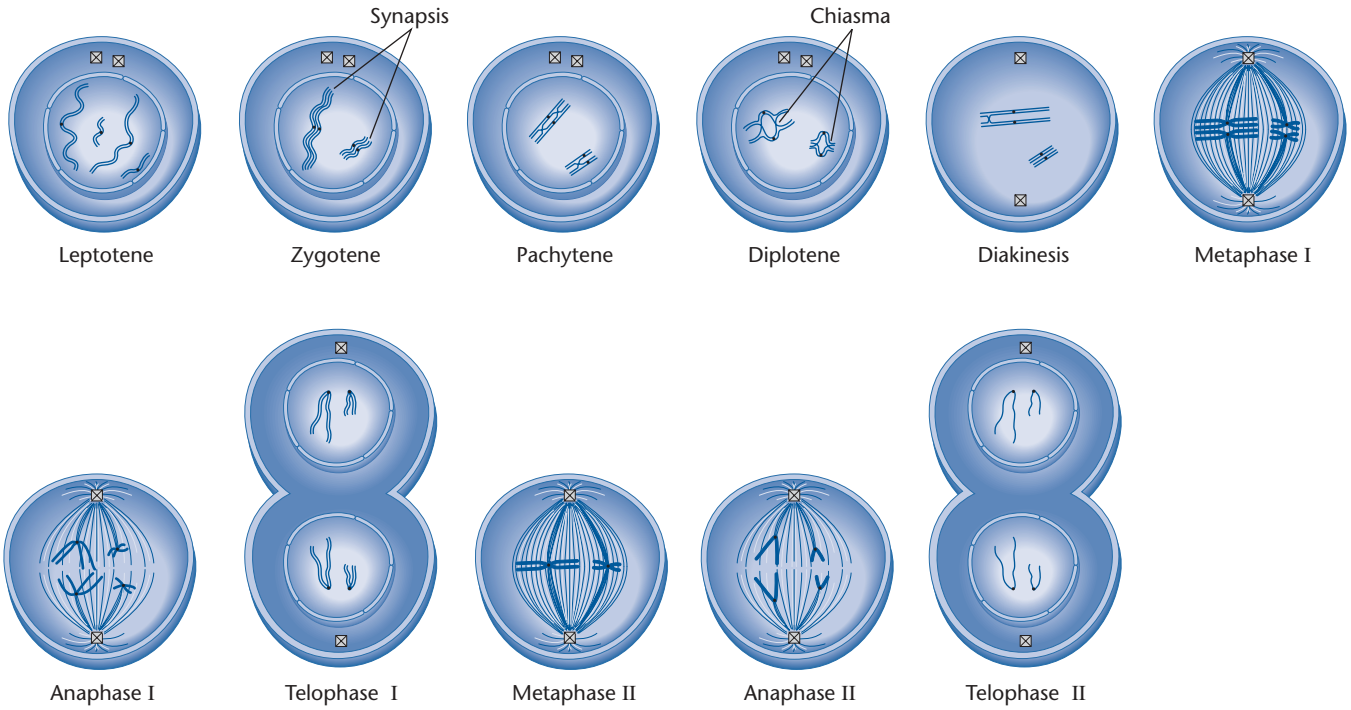


Figure 2.5 Essential features of chromosome distribution during the phases of the meiotic process of a cell with two pairs of chromosomes.

Meiosis II has no S phase but, in all other aspects, resembles closely the phases of a mitotic cell division cycle. At metaphase II, the condensed, duplicated chromosomes are aligned at the spindle equator (Figure 2.5). During anaphase II, the duplicated chromosomes separate at the centromere attachment site, and a single chromatid of each chromosome moves to a spindle pole. After telophase II and cytokinesis, cells are produced that, in humans, have 23 elongated, single chromosomes representing one from each pair of chromosomes.

The alignment and orientation of chromosomes at the spindle equator during both metaphase I and II are random. The meiotic process, then, ensures that chromosome constancy is maintained from one generation to the next and that various combinations of maternal and paternal chromosomes are present in the gametes. In addition, crossing over creates new linear combinations of genes on a chromosome. Traditionally, meiosis I is called reductional segregation and meiosis II is called equatorial segregation.

Characterizing Human Chromosomes

A comparison of the lengths and overall morphology of the mitotic metaphase chromosomes of human males and females shows that 22 pairs are held in common. These chromosomes, not all the same length, are called autosomes. The 23rd pair includes the sex chromosomes. In females, both sex chromosomes have the same length and are designated as X chromosomes. In males, the sex chromosomes consist of one X chromosome and a smaller chromosome designated as Y.

Generally, human chromosome studies are carried out with cells grown in tissue culture. These cells are blocked during metaphase by the addition of a

chemical that prevents spindle fiber formation. After fixation, staining, and preparation of blocked metaphase cells, entire chromosome sets are viewed through a light microscope. The site of the centromere (primary constriction) is a consistent feature of a metaphase chromosome, dividing each chromosome into two components called arms. Rarely is the centromere precisely in the middle. In most chromosomes, this results in readily identifiable short and long arms. The shorter chromosome arm is designated as the p (petite) arm and the longer arm as q, because, for lack of a better reason, q is the letter in the alphabet that follows p. When the centromere is near the middle of a chromosome, the chromosome is called metacentric. When it is positioned some distance from a chromosomal end, but not at or near the center, the chromosome is submetacentric. An acrocentric chromosome has its centromere located near a chromosome end.

The ratio of the arm lengths (p/q), the centromere index $[p/(p + q) \times 100]$, and the length of each chromosome relative to the total length of a haploid set were used initially to classify different chromosomes. By convention, the longest autosomal chromosome is designated as chromosome 1, the next longest as chromosome 2, and so on. When, with a photograph, the chromosomes from a particular metaphase spread are cut out and arranged into aligned pairs of the same length, the result is called a karyotype. In some cases, the originally assigned chromosome numbers do not reflect the actual chromosome lengths. For example, it is now known that chromosome 22 is slightly longer than chromosome 21. However, the original numbers are so entrenched that cytogeneticists have retained them. Some of the chromosomes have distinguishing features other than variations in the location of centromeres. For example, distinctive secondary constrictions are present during metaphase on five chromosomes, including chromosomes 13, 14, 15, 21, and 22. These regions do not readily bind standard chromosome stains.

Relative chromosome length and other features are not entirely satisfactory in identifying each specific chromosome. Variations in length in chromosomes can occur with different treatments and staining procedures. In addition, certain groups of chromosomes, for example, chromosomes 8, 9, 10, and 11, have very similar lengths and centromere positions. With a homogeneous (solid) stain, it is almost impossible to recognize with consistency a specific member of such a similar group. This problem was resolved when various cytochemical procedures were introduced to give each chromosome a distinctive staining pattern of light and dark transverse bands (Figure 2.6). A band is defined formally as "that part of a chromosome which is clearly distinguishable from its adjacent segments by appearing darker or lighter by one or more banding techniques." For example, when either a Giesma staining procedure or a quinacrine stain is used, different sets of bands called G-bands and Q-bands, respectively, are observed. A band can be dark, pale, diffuse, thick, or thin. But, with any one technique, the characteristics of a band are usually the same.

The physical basis of the banding patterns is not well understood. The bands may represent—among other possibilities—differences of DNA sequences (nucleotide composition) along a chromosome, the relative amount of condensed DNA in different segments of a chromosome, localized structural fea-

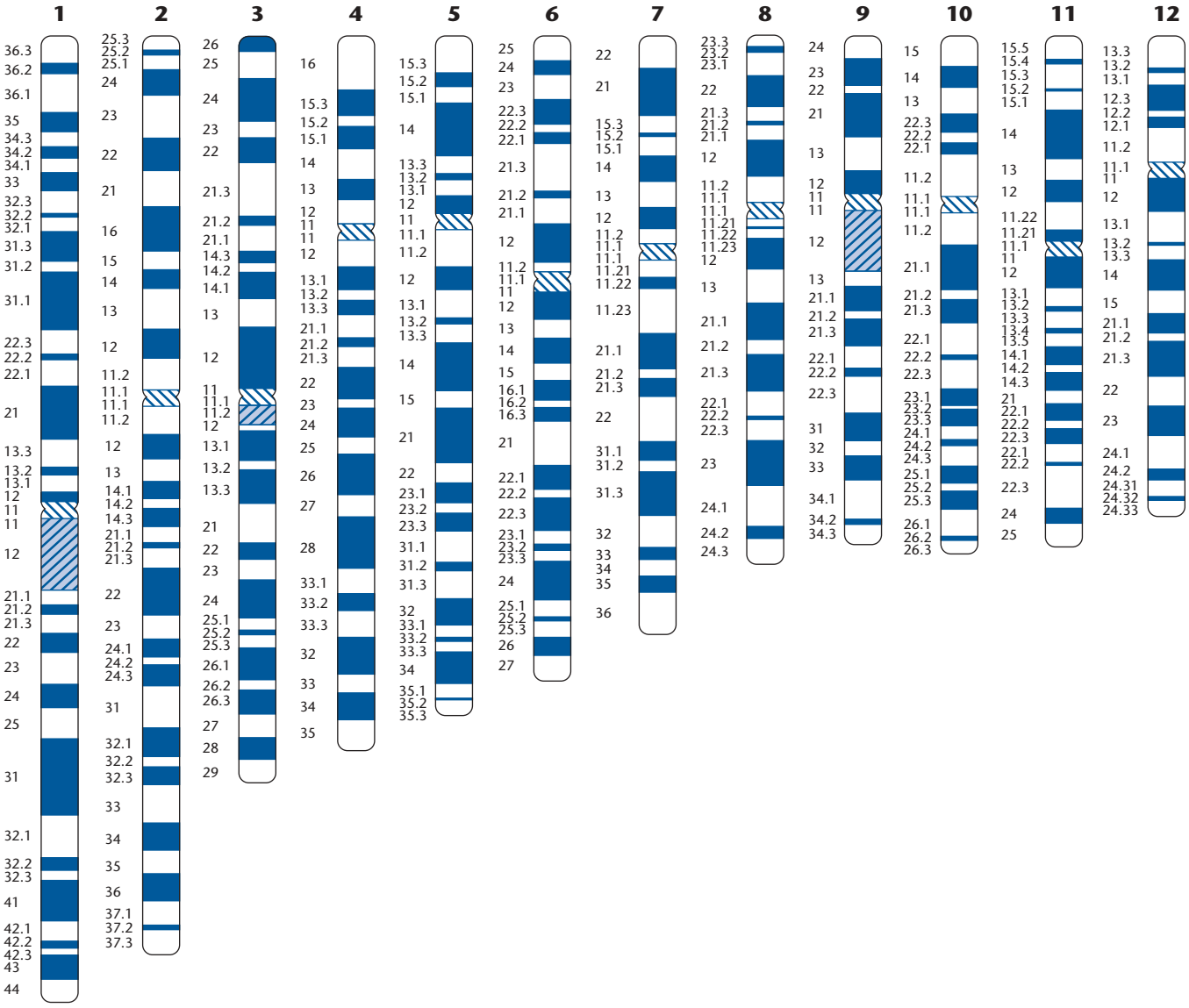
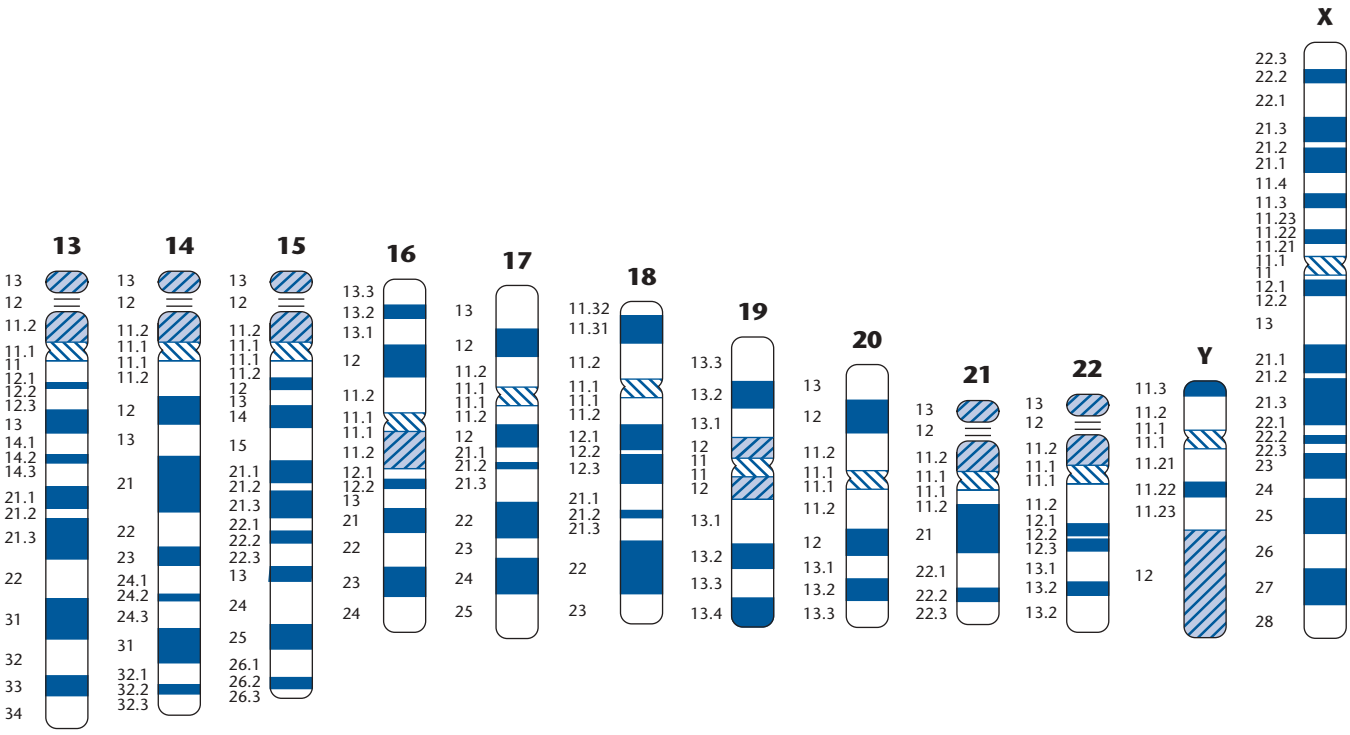


Figure 2.6 Ideograms with band designations of all human chromosomes. The level of resolution is about 500 total bands. Metaphase chromosomes are represented diagrammatically. The downward diagonal pattern marks the centromere of each chromosome; the upward diagonal pattern marks variable staining regions. The two parallel horizontal lines in chromosomes 13, 14, 15, 21, and 22 indicate secondary constrictions.

tures such as DNA loop formation within sectors of a chromosome, or the presence of specific proteins that bind to designated sections of a chromosome.

Regardless of the physical basis of chromosomal band formation, the patterns in each arm allow each chromosome to be identified and regions within each arm to be recognized routinely (Figure 2.6). As the procedures for generating band patterns were refined and new methods were devised, a nomenclature for standardizing the identification of chromosome regions and specific bands was developed.

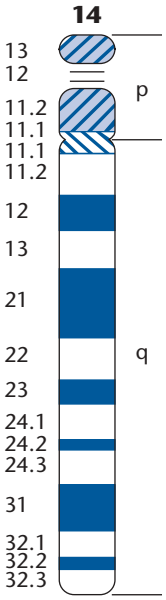
The band numbering system was initially created when about 400 bands for an entire haploid human chromosome set were known. To identify specific chromosome segments, the chromosome arms, if possible, were divided into regions. Consistent and distinct morphological features (landmarks) were used to designate each chromosome region. A region is defined as a segment of a chromosome arm lying between the midpoints of two landmarks. Regions are



denoted with arabic numerals starting from the centromere and proceeding to the end (terminus or ter) of each chromosome arm. For example, chromosome 1, the longest in the human chromosome set, has three regions on its short (p) arm and four on the long (q) arm. However, for many chromosomes, the entire short arm and, in some instances, the long arm were designated as single regions. With the 400-band staining procedures, a region rarely had more than five bands. Bands within each region were numbered in succession from the centromere to the end of each arm. Accordingly, the designation 14q21, when translated in reverse order, would represent the first band (---1) in the second region (--2-) of the long arm (-q--) of chromosome 14 (14---) (Figure 2.7).

As the number of resolvable bands that could be stained reliably increased from 400 to 550 and then to approximately 850, a number of the original bands were found to be made up of more than one band. Thus, some of the original bands were divided into subbands, and additional bands were resolved within some of these subbands. The nomenclature was expanded by retaining the rule of sequential numbering from the centromere to the terminus of a chromosome arm for new subbands of an original band and by adding a decimal point to distinguish an original band of the 400-band data set from subbands revealed by higher resolution techniques. Similarly, when subbands showed additional bands, they were numbered sequentially from the centromere to the terminus of a chromosome arm, although no additional decimal point was used. For example, if band 2 of region 1 (that is, band 12) of a hypothetical chromosome (Figure 2.8A) subdivides into three bands with an enhanced staining procedure, then the designation becomes 12.1, 12.2, and 12.3, where the numbers preceding the decimal point represent the region (1) and the original band number (2), with the subband numbers following the decimal point (Figure

Figure 2.7 Ideogram of human chromosome 14. The level of resolution is at 500 bands.



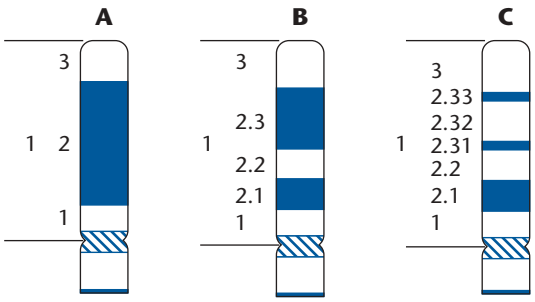


Figure 2.8 The International System for Human Cytogenetic Nomenclature (ISCN) designation of additional chromosomal bands. (A) At low band resolution, the chromosomal arm of a hypothetical chromosome has one region (1) and three bands (11, 12, and 13). (B) At a higher band resolution than in A, band 12 consists of three subbands, designated 12.1, 12.2, and 12.3. The new band numbers ascend from the centromere to the terminus of the chromosomal arm. (C) At a higher band resolution than in B, band 12.3 consists of three additional bands, numbered 12.31, 12.32, and 12.33.

2.8B). And, if subband 3 from band 12.3 subdivides into three additional bands with higher-resolution staining, then the designation becomes 12.31, 12.32, and 12.33 (Figure 2.8C).

This nomenclature can be expanded as more bands within bands are discovered, while retaining designations used previously for lower levels of band resolution. With this system, specific chromosome locations can be depicted readily on diagrammatic representations (ideograms, idiograms) of banded chromosomes (Figure 2.6). An ideogram represents most bands as either black or white transverse components. Variations in staining intensities and band widths are ignored. The centromere region and consistently variable-staining chromosomal regions are often noted by hatched lines (Figure 2.7). By convention, the upper chromosome arm in an ideogram is always the p arm.

Chromosome Abnormalities

Whole Chromosome Changes: Aneuploidy

The mitotic and meiotic processes are not error free. Although the distribution of chromosomes is remarkably efficient, occasionally whole chromosomes may be lost or gained because of defects in spindle function. A change in the chromosome number (aneuploidy) of a dividing somatic cell can be perpetuated in some instances, although often such cells lose the capacity to divide. Some aneuploid somatic cells, in combination with other cellular and chromosomal changes, develop deranged cell division cycles that lead to various cancers.

On the other hand, the failure of chromosomes to segregate properly during meiosis I or meiosis II can result in gametes with an extra chromosome or the loss of a whole single chromosome. The occurrence of an extra autosome (trisomy), in most cases, prevents any significant development of the embryo and leads to its loss in utero. There are very few known conditions in which a whole single autosome of a pair (monosomy) can be missing and still result in a live birth.

The presence of an extra chromosome 21 (21/21/21 or trisomy 21) in a zygote can lead, in some instances, to the completion of development and birth. A female with an extra chromosome 21 is designated with the code 47,XX,+21. In a male the symbolization is 47,XY,+21. Unfortunately, an array of symptoms, including some degree of mental retardation, slowed growth, shortening of the bones, and a characteristic broad, flat face with a small nose and distinctive eyefolds accompanies trisomy 21. This condition has been designated medically as Down syndrome (DS). The frequency of DS is approximately 1

out of 800 live births and increases with the age of the mother, so that by maternal age of 40 years, there is a 1% chance that a child will have an extra chromosome 21. Life expectancy for those with DS has improved over the past few decades, and today many DS individuals live to be 40 or older.

Other human autosomal trisomies that result in live births are uncommon. Trisomy 18 (Edwards syndrome) occurs approximately once in 8000 live births. Approximately 5% of zygotes with trisomy 18 develop to term. At birth, infants with trisomy 18 have severe abnormalities, including mental retardation and malformation of the heart. Most trisomy 18 patients fail to thrive, although some have lived to be 20 years old. The incidence of trisomy 13 (Patau syndrome) is 1 out of 25,000 births. This condition causes a range of severe defects, with most trisomy 13 infants dying within a month of birth. Few survive beyond 12 months. Central nervous system defects and cleft palates are consistent features of Patau syndrome. Trisomy 9 is quite rare, with most children dying within their first year from multisystem abnormalities affecting the eyes, nose, limbs, and various organ systems.

The presence of an extra sex chromosome has less biological impact than an autosomal trisomy. The XXY constitution (47,XXY; Klinefelter syndrome) occurs in 1 out of 600 males. These men are uniformly incapable of producing sperm (infertile), but, in other respects, the condition is quite variable. Men with Klinefelter syndrome tend to be tall, with long arms and large hands and feet. Occasionally, they have decreased mental capabilities but are sexually competent. Trisomy X (47,XXX) occurs in 1 out of 1000 females. There is no consistent set of features accompanying trisomy X, although many trisomy X women experience major learning problems. An extra Y chromosome (47,XYY) occurs in 1 out of 1000 males. No significant biological disabilities are associated with XYY males. They are fertile, with normal intelligence and a tendency to be tall.

The only condition with just one sex chromosome, the X chromosome, is Turner syndrome (45,X), which occurs in 1 out of 10,000 females. Women with Turner syndrome are short and infertile, have distinctive physical features, including a thick neck, and, in some cases, suffer from kidney and cardiovascular abnormalities. Intelligence is not impaired, although spatial perception and delicate motor skills may be affected. A single Y chromosome constitution (45,Y) has never been observed in a live birth. Multiple sex chromosomes, such as XXYY, XXXY, and other combinations of X and Y chromosomes occur very rarely. The affected individuals usually have more pronounced mental retardation and infertility than those with single sex chromosome abnormalities. The relatively mild consequences of one additional X chromosome is due to a regulatory system that inactivates most of the gene expression of extra X chromosomes. This mechanism limits the overproduction of gene products. With three or more X chromosomes, however, some gene products are in excess and the biological impact is more severe. In this context, there is no compensatory mechanism for extra autosomes; consequently, the genes of all three chromosomes of an autosomal trisomy are active. (The molecular features of X chromosome inactivation are discussed in a later chapter.)

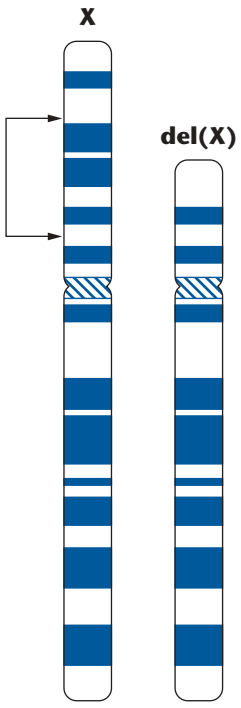
Chromosome trisomies and other chromosome abnormalities do not always occur in all the cells of an individual. In other words, cell populations of various organs are mixtures. Some cells have normal chromosome complements,

whereas others carry chromosome abnormalities. The occurrence of these kinds of mixtures is called mosaicism. Although the distribution and frequency of cells with chromosome abnormalities is important biologically, mosaicism generally leads to longer survival times and less severe symptoms in comparison to individuals with a chromosome aberration in all of their cells. Mosaicism may result from a mitotic error that creates a cell with a normal (disomic) chromosome complement from a chromosomally abnormal zygote early in development. A mitotic chromosome error (nondisjunction) also can occur early in the development of a chromosomally normal zygote that produces a trisomic cell. If both disomic and trisomic cells are present early, then multisystem mosaicism results. On the other hand, if trisomy occurs late in development, most of the organ systems will contain disomic cells, and the overall impact of the trisomy will probably be negligible.

Chromosome Structural Changes

Chromosome structural changes occur when the DNA of a chromosome breaks and is rejoined to another broken piece of chromosome DNA, forming an unusual rearrangement. Most of these alterations occur during S phase and involve faulty repair of DNA molecules or mistakes during DNA replication. Environmental agents, such as X rays or chemicals, also induce chromosome breaks. Chromosome structural alterations can affect a single chromosome and lead to a loss of chromosomal material from the end of an arm (terminal deletion), a loss of material within a chromosomal arm (interstitial deletion, deletion) (Figure 2.9), the 180° flipping of an internal portion of a chromosome (inversion) (Figure 2.10) or the addition of chromosomal material into a chromosomal arm (insertion). When these kinds of changes occur, and if they are large enough, chromosome banding patterns can be used to define the limits and nature of the alteration. A nomenclature (International System for Human Cytogenetic Nomenclature, ISCN) provides a shorthand description of chromosome abnormalities that result from structural alterations. For example, 46,XY,del(5)(p13) indicates that in a male (XY) with 46 chromosomes, by count, the short arm (p) of chromosome 5 has been deleted (del) from its end to band p13.

Figure 2.9 Chromosome deletion. The arrowheads of joined arrows mark two sites of chromosome breakage and the material lost from an intact X chromosome (X). The chromosome with the deletion is designated del(X). The ISCN short description for this deletion is del(X)(p11.23p22.1).



Any terminal deletion of 5p that includes band 5p15 is associated with the cri-du-chat ("cry of the cat") syndrome. Infants with this condition make sounds similar to a cat's mewing and have abnormally small heads (microcephaly), a very wide space between the eyes (ocular hypertelorism), small lower jaws (micrognathia), extreme mental retardation, and other physical abnormalities. The loss of most of 4p also causes severe mental retardation and, as with the loss of part of 5p, a specific spectrum of physical abnormalities. Other than losses of the short arms of chromosomes 4 and 5, live births with a large terminal deletion of any autosome or the X chromosome are exceptionally rare.

The inversion inv(9)(p11q12), which entails the 180° flipping of the chromosome segment between p11 and q12 of chromosome 9, occurs in approximately 1 out of 100 people and has no associated physical abnormalities. Inversions change the order of the sequence of information along a chromosome. If breakpoints occur in noncritical regions, then all the genetic information and all normal gene functions are retained after the inversion is established.

On the other hand, both large terminal and interstitial deletions can lead to a loss or disruption of genetic information, which usually causes multiple physical and physiological abnormalities. In addition, without a centromere, a chromosome or fragment of a chromosome is doomed, because it cannot participate in metaphase and anaphase movements. Consequently, "centromere-less" (acentric) chromosomal material is not included in the nuclei of daughter cells.

Structural changes can occur between different (nonhomologous) chromosomes. In some instances, parts of two nonhomologous chromosomes are interchanged without any apparent loss of chromosomal material. The occurrence of such a mutual exchange is called a reciprocal translocation (balanced translocation). A translocation is abbreviated as t in the ISCN system. Accordingly, t(7;19)(q22;q13.1) denotes a translocation between chromosome 7 at band q22 and chromosome 19 at band q13.1. In this case, the chromosome segment from 7q22 to the end of the 7q (7qter) was translocated to a break in chromosome 19 at band q13.1. At the same time, the chromosome segment from 19q13.1 to 19qter was joined to chromosome 7 at q22 (Figure 2.11). Overall, a reciprocal translocation of some kind occurs about once in 800 newborns. Most translocations are family-specific, that is, private, although t(11;22)(q23;q11.2) has been observed in more than 100 unrelated families. Because all chromosome material is retained, despite some material in alien locations, reciprocal translocations, in most instances, have no significant impact on physical and mental capabilities. However, there are some examples of disease-specific chromosome rearrangements. Many of these are found in somatic cells that have become cancerous. For example, the chromosomal rearrangement

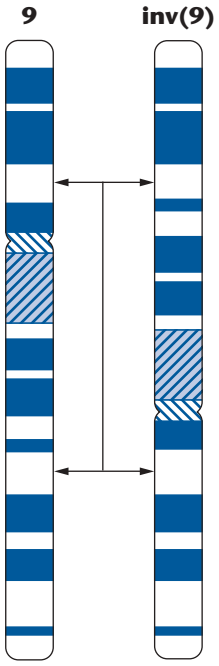


Figure 2.10 Chromosome inversion. The double-headed arrows mark the break points on a normal chromosome (9) and the rejoined points of the inverted chromosome [inv(9)]. The vertical line shows the extent of the inversion. An inversion that includes the centromere is a pericentric inversion. Although not shown here, an inversion within a chromosomal arm is a paracentric inversion. The ISCN short description for this inversion is inv(9)(p13q22.3).

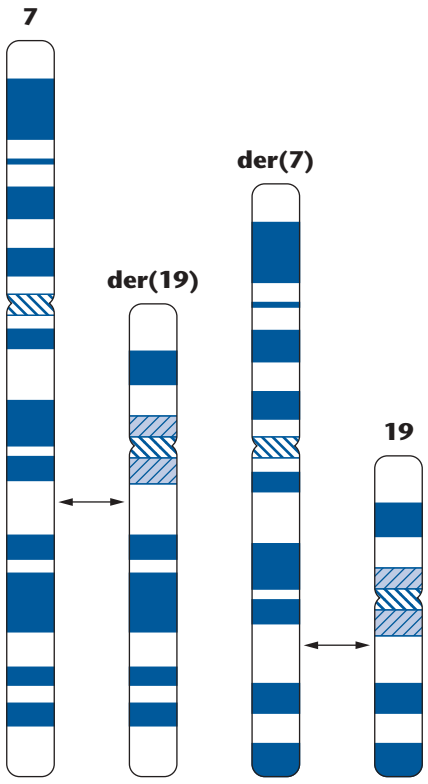


Figure 2.11 Balanced reciprocal translocation between chromosomes 7 and 19. The double arrows mark the break points on each normal chromosome (7, 19) and the rejoining points to produce the translocated chromosomes der(7) and der(19). The term "der" represents derivative chromosome, which, according to ISCN is "one of the structurally rearranged chromosomes generated by a rearrangement involving two or more chromosomes." The ISCN short description for this translocation is t(7;19)(q22;q13.1).

from the HUMAN GENETICS files

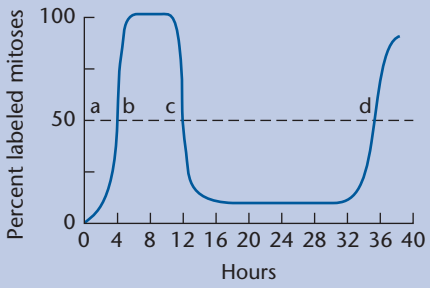
Determining the Phases of the Cell Cycle

Historically, the period from one cell division to the next was divided into two phases—interphase and mitosis—based on the presence of stained chromosomes under the microscope. Interphase was the name given to the interval when the chromosomes were not evident. It was erroneously assumed that interphase was a resting phase. Mitosis, which featured the "dance of the chromosomes" (as some poetic cytogeneticists have described it), included the part of the cell division cycle in which the chromosomes were visible and distributed equally to newly formed daughter nuclei, followed immediately by cell division. Quantitative analysis of the nuclear DNA content of dividing cells showed that the amount of DNA doubled precisely during interphase. This result was not unexpected because DNA is found, almost exclusively, in chromosomes. The question of interest, which could not be resolved readily by quantitative DNA measurements, was whether DNA synthesis, that is, chromosome duplication, was continuous or whether it occurred within a discrete interval during interphase.

This issue was settled with "pulse-chase" experiments. Radiolabeled thymidine (thymidine labeled with tritium, tritiated thymidine, ^{3}H-thymidine) is added to growing cells in culture. The purpose of this step is to mark (label) newly synthesized DNA with ^{3}H-thymidine. Thymidine is a component of DNA and is incorporated into DNA when it is synthesized. After 10 min in the presence of ^{3}H-thymidine (pulse), the cells are thoroughly washed and placed into a culture medium with unlabeled thymidine (chase). In this way, only the DNA that was synthesized during the labeling period will incorporate ^{3}H-thymidine. Thereafter, samples of cells are withdrawn every hour for approximately 40 h. The cell samples are placed on microscope slides and air dried, which spreads out metaphase chromosomes. The chromosomes are stained, and, in the dark, the sample is covered with a thin layer of liquefied photographic emulsion. After the photographic emulsion dries, the slides are stored in the dark at 5°C for about 7 days, when they are developed to reveal silver grains produced by radioactive emissions from radiolabeled chromosomes. The number of labeled mitotic chromosomes (mitotic cells) per sample are viewed with a microscope and scored. The data are plotted as percent labeled mitoses against time in hours (Figure A). Overall, after a lag, the proportion of labeled mitotic chromosomes forms a flat-topped, steep-sided peak, followed by a low level of labeled mitoses for a number of hours, before a second increase later in the experiment.

How are these results interpreted? First, DNA synthesis occurs during a discrete time period within interphase; otherwise, the same proportion of labeled mitoses would be observed throughout the experiment. The period of DNA synthesis is called the S phase. Second, the duration of the S phase is the time spanned between the 50% points of the first peak, that is, reading from the *x*-axis the number of hours from b to c in Figure A. Ideally, the 100% level should be used to

Figure A Percent labeled mitotic chromosomes at various times (hours) after a 10-min pulse with ^{3}H-thymidine to cells in culture followed by a chase in unlabeled thymidine. The durations of the G_2 phase, S phase, and total cell cycle are approximately 4 h (a to b), 8 h (b to c), and 24 h (c to d), respectively.



t(9,22)(q34;q11) is present in about 90% to 95% of cases of chronic myeloid leukemia (CML), a cancer that causes an overabundance of certain types of white blood cells called granular leukocytes.

Regardless of whether there is a disease-specific relationship, balanced reciprocal translocations have important genetic consequences. During the meiotic process, when a reciprocal translocation is present, synapsis will join the two translocated chromosomes with the two normal chromosomes to form a unique structure called a quadrivalent, consisting of four duplicated chromosomes (Figure 2.12). During segregation at meiosis I, different combinations of chromosomes of a quadrivalent will go to the spindle poles. After meiosis II, some gametes will have a normal complement of genetic information, whereas others, after fertilization, will produce zygotes with one or three copies of the same chromosome segments instead of the required two. For example, the cells entering the meiotic process of a person with t(7:19)(q22;q13.1) would have intact, normal versions of chromosomes 7 (7) and 19 (19) as well as the translocated chromosomes 19 [der (19)] and 7 [der (7)]. The designation "der" denotes

determine the duration of the S phase and the other phases, but, in practice, calculations are based on the 50% level because of experimental and biological variability inherent in the procedure. For example, it is impossible to control the pulse and chase regimens perfectly; therefore, cells are labeled for different times, which causes the observed results to deviate from theoretical expectations. Generally, under normal conditions, there is a narrowing of the top of the first peak, and the time span at the 100% level is an underestimate of the duration of the S phase. Third, the initial lag before the appearance of labeled mitoses is equivalent to the time period from the end of the S phase to the formation of metaphase chromosomes, that is, a to b in Figure A. If labeled prophase chromosomes are scored, then the value of a to b approximates the duration of the G_2 phase. The rationale for the experiment is that the labeled chromosomes that first appear after the chase contain the DNA that incorporated ^{3}H-thymidine during the last 10 minutes of the S phase. The labeled mitoses on the downward part of the first peak (c) represent chromosomes labeled at the start of the S phase. Fourth, the duration of the cell cycle, which includes each phase, is the time span from c to d in Figure A. Fifth, the period that precedes the initiation of the S phase, that is, the G_1 phase, is equal to the duration of the total cell cycle minus the sum of the durations of the G_2, S, and M phases. The duration of the M phase, which is usually 1 h, is equivalent to the proportion of cells in the overall cell population that are in mitosis multiplied by the duration of the total cell cycle.

In sum, this type of pulse-chase experiment established that interphase has three successive phases and that DNA replication, which is synonymous with chromosome doubling, occurs during a discrete interval within interphase. Usually, the durations of a cell cycle are represented pictorially as a circle with the G_0 phase, the true quiescent state, entering and leaving the G_1 phase (Figure B). The generation time of a cell cycle often differs from one cell type to another because of variations in the duration of the G_1 phase, whereas the time spans of the S, G_2, and M phases tend to remain the same. The generation time of mammalian cells in culture is approximately 24 h, with the G_1, S, G_2, and M phases being about 11 h, 8 h, 4 h, and 1 h, respectively.

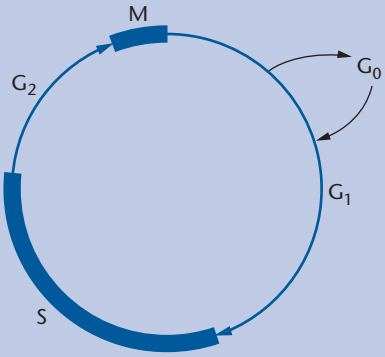


Figure B The durations of the phases of a cell cycle with a 24-h generation time. The G_1, S, G_2, and M phases are 11 h, 8 h, 4 h, and 1 h, respectively.

The ^{3}H-thymidine pulse-chase strategy has been superseded by automated systems that determine the amount of nuclear DNA in individual cells of a growing cell population. In this context, cells in the G_1 phase will have x amount of DNA; those in S phase, between x and 2x; and those in G_2 and M, 2x. The time periods of the G_1, S, and G_2 phases are calculated on the basis of the proportion of each phase in the cell population. The durations of the M phase and the total cell cycle are determined independently.

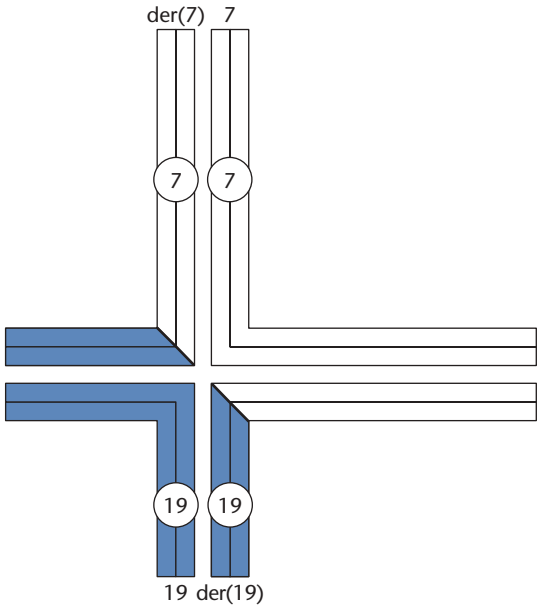


Figure 2.12 Synapsis of a reciprocal translocation. The translocation t(7;19)(q22;q13.1) is shown schematically with chromosomal material from chromosome 7 as the open regions and chromosome 19 as the solid regions. The normal chromosomes are marked as 7 and 19, and the translocated chromosomes as der(7) and der(19). The numbered circles indicate the centromeres of chromosomes 7 and 19.

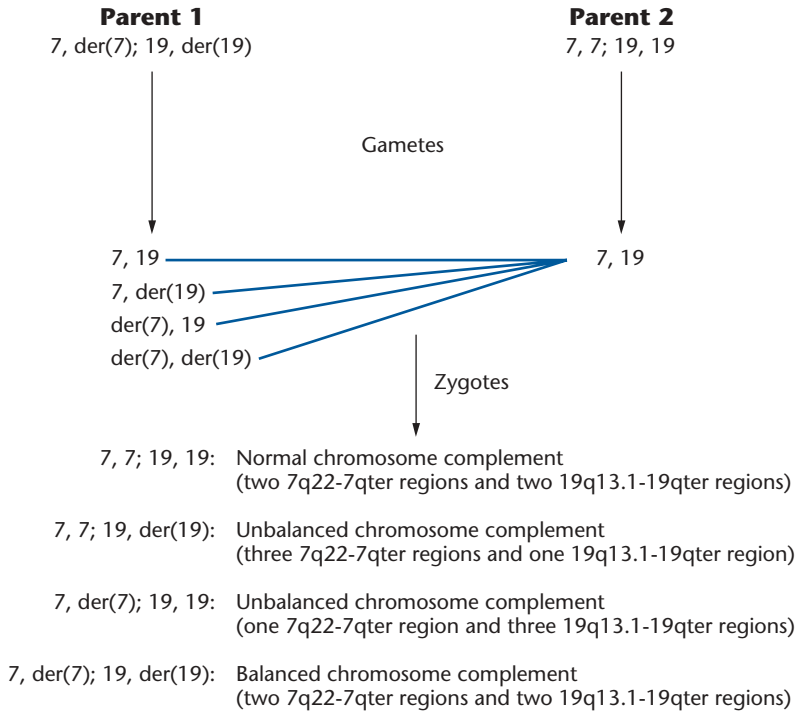


Figure 2.13 The theoretical consequences among all the potential zygotes produced from a mating between a person (parent 1) with the reciprocal translocation t(7;19)(q22;q13.1) and a spouse (parent 2) with a normal chromosome constitution.

a derivative chromosome and is used here to represent a chromosome with a translocation. As a consequence of meiosis, gametes with four chromosome combinations—[7, 19]; [7, der(19)]; [der(7), 19] and [der(7), der (19)]—will be produced. For brevity, this type of simplified notation, common in genetics, ignores the fact that the normal haploid complement of all of the other chromosomes is present in each gamete. The chances are insignificant that a couple will both have the same reciprocal translocation. Therefore, all the gametes of the spouse with the unrearranged chromosomes will carry the 7, 19 chromosomes. Theoretically, in this case, fertilization can lead to four different kinds of zygotes (Figure 2.13). The zygotes with either an intact, normal chromosome complement (7,7/19,19), or a reciprocal translocation [7, der(7)/19, der(19)] will develop normally. By contrast, development of zygotes with unbalanced chromosome sets will lead to spontaneous abortions. For example, the 7, 7/19, der(19) constitution will have three versions of chromosome material from 7q22 to 7qter (7q22–7qter) and only one segment of the chromosomal region 19q13.1–19qter. This condition creates both a partial trisomy and a partial monosomy. Similarly, the combination 7, der(7)/19, 19 will have three versions of 19q13.1–19qter and one of 7q22–7qter. Thus, reciprocal translocations cause a 50% reduction in fertility.

In rare cases, translocations are not reciprocal; exchanges occur among three or more chromosomes; and combinations of deletions, inversions, and translocations affect different chromosomes. When present in a zygote, these types of complex chromosomal alterations are often lethal. Clearly, both the correct chromosome number and the full amount of genetic information are essential for normal development. On the other hand, as discussed in a later chapter, multiple chromosome rearrangements are common in cancer cells.

key terms

anaphase
aneuploidy
autosome
cell division cycle
centromere
chromatid
chromosome
daughter cell
deletion
diakinesis
diplotene
G_1 phase
G_2 phase
gametes
inversion
leptotene
meiosis I
meiosis II
metaphase
mitosis
monosomy
mosaicism
nondisjunction
p arm
pachytene
prophase
q arm
reciprocal translocation
S phase
somatic cells
synapsis
telophase
translocation
trisomy
X chromosome
Y chromosome
zygotene

summary

Chromosomes are threadlike microscopic structures that reside in the nuclei of eukaryotic cells and carry almost all of the genetic information of the organism. In humans, somatic cells have 23 pairs of chromosomes. Of these 23 pairs, 22 are present in both males and females and are called autosomes. The remaining pair constitutes the sex chromosomes. In females, the two sex chromosomes are the same morphologically and are designated as X chromosomes. In males, the sex chromosomes consist of one X chromosome and a male-specific chromosome called the Y chromosome.

The cell division cycle, which is responsible for the proper distribution of a full complement of chromosomes to each daughter cell, consists of a series of coordinated events divided into phases that include the G_1 phase, S phase, G_2 phase, and mitosis (M phase). The stages of mitosis are prophase, metaphase, anaphase, and telophase.

In sexually reproducing organisms, the meiotic process produces gametes that have one member of each pair of chromosomes. This chromosome reduction is accomplished by two successive cell divisions, termed meiosis I and meiosis II. The G_1, S, and G_2 phases of meiosis I resemble closely the same phases during the cell division cycle. The prophase of meiosis I, called prophase I, consists of five substages: leptotene, zygotene, pachytene, diplotene, and diakinesis. During zygotene, homologous chromosomes synapse. During pachytene, genetic material may be exchanged between nonsister chromatids of synapsed chromosomes. Cells produced by meiosis I carry one duplicated chromosome of each pair of chromosomes. During meiosis II, there is no S phase. Chromosomal activities during prophase II, metaphase II, anaphase II, and telophase II are essentially the same as during mitosis of the cell division cycle. In human males, the four cells produced by the meiotic process develop into sperm. By contrast, in human females only one of the products of meiosis becomes an unfertilized egg.

Human metaphase autosomes are numbered from 1 to 22 in descending order of length, with the longest chromosome designated as 1. The X and Y chromosomes differ in length. However, length alone is not an effective way to identify individual chromosomes, because some groups of chromosomes have similar lengths. The site of the centromere, a fixed location, divides a chromosome into two arms. The shorter chromosome arm is called the p (petite) arm and the longer one the q arm. Both the lengths and ratios of arm lengths of some chromosomes are the same. Definitive identification of each member of the human chromosome complement was achieved by special staining procedures of metaphase and, in some cases, late prophase chromosomes to produce a distinctive set of dark and light transverse bands for each chromosome. A notational system has been devised for describing the location of each chromosome band. For example, 12q24.32 signifies a specific band, namely, band 2 within subband 3 of band 4 of region 2 of the long arm (q) of chromosome 12.

Mistakes in the distribution of chromosomes during the cell division cycle and the meiotic process can produce cells that lack one chromosome or have an extra single chromosome. Usually, a somatic cell with an abnormal chromosome complement is not able to persist, although, in combination with other genetic changes, these kinds of aneuploid cells may, in some instances, become cancer cells. Gametes with whole chromosome abnormalities are responsible for aneuploid zygotes. In humans, it is usually

continued

lethal when a zygote lacks a single autosome (monosomy). And the presence of an extra autosome (trisomy) in a human zygote is usually lethal as well, but not with every autosome. For example, some zygotes with an extra chromosome 13, 18, 19, or 21 undergo complete development. Trisomy 21 is called Down syndrome (DS) and gives rise to a spectrum of defects. Some individuals with DS can live for more than 40 years. Newborns with any of the other autosomal trisomies have severe physical abnormalities and very low probabilities for survival.

Sex chromosome complements such as XXY, XXX, or XYY in all cells of an individual occur relatively frequently and have less biological impact than autosomal trisomies. Of these whole chromosome abnormalities, XXY individuals are male and often sterile but otherwise generally normal. XXX individuals are females with no consistent physical abnormalities, and XYY individuals are males with no specific associated syndrome. Individuals with a single X chromosome have some distinctive biological features, including infertility and cardiac abnormalities. A single-Y condition has never been observed in a live-born human.

In addition to whole chromosome abnormalities, parts of chromosomes can be altered by deletion, inversion, or translocation. Deletion of the terminal portion of one of the p arms of either chromosome 4 or 5 occasionally results in the live birth of an individual with severe defects. If vital genetic information is not disrupted by an inversion or a reciprocal translocation, development may proceed normally. However, individuals with reciprocal translocations have reduced fertility, because half of their zygotes will have an unbalanced set of chromosomes with a partial monosomy and a partial trisomy.

References

Bloom, K. 1993. The centromere frontier: kinetochore components, microtubule-based motility, and the CEN-value paradox. *Cell* **73**:621–624.

Gorbsky, G. J. 1992. Chromosome motion in mitosis. *Bioessays* **14**:73–80.

Holmquist, G. P. 1992. Chromosome bands, their chromatin flavors, and their functional features. *Am J Hum Genet* **51**:17–37.

McIntosh, J. R. 1991. Structural and mechanical control of mitotic progression. *Cold Spring Harb Symp Quant Biol* **56**: 613–619.

McIntosh, J. R. and G. E. Hering. 1991. Spindle fiber action and chromosome movement. *Annu Rev Cell Biol* **7**:403–426.

McIntosh, J. R. and C. M. Pfarr. 1991. Mitotic motors. *J Cell Biol* **115**:577–585.

Miller, O. J. and E. Therman. 2001. *Human Chromosomes*. 4th ed. Springer, New York.

Mitelman, F. (ed.). 1995. *ISCN 1995: An International System for Human Cytogenetic Nomenclature*. Karger Monographs, Basel, Switzerland.

Saitoh, Y. and U. K. Laemmli. 1994. Metaphase chromosome structure: bands arise from a differential folding path of the highly AT-rich scaffold. *Cell* **76**:609–622.

Schinzel, A. 2001. *Catalogue of Unbalanced Chromosome Aberrations in Man*. Walter de Gruyter, Berlin.

Shaffer, L. G. and J. R. Lupski. 2000. Molecular mechanisms for constitutional chromosomal rearrangements in humans. *Annu Rev Genet* **34**:297–329.

review questions

1. Compare and contrast the numbers of chromosomes and their physical organization at metaphase, metaphase I, and metaphase II. What is the biological significance of the organization of the chromosomes at each of these metaphases?
2. Write a short essay on the significance of synapsis.
3. Discuss why there is no S phase during meiosis II.
4. What are the differences between monosomy and trisomy?
5. Describe the chromosomal basis of the Patau, Edwards, Down, Klinefelter, and Turner syndromes.
6. What are the consequences of nondisjunction of chromosome 7 during a cell division cycle?
7. Discuss the origins of somatic cell chromosome mosaicism.
8. Using Figure 2.6A as a guide, accurately construct inv(2)(p21q31) and del(1)(q21.1q25).
9. Account for the observation that chromosome inversions often have less biological impact than chromosome deletions.
10. Explain fully the notation 46,XY,t(2;5)(q21.1;q31.1).

chapter 3

The Genetic System: Mendel's Laws of Inheritance and Genetic Linkage

Not haphazard experimentation, but the combination of theoretical ideas with planned experiments has made possible Mendel's achievement and the subsequent advance of genetics.

L. VON BERTALANFFY (1901–1972)

WELL-DESIGNED EXPERIMENTS and a conceptual point of view enabled Mendel to formulate the fundamental principles of inheritance. The garden pea, which he selected for his studies, normally reproduces by self-fertilization (self-pollination), because its reproductive organs are securely enclosed within the petals of the flower. This feature had a twofold benefit for Mendel. On the one hand, it allowed him to use self-fertilization without concern about cross-fertilization (cross-pollination), which would have led to genetic contamination among the offspring plants. On the other hand, for some experiments, he could create genetically precise crosses by removing the stamen from a plant before the formation of pollen grains and then dusting the remaining female part (pistil) with pollen from a strain of his choosing. In the latter cases, the tight packaging of the flower prevented cross-pollination.

Another of Mendel's critical decisions was to study the inheritance of simple, contrasting characteristics (traits) from one generation to the next. He examined seeds that were round or wrinkled (seed shape), seeds that were yellow or green (seed color), stems that were tall or short (stem height), flowers that were white or purple (flower color), pods that were inflated or pinched (pod shape), pods that were green or yellow (pod color), and flowers that were axial or terminal (flower position). With brilliant insight, he used strains (lines) of plants that bred true for each of the traits he had picked to study. In other words, for his genetic experiments, he cross-bred plants that gave rise only to round seeds with those that produced only wrinkled seeds, those that gave rise only to yellow seeds with those that produced only green seeds, and so on. He also approached his data from a quantitative perspective by counting the

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number of progeny with the traits of the parents after the first generation and those that had the traits of the parents and grandparents after two generations. After conducting many different crosses, he observed common patterns of inheritance regardless of the trait that was studied. In each case, the results conformed to simple, consistent mathematical relationships. With this information, Mendel formulated a generalized explanation of how genetic determinants are inherited.

Dominance, Recessiveness, and Segregation

When Mendel crossed true-breeding plants that gave rise to round seeds with those that always produced wrinkled seeds, all the seeds of the first generation plants were round. Next, he let these first-generation plants self-fertilize. In the following generation, he observed plants that produced both round and wrinkled seeds. Moreover, the proportion of round to wrinkled seeds in the second generation was 3 to 1 (3:1). When each of the other pairs of traits was tested in this manner, the same pattern of inheritance was observed. Mendel realized that inheritance was a consistent biological phenomenon that did not depend on the trait that was studied. A thorough explanation of the results of any genetic cross requires a clear, uniform genetic vocabulary. In genetic terms, the original members of a mating (cross) of a genetic study are called the parental (P) generation. The progeny of a parental cross make up the first filial (F_1) generation, and the progeny of a cross that includes either one or both of the F_1 parents comprise the second filial (F_2) generation. In Mendel's study of the inheritance of seed shape, the cross between true-breeding lines for round and wrinkled seeds gave rise to F_1 plants that all produced round seeds. The wrinkling trait present in one of the parental plants was not evident in the F_1 generation. After self-fertilization of the F_1 plants ($F_1 \times F_1$, where the $\times$ denotes "mated with"), plants that produced wrinkled seeds were observed in the F_2 generation. The appearance of "wrinkledness" of seeds from the F_2 plants was identical to that produced by the P-generation plants. Mendel deduced that a seed-wrinkling factor was present in the F_1 plants that did not affect seed shape in these plants but was passed on as an intact entity to the F_2 progeny.

In addition, he postulated that a different genetic factor was responsible for the roundness of the seeds of the F_1 plants. The simplest interpretation was that the F_1 plants had two factors (genes) for seed shape, one for roundness and one for wrinkling. Following this reasoning, the original parental strain that produced only round seeds must have had two genes that were the same, and each alone would be sufficient to cause seeds to be round. Similarly, the other parental line that produced only wrinkled seeds had two genes that were alike but different from the genes that determined the roundness of the seeds. By maintaining logical consistency, Mendel concluded that the number of factors that determined a particular trait remained the same from one generation to the next. Consequently, he deduced that there was a process that ensured that pollen and egg cells each received one member of a pair of genes from each parent. Although Mendel had no knowledge of chromosomes, we can appreciate that his conjecture about the distribution of genes from one generation to the next perfectly parallels the meiotic process.

Mendel realized that in the F_1 plants only a single gene for seed roundness was necessary to produce round seeds. And, although a gene for wrinkling was present in the F_1 plants, it had no apparent effect on seed shape. Accordingly, he understood that the appearance of a trait does not necessarily reflect the underlying genetic constitution of an organism. Today, the appearance of a trait is called the phenotype, and the genetic constitution is the genotype. In Mendel's F_1 plants, the genotype for seed shape consisted of one gene responsible for round seeds and one for wrinkled seeds. When a particular genotype is characterized, all the gene pairs that are not under study are ignored. The genes for seed roundness and wrinkledness are alternative forms (alleles) of the gene that contributes to seed shape in the pea plant. Because the phenotype of all the seeds of the F_1 plants was round, the allele for round seeds is said to be dominant to the allele for wrinkled seeds. On these grounds, the wrinkling allele is a recessive gene. An organism with two different alleles of a gene is called a heterozygote. A homozygote has the same two alleles. If a gene behaves as a dominant in the heterozygous state, then an organism with two dominant genes is said to be homozygous dominant. An organism with two of the same recessive genes is homozygous recessive.

In addition to genetic terminology, symbols for alleles act as a convenient shorthand code. For plant and animal genetic studies, a single letter, abbreviation, or identifiable shortened form often is used as a symbol for a gene, with the dominant allele represented by an uppercase italic letter(s) and the recessive allele with a lowercase italic letter(s). Frequently, the letter(s) is chosen to denote some aspect of the phenotype determined by the dominant allele. Accordingly, the dominant allele for the rounding of seeds could be *R* and the recessive allele *r*. Genotypically, for the seed shape gene in peas, heterozygous plants would be designated as *Rr*, homozygous dominants as *RR*, and homozygous recessives as *rr*.

In strict genetic terms, dominance and recessiveness are descriptions of phenotypes and not of genes. However, few genetic textbooks bother to make this distinction, because it is both convenient and highly ingrained for geneticists and others to refer to dominant and recessive alleles. In this book, the conventional (inaccurate) use of these terms will be perpetuated, and alleles, when necessary, will be referred to as dominant or recessive.

In Mendel's study of the inheritance of seed shape, the $F_1 \times F_1$ cross was between two heterozygotes ($Rr \times Rr$). For each heterozygous parent, half the gametes carried an *R* gene and the other half an *r* gene (Figure 3.1). In other words, during gamete formation, the alleles of a gene pair segregate from each other. The fusion of gametes during fertilization is completely random. And, if there are enough gametes, all possible combinations, that is, fertilizations, will occur (Figure 3.1). Consequently, the F_2 progeny will have three genotypes (*RR*, *Rr*, and *rr*), and among these progeny there will be two phenotypes (round and wrinkled) with respect to seed shape.

According to one of the rules of probability, the expected frequency of gametic combinations (fertilized eggs) is the product of the individual frequencies of the gametes that unite. Consequently, 1/4 of the progeny in this cross are expected to be *RR*, because the chance that two *R*-containing gametes will combine is 1/2 times 1/2. Similarly, 1/4 of the progeny will be *rr*. Because

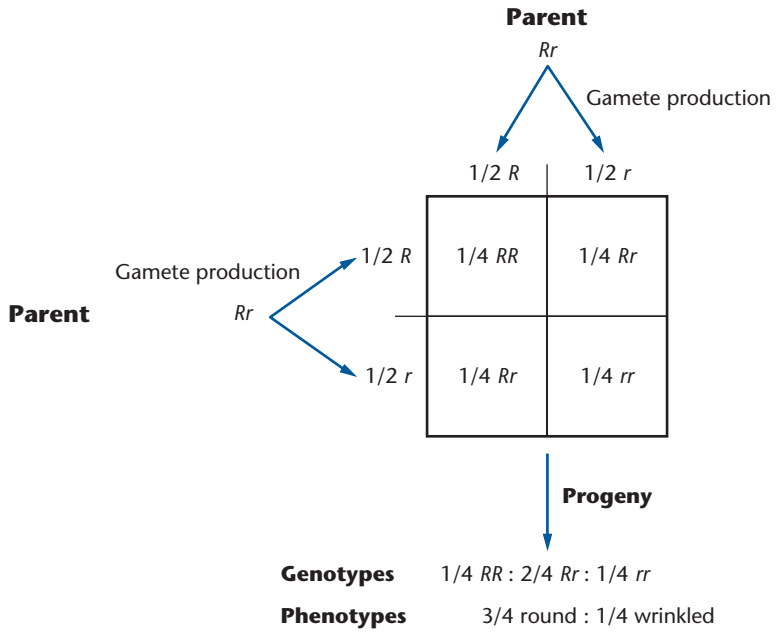


Figure 3.1 The results of a cross between pea plants that are both heterozygous (*Rr*) for round seeds, including the frequencies of the gametes and the genotypes and phenotypes of the F_1 progeny.

a heterozygote can occur in two ways, with either the *R* or the *r* allele being contributed by either parent, the expected frequency of heterozygotes is [(1/2 *r* times 1/2 *R*) + (1/2 *R* times 1/2 *r*)], which results in (1/4 *Rr* + 1/4 *Rr*) or 1/2 *Rr*. In this cross, the F_2 genotypic frequencies would be 1/4 homozygous dominant (*RR*), 1/2 heterozygous (*Rr*), and 1/4 homozygous recessive (*rr*) plants, a genotypic ratio of 1:2:1. The phenotypic ratio, which Mendel observed, would be 3:1 for plants that produced round (*RR* and *Rr*) and wrinkled (*rr*) seeds, respectively.

To test whether the deduced relationship between genotype and phenotype was correct, Mendel crossed heterozygous (*Rr*) F_1 plants with homozygous recessive (*rr*) plants. The heterozygous parents are expected to produce equal numbers of *R*- and *r*-containing gametes. All the gametes produced by the homozygous recessive plants carry an *r* gene. The anticipated genotypic ratio in the F_1 generation would be 1:1 for the *Rr* and *rr* genotypes, respectively (Figure 3.2). Because of the dominant behavior of the *R* allele, the phenotypic ratio would be 1:1. Mendel's results from this cross confirmed his prediction.

Mendel was extremely fortunate in the alleles he chose to study. For example, all the dominant genes of his contrasting traits tended to be completely dominant. In each instance, both the homozygous dominant and heterozygous genotypes gave virtually the same phenotype. Later, after the rediscovery of Mendel's work, as researchers studied other single-gene traits in different organisms, it became clear that heterozygotes often have phenotypes that differ from those with either the homozygous dominant or homozygous recessive genotypes. Dominance, in most instances, is neither a complete nor a simple phenomenon. A recessive gene in a heterozygote, for example, may make some contribution to the phenotype, although this may not be observed readily without biochemical or microscopic analyses. It is also erroneous to think of a dominant gene as being "better" than its recessive allele. Dominance

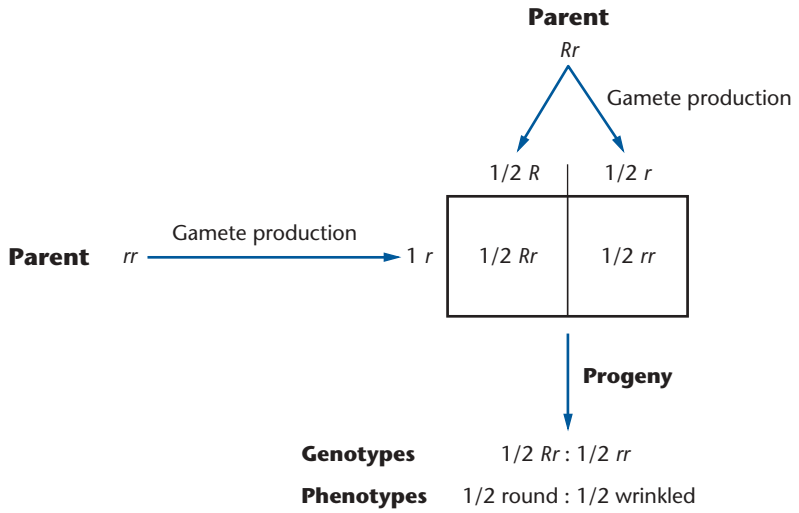


Figure 3.2 The results of a cross between a pea plant that is heterozygous (*Rr*) for round seeds and one that is homozygous recessive (*rr*) for wrinkled seeds, including the frequencies of the gametes and the genotypes and phenotypes of the F_1 progeny.

is merely defined by the behavior of a gene in the heterozygous condition. In humans, some diseases result from dominant genes and some from recessive genes. For some human genetic diseases, the terms dominant and recessive are used out of habit and convenience, even when complete dominance does not occur.

Although it may be obvious, it should be noted that it is impossible to carry out genetic analyses without alleles. If all members of an organism were homozygous for a single gene, then only one genotype would exist, and breeding experiments could reveal nothing about the mode of inheritance for that gene.

Independent Assortment

In addition to conducting crosses with single gene pairs (monogenic crosses), Mendel studied the simultaneous fates of two different traits determined by two gene pairs (digenic crosses). He assumed that the two traits would either be transmitted independently of each other or would remain together from one generation to the next. For example, when true-breeding plants with round and yellow seeds (*RRYY*) were crossed with plants that only produced wrinkled and green seeds (*rryy*), all the F_1 plants were doubly heterozygous (*RrYy*) and formed round and yellow seeds. After self-fertilization of these F_1 plants (*RrYy* × *RrYy*), the F_2 plants produced four kinds of seeds in a ratio of 9:3:3:1. Specifically, Mendel observed 315 round and yellow, 108 round and green, 101 wrinkled and yellow, and 32 wrinkled and green seeds. At first glance, the ratio 9:3:3:1 appears strange. However, if seed shape is considered alone, then the proportion of round to wrinkled seeds from the F_2 progeny was, as expected, 3:1, that is, (315 + 108):(101 + 32), or 3.2:1. Similarly, the ratio of yellow to green seeds is also 3:1, that is, (315 + 101):(108 + 32), or 2.9:1. When taken separately, each set of characteristics behaved exactly as it would from a cross between two heterozygotes. And, when considered together, the alleles of both traits were distributed randomly among the offspring. In other words, during

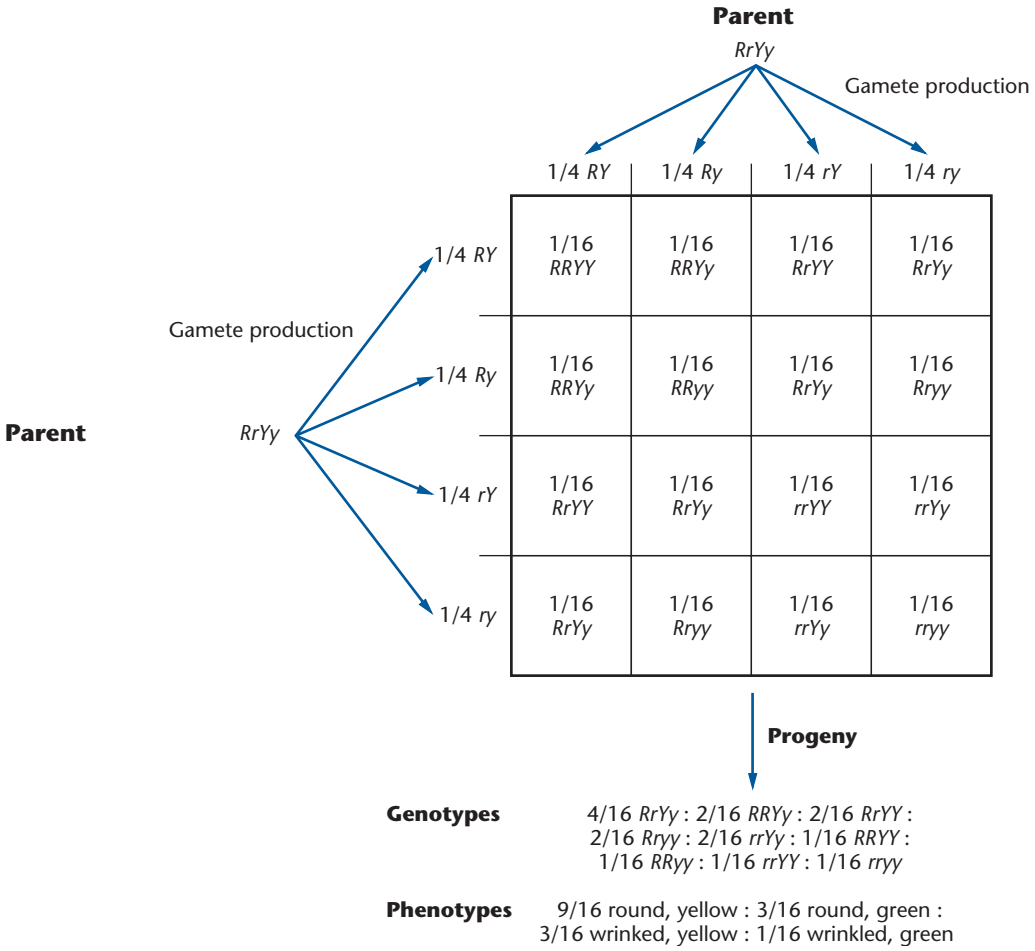


Figure 3.3 The results of a cross between pea plants that are both doubly heterozygous (*RrYy*) for round and yellow seeds, including the frequencies of the gametes and the genotypes and phenotypes of the F_1 progeny.

gamete formation any one allele of one trait was equally likely to be present with an allele of the other trait.

On this basis, Mendel realized that each double heterozygote parent produced four kinds of gametes (*RY*, *Ry*, *rY*, *ry*) in equal numbers. Accordingly, after fertilization, the random union of all gametic types would produce a phenotypic ratio of 9:3:3:1 when the dominant effects are considered and a genotypic ratio of 4:2:2:2:2:1 (Figure 3.3). This pattern of inheritance is called independent assortment. Mendel's results are consistent with the meiotic behavior of nonhomologous chromosomes. In genetic parlance, the location of a gene site on a chromosome is called a locus (loci, pl.). Independent assortment occurs when two gene loci are on different chromosomes. In addition, Mendel's digenic cross showed that new genotypes can be formed that are unlike the genetic constitutions of either parent. Because of the consequences of dominant alleles, different genotypes (e.g., *RRYY*, *RrYy*, *RrYY*, *RRYy*) can be responsible for the same phenotype (in this case, round and yellow seeds).

A cross between a double heterozygote (*RrYy*) and a doubly homozygous recessive (*rryy*) produces four kinds of genotypes and phenotypes among the offspring, with 1:1:1:1 as both the genotypic and phenotypic ratios (Figure 3.4). This result is expected, because a doubly heterozygous parent produces

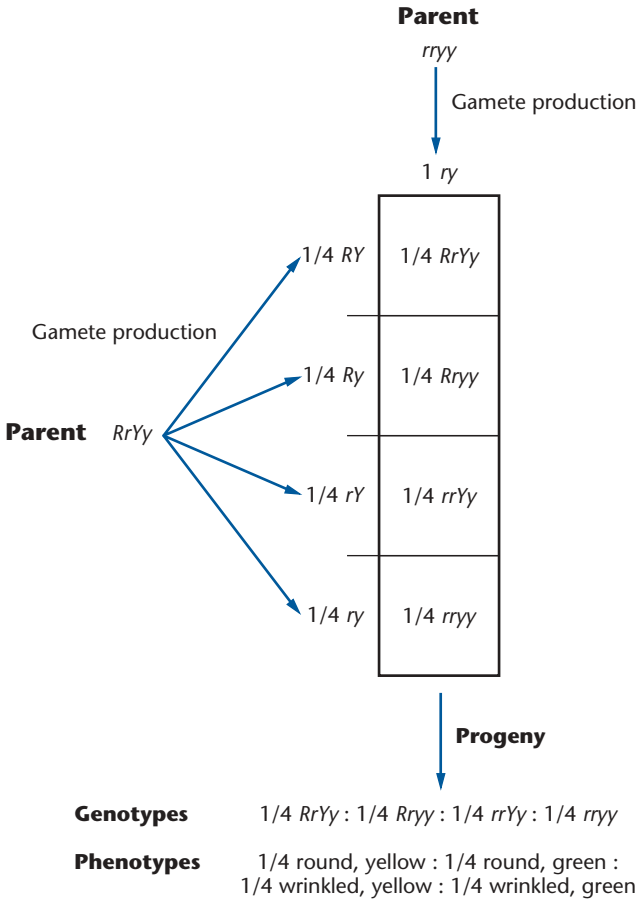


Figure 3.4 The results of a test cross between a pea plant that is doubly heterozygous (*RrYy*) for round and yellow seeds and one that is doubly homozygous recessive (*rryy*) for wrinkled and green seeds, including the frequencies of the gametes and the genotypes and phenotypes of the F_1 progeny.

four genetic kinds of gametes in equal numbers and a doubly homozygous recessive individual produces only one kind of gamete with respect to these alleles. An examination of the offspring from the *RrYy* × *rryy* cross shows that 50% of the progeny (*RrYy*, *rryy*) have the same genotype as the parents (parental genotypes), and 50% (*Rryy*, *rrYy*) represent new genetic combinations not present in either parent (Figure 3.4). A cross where each phenotypic class among the progeny corresponds to a specific genotype is very helpful to geneticists and is called a backcross by some and a test cross by others. In this instance, from a genetic perspective, the doubly homozygous recessive parent is irrelevant (noninformative), because all its gametes carry the same alleles.

All the pairs of genes that Mendel tested with digenic crosses showed independent assortment. On this basis, it would have been natural to assume that genes existed as separate, intact entities. However, unknown to Mendel, the dispersal of genes from one generation to the next parallels precisely the meiotic distribution of chromosomes during gamete formation. Consequently, it was logical for the researchers who followed Mendel to conclude that genes and chromosomes were closely associated with each other. In addition, it also became clear by the end of Mendel's century that all organisms have far fewer chromosomes than genes. Therefore, it was anticipated that a chromosome would carry a large number of different gene loci. On this basis, one would

expect that all of the gene loci of a chromosome would form a linear array and tend to be inherited as a group. On the other hand, genes on different chromosomes would be expected to assort independently. The question that Mendel never encountered was: What is the pattern of inheritance of different genes that occupy sites close to each other on the same chromosome?

Genetic Linkage

One of the main objectives of genetic studies is to determine the linear order of genes along a chromosome. Theoretically, if the genes of a chromosome always remained together, or, in genetic terminology, if they were completely linked, then they would be passed on to the sex cells as an intact block, with no new genetic combinations formed during the meiotic process. Genetic linkage is symbolized with either a single straight line or a single forward slash separating the genes that reside on different members of a pair of homologous chromosomes, for example,

$$\frac{AB}{ab}, \frac{Ab}{aB}, \frac{AB}{AB}, \text{etc.} \quad \text{or} \quad AB/ab,\ Ab/aB,\ AB/AB, \text{etc.}$$

The notation *AB*/*ab*, for example, signifies that the dominant alleles (*A*, *B*) of two different gene loci are on one chromosome and the recessive alleles (*a*, *b*) are on the homologous chromosome. With complete linkage, a double heterozygote (*AB*/*ab*) would produce only two types of gametes (1/2 *AB* and 1/2 *ab*), and the cross *AB*/*ab* × *ab*/*ab* would yield only two kinds of genotypes (1/2 *AB*/*ab* and 1/2 *ab*/*ab*) among the offspring (Figure 3.5). In this case, there are no (0%) new (nonparental) genetic combinations. By contrast, a cross of a double heterozygote with a double homozygous recessive with the two gene loci on nonhomologous chromosomes produces 50% new genetic combinations. Accordingly, with this specific cross, the uppermost limit for new genetic combinations is 50%, which represents independent assortment. And, depending on the cross, the lowest possible value for new genetic combinations is 0%, which occurs when linkage is complete.

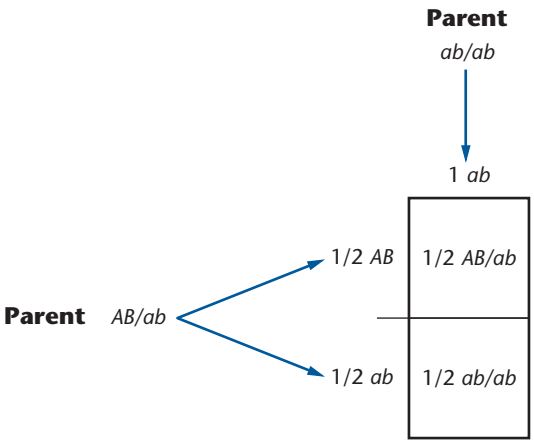


Figure 3.5 The results of a test cross with two gene loci that are completely linked, including the frequencies of the gametes and the genotypes of the F_1 progeny.

However, gene linkage is seldom complete, because during the meiotic process, crossing over occurs between gene loci on nonsister chromatids to create new genetic combinations. For example, a cross, with complete dominance, between a double heterozygote (*SsTt*) and a doubly homozygous recessive (*sstt*) produces four phenotypic classes representing four genotypes, of which two are new genetic combinations (*Sstt*, *ssTt*). In this case, unlike independent assortment, repeated experiments show that each new genetic combination makes up 10% of the total progeny and each parental genetic combination is always 40% of the total progeny (Table 3.1). What is the explanation for these results?

First, because the total of new genetic combinations from this cross is less than 50%, the two gene loci are not assorting independently, which means they are genetically linked on the same chromosome. Second, the frequency of the new genetic combinations is greater than 0%. Therefore, the two gene loci are not completely linked. Third, the total frequency of new genetic combinations is 20%, which is the sum of the frequencies of each new genetic combination class. The proportion of new genetic combinations among the progeny of a cross is the consequence of the frequency of reciprocal physical exchanges between the two gene sites on nonsister chromatids during meiosis.

To explain how the symmetry of both the new (10% each) and parental (40% each) combinations occurs, it is necessary to consider what happens during meiosis of the double heterozygous parent. During one meiosis, a single crossover event between two loci in a double heterozygote produces two crossover chromatids and two noncrossover chromatids (Figure 3.6). After

Table 3.1 Results from a cross between *SsTt* × *sstt* genotypes.

Phenotype	Genotype	Frequency
ST	*SsTt*	40%
St	*Sstt*	10%
sT	*ssTt*	10%
st	*sstt*	40%

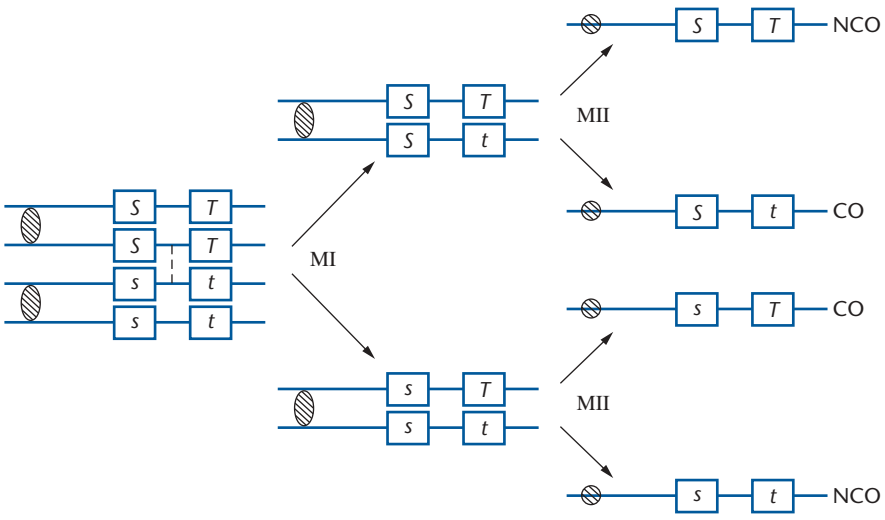


Figure 3.6 Schematic representation of the consequences of a single crossover between two loci. The vertical dashed line marks the location of the crossover, the distribution of the chromosomes after meiosis I (MI) and meiosis II (MII) are shown, and the noncrossover (NCO) and crossover (CO) chromosomes are noted.

meiosis, each gamete receives one of these chromatids. When 40 out of 100 meioses have a single crossover between two loci, 80 gametes receive crossover chromatids and 80 receive noncrossover (nonrecombined) chromatids. From the remaining 60 meioses, 240 gametes with noncrossover (parental) chromatids are produced. Accordingly, the recombination frequency (fraction) is the number of gametes with new genetic combinations divided by the total number of gametes, or 80/(80 + 80 + 240), which equals 0.20.

When the numbers are large, the frequencies of the progeny from a backcross represent the frequency of the gametes produced by the double heterozygous parent. Because the crossover event is a reciprocal exchange, the two resulting crossover chromatids always occur with equal frequencies. And, because of the meiotic process, equal numbers of nonsister chromatids are distributed to the gametes. By convention, 1% recombination is equivalent to 1 map unit (mu). Therefore, in our example, the genetic distance between the gene loci is 20 mu. The centimorgan (cM) is a third unit of measure used for map distances. Generally, a 1% recombination is equivalent to 1 cM for distances of 10 mu or less. When recombination between two loci is greater than 10%, the measure given as centimorgans represents a statistically corrected value. This correction is necessary because at greater map distances the observed percent recombination is an underestimate of the "true" map distance between two loci. A corrected map distance for two loci that recombine with a frequency of 20% (20 mu) is 21.2 cM. Despite the subtleties of fine-tuning an observed recombination frequency, a genetic distance between two loci represents the beginning of the construction of a genetic map of a chromosome.

In summary, the analysis of the *SsTt* × *sstt* cross shows that the two loci are linked and are 20 mu apart. Because the *ST* and *st* phenotypic classes represent the majority classes, they each represent nonrecombined chromosomes. Consequently, the genetic arrangement of the doubly heterozygous parent is *ST*/*st*, and the cross should be represented as *ST*/*st* × *st*/*st*.

Crossing over always occurs between two genes with the same frequency, regardless of the genotype. However, in doubly homozygous individuals (e.g., *AB*/*AB*, *ab*/*ab*, *Ab*/*Ab*, etc.), the crossover chromosomes will have the same genetic constitution as the parental (noncrossover) chromosomes and cannot be detected genetically. Therefore, double heterozygosity (*AB*/*ab*, *Ab*/*aB*) in one of the parents of a cross is required to score the occurrence of recombination.

Linkage analysis can be carried out with crosses between double heterozygotes. For example, with an *AB*/*ab* × *AB*/*ab* cross, if linkage is present, then the observed phenotypic ratio would deviate from the 9 : 3 : 3 : 1 that is expected for independent assortment. In this case, it is not possible to calculate total recombinants and nonrecombinants directly from the progeny, because dominance masks various genotypes. However, because the genetic constitution of both parents is *AB*/*ab*, the square root of the frequency of the *ab*/*ab* class among the progeny will give the frequency of the *ab* gametes (y) produced by each parent. In this cross, these gametes represent noncrossover types. Therefore, the frequency of *AB* gametes, which are also noncrossover types, will be equal to the frequency of the *ab* gametes. Accordingly, 1 – 2y gives a close approximation of the frequency of recombination between the two loci. On the other hand, if the genetic constitution of the parents is *Ab*/*aB* × *Ab*/*aB*, then the rationale for the calculation of the frequency of recombination is slightly different

because the *ab*/*ab* class, in this case, represents the union of two recombined chromosomes. Accordingly, doubling the frequency of this *ab*/*ab* class will give an estimate of the frequency of recombination.

Finally, a map distance from an *AB*/*ab* × *Ab*/*aB* cross cannot be calculated easily from the observed data because the *ab*/*ab* class, in this case, is the combination of a recombinant and a nonrecombinant chromosome. Although mathematical methods have been devised to give estimates of linkage in this type of cross, most experimenters prefer to use a backcross because all recombinants and nonrecombinants among the offspring can be identified and counted.

Genetically linked double heterozygotes have two different configurations with respect to the positioning of the dominant and recessive alleles. First, both dominant alleles of the two gene loci can be on the same chromosome and the two recessive alleles on the homologous chromosome (*AB*/*ab*). Second, a dominant and a recessive allele of two gene loci can be on one homologous chromosome and a recessive and a dominant allele, respectively, on the other homologous chromosome (*Ab*/*aB*). The *AB*/*ab* configuration is called the *cis* (coupling) phase, and the *Ab*/*aB* configuration is the *trans* (repulsion) phase. The frequencies of gametic genotypes depend on the configuration of the double heterozygous parent. With a *cis* configuration, the *AB* and *ab* gametes will be nonrecombinant, and the *Ab* and *ab* gametes will be recombinant. Conversely, with a *trans* configuration, the *Ab* and *ab* gametes will be nonrecombinant, and the *AB* and *ab* gametes recombinant (Table 3.2). Regardless of the genetic phase of two specific gene pairs, of course, the map distance, based on the frequency of recombinants, is always the same for the same two gene loci.

Constructing Genetic Maps

The order of the gene sites (loci) along a chromosome arm can be generated from the results of a series of pairwise (two point) backcrosses. For example, if locus *A* is 20 mu from locus *B*, and locus *C* is 5 mu from locus *B* and 15 mu from locus *A*, then locus *C* must lie between loci *A* and *B* (Figure 3.7). The map distances between successive gene loci are cumulative. Consequently, with this linkage group, the map distances from locus *A* to locus *C* and from locus *C* to locus *B* are 15 and 5 mu, respectively, and therefore the distance from locus *A* to locus *B* is 20 mu, that is, 15 + 5 = 20 (Figure 3.7). A set of linearly arranged gene loci labeled with map distances is called a genetic map. At this preliminary stage of a genetic mapping program, the order of the genes could be *ACB*

Table 3.2 The gametic frequencies produced by *cis* and *trans* configurations of double heterozygotes.

Genotype	Gametic frequencies			
	AB	*ab*	*aB*	*Ab*
AB/ab	1/2(1 – r)	1/2(1 – r)	1/2r	1/2r
Ab/aB	1/2r	1/2r	1/2(1 – r)	1/2(1 – r)

Note: The letter r represents the recombination fraction between the *A* and *B* loci. If, for example, r equals 0.10, then the gametic ratios from a parent with the *cis* (*AB*/*ab*) configuration would be 0.45 *AB*, 0.45 *ab*, 0.05 *aB*, and 0.05 *Ab*.

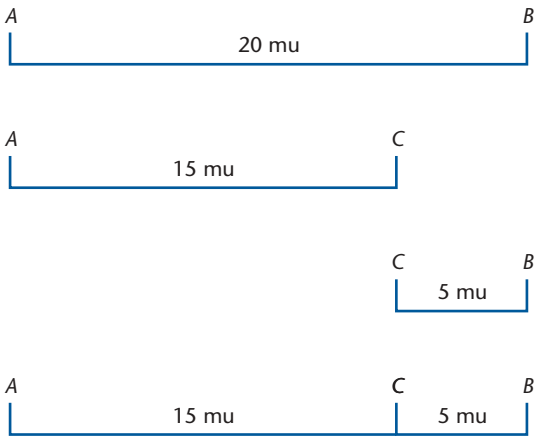


Figure 3.7 Two-point genetic maps between loci *A* and *B*, *A* and *C*, and *B* and *C* and a combined genetic map of the three loci. The distances between the loci are expressed as map units (mu).

or *BCA* because the genes have not been oriented within a chromosome arm. Genetic distances between loci are not converted readily to physical distances or to units of DNA. Various estimates of the chromosomal material between two mapped human genes has led to a rule-of-thumb measure where 1 cM is equivalent to about one million DNA base pairs.

A genetic map can be expanded by carrying out two-point crosses between additional members of a set of linked genes. However, genetic mapping with two gene loci at a time can be tedious and imprecise. The principal objective of genetic mapping of a chromosome is to score all the possible crossover events between two gene loci. In a double heterozygote, an even number of crossovers between two loci does not produce new genetic combinations, because genetic material that lies between the loci is exchanged while the original alleles remain together (Figure 3.8A). Only an odd number of crossovers between two gene loci generates new gene combinations. Because not all crossover events are scored with a two-point cross, the observed map distances are often underestimates. In addition, when two loci at the extremes of a large map are involved in a cross, they often appear to assort independently. In these instances, the loci are far enough apart that the frequency of odd and even crossovers between them is the same. This equivalence creates 50% recombinant genotypes, which genetically yields the same outcome as when the loci are on different chromosomes. The availability of intervening genes determines which genes belong to a particular linkage group.

Because of the limitations of two-point crosses, mapping experiments are conducted, when feasible, with three or more loci at a time. When three close loci are analyzed together (three-point cross), a single crossover on either side of the middle locus will create novel recombinants that can be identified among the progeny (Figure 3.8B). Without the middle locus, these crossovers would not have been detected. A more precise map is created if the loci to be mapped are close to each other, because the closer two genes are, the less likely the chance of crossing over between them.

Three-Point Cross

A cross between a triple heterozygote (*AaBbCc*) and a triple recessive (*aabbcc*) will be analyzed here to show the usefulness of a three-point cross for con-

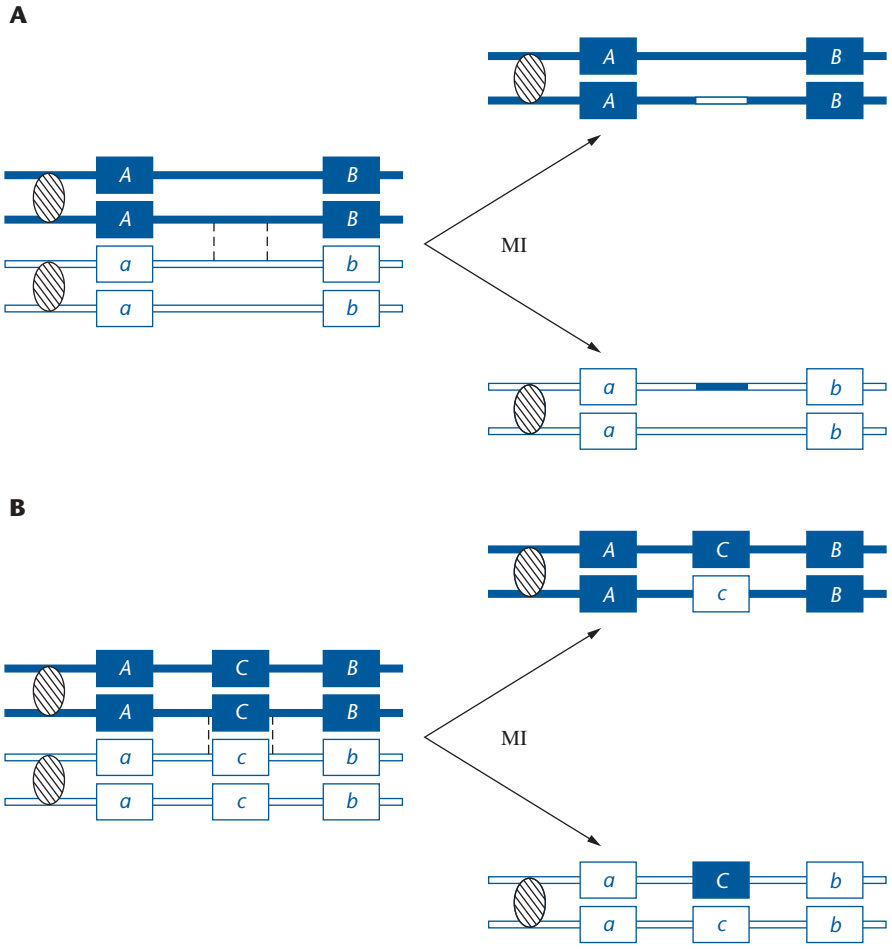


Figure 3.8 The consequences of a double crossover between two loci. (A) The vertical dashed lines mark the two crossovers. After meiosis I (MI), genetic material between the two loci has been exchanged, whereas the original allelic arrangements remain the same on each chromatid. (B) A crossover (dashed line) occurs on each side of the middle locus. After meiosis I (MI), genetic material containing the middle loci has been exchanged, which results in a new allelic arrangement on two of the chromatids.

structing a genetic map. If the three genes in this cross are linked, the gene order (*ABC*, *CAB*, or *ACB*) and the phase (*ABC*/*abc*, *ABc*/*abC*, *AbC*/*aBc*, or *Abc*/*aBC*) of the triple heterozygous parent can be determined from the results of the backcross. The first question to be answered from a three-point cross is whether the three genes are linked. If so, then the locus in the middle can be established. Finally, the map distances between the loci are calculated. The genetic contribution of the triple homozygous recessive parent is not relevant to this analysis, because all of its (or his or her) gametes carry the same set of recessive alleles. As mentioned previously, the progeny of any backcross reflect the meiotic events that occurred in the heterozygous parent.

If the three gene loci of this backcross were on different chromosomes, eight equal phenotypic and genotypic classes would be observed among the progeny. As expected for a triple heterozygous backcross, the progeny of the sample cross consist of eight classes (Table 3.3). However, because the numbers of the progeny within each class are not equal, linkage exists between at least two of the three loci. To examine whether all three loci are linked, two loci among the offspring can be analyzed at a time. For example, only the *AC* types (21 + 395 = 416), the *AB* types (21 + 77 = 98), and the *BC* types (21 + 3 = 24) need to be considered. None of these two-gene types, which represent a single pheno-

Table 3.3 Results from a cross between *AaBbCc* × *aabbcc* genotypes.

Phenotypes	Genotypes	Numbers of progeny
ABC	*ABC*/*abc*	21
ABc	*ABc*/*abc*	77
AbC	*AbC*/*abc*	395
Abc	*Abc*/*abc*	4
aBC	*aBC*/*abc*	3
aBc	*aBc*/*abc*	397
abC	*abC*/*abc*	73
abc	*abc*/*abc*	30
		Total = 1000

typic class from the *AaCc* × *aacc*, *AaBb* × *aabb*, and *BbCc* × *bbcc* crosses, respectively, contained within the original cross, comes close to the predicted 25% (250) of total offspring expected if these gene pairs were assorting independently. In other words, the absence of independent assortment demonstrates that locus *A* is linked to locus *C*, locus *A* to locus *B*, and locus *B* to locus *C*. If locus *A* is linked to locus *B* and locus *B* to locus *C*, then all three are linked.

The locus in the middle is determined by comparing the genetic constitutions of the most frequent progeny with those that are least frequent. The observed data can be grouped into four classes based on numerical similarities. In this example, the most frequent class comprises the *AbC* (395) and *aBc* (397) phenotypes. The least frequent class contains the *aBC* (3) and *Abc* (4) phenotypes. Because recombination is rare, the two types of the most frequent class represent noncrossover (NCO) chromosomes from the triple heterozygous parent. Accordingly, the phase of the genes in the triple heterozygous parent is *AbC*/*aBc*, *ACb*/*acB*, or *CAb*/*caB*. At this point in the analysis, it is not known which locus is in the middle. It is assumed that the chromosomes represented in the least frequent class (*Abc*, *aBC*) are the result of simultaneous single crossover events on each side of the middle gene, because such a double crossover (DCO) is rarer than a single crossover (SCO) event. The numerical similarity within each class is the result of the distribution of chromosomes during meiosis and the reciprocal nature of recombination (if it has occurred).

When a crossover occurs on each side of a middle locus, the alleles in the middle are rotated (flipped) relative to the two flanking loci. If the *B* locus is in the middle, then, based on the parental genotype *AbC*/*aBc*, the DCO class would be *ABC* and *abc* (Figure 3.9A). However, the data show that the *ABC* and *abc* chromosomes are not in the least frequent class. Consequently, locus *B* does not lie between the *A* and *C* loci. The gene order that conforms to expectation has the *C* locus in the middle (Figure 3.9B). Thus, the phase and gene order of the triple heterozygous parent is *ACb*/*acB*.

Map distances are calculated from the data by summing all crossovers between two loci and dividing by the total number of recombinant and nonrecombinant chromosomes. The total crossovers in the interval between loci *A* and *C* include the SCOs, which generated the class containing the *AcB* (77) and *aCb* (73) chromosomes, and the crossovers in this region from the DCO class [Acb (4), *aCB* (3)]. The recombination fraction between loci *A* and *C* is

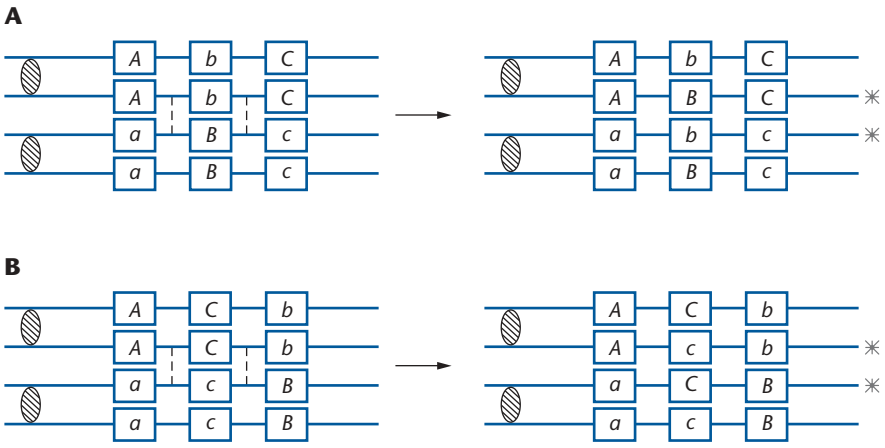


Figure 3.9 Using the double crossover class from a triple heterozygote backcross to determine gene order. (A) A double crossover (dashed lines) including the middle locus of the genotype *AbC*/*aBc* leads to the formation of two double crossover chromatids (*) with the allelic order *ABC* and *abc*, respectively. If locus *B* is in the middle, then the *ABC* and *abc* types would be members of the least frequent class among the progeny. (B) A double crossover (dashed lines) including the middle locus of the genotype *ACb*/*acB* leads to the formation of two double crossover chromatids (*) with the allelic order *Acb* and *aCB*, respectively. If locus *C* is in the middle, then the *Acb* and *aCB* types would be members of the least frequent class among the progeny.

[(77 + 73 + 4 + 3)/1000] × 100 or 15.7 mu. Total crossovers between loci *C* and *B* include the SCO class containing the *ACB* (21) and *acb* (30) chromosomes and the crossovers in this region from the DCO class [*Acb* (4), *aCB* (3)]. The recombination fraction for the interval between the *C* and *B* loci is [(21 + 30 + 4 + 3)/1000] × 100 or 5.8 mu. The map distance between the *A* and *B* loci is 21.5 mu (15.7 mu + 5.8 mu). If the data from this cross are analyzed as a two-point cross by ignoring the middle locus, then the map distance between loci *A* and *B* would be [(21 + 30 + 77 + 73)/1000] × 100 or 20.1 mu. The two-point map is shorter than the three-point map, because the DCOs between the *A* and *B* loci are not observed as recombinants and, therefore, are not included in the tally of recombination events.

Extremely precise map distances between loci are difficult to obtain, because large numbers of different allelic gene pairs must be tested in a concentrated chromosomal region. In addition, with multiple crossover events, often the occurrence of a crossover in one region diminishes the probability that a second crossover will take place in an adjacent segment. This phenomenon is called interference. Accordingly, the observed frequency of DCOs is often less than the expected frequency.

The frequency of crossovers may differ between the sexes of an organism. In the fruit fly *Drosophila melanogaster*, crossing over only occurs in the female. In humans, the recombination rate in female meioses is higher than in males. Overall, the human female genetic map is 1.5 times longer than the male genetic map. Human female and male genetic maps are calculated from crossover frequencies based on chromosomes that originate from females and males, respectively. Generally, it is convenient to compile a sex-averaged map by averaging the map distances between the same two loci from female and male genetic maps. The biological basis for the sex difference in recombination frequencies is not known.

A four-point cross (e.g., *ABCD*/*abcd* × *abcd*/*abcd*) for genetic mapping would produce 16 phenotypic and genotypic types. The two rarest types, in this case, would be the result of a triple crossover event. And, if all the types are present among the offspring, the map distances between loci can be calculated from the appropriate single, double, and triple crossover classes. As more gene pairs

are added, multipoint crosses become more laborious to analyze. Often, in these instances, the data are incomplete, which precludes the determination of a complete set of map distances. However, a probable gene order can be deduced by comparing the genetic constitutions of chromosomes of the rarest types with those that occur most frequently and assigning an order consistent with the maximum number of crossovers.

Genetic maps are extremely useful. They provide a detailed linear representation of the gene organization of an organism, with each mapped locus equivalent to a genetic address. With appropriate crosses, newly discovered genes can be assigned to specific linkage groups. In humans, maps can be used as springboards for identifying locations of disease-causing genes. Comparing genetic maps of diverse organisms provides insights into evolutionary relationships and, possibly, into the ways in which genetic systems evolved.

Chi-Square Distribution: Testing for Significance

For most genetic analyses, observed data are expected to conform to an anticipated result. When a data set is large, observed numbers alone can be convincing testimony that the expected outcome will be confirmed. However, when sample sizes are small or the observed values appear to support alternative interpretations, objective statistical tests are needed to help investigators either reject or substantiate a specific hypothesis.

The chi-square (χ^2) test is a statistical procedure often applied to the results of genetic crosses. With this method, the enumerated values from an experiment are compared with those that would be expected from a specific mode of inheritance. The results are assessed to determine whether the observed results reflect acceptable differences from the expected values or whether the proposed hypothesis is inappropriate.

For example, from a cross with 1000 progeny, 720 offspring have one phenotype and 280 have a contrasting phenotype. The observed ratio is 2.6:1. Is this ratio consistent with a ratio of 3:1? If the results from this cross occur with a ratio of 3:1, the expected numbers for the two phenotypic categories would be 3/4 and 1/4 of the total progeny, that is, 750 and 250, respectively. The formula for χ^2 is

$$\sum \frac{(O-E)^2}{E}$$

where O is the number of observed cases and E is the number of expected cases. The χ^2 value is determined by calculating a value for each category and then summing these results. In our example, the χ^2 value for the two categories is

$$\left[\frac{(720-750)^2}{750}+\frac{(280-250)^2}{250}\right]=2.4$$

The interpretation of a calculated χ^2 value is obtained by consulting a table of χ^2 values that correspond to various probabilities with a specified number of

Table 3.4 Table of chi-square values with various probabilities (levels of significance) and degrees of freedom (df).

	Probability							
DF	0.99	0.90	0.70	0.50	0.20	0.05	0.01	0.001
1	0.00016	0.016	0.15	0.46	1.64	3.84	6.64	10.83
2	0.02	0.21	0.71	1.39	3.22	5.99	9.21	13.82
3	0.12	0.58	1.42	2.37	4.64	7.82	11.34	16.27
4	0.30	1.06	2.20	3.36	5.99	9.49	13.28	18.46
5	0.55	1.61	3.00	4.35	7.29	11.07	15.09	20.52
6	0.87	2.20	3.83	5.35	8.56	12.59	16.81	22.46
7	1.24	2.83	4.67	6.35	9.80	14.07	18.48	24.32
8	1.65	3.49	5.53	7.34	11.03	15.51	20.09	26.12
9	2.09	4.17	6.39	8.34	12.24	16.92	21.67	27.88
10	2.56	4.86	7.27	9.34	13.44	18.31	23.21	29.59

degrees of freedom (Table 3.4). The degrees of freedom parameter takes into consideration the number of categories that contribute to the calculation of the χ^2 value. The number of degrees of freedom for a particular experiment is equal to the number of categories that need to be known numerically before the remaining category can be calculated. With two categories, knowledge of the number of observations in one category is sufficient, because the total number of observations, a known quantity, minus the number of observations in the first category equals the number of observations that must make up the second category. For two categories, the degrees of freedom (df) = 1. Generally, for most χ^2 tests, the number of df is k – 1, where k is the total number of categories.

The χ^2 table indicates that a value of 3.84 or greater with 1 df can be expected 5 times or fewer out of 100 replications of the same experiment. By convention, the 0.05 (5%) level of probability is used as a cutoff point. For χ^2 values with 1 df that are less than 3.84, the genetic ratio being tested is considered to be confirmed. In these cases, the observed numbers fall within the range that can be attributed to chance variation. For χ^2 values with 1 df that are greater than 3.84, the proposed genetic ratio is unlikely and should be rejected. If the probability for a χ^2 value is less than 0.05, but greater than 0.01, the difference between the observed and expected results is considered to be significant. If the probability is less than 0.01, the difference is highly significant. Thus, in our example, a ratio of 3 : 1 is an appropriate hypothesis, because the calculated χ^2 value of 2.4 is less than 3.84 with 1 df. Moreover, on this basis it can be inferred that the cross is between two heterozygotes where one allele has a dominant effect and the other is recessive. If, on the other hand, a χ^2 test is carried out under the presumption of a phenotypic ratio of 2 : 1 with the same data set, the χ^2 value is

$$\left[\frac{(720-666.7)^2}{666.7}+\frac{(280-333.3)^2}{333.3}\right]=12.78$$

With 1 df, this value is greater than the χ^2 value, with a probability of 0.001. The difference is highly significant, and it is extremely unlikely that a pheno-

typic ratio of 2:1 accounts for the observed values. Although not encountered by Mendel, a phenotypic ratio of 2:1 can occur when two heterozygotes are crossed and the homozygous dominant genotype is lethal or, in some instances, when the heterozygous and homozygous dominant genotypes are responsible for different phenotypes and the homozygous recessive genotype is lethal.

After the self-fertilization of *RrYy* plants, Mendel observed 315 plants that produced round and yellow seeds, 108 with round and green seeds, 101 with wrinkled and yellow seeds, and 32 with wrinkled and green seeds. The χ^2 value, assuming a ratio of 9:3:3:1, is

$$\left(\frac{(315-312.75)^2}{312.75}+\frac{(108-104.25)^2}{104.25}+\frac{(101-104.25)^2}{104.25}+\frac{(32-34.75)^2}{34.75}\right)=0.455$$

With 3 df, this χ^2 value has a probability greater than 0.90, which supports the hypothesis that a ratio of 9:3:3:1 is an appropriate description of the observed data. Chi-square tests of other data assembled by Mendel also show convincing agreement with expected ratios. To some statisticians, these results seemed "too good to be true." On this basis, it was thought Mendel might have manipulated his counts to conform to his hypotheses. Although this controversy will never be resolved completely, the biases in Mendel's data appear to be due to statistical and botanical issues and not deliberate falsification to accommodate theoretical expectations.

Multiple Alleles

In theory, any gene locus can have a large number of different alleles. At the DNA level, genes are sequences of nucleotide pairs that encode information for a product that, in most instances, is a protein. Any change of any one of the thousands of nucleotide pairs that comprise most normal genes creates an allele. But, for practical reasons, only nucleotide changes that cause observable phenotypic variations from the normal phenotype are usually designated as alleles.

Human genes are officially assigned symbols consisting of uppercase italicized letters by the International System for Human Gene Nomenclature (ISGN; http://www.gene.ucl.ac.uk/nomenclature/). These codes should reflect a feature of the condition that is determined by an allele of the normal gene, the name of the condition, or, preferably, the product of the normal gene. An asterisk (*) following a gene symbol is used to indicate that an allele of the gene is being considered. With few exceptions, which include various blood group gene systems, the alleles of a gene are presented as arabic numerals. The first two alleles of the adenosine deaminase gene are designated as *ADA*1* and *ADA*2*. A phenotype is represented by the same letter codes as the determining gene and its alleles, except that italics are not used and a space replaces the asterisk. The phenotype designation for the *ADA*1/ADA*1* genotype is ADA 1. For the heterozygote *ADA*1/ADA*2*, the designation for the phenotype is ADA 1, 2.

The names of genetic diseases such as Huntington disease, Alzheimer disease, and cystic fibrosis are often abbreviated and used as symbols for gene loci. Although any allele can occupy a locus, the expression "Huntington disease

gene," for example, can be confusing, because it is not always clear whether the normal (wild type) gene or an allele(s) responsible for the condition is being considered. When required, the normal allele of a gene is identified by adding **N* to the gene symbol, in this case, *HD*N*. Adding **D* or **R* to a gene symbol designates a dominant or recessive allele, respectively. When the product of a gene is determined, a more precise gene symbol is assigned to replace the more general designation. Originally, *CF* was the locus symbol for cystic fibrosis, but, after the protein that is encoded by the normal *CF* gene was discovered and named cystic fibrosis transmembrane conductance regulator (CFTR), the gene symbol was changed from *CF* to *CFTR*.

The ABO blood group gene locus at chromosome 9q34.2 in humans is one of the best known and most frequently cited examples of a multiple allele system. There are three major alleles, *ABO*A*, *ABO*B*, and *ABO*O*, that give rise to the A, B, AB, and O blood groups. The *ABO*A* and *ABO*B* alleles are dominant to *ABO*O*, and, when the *ABO*A* and *ABO*B* alleles occur together, both gene products can be distinguished phenotypically (codominance). Because of the dominance and codominance behavior of the alleles, four ABO blood group phenotypes are produced by the six genotypes (Table 3.5). The ABO blood groups are oligosaccharide chains linked to a lipid on the surface of red blood cells and other cell types. Almost everyone synthesizes the O type oligosaccharide (H substance, H antigen). The *ABO*A* allele encodes an enzyme, glycosyltransferase, which adds an *N*-acetylgalactosamine group to the H substance to form the A-type oligosaccharide. The *ABO*B* allele encodes a variant of the glycosyltransferase, which adds only a galactose group to the H substance to form the B-type oligosaccharide. The *ABO*O* allele produces an inactive protein that is not able to use the H substance as a substrate. With the genotype *ABO*A/ABO*B*, both glycosyltransferases are synthesized and both the A and B oligosaccharides are formed. At the level of the DNA sequences of the *ABO* gene locus, the *ABO*A* and *ABO*B* alleles differ from each other by four nucleotide pairs and the *ABO*O* allele has a single nucleotide pair deletion.

Human Genetics

Strategies for determining the inheritance of human traits differ markedly from those used to study the genetics of peas, fruit flies, roundworms, corn, or mice. Humans have small families and long generation times. Mendelian proportions can not be expected among the progeny of a single human marriage (mating).

Table 3.5 Genotypes and phenotypes of the ABO blood group.

Genotypes	Phenotypes
*ABO*A/ABO*A*	ABO A
*ABO*A/ABO*B*	ABO AB
*ABO*A/ABO*O*	ABO A
*ABO*B/ABO*B*	ABO B
*ABO*B/ABO*O*	ABO B
*ABO*O/ABO*O*	ABO O

And, of course, there is no possible way—taking into account ethical, moral, and practical considerations—that human matings can be controlled on the basis of the genotypes of the parents.

Yet genetic analysis of human traits can be studied effectively from two perspectives. First, data from large populations can be collected and mathematical methods applied to deduce whether a particular trait is inherited. Although adequate, this approach requires the examination of many individuals, an approach that can be arduous, expensive, and time consuming. Second, the pattern of inheritance of a particular trait can be followed within individual families (kindreds, kinships), preferably within large, extended multigeneration families. The examination of sets of relatives is often easier to conduct than population studies. Today, family studies are used widely for investigating the molecular genetics of human diseases.

The patterns of inheritance of a trait within families are visualized by constructing family trees (pedigrees). The term pedigree is an English idiom for the French phrase *pied de grue*, which means "foot of a crane" and was a whimsical way for the French to describe the lines of a genealogical chart. A pedigree used for genetic studies consists of a set of symbols describing both individual relationships and the history of a trait within a single or extended family (Figure 3.10). Pedigrees are essential experimental data for human geneticists.

A reliable set of clinical features that accurately categorizes a human phenotype (condition) is essential for determining whether the condition is inherited and for developing specific treatments. If the description of a condition is imprecise, then different genetic and nongenetic disorders may be lumped together, confusing analysis and treatment. Since the nineteenth century, clinicians have sought to create exact, inclusive descriptions of each human disease. A consistent, definitive set of signs and symptoms that accompanies a condition is commonly called a syndrome or, sometimes, a disease. Some syndromes are easy to diagnose, whereas others require a comprehensive medical assessment.

As a form of recognition and honor, often a syndrome or disease is given the last name(s) of the person(s) who first identified the symptoms that make up its significant and invariant features (hallmarks). For example, on the basis of initial descriptions, the names of John Down (1828–1896), George Huntington (1850–1916), and Alois Alzheimer (1864–1916) have become associated with specific human disorders. In the past, the possessive form (such as Down's syndrome, Huntington's disease, Alzheimer's disease, and many others) was used routinely. Today, in OMIM, the major human genetic database, and in some technical publications, the possessive case is omitted. In this way, the recognition of the discovery is retained, but the implied "ownership" of the condition is relinquished. In written form, the dropping of the apostrophe and letter "s" is easy to implement. However, in spoken language, "Alzheimer disease" just does not seem to roll off the tongue as easily as "Alzheimer's disease." Despite this dissonance, the possessive form for all syndromes, disorders, and diseases has been abandoned in this book.

For some studies, many different pedigrees must be assembled. In others, a single, large multigeneration pedigree may provide extensive genetic

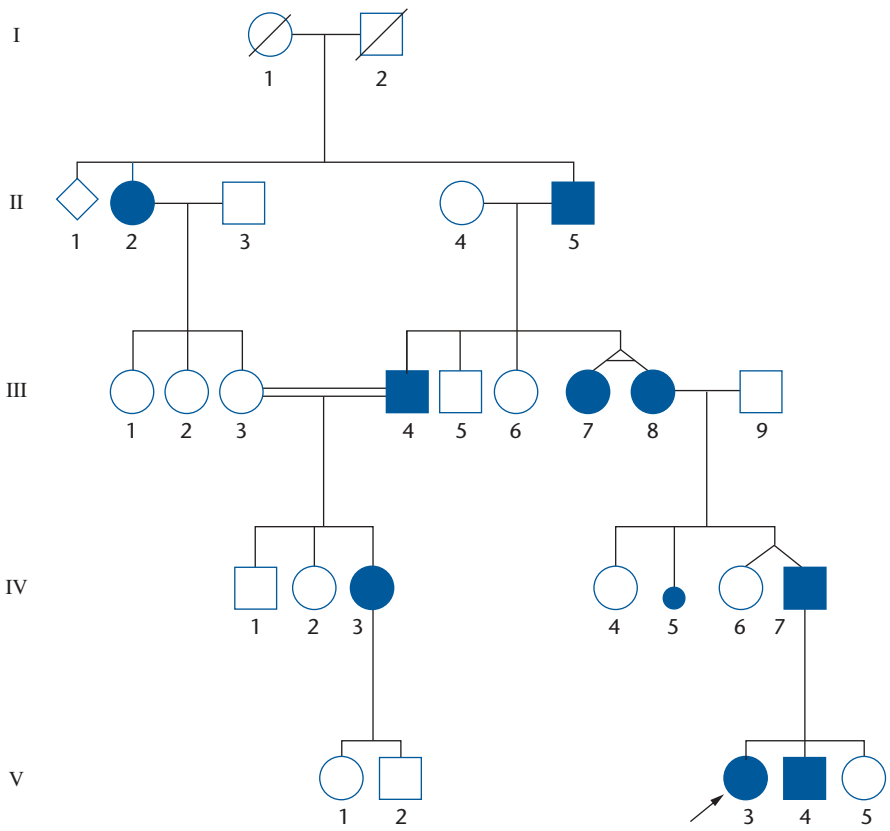


Figure 3.10 Interpreting standard pedigree notation. Circles represent females and squares represent males. Completely solid (filled in) symbols mark affected individuals. Roman numerals (I, II, III, etc.) designate the generations. Arabic numbers (1, 2, 3, etc.) specify the individuals in a particular generation. A binary code, such as II-5, is used to identify each individual within a pedigree. A horizontal line that joins male and female symbols is a relationship line and denotes a marriage. A double relationship line designates a marriage between closely related individuals. For example, III-3 and III-4 are first cousins. All the progeny with at least one common parent form a sibship. The horizontal line above a sibship is called a sibship line; a vertical line joining a relationship line to a sibship line is a descent line (line of descent). A vertical line to a symbol is an individual's line. A sibship is arranged in descending birth order, with the oldest offspring of a sibship on the left. A slash through a symbol (e.g., I-1) indicates that the particular individual was deceased at the time the pedigree was assembled. A diamond symbol (e.g., II-1) is used when the sex of the individual is not known. Diamond symbols used throughout a published pedigree indicate that researchers have complied with the request of pedigree members for anonymity. Monozygotic twins (III-7 and III-8) are shown with a forked line to each symbol from a single individual's line and a horizontal line meeting the two forked lines. For dizygotic twins (IV-6 and IV-7), only a forked line is drawn from a single individual's line. Symbols with reduced size (e.g., IV-5) indicate that the individual died in infancy or early childhood. In some instances, the age of an individual at the time the pedigree was created, date of birth, age at death, certain genotypes, or other relevant information are noted in text format below an individual's symbol. Often, to conserve space and because the genetic contribution of a spouse may not be relevant for a particular analysis, spouses are omitted from a pedigree and descent lines are drawn directly from a biological parent to a single offspring or a sibship line (e.g., IV-3 to V-1 and V-2). An arrow marks the individual (propositus if male, proposita if female, or proband) who acted as the initial source of the pedigree.

information. With unambiguous information, a human geneticist selects from only four different familial patterns to determine the mode of inheritance of a trait that resides at a single gene locus. Included in this category of single-gene (monogenic) traits are autosomal dominant, autosomal recessive, X-linked recessive, and X-linked dominant conditions.

Autosomal Dominant Inheritance

There are approximately 200 human genetic conditions known to be the result of dominant genes with loci disbursed among all of the autosomes. These autosomal dominant (ad, AD) disorders can affect any organ system and occur with different frequencies (Table 3.6).

A typical pedigree of an autosomal dominant condition has distinguishing features, which include the presence of affected individuals in successive generations, equal numbers of affected males and affected females, each affected individual usually having at least one affected parent, two affected parents having unaffected offspring, and unaffected individuals of the pedigree who marry unaffected individuals rarely having affected offspring (Figure 3.11).

Table 3.6 Catalog of some human autosomal dominant disorders.

Disorder	Prevalence	Description
Huntington disease	1/10,000	Late onset; degeneration in the cerebral cortex and basal ganglia; involuntary movement (chorea); dementia
Neurofibromatosis type 1	1/5,000	Multiple neurofibromas on the nerves of the head, neck, and body; pigmented (café-au-lait) spots
Tuberous sclerosis	1/5,800	Multisystem; growths (hamartomas) within the brain, eyes, skin, kidneys, heart, lungs, and skeleton
Myotonic dystrophy	1/8,000	Multisystem; prolonged muscle contraction (myotonia); variable muscle weakening and wasting (atrophy); cataracts; defective impulse conduction by the heart; inadequate gonadal function (hypogonadism)
Polycystic kidney disease	1/1,000	Genetically heterogeneous; variable age of onset; kidney cysts; decreased kidney concentrating ability; enlarged kidneys; hypertension
Retinitis pigmentosa	1/4,000	Genetically heterogeneous; progressive loss of night vision and visual acuity
Marfan syndrome	1/10,000	Multisystem; long fingers and toes (arachnodactyly); skeletal deformities; loose joints; ocular lens dislocation; impaired vision; cardiovascular disorders; lateral curvature of the spine (scoliosis); rupture of the aorta
Waardenburg syndrome	1/100,000	White forelock; premature graying; different-colored eyes; deafness
Hypercholesterolemia	1/500	High serum cholesterol levels; early-onset coronary artery disease
Osteogenesis imperfecta	1/10,000	Genetically heterogeneous; clinically heterogeneous; bone deformity; brittle bones; deafness; blue sclerae

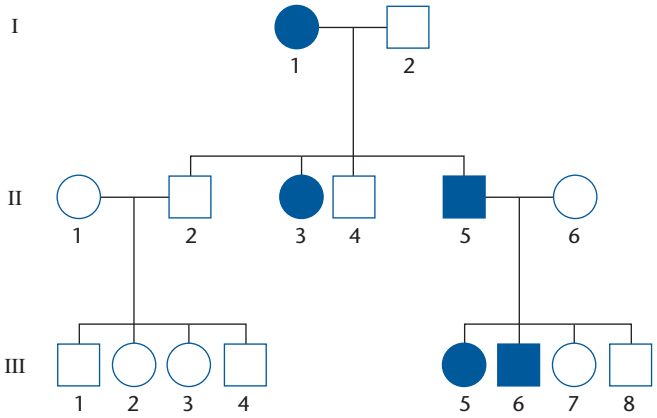


Figure 3.11 A sample pedigree with features of an autosomal dominant disorder.

Table 3.7 Catalog of some human autosomal recessive disorders.

Disorder	Prevalence	Description
Cystic fibrosis	1/2,500	Multisystem; defective chloride transport in epithelial tissues; blockage of ductules and small airways; severe lung disease; pancreatic insufficiency; chronic inflammation of the nasal sinuses (sinusitis); infertility
Lamellar ichthyosis	1/25,000	Disorder of the skin; disfigurement; large scales; variable redness
Wilson disease	1/40,000	Chronic liver disease; progressive neurological impairment; accumulation of copper in the liver, brain, and other tissues
Gaucher disease type 1	1/50,000	Increased blood cell breakdown in the spleen (hypersplenism); enlargement of the liver and spleen (hepatosplenomegaly); fragile bones
Friedreich ataxia	1/50,000	Onset at puberty; inability to coordinate muscles for voluntary movement (ataxia); inability to speak (dysarthria); muscle atrophy
Childhood spinal muscular atrophy	1/10,000	Genetically heterogeneous; variable; degeneration of the anterior horns of the spinal cord; weakness and wasting of the muscles; often fatal by 20 years of age or earlier
Phenylketonuria	1/10,000	Deficiency of liver enzyme phenylalanine hydroxylase; brain damage; mental retardation; excess phenylpyruvate in the urine; accumulation of phenylalanine in the blood
β-Thalassemia	1/20,000	Severe depletion of red blood cells (anemia); enlarged spleen (splenomegaly); bone deformities
Galactokinase deficiency	1/40,000	Inability to utilize galactose; cataracts; mild mental retardation
α_1-Antitrypsin deficiency	1/3,500	Breathlessness (emphysema); cirrhosis of the liver

Autosomal Recessive Inheritance

Approximately 900 different autosomal recessive (ar, AR) disorders that affect a range of organ systems are scattered among all the autosomes (Table 3.7). Two defective alleles at the same locus cause these disorders.

The distinctive features of a pedigree with an autosomal recessive disorder include unaffected parents who have affected offspring, equal numbers of affected males and females, all offspring affected when both parents are affected, and, in rare disorders, affected children who are the offspring of marriages between first cousins or other closely related family members (Figure 3.12).

The interpretation of the mode of inheritance from a single pedigree is not always reliable. If a defective recessive allele is frequent, then homozygous recessive individuals have a high probability of marrying heterozygotes. In these cases, an autosomal recessive condition may mimic an autosomal dominant pattern (Figure 3.13). Usually, the nature of the mode of inheritance becomes apparent after pedigrees from many different families with the same condition are assembled and examined.

X-Linked Inheritance

In fertile human males, half the sperm carry a Y chromosome and the other half an X chromosome. All unfertilized eggs of a female receive a single X chromosome. Consequently, within a population, half of the next generation will be XX (females) and the other half will be XY (males) (Figure 3.14). The segregation of the sex chromosomes during meiosis is an effective way to main-

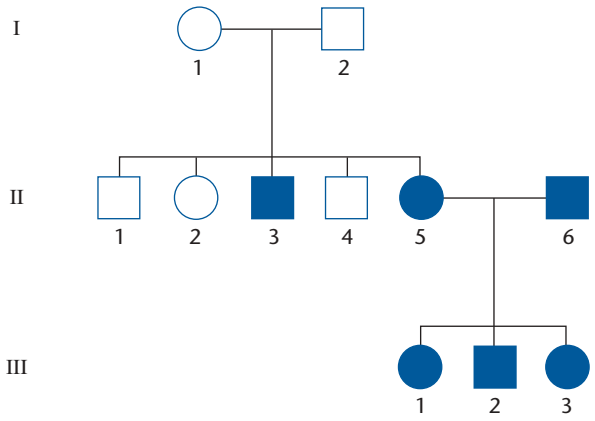


Figure 3.12 A sample pedigree with features of an autosomal recessive disorder.

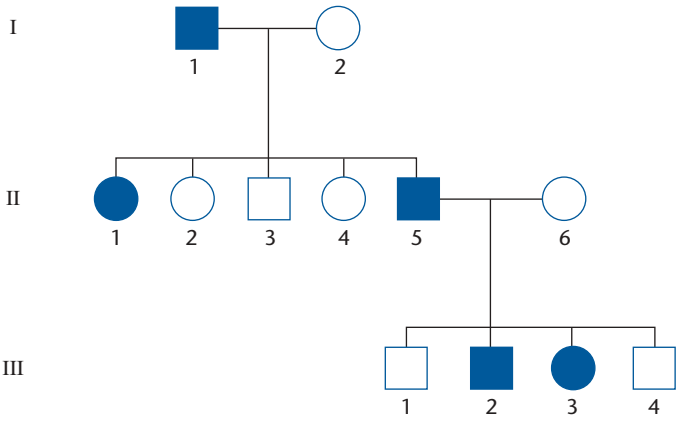


Figure 3.13 A pedigree showing a pseudodominant effect where the pattern of inheritance of a prevalent autosomal recessive disorder resembles an autosomal dominant condition.

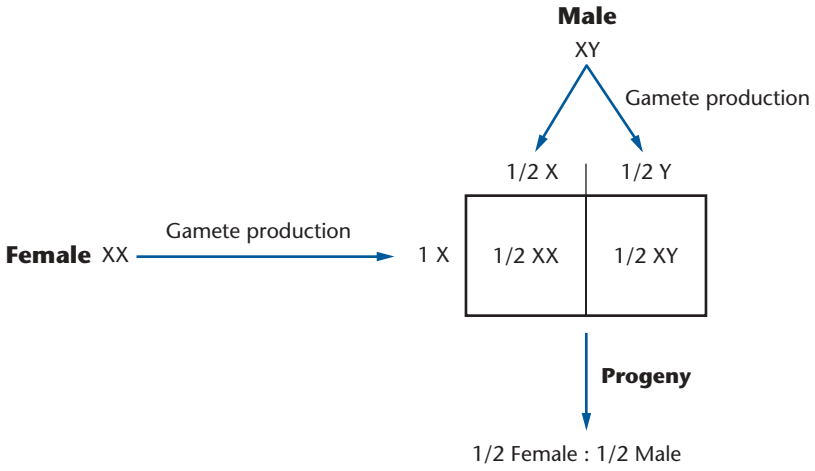
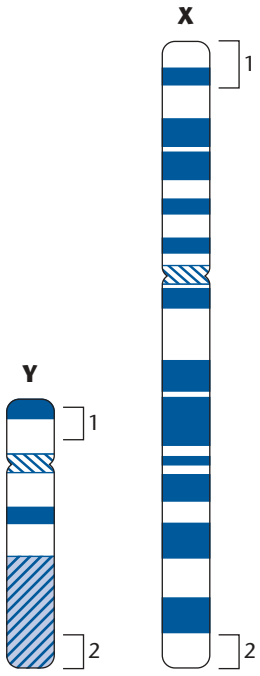


Figure 3.14 Distribution of sex chromosomes and determination of the sex ratio in humans.

tain a sex ratio close to 1:1 from one generation to the next. With this sex determination system, all sons inherit a Y chromosome from their fathers. In humans, an important gene for determining testis formation (*SRY*, sex determining region of the Y chromosome) is located at chromosome Yp11.3.

Except for two segments (at least), which are designated pseudoautosomal regions (Figure 3.15), the sex chromosomes do not share much chromosomal material. The pseudoautosomal regions are locations where synapsis and crossing over occur between the X and Y chromosomes. Synapsis of the X and Y chromosomes ensures the correct segregation of the sex chromosomes during meiosis. Both synapsis and crossing over are more common between the Xp-Yp (1-1) than the Xq-Yq (2-2) pseudoautosomal regions. Approximately 23 genes have been localized to the non-pseudoautosomal portion of the Y chromosome. These Y-linked genes have been called holandric genes. The genetic makeup of the X chromosome is well known. More than 285 X-linked genes have been identified (see, e.g., Table 3.8).

Figure 3.15 Human sex chromosomes. The numbered brackets mark the approximate locations of two pseudoautosomal regions. The Xp-Yp pseudoautosomal regions (1-1) synapse more frequently than the Xq-Yq pseudoautosomal regions (2-2).



A single X-linked recessive allele is sufficient to produce a phenotype in human males, because there is little homologous genetic information shared between the X and Y chromosomes. Consequently, in these cases, there is virtually no chance of a normal dominant allele masking the effect of a recessive allele. The dominant or recessive effect of an X-linked gene is determined by the phenotype in females because they have two X chromosomes. In pedigrees with an X-linked dominant condition, each generation usually has an affected individual, all daughters of affected males are affected, both sons and daughters of an affected heterozygous female may be affected, and, generally, twice as many females as males are affected (Figure 3.16). In pedigrees with X-linked recessive disorders, all sons of an affected mother are affected, affected fathers never transmit the trait to their sons, unaffected parents may have affected offspring, and, generally, there are more affected males than females (Figure 3.17).

Using Pedigrees to Study Human Genetic Disorders

The study of a disorder often begins when an individual with an unusual set of symptoms is referred to a clinician, who assesses the array of abnormalities and compares them with those of known syndromes. If there is a close match

Table 3.8 Catalog of some X-linked disorders.

Disorder	Prevalence	Description
Duchenne muscular dystrophy	Males: 1/3,500 Females: rare	Early onset; progressive muscle weakness; severe skeletal muscle degeneration
Fragile X syndrome	Males: 1/1,500 Females: 1/2,500	Mental retardation; long head; prominent jaw and forehead; long ears; loose joints; enlarged testes (macroorchidism) in males
Adrenoleukodystrophy	Males: 1/20,000 Females: very rare	Variable onset; progressive dementia; spastic paralysis; intellectual and neurological disturbances; adrenocortical insufficiency
Hemophilia A	Males: 1/5,000 Females: rare	Deficiency of blood clotting factor VIII; excessive bleeding from minor traumas; internal bleeding
Lesch–Nyhan syndrome	Males: 1/10,000 Females: rare	Deficiency of hypoxanthine-guanine phosphotransferase (HGPRT); mental retardation; muscular spasms, self-mutilation
Alport syndrome	Males: 1/5,000 Females: rare	Defective α5 chain of type IV collagen; clinically heterogeneous; progressive degeneration of the kidneys; deafness; ocular defects
Color vision defects	Males: 8/100 Females: 1/100	Deficiency in red or green or both red and green color vision
Ichthyosis	Males: 1/6,000 Females: rare	Deficiency of steroid sulfatase; dryness and fishlike scaling of the skin
Hypophosphatemia	Males: 1/20,000 Females: rare	Phosphate deficiency; softening of the bones; retarded growth; skeletal deformities; unresponsive to vitamin D treatment
Norrie disease	Males: rare Females: very rare	Blindness at birth as a result of abnormal proliferation of retinal tissue; later onset of deafness; mental retardation

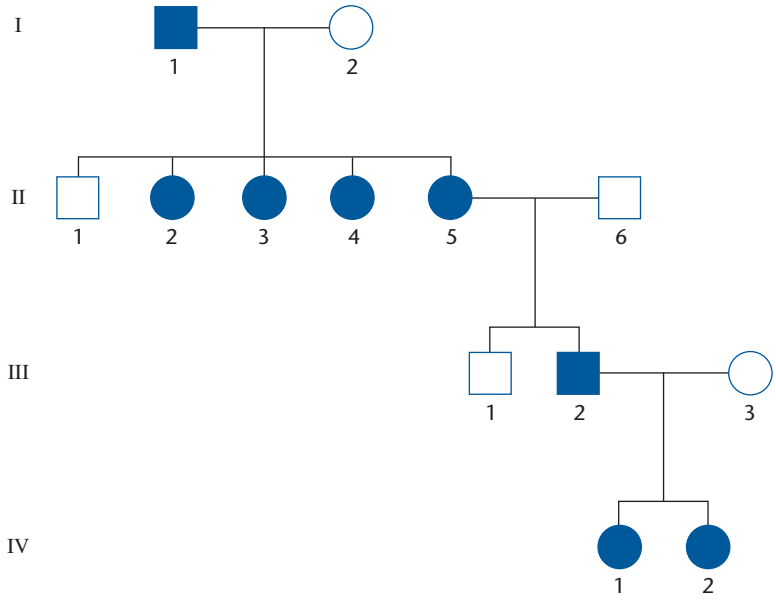


Figure 3.16 A sample pedigree with features of an X-linked dominant inherited disorder.

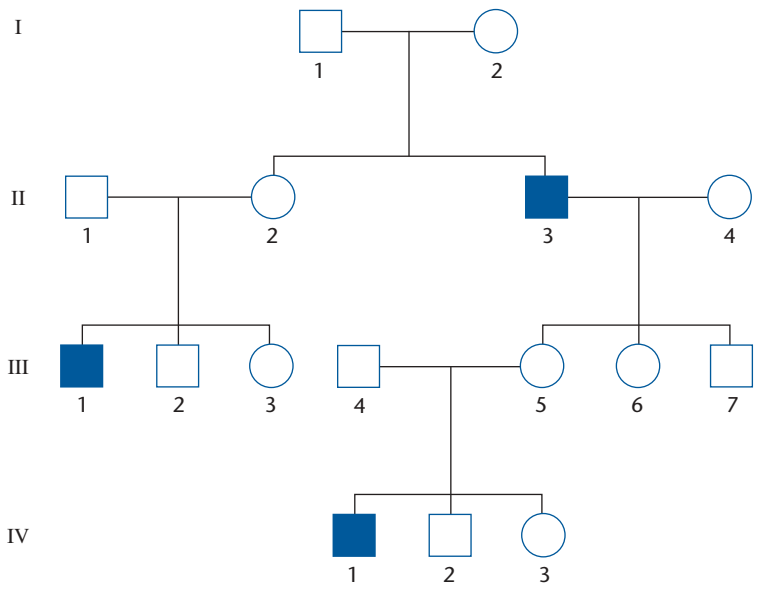


Figure 3.17 A sample pedigree with features of an X-linked recessive inherited disorder.

with an existing syndrome, then it is assumed that the condition is identified. If the signs of the condition are novel, further study may be undertaken to determine whether the condition is inherited or the result of an environmental factor. Accurate knowledge of the cause of a human disorder leads to the development of an effective treatment.

As a first step, the immediate family members of the first individual (proposita, propositus, proband) found to have a new or interesting condition are examined, and a pedigree is constructed. The pedigree is enlarged, if possible, by studying family archives, interviewing additional family members, and having others fill in questionnaires. The pattern of inheritance may be revealed at this stage, but a single pedigree, unless it is quite large (more than 50 members) and has many affected individuals, usually does not provide sufficient information to deduce the mode of inheritance. If the initial pedigree does not produce a definitive genetic interpretation, the clinician may seek other families with the same condition and other clinical geneticists who may have encountered either the same or a similar syndrome. In this way, the numbers of pedigrees may be increased and collaborative research established. The mode of inheritance should become evident after a number of different pedigrees have been assembled.

In the real world, analyses of human genetic disorders are not always straightforward. In some instances, different pedigrees with the same set of clinical symptoms may show completely different modes of inheritance. For example, retinitis pigmentosa (RP), which is the progressive degeneration of the retina of the eye, occurs as an autosomal dominant, an autosomal recessive, and a X-linked abnormality. There are at least 25 autosomal and 3 X-linked RP loci in humans. Genetic heterogeneity is the term used to describe different, nonallelic loci that produce the same phenotype. When faced with genetic heterogeneity, which appears to be more the rule than the exception, clinicians look for subtle physiological and physical differences that may distinguish the

effect of one locus from the others. In this way, the overall phenotype is often divided into various subtypes.

It is seldom apparent why different genes cause the same syndrome. However, conceptually, if somewhat simplistically, developmental processes or the proper functioning of organ systems can be viewed as pathways, similar to metabolic pathways. If there are a number of sequential steps that are required for a biological end result, where each step is mediated by the product of a different gene, then the loss of any one of these steps prevents the end result from being realized. In this way, nonallelic mutant genes that are involved in the same pathway could produce the same phenotype.

Other factors may confound pedigree analysis. In some instances, although an individual has a certain genotype, the expected phenotype is not produced. This phenomenon is called incomplete penetrance. More specifically, penetrance is a quantitative measure of the frequency of occurrence of the phenotype when a particular genotype or allele is present. With most genetic disorders, each time an individual has a specific genotype, the phenotype is evident. This represents complete (100%) penetrance. For other conditions, the penetrance of a genotype is incomplete (less than 100%). For example, if 87 out of 112 people with the same genotype show the condition and the remainder do not, then the penetrance for this disorder is 78% $[(87/112) \times 100]$ and nonpenetrance is 22% $\{[(112 - 87)/112] \times 100\}$.

Another confounding aspect of some genetic disorders is that onset of the condition does not always occur at birth. The age of onset of a disorder can range over decades, and with some genetic diseases, the condition does not appear until the fourth, fifth, or sixth decade after birth. Disparity in the age of onset of a disorder can cause members of a pedigree to be wrongfully classified at the time the pedigree is assembled.

Another potentially confusing circumstance occurs when the same allele in different individuals gives rise to diverse phenotypes. For example, the autosomal dominant disorder osteogenesis imperfecta (OI) is a condition that causes brittle bones, loose joints, fragile skin, deafness, soft teeth, and a blue tint to the outer layer (sclera) of the eyeball. Within a single family, the phenotype can vary considerably. Some family members show a minimal effect, such as blue sclerae, whereas others are almost totally incapacitated because of brittle bones. This type of phenotypic variation is called variable expressivity, variability, or clinical heterogeneity. With incomplete penetrance, a particular genotype has no apparent effect on some individuals. By contrast, with clinical heterogeneity, some aspect of the phenotype is observed in all individuals with the same genotype, although the degree of severity varies.

There are no simple explanations for incomplete penetrance, age-dependent penetrance, or clinical heterogeneity. Incomplete penetrance may be the result of the activity of a gene product of a nonallelic gene locus nullifying the effect of the disease-causing allele. Age-dependent penetrance may result over a period of time from cumulative tissue damage from an altered gene product. In these cases, the definitive clinical features that mark a disorder may not be evident until a certain level or threshold of tissue damage or cell death is reached or surpassed. Clinical heterogeneity can occur if nonallelic gene products modify the consequences of a disease-causing allele. Regardless of the basis

from the HUMAN GENETICS files

Calculating Mendelian Frequencies

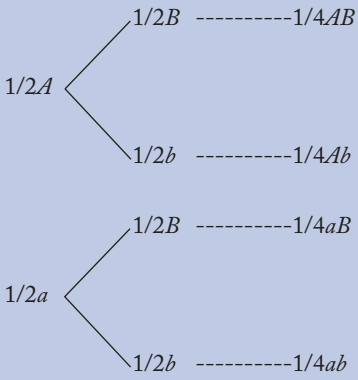
Solving problems is a practical skill like, let us say, swimming. We acquire any practical skill by imitation and practice. Trying to swim, you imitate what other people do with their hands and feet to keep their heads above water, and, finally, you learn to swim by practicing swimming.

G. POLYA (1887–1985)

There are simple rules and procedures for determining the frequencies of gametes, genotypes, and phenotypes for any cross with two or more gene pairs that assort independently. These methods use basic mathematical operations that are grounded on the predictable consequences of Mendel's laws of segregation and independent assortment. It is important to remember that most genetic calculations are concerned with combinations of independent events that have separate frequencies. One of the laws of probability states that the probability of independent events that occur together is the product of the frequencies of the individual events. The calculation of Mendelian frequencies depends on this multiplication rule. Also, it is helpful, although not necessary, to know the expected frequencies of the gametes and genotypes of the progeny from all monohybrid crosses, that is, $AA \times AA$, $AA \times Aa$, $AA \times aa$, $Aa \times aa$, and $aa \times aa$. Gametic, genotypic, and phenotypic frequencies are determined using either the branching (algebraic) or Punnett square (checkerboard) method.

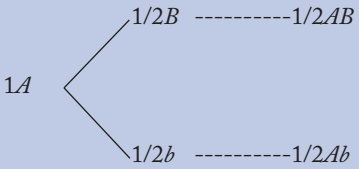
However, the Punnett square approach, which is used in the text of this chapter, has drawbacks when more than two loci are being tracked. First, the cells of Punnett squares from genotype and phenotype determinations must be checked for redundancy and those with the same result must be summed. Second, for many crosses the number of cells in a Punnett square table becomes difficult to handle efficiently. For example, determination of the genotypic ratio for *AaBbCc* × *AaBbCc* would require an 8-by-8 table. The branching method, which will be illustrated here with a two-locus cross, is more convenient because complete gamete, genotype, or phenotype information is obtained directly. The question is: What are the kinds and frequencies of gametes, genotypes and phenotypes from the cross *AaBb* × *AABb*, where *A* and *B* are completely dominant and the *A* and *B* loci are on nonhomologous chromosomes?

I. The kinds and frequencies of gametes produced by the *AaBb* parent.



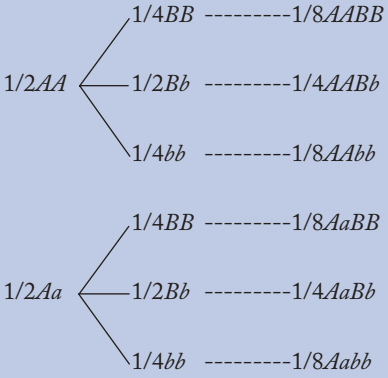
Rationale: Gametes contain only one allele of each gene pair because of segregation, and all gamete combinations are equally likely to occur. Thus, with an *Aa* genotype half the gametes will carry the *A* allele (1/2*A*) and the other half the *a* allele (1/2*a*). Similarly, half the gametes will carry the *B* allele (1/2*B*) and the other half the *b* allele (1/2*b*). And the *A* allele can be accompanied in a gamete by either a *B* or *b* allele. These possibilities are represented by the forked lines (branches) leading from the *A* (1/2*A*) and *a* (1/2*a*) alleles. The frequency of a particular kind of gamete is obtained by both multiplying the individual frequencies and combining all of the alleles along a branch. This information is noted to the right of the tally line (dashed line) for each branch.

II. The kinds of gametes produced by the *AABb* parent.



Rationale: All the gametes from the *AABb* parent will carry an *A* allele, hence the use of unity (1) and the *Bb* genotype produces two kinds of gametes in equal numbers. The gamete types are generated by two branches from 1*A*, one to 1/2*B* and the other to 1/2*b*.

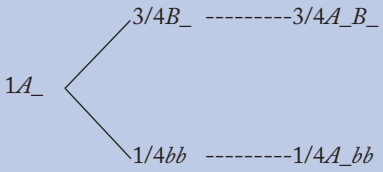
III. The kinds and frequencies of the genotypes produced by a *AaBb* × *AABb* cross.



Rationale: Initially, each pair of genes is considered separately. The $Aa \times AA$ component of the cross produces two types of genotypes in equal frequency (1/2*AA*, 1/2*Aa*). The $Bb \times Bb$ component of the cross produces three genotypes (1/4*BB*, 1/2*Bb*, 1/4*bb*). Each of the *AA* and *Aa* genotypes is expected to occur with each of the *BB*, *Bb*, and *bb* genotypes which is reflected in the branching pattern where each of the three possible combinations for the *B* locus occurs with either *AA* or *Aa*. The tally of the frequency and genotype for each branch is recorded. The fractions are converted to whole numbers to give, in this case, a genotypic ratio of 2:2:1:1:1:1.

continued

IV. The kinds and frequencies of the phenotypes produced by a *AaBb* × *AABb* cross.



Rationale: The genotypes of the offspring of the cross will be either *AA* or *Aa*, therefore, all the offspring will have the A phenotype. The dash denotes the presence of either allele. Consequently, the starting point for the branching method is 1*A*_. From the *Bb* × *Bb* part of the cross, 3/4 and 1/4 of the offspring will be *B*_ and *bb*, respectively. Interestingly, the phenotypic ratio is 3 : 1. In other words, this two-locus cross produces the same ratio as a cross between two heterozygotes, for example, *Aa* × *Aa*, with complete dominance.

Conclusion: The calculation of Mendelian frequencies for multiple locus crosses should not be intimidating. All that is required to solve these kinds of problems, in addition to an awareness of the consequences of monohybrid crosses, segregation, and independent assortment, is allocating enough space on a blank page to accommodate a branching pattern as it spreads out. Answering the question What are the kinds and frequencies of the gametes, genotypes, and phenotypes from the cross *AaBBCcDdeeFfGG* × *aabbCcDDEeFfgg*, where all alleles are codominant and each locus is on a different chromosome? may be tedious, but figuring it out is elementary. Give it a try!

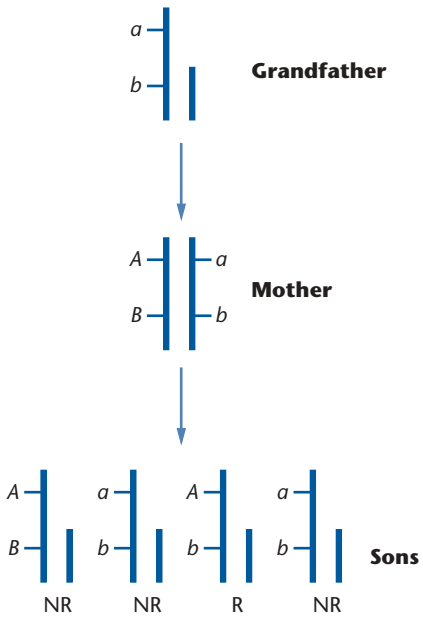
of each of these confounding factors, they make the interpretation of the mode of inheritance of a particular disease with pedigree analysis more troublesome.

Detection and Estimation of Genetic Linkage in Humans

Before the advent of recombinant DNA technology in the early 1980s, the detection and estimation of genetic linkage in humans was difficult, laborious, and often unsuccessful. In general, linkage studies in humans had inherent problems. The genetic phase of either parent was rarely known, which obscures the distinction between recombinant and nonrecombinant types among the progeny. In addition, the statistical reliability of recombination fraction determinations was limited by the small size of most families. Despite these restrictions, a number of approaches were devised to analyze human linkage involving two gene loci.

Theoretically, the presence of a single X chromosome in human males provides an excellent opportunity for estimating map distances between two or more gene loci. In this special circumstance, the alleles of loci on the X chromosome are registered directly as phenotypes in males. Mothers who are doubly heterozygous for X-linked loci will have sons who carry either recombinant or nonrecombinant X chromosomes. If the phase of the alleles of two gene loci in these mothers is known, then recombinant and nonrecombinant types can be directly distinguished among the sons. The genotypes of the fathers, in these cases, are irrelevant, because sons inherit X chromosomes only from their mothers. In some instances, the phase of a doubly heterozygous mother can be ascertained from the phenotype of her father. For example, if a father shows two X-linked recessive traits and one of his daughters exhibits the dominant phenotype for both of these traits, then she would be a *cis*-phase, double heterozygote (*AB*/*ab*) (Figure 3.18). This so-called "grandfather method" of linkage determination depends on scoring the phenotypes for two loci of sons of large numbers of different, phase-known doubly heterozygous mothers. In this case, the recombination fraction between two specific gene loci would be equivalent to the total recombinant chromosomes (R) divided by the

Figure 3.18 Mapping the X chromosome with the "grandfather" method. The genetic phase of two or more X-linked loci in a daughter (Mother) is derived from knowledge of the X-linked alleles carried by her father (Grandfather). In turn, this information is used to determine which of the mother's sons (Sons) received a nonrecombinant (NR) or recombinant (R) X chromosome. Here, the grandfather carries two recessive genes at loci *A* and *B* on his X chromosome, and the daughter is a double heterozygote in the *cis* phase. The X chromosome is shown with alleles at the *A* and *B* loci, and the Y chromosome is the shorter solid line.



total recombinant chromosomes plus the total nonrecombinant chromosomes (NR), or

$$\frac{\Sigma R}{\Sigma R + \Sigma NR}$$

There are constraints to this strategy. Grandfathers are not always available to be typed. As a result, the phase of a presumptive doubly heterozygous mother is unknown. Moreover, the occurrence of heterozygosity at two specific loci in a large number of families is not often encountered. Consequently, before the 1980s, it was not possible to assemble an extensive, unequivocal linkage map of the human X chromosome with traditional genetic approaches. At the time, there were too few loci and not enough alleles.

The Logarithm of the Likelihood Ratio Method of Linkage Analysis: LOD Score

In addition to the direct method of counting large numbers of recombinants and nonrecombinants to determine the recombination fraction between two loci in humans, a broader-based, indirect method was needed that (a) could rigorously distinguish between independent assortment and linkage, (b) was not necessarily dependent on knowledge of the actual phase of the doubly heterozygous parent, (c) could combine information from a number of different families, and (d) if linkage was evident, could give an estimate of the recombination fraction. In 1955, N. E. Morton (b. 1929) formulated such a method, which has become the standard way of determining linkage in humans.

In human linkage studies the recombination fraction is symbolized by the Greek letter theta (θ). Morton's method compares the likelihood [$L(\theta)$], for any recombination fraction (θ), that two loci within a group of siblings (sibship) are linked to the likelihood [L(0.50)] that the two loci are not linked. In the case of linkage, because the recombination fraction (θ) is unknown, it can have any value less than 0.50 and greater than or equal to zero ($0 \leq \theta < 0.50$). When two loci assort independently, the value of θ, by definition, is 0.50. In other words, when half of the gametes produced by a double heterozygote contain new genetic combinations, the two loci are either on nonhomologous chromosomes or so far apart on the same chromosome that they appear to be on different chromosomes. Consequently, if $L(\theta)$ equals L(0.50), then the two loci are not linked. The common logarithm of the ratio of these two likelihoods, $\log[L(\theta)/L(0.50)]$, is the log-of-odds ratio, which, in a contracted form, is called the LOD score. The letter Z is used to symbolize a LOD score, and the term $Z(\theta)$ represents a LOD score for a particular value of θ, where $0 \leq \theta < 0.50$.

Part of the determination of $L(\theta)$ depends on the probability of occurrence of a particular combination of recombinant and nonrecombinant chromosomes among the members of each sibship under study. The probability that an offspring will receive either nonrecombinant chromosome from a doubly heterozygous parent is $1/2(1 - \theta) + 1/2(1 - \theta)$, or $1 - \theta$. Similarly, the probability that an offspring will receive either recombinant chromosome from a doubly

heterozygous parent is $1/2\theta + 1/2\theta$, or θ. If each of the individuals of a five-member sibship has a nonrecombined chromosome that originated from a doubly heterozygous parent, then the probability of this happening would be $K(1 - \theta)^5$, where $(1 - \theta)$ represents the frequency of receiving a nonrecombined chromosome; the exponential term indicates the number of sibs with a nonrecombined chromosome or, more precisely, the number of nonrecombinant chromosomes in the sibship; and K is the coefficient for this case. When all the components of a term are the same (either all nonrecombinants or all recombinants), the coefficient equals 1. If all individuals of a four-member sibship receive a recombined chromosome from a doubly heterozygous parent, then the probability of this occurring would be $(\theta)^4$. And, if a nine-member sibship has five nonrecombinant types and four recombinant types, then the overall probability of this combination is $K(1 - \theta)^5(\theta)^4$, where K is 126 [9!/(5!4!)]. The calculation of a LOD score is based on a ratio where the coefficients of the terms of the numerator and denominator are the same and, therefore, factor out. The coefficients are commonly disregarded in linkage analyses.

A single sibship will be used to illustrate how LOD scores are calculated (Figure 3.19). The notations *B* and *O* in Figure 3.19 represent the alleles *ABO*B* and *ABO*O* of the ABO blood group, and the solid symbols denote the presence of a completely penetrant, autosomal dominant trait called nail-patella syndrome (NPS). The principal features of NPS are abnormal growth of fingernails and toenails and a reduced or absent kneecap (patella). The gene symbol for NPS is *LMX1B*, formerly *NPS1*. In this section, the *NPS1* designation will be used and the normal and dominant alleles are *NPS1*N* and *NPS1*D*, respectively. NPS is a good trait for linkage studies, because it is easily diagnosed, does not affect viability or fertility, and is present at birth.

Individual I-2 in Figure 3.19 is heterozygous for NPS, because among his offspring there are both affected and unaffected individuals. In addition, I-2 is heterozygous at the ABO locus (*ABO*B*/*ABO*O*), because among his offspring are both type B and type O phenotypes, which is consistent with his spouse (I-1) being *ABO*O*/*ABO*O*. Consequently, I-2 is a double heterozygote (*NPS1*D*/*NPS1*N*, *ABO*B*/*ABO*O*) for these two autosomal loci. If the *ABO* and *NPS1* loci were linked, then the phase of I-2 is unknown (phase unknown). It could be either *ABO*B NPS1*D*/*ABO*O NPS*N* (phase 1) or *ABO*B NPS1*N*/*ABO*O NPS1*D* (phase 2). Or, with an abbreviated notation, the two possible phases would be *BD*/*ON* (phase 1) or *BN*/*OD* (phase 2).

If it is assumed that both the *ABO* and *NPS1* loci are linked and the genotype of I-2 is in phase 1 (*ABO*B NPS1*D*/*ABO*O NPS1*N*), then, among his

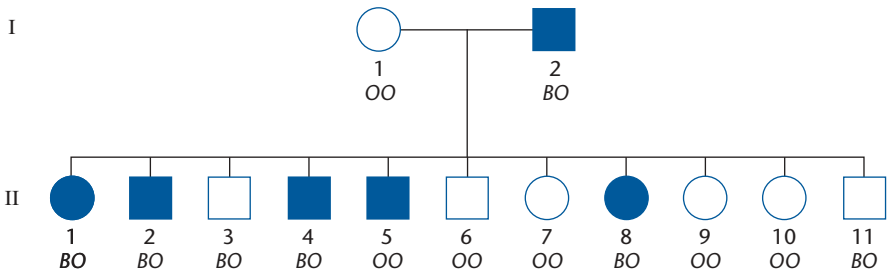


Figure 3.19 A sample pedigree for demonstrating the LOD score method. Solid symbols represent individuals with nail-patella syndrome. The letters below each symbol designate the ABO blood group genotype. The ABO blood group alleles are abbreviated with *O* and *B* for *ABO*O* and *ABO*B*, respectively.

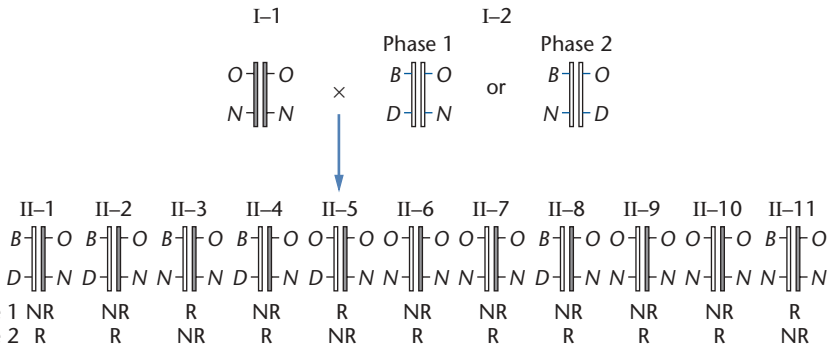


Figure 3.20 The genetic organization of nail-patella and ABO blood group alleles of each member of the pedigree shown in Figure 3.19, under the assumption that these two loci are linked. The ABO blood group allele designations are abbreviated with *O* and *B* for *ABO*O* and *ABO*B*, respectively. The normal and dominant alleles for the nail-patella syndrome locus are presented as *N* and *D*, respectively. Individual I-2 could be in either of two genetic phases (phase 1, phase 2). The bars with no fill represent the chromosomes of individual I-2 and the chromosomes inherited by the offspring from I-2. The filled bars represent chromosomes of individual I-1 and the chromosomes inherited by offspring from I-1. For phase 1 and phase 2, the chromosome inherited from individual I-2 by each offspring is identified as nonrecombinant (NR) or recombinant (R).

progeny, individuals II-1, II-2, II-4, II-6, II-7, II-8, II-9, and II-10 would be nonrecombinant types, because they received either the *ABO*B NPS1*D* or *ABO*O NPS1*N* chromosome from him (Figure 3.20). All the offspring receive an *ABO*O NPS1*N* chromosome from I-1, because she is doubly homozygous (*ABO*O NPS1*N/ABO*O NPS1*N*). In this context, the genetic contribution of I-1 to her offspring is known and irrelevant for the analysis of linkage. Based on the assumption of a phase 1 genetic organization of I-2, individuals II-3, II-5, and II-11 each have received a recombinant chromosome. By assuming phase 1, then, the combination of nonrecombinant and recombinant chromosomes is $(1 - \theta)^8(\theta)^3$.

The genotype of I-2 could be in phase 2 (*ABO*B NPS1*N/ABO*O NPS1*D*) as well. Accordingly, II-3, II-5, and II-11 would have received nonrecombinant chromosomes, and each of the remaining members of the sibship would have inherited a recombined chromosome from I-2 (Figure 3.20). The combination for this sibship is $(1 - \theta)^3(\theta)^8$.

Because the two possible genetic phases for I-2 are equally likely, then the overall likelihood [$L(\theta)$] for the sibship is $1/2(1 - \theta)^8(\theta)^3 + 1/2(1 - \theta)^3(\theta)^8$. The value for this expression can be computed for different values of θ. A commonly used set of θ values is 0, 0.001, 0.05, 0.10, 0.2, 0.3, 0.4, and 0.50. If time is not an issue, a full range of values for θ between 0 and 0.50 can be used. After a set of values is chosen, the logarithm of the ratio of the likelihood of the sibship for each value of θ other than 0.50 to the likelihood for θ set to 0.50 is calculated. For example, with $\theta = 0.10$, then

$$\frac{L(0.10)}{L(0.50)} = \frac{1/2(1-0.10)^8(0.10)^3 + 1/2(1-0.10)^3(0.10)^8}{1/2(1-0.50)^8(0.50)^3 + 1/2(1-0.50)^3(0.50)^8} = \frac{2.152 \times 10^{-4}}{4.883 \times 10^{-4}} = 0.441$$

The common logarithm of 0.441 is –0.356, which represents the LOD score for this ratio. In other words, Z(0.10) equals –0.356.

If the phase of I-2 is known (phase known), then the likelihood for the sibship will also be known. For example, if I-2 has the phase 1 genotype (*ABO*B NPS1*D/ABO*O NPS1*N*), then, as noted above, the likelihood for the sibship would be $(1 - \theta)^8(\theta)^3$. The Z score with θ set to 0.10 would be

$$\log \frac{(1-0.10)^8(0.10)^3}{(1-0.50)^8(0.50)^3} = \log \frac{4.305 \times 10^{-4}}{4.883 \times 10^{-4}} = -0.055$$

Or, if I-2 has the phase 2 genotype (*ABO*B NPS1*N/ABO*O NPS1*D*), then the value for Z(0.10) would be

$$\log \frac{(1-0.10)^3(0.10)^8}{(1-0.50)^3(0.50)^8} = -4.826$$

For the phase-unknown condition, the Z scores for the sibship shown in Figure 3.19 range from –5.993 for θ equal to 0.001 to +0.029 for θ equal to 0.45 (Table 3.9). When at least one recombinant type is present in a sibship and θ is set to 0, mathematically the Z score is minus infinity (–∞). The results in Table 3.9 indicate that the maximum LOD score (Z_{max}) falls near θ equal to 0.30. Additional calculations with a full range of θ values from 0.20 to 0.40 establish that the Z_{max} is +0.214 at θ equal to 0.276. A Z_{max} of +0.214 is not sufficient to support the likelihood that the *ABO* and *NPS1* loci are linked. By convention, a maximum LOD score of +3.000 or greater is used as the criterion that two autosomal loci are linked, because the odds in favor of linkage with this LOD score are 1000 to 1 or greater. For X-linked genes, because both loci are known to be on the same chromosome, the standard for linkage is set to a maximum Z score of +2.000 or greater, which represents odds of 100 to 1 or greater in support of linkage. A LOD score of –2.000 is considered to be sufficient to exclude linkage between two loci, because there is only 1 chance in 100 that the two loci are linked.

To detect and estimate linkage, Z scores for the range of values for θ from a number of different sibships must be determined before the maximum Z score for a particular value of θ is obtained. The conversion of the ratio of the likelihood for each sibship to the common logarithm allows each $Z(\theta)$ value to be summed. In a thorough study of the linkage between the *ABO* and *NPS1* loci, 25 pedigrees, including several sibships, gave a Z(0.10) of +31.235 (Table 3.10). These data show clearly that the two loci are linked. Had they not been linked, all values of $Z(\theta)$ would have been less than +3.000.

The value of θ at Z_{max} gives a rough estimate of the recombination fraction between two linked loci. Although a precise Z_{max} was not cited in the original study of the *ABO* and *NPS1* loci, the Z score with θ set to 0.10 was the highest for the range of calculated recombination fractions, which suggests that these two loci are approximately 10 cM apart. It should be stressed that with the various NPS pedigrees in the study, different *ABO* alleles were linked to the

Table 3.9 Z scores, based on the phase-unknown condition, for various values of θ for the sibship shown in Figure 3.19.

θ	0	0.001	0.01	0.05	0.10	0.20	0.30	0.40	0.45
Z score	–∞	–5.993	–3.025	–1.071	–0.356	0.138	0.209	0.095	0.029

Table 3.10 Cumulative Z scores for various values of θ for the *ABO* and *NPS1* loci.

θ	0.05	0.10	0.15	0.20	0.25	0.30	0.40
Z score	28.159	31.235	30.405	27.756	23.983	19.434	9.048

Adapted from Renwick JH, Schulze J (1965). Male and female recombination fractions for *nail-patella*:*ABO* linkage in man. *Ann Hum Genet* **28**:379–392.

NPS1 locus. In other words, there is no specific association between one particular *ABO* allele and the *NPS1* locus. Unless proven otherwise, genetic linkage occurs between loci and not certain alleles. It should also be noted that the LOD score method does not reveal the autosomal location of two linked loci. Additional studies, as described in a later chapter, were required to show that the *ABO* and *NPS1* (*LMX1B*) loci are located at 9q34.2 and 9q33.3, respectively.

The reliability of LOD score calculations relies on a clear understanding of the mode of inheritance of the human disease being studied. In addition, erroneous LOD scores can occur if an individual is considered to be unaffected at the time of the study but later develops signs of the disease. Other factors, such as incomplete penetrance, unequal male and female recombination frequencies, missing information, and misdiagnoses can also undermine the accuracy of LOD score determinations.

The impact of a single misdiagnosis on a LOD score can be significant. For example, with a sibship of six progeny where the phase is known and all offspring are scored as nonrecombinants, the Z_{max} is 1.806 at θ of 0. If one member of this sibship is actually a recombinant type, then the Z_{max} becomes 0.631 at θ of 0.1075. The decrease in the Z_{max} for this sibship is 1.175. Two or three misdiagnoses in an extended pedigree can change Z_{max} from a value that supports linkage to one that indicates linkage is unlikely. From a practical standpoint, it has been recommended that where diagnosis, incomplete penetrance, and age of onset may be important factors in a linkage study, the data should be reanalyzed by arbitrarily changing the diagnostic status of each member, one at a time, in each pedigree of the study. Under these conditions, the recalculation of the Z_{max} will pinpoint, if present, a particular misdiagnosed individual or individuals, who contribute significantly to the LOD score. For the most part, LOD scores are calculated by computer programs with processing options that attempt to take into account the impact of the various complicating factors. These programs also calculate the full range of LOD scores and determine the maximum LOD score.

key terms

allele
autosomal dominant
autosomal recessive
backcross
χ^2 test
chromatid
***cis* phase**
clinical heterogeneity
codominance
complete dominance
coupling
crossover
digenic
dizygotic
dominance
dominant
double crossover (DCO)
F_1 generation
F_2 generation
fertilization
gamete
gene
genetic distance
genetic heterogeneity
genetic map
genotype
grandfather method
heterozygote
homozygote
independent assortment
interference
ISGN
linkage
locus (loci)
LOD score
map unit (mu)
monogenic
monozygotic
multiple alleles
nonrecombinant chromosome
P generation
pedigree
penetrance
phenocopy
phenotype
polygenic inheritance
proband
progeny
proposita
propositus
pseudoautosomal region
recessive
recombinant chromosome
recombination frequency
repulsion
single crossover (SCO)
segregation
sibship
test cross
three-point cross
***trans* phase**
variable expressivity
X-linked dominant
X-linked recessive
$Z(\theta)$
zygote

summary

Certain biological traits are determined by discrete entities called genes that are passed on from one generation to the next in precise, predictable patterns. Genes exist as pairs on homologous chromosomes, with each gene occupying a locus on a chromosome. Alternative forms of a gene are known as alleles. Some alleles have a recessive effect, where two copies of the same allele are required to produce a certain trait. Other alleles have a dominant effect, where a single copy of such an allele in the presence of a recessive allele is sufficient to create the same biological effect as two copies of the dominant allele.

Each gamete receives one gene of each pair (segregation), and, during fertilization, any one gamete is equally likely to combine with any other gamete, producing zygotes with two copies of all genes and all possible gene combinations. When gene loci are on nonhomologous chromosomes, all possible combinations of single alleles occur in the gametes (independent assortment) and, after fertilization, theoretically, all possible genetic combinations will be formed. Because of the quantitative aspects of Mendelian genetic analysis, the χ^2 test is often used to test the validity of a genetic hypothesis indicated by the observed data from a particular cross.

Gene loci on the same chromosome arm tend to be inherited together and usually do not assort independently from one another. Such loci are not absolutely linked to each other, because reciprocal physical exchanges (crossovers) occur at fixed frequencies between gene loci during meiosis. The frequency of recombination between two loci represents a genetic distance. For loci that are linked at 10% recombination or less, 1% recombination is equivalent to 1 map unit (mu). For distances that are greater than 10 mu, the observed frequency of recombination is an underestimate of the actual genetic distance, and a map function can be used to correct the map distance. High-resolution genetic maps can be constructed from test crosses with three or more closely linked gene loci. Three-point test crosses allow double crossovers between the outside loci to be scored as recombinants, if the crossovers flank a middle locus. Otherwise, a double crossover between two loci does not produce a novel recombinant chromosome.

Mendelian genetic analysis, which depends on calculating the proportions among many offspring, is not effective for studying the inheritance of traits in humans. Pedigree analysis of large multigeneration families or many small families with the same condition is often used to deduce whether a trait is inherited. The pattern of inheritance of the same condition in many pedigrees reveals whether the trait is autosomal dominant, autosomal recessive, X-linked dominant, or X-linked recessive. Misdiagnoses, incomplete penetrance, and variable age of onset of a condition may obscure the exact mode of inheritance of a human trait.

Genetic linkage in humans is often detected by implementing the LOD score method. With this test, the logarithm of the ratio of the likelihood of linkage at various recombination fractions (θ) to the likelihood of independent assortment between the two loci under study is determined for a number of sibships from different families. For an autosomal or an X-linked trait, the maximum LOD score must be at least +3.000 or +2.000, respectively, if the two loci are to be considered linked. Linkage is excluded if a maximum LOD score of –2.000 or less is obtained.

References

Bennett, R. L. et al. 1995. Recommendations for standardized human pedigree nomenclature. Pedigree Standardization Task Force of the National Society of Genetic Counselors. *Am J Hum Genet* **56**:745–752.

Fairbanks, D. J. and B. Rytting. 2001. Mendelian controversies: a botanical and historical review. *Am J Bot* **88**:737–752.

Hartl, D. L. and V. Orel. 1992. What did Gregor Mendel think he discovered? *Genetics* **131**:245–253.

Hodge, S. E. and D. A. Greenberg. 1992. Sensitivity of lod scores to changes in diagnostic status. *Am J Hum Genet* **50**: 1053–1066.

Morton, N. E. 1955. Sequential tests for the detection of linkage. *Am J Hum Genet* **7**:277–318.

Morton, N. E. 1995. LODs past and present. *Genetics* **140**:7–12.

Olby, R. 1985. *Origins of Mendelism*. 2nd ed. University of Chicago Press, Chicago and London.

Ott, J. 1992. *Analysis of Human Genetic Linkage*. Johns Hopkins University Press, Baltimore and London.

Renwick, J. H. and J. Schulze. 1965. Male and female recombination fractions for *nail-patella*: *ABO* linkage in man. *Ann Hum Genet* **28**:379–392.

Risch, N. 1992. Genetic linkage: interpreting lod scores. *Science* **255**:803–804.

Risch, N. and L. Giuffra. 1992. Model misspecification and multipoint linkage analysis. *Hum Hered* **42**:77–92.

Shows, T. B. et al. 1987. Guidelines for human gene nomenclature. An international system for human gene nomenclature (ISGN, 1987). *Cytogenet Cell Genet* **46**:11–28.

Steinhaus, K. A. et al. 1995. Inconsistencies in pedigree symbols in human genetics publications: a need for standardization. *Am J Med Genet* **56**:291–295.

Terwilliger, J. D. and J. Ott. 1994. *Handbook of Human Genetic Linkage*. Johns Hopkins University Press, Baltimore and London.

Wain, H. M. et al. 2002. Guidelines for human gene nomenclature. *Genomics* **79**:464–470.

Wain, H. M. et al. 2002. Genew: the human gene nomenclature database. *Nucleic Acids Res* **30**:169–171.

Weiling, F. 1991. Historical study: Johann Gregor Mendel 1822–1884. *Am J Med Genet* **40**:1–25.

Yamamoto, F. et al. 1990. Molecular genetic basis of the histo-blood group ABO system. *Nature* **345**:229–233.

review questions

1. What are the expected gamete types, gamete frequencies produced by each parent, and frequencies of genotypes and phenotypes among the offspring from the cross *AaBbcc* × *aaBBCc*, where each locus is on a different chromosome and each dominant allele is completely dominant?
2. The *aabb* class among the offspring from the cross *AaBb* × *AaBb* makes up 16% of the total offspring. Describe the genetic basis for this result.
3. From a test cross with two linked loci, the observed numbers of the four classes of the progeny are 420, 440, 80, and 60. Using the χ^2 test, show whether the map distance between the two loci is likely to be 14 map units.
4. From the cross *AaBbCc* × *aabbcc*, the following phenotypic results were observed:

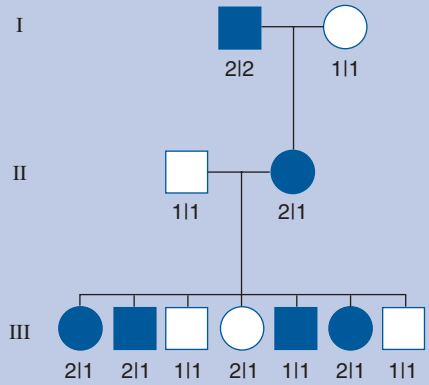
ABC	100	aBC	400
ABc	400	aBc	100
AbC	100	abC	400
Abc	400	abc	100

   What are the genetic relationships among these three loci?
5. The map distance from locus *A* to locus *B* is 5 map units and from locus *B* to locus *C* is 10 map units. Locus *B* is in the middle of the three loci. Assuming no interference, what phenotypes and genotypes and how many of each are expected among 2000 progeny from the cross *ABC*/*abc* × *abc*/*abc*?
6. With a five-point test cross, the two most frequent phenotypic classes among the progeny are ABCDE and abcde and the two least frequent classes are ABCde and abcDE. If locus *A* is at one end of this linkage group, what is the likely order of the five loci?
7. A woman who is heterozygous for an autosomal dominant disorder and homozygous recessive for an X-linked recessive condition marries a man who is normal for both of these traits. List all the possible genotypes and phenotypes with respect to these two traits that might occur among their male and female offspring.
8. What is genetic heterogeneity?
9. What is pseudodominance?
10. What is expressivity?
11. What criteria does the LOD score method fulfill for linkage analysis in humans?
12. What is a significant LOD score? Explain.
13. Distinguish between *cis* and *trans* genotypes.
14. What are the drawbacks of "grandfather" mapping?
15. Draw a three-generation pedigree illustrating the features of autosomal dominant inheritance.
16. Draw a pedigree with a first-cousin marriage.
17. Determine the Z(0.01) values for the two loci in both of the phase-known autosomal dominant pedigrees shown below. Affected individuals are shown with solid symbols, and alleles of the second locus are presented as numbers separated by a vertical line beneath each symbol in the pedigrees. Which of

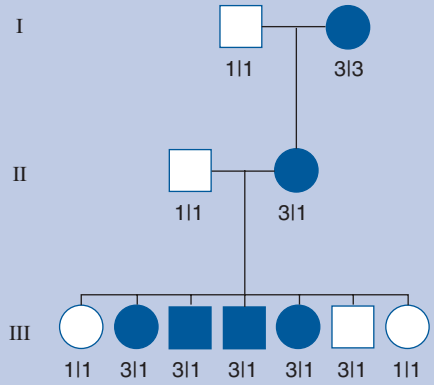
continued

the offspring in generation III of both pedigrees are nonrecombinant types? Which are recombinant types? What conclusion can be reached about genetic linkage in this case? How should this study proceed?

A



B



18. Discuss the advantages and disadvantages of crosses with three or more loci at a time.

19. Discuss how incomplete penetrance, age-of-onset differences, and misdiagnoses can affect LOD score determinations.

20. Determine the genetics, chromosome location, and other features of the *PAH* gene. Go to http://www.gene.ucl.ac.uk/nomenclature/ and submit PAH and then examine the various links such as GeneCards, LocusLink, OMIM, etc.

chapter 4

The Molecular Biology of the Gene

. . . nothing is more monarchial than a molecule of DNA.
SALVADOR DALI (1904–1989)

The central generalization of modern genetics is that all hereditary "know-how" at the molecular level is embodied in nucleic acids.
SALVADOR LURIA (1912–1991)

IN THE FIRST 40 YEARS OF THE TWENTIETH CENTURY, genetic studies of corn, mice, fruit flies, humans, and other organisms established that genes are integral components of chromosomes. The question at the molecular level was whether a gene was deoxyribonucleic acid (DNA) or protein, because both of these multiunit (polymeric) molecules are the major constituents of chromosomes. As early as 1899, E. B. Wilson (1856–1939), a noted developmental biologist, suggested that nucleinic acid, later called DNA, could be the genetic material. Despite Wilson's insight, the focus of attention shifted from DNA to protein. Chemical studies led researchers to postulate that a DNA strand was made up of a set of four different basic units repeated over and over. On this basis, they concluded that such a redundant molecule could not possibly contain encoded information. By contrast, proteins were considered more likely to carry genetic information, because not only were they quite diverse but many of them catalyzed the synthesis of other biological compounds. Moreover, based on Garrod's explanation of certain inherited metabolic disorders, there appeared to be an important causal relationship between genes and enzymes.

Properties of Genetic Material

In 1920, H. J. Muller (1890–1967), who would win a Nobel Prize in 1945 for demonstrating that X rays change (mutate) genes, postulated that genetic material, regardless of chemical composition, must have three fundamental properties. First, it must encode information for the production of compounds that determine phenotypic features. Second, it must be able to replicate. Third, it must undergo change that can be perpetuated.

By 1945, considerable experimental effort showed that when purified DNA was transferred from a donor bacterial strain with a specific biochemical

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characteristic to a recipient bacterial strain lacking this feature, some of the treated bacteria both displayed and perpetuated the same trait as the donor strain. This work established that DNA directed the synthesis of specific cellular products that contribute to the phenotype of an organism. Thus, at midcentury, two questions in genetics were paramount: What was the structure of DNA? How could the structure of DNA account for the fundamental properties of genetic material?

Structure of DNA

The chemistry of DNA has been studied since 1868. By the 1940s, it was known that DNA is made up of individual units called nucleotides, linked to each other to form long chains. A nucleotide consists of an organic base (base), a five-carbon sugar (pentose), and a phosphorus group (Figure 4.1A). The phosphorus group and organic base are attached to the 5′-carbon and 1′-carbon atoms of the sugar moiety, respectively. In DNA, the four kinds of organic bases are called adenine (A), guanine (G), cytosine (C), and thymine (T) (Figure 4.1B). The pentose sugar of DNA is 2′-deoxyribose, because, in contrast to ribose (another five-carbon sugar with hydroxyl [OH] groups at both of the 2′- and 3′-carbons of the pentose), deoxyribose has only a hydroxyl group on the 3′-carbon. The nucleotides of DNA are joined by phosphodiester bonds,

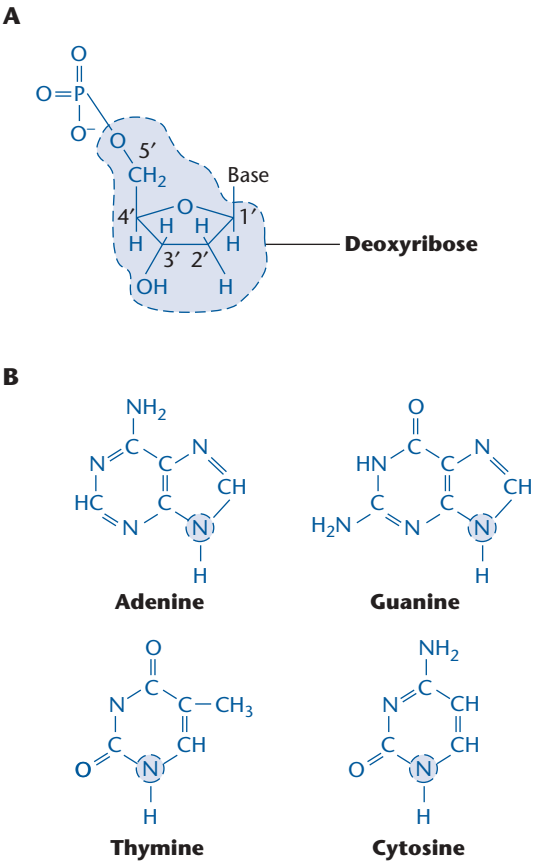


Figure 4.1 Chemical structures of the components of DNA. (A) A representative nucleotide. The term base denotes any of the four bases—adenine, guanine, cytosine, or thymine—that are found in DNA. The deoxyribose sugar is enclosed by dashed lines. The numbers with primes mark the carbon atoms of the deoxyribose moiety. (B) The bases of DNA. The circled nitrogen atom is the site of attachment of the base to the 1′-carbon atom of the deoxyribose moiety.

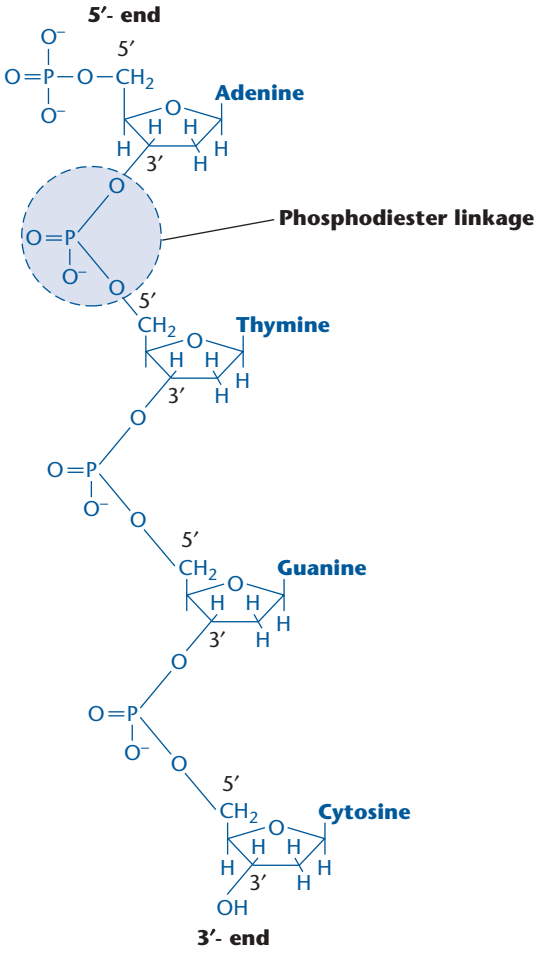


Figure 4.2 Chemical structure of a single strand of DNA.

Figure 4.3 A rod-ribbon model of double-helical DNA. The rods represent the complementary base pairs, and the ribbons represent the deoxyribose-phosphate backbones.

with the phosphate group of the 5′-carbon of one nucleotide linked to the 3′-OH group of the deoxyribose of the adjacent nucleotide (Figure 4.2). A polynucleotide strand has a 3′-OH group at one end (3′-end) and a 5′-phosphate group at the other (5′-end).

In 1953, James Watson (b. 1928) and Francis Crick (1916–2004), using X-ray diffraction analysis of crystallized DNA, discovered that native DNA consists of two long chains (strands) forming a double-stranded helix (Figure 4.3). The coiled polynucleotide chains of DNA are held together by hydrogen bonds between the bases of the opposite strands. The bases occur as specific sets of complementary pairs (Figure 4.4). Adenine (A) pairs only with thymine (T), and guanine (G) pairs only with cytosine (C). An AT base pair is held together by two hydrogen bonds, and a GC base pair by three. The number of complementary base pairs (bp) is often used to describe the length of a double-stranded DNA molecule. For DNA molecules with thousands or millions of base pairs, the designations kilobase pairs (kb) or megabase pairs (Mb) are used, respectively. For example, the DNA of human chromosome 1 is one double-stranded helix with approximately 263 Mb.

The AT and GC base pairs lie within the interior of the molecule, and the 5′- to 3′-linked phosphorus and deoxyribose components form the backbone

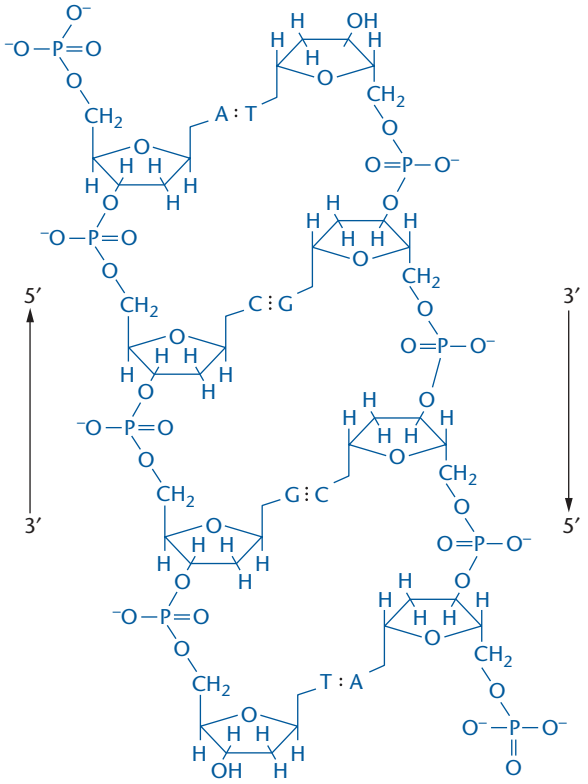


Figure 4.4 Chemical structure of double-stranded DNA.

of each strand (Figure 4.4). The two strands of a duplex DNA molecule run in opposite directions (antiparallel chains). One chain is oriented in a 3′ to 5′ direction and the other in a 5′ to 3′ direction. Because of base pairing requirements, when one strand of DNA has the sequence of bases that is 5′-TAGGCAT-3′, for example, the complementary strand must be 3′-ATCCGTA-5′. In this case, the double-stranded form would be

5′-TAGGCAT-3′
3′-ATCCGTA-5′

By convention, when DNA is drawn on a horizontal plane, the 5′ end of the upper strand is shown on the left.

In theory, genetic material must encode information for the production of proteins and be reproduced (replicated) with a high degree of accuracy, although occasionally it may undergo a permanently retained coding change. The Watson–Crick model of DNA fully met these important requirements. First, the genetic information of a gene could be encoded by a sequence of nucleotides, which could be decoded by cellular machinery and lead to the production of a specific protein. Second, with base complementarity, each preexisting DNA strand could act as a template for the production of a new complementary strand. After one round of replication, two daughter molecules would be produced, each with the same sequence of nucleotide pairs as the original DNA molecule. Third, a change in one or more base pairs not only would alter the encoded information but would be perpetuated by the DNA

replication process. Within a decade of the demonstration of the double-helical nature of DNA and its complementary base pairs, the molecular aspects of DNA replication were known; the cellular processes responsible for both decoding and regulating synthesis of gene products were understood; and many of the kinds of changes that lead to altered gene products were recognized.

DNA Replication

The process of DNA synthesis is called replication. As predicted by the Watson–Crick model of DNA, each strand of an existing DNA molecule acts as a template for the production of a new strand, and the sequence of nucleotides of a synthesized (growing) strand is determined by base complementarity. During replication, the phosphorus group of each incoming nucleotide is joined enzymatically by a phosphodiester linkage to the 3′-OH group of the last nucleotide incorporated in the growing strand (Figure 4.5). The nucleotides used for DNA replication are triphosphate deoxyribonucleotides, which have three consecutive phosphate groups attached to the 5′-carbon of the deoxyribose sugar moiety. The phosphate attached to the 5′-carbon is designated as the α-phosphate, the next phosphate is the β-phosphate, and the third is the γ-phosphate. During the replication process, the β- and γ-phosphates are cleaved off as a unit, and the α-phosphate is linked to the 3′-OH group of the previously incorporated nucleotide (Figure 4.5). The DNA synthesis machinery includes a large number of different proteins that bind to single-stranded DNA, unwind duplex DNA, initiate DNA synthesis, detect and correct replication errors, and synthesize DNA, among other activities. Of these, DNA polymerases are responsible for binding deoxyribonucleotides, fitting the correct nucleotide into place by the base pairing requirements of the template strand, and forming the phosphodiester linkage.

During the S phases of the cell division cycle and meiosis I, the duplex DNA of each chromosome is replicated to give two double-stranded DNA molecules. For mammalian chromosomal DNA replication, approximately 3000 nucleotides are added each minute, with an accuracy of replication that is probably greater than 99.98%. Mistakes, although very rare, do occur and often result in changes in single base pairs. In some cases, larger segments of DNA may be deleted or expanded.

Many different sites of initiation of replication (origins of replication) along each chromosome are required to replicate the entire 3.3×10^9 bp of DNA (genome) in the nucleus of each dividing human cell within a reasonable period of time. Because of these multiple origins of replication, part of the replication process includes enzymatically joining (ligating) segments of newly synthesized DNA together with phosphodiester bonds. In addition, a special replication enzyme called telomerase is used for the completion of the ends (telomeres) of each chromosome.

Decoding Genetic Information: RNA and Protein

The vast majority of genes encode information for the production of protein chains. Proteins are essential polymers involved in almost all biological

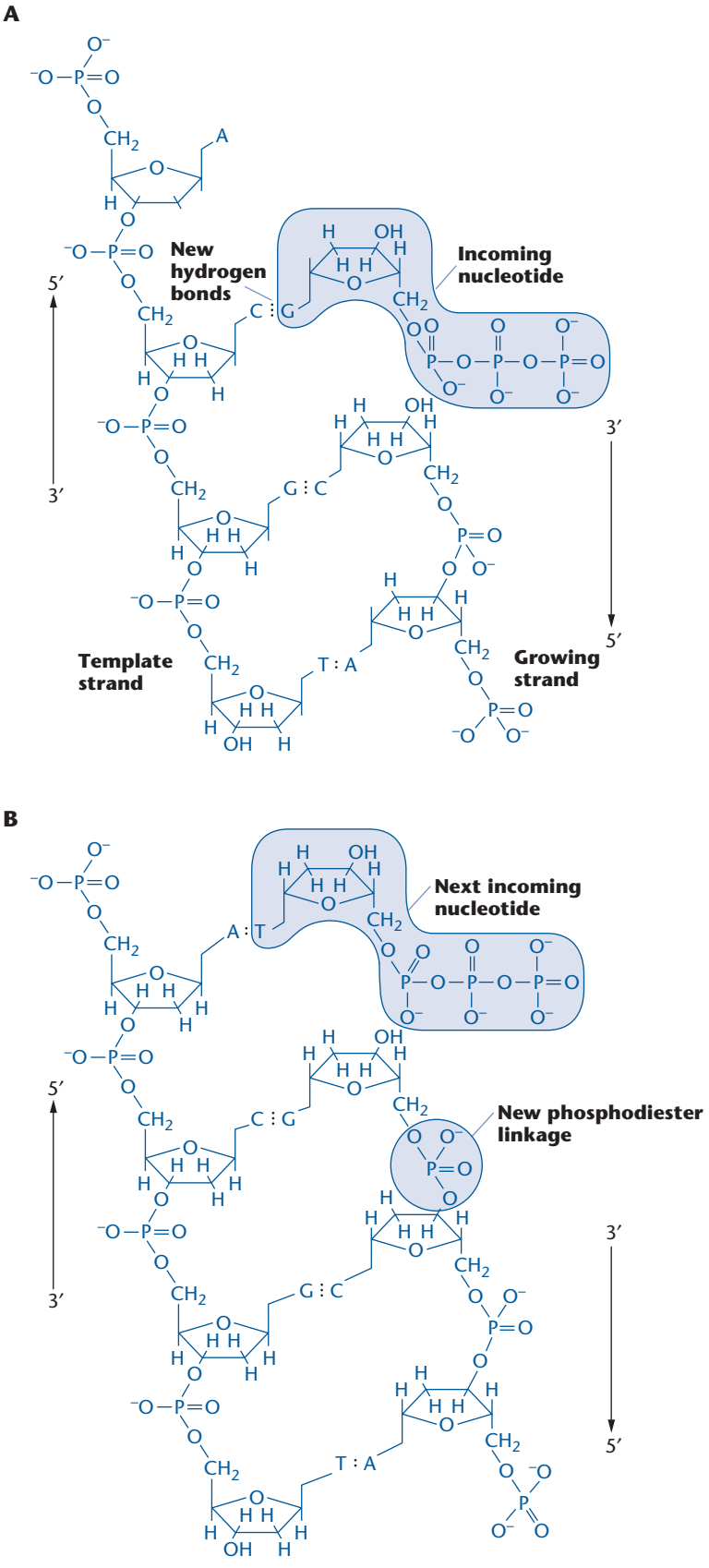


Figure 4.5 DNA replication. (A) The incoming nucleotide is a deoxyribonucleotide triphosphate directed by DNA polymerase to pair with the complementary base of the template strand. The letters A, G, C, and T denote the bases adenine, guanine, cytosine, and thymine, respectively. (B) The α-phosphate of the incoming nucleotide forms a phosphodiester bond with the 3′-hydroxyl group of the growing strand. The next incoming nucleotide of the growing strand that is complementary to the nucleotide of the template strand is positioned by DNA polymerase.

functions. They catalyze chemical reactions; transport molecules within cells; escort molecules between cells; control membrane permeability; give support to cells, organs, and body structures; cause movement; provide protection against infectious agents and toxins; and regulate the differential production of other gene products. A protein chain consists of a specific sequence of units called amino acids. All amino acids have the same basic chemical organization. There is a central carbon atom (α-carbon) with a hydrogen (H), carboxyl group (COO^-), amino group (NH_3^+), and an R group attached to it (Figure 4.6A). An R group can be any one of 20 different side chains (groups) that make up the 20 different amino acids found in proteins. When R, for example, is a methyl group (CH_3), then the amino acid is alanine. The amino acids of proteins are designated by either a three-letter or a single-letter notation (Table 4.1). In a protein, each amino acid is linked to an adjacent amino acid by a peptide bond that joins the carboxyl group of one amino acid to the amino group of the adjacent amino acid (Figure 4.6B). The first amino acid of a protein has a free amino group (N-terminus), and the last amino acid in the polypeptide chain has a free carboxyl group (C-terminus).

Proteins range in length from approximately 40 to more than 1000 amino acid residues. A protein folds into a particular shape (configuration) depending on the location of specific amino acid residues and the overall amino acid composition. In addition, many functional proteins consist of two or more polypeptide chains. In some cases, a set of multiples of the same polypeptide chain is required for an active protein molecule (homomeric protein). In other instances, a set of different protein chains (subunits) assembles to form a functional protein (heteromeric protein). Finally, large complexes made up of many different proteins often perform important cellular functions.

The biological decoding of genetic information is carried out through intermediary ribonucleic acid (RNA) molecules synthesized from segments of the

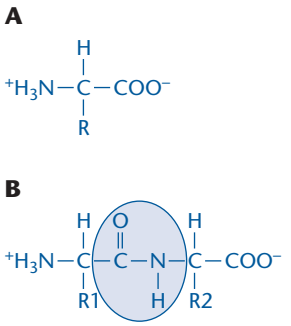


Figure 4.6 Generalized structure of an amino acid and peptide bond. (A) An amino acid. The R represents the location of the side chain. (B) A peptide bond. The peptide bond is circled, and R1 and R2 represent different side chains.

Table 4.1 Amino acids of proteins and designations.

Amino acid	Three-letter designation	Single-letter designation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	Q
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	N
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

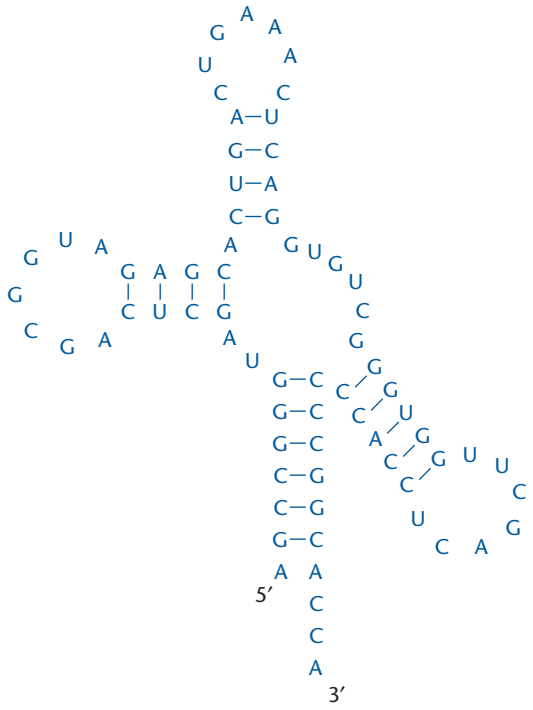


Figure 4.7 Secondary structure of an RNA molecule. The lines represent hydrogen bonding between complementary base pairs. The ribose-phosphate backbone is omitted.

DNA. RNA molecules are linear polynucleotide chains that differ from DNA in two chemical aspects. The sugar moiety of the nucleotides of RNA is ribose, which has hydroxyl groups on both the 2′- and 3′-carbons of the sugar. And, instead of thymine, the base uracil (U) is found in RNA. Most RNA molecules are single stranded, although often, within a single chain, segments of nucleotides are complementary to each other and form double-stranded regions (Figure 4.7). Base pairing within a single RNA strand follows the same principles as base pairing between complementary sequences of DNA, except that uracil pairs with adenine in an RNA molecule. In addition, base pairing can occur between two RNA molecules if they contain complementary sequences of base pairs.

The major kinds of RNA molecules essential for decoding genetic information are messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA). The production of RNA from DNA is called transcription. In eukaryotic organisms, mRNA, rRNA, and tRNA are each transcribed by a different RNA polymerase. In many of its features, transcription resembles replication. Briefly, one strand of the DNA of a specific region acts as a template for transcription. By forming 3′- and 5′-phosphodiester linkages, RNA polymerase sequentially joins together a string of ribonucleotides that are complementary to the nucleotides of the template DNA strand (Figure 4.8). As transcription proceeds, the newly synthesized RNA is released from the DNA, and the DNA helix is reconstituted. Because only segments of DNA molecules are transcribed, sets of short stretches of base pairs (signal regions) within the DNA are required to ensure that transcription is initiated at the correct nucleotide and that it terminates at a specific nucleotide. The sequences that control the initiation of transcription usually precede the coding sequence, and

Figure 4.8 Schematic representation of transcription. The arrow indicates the direction of transcription.

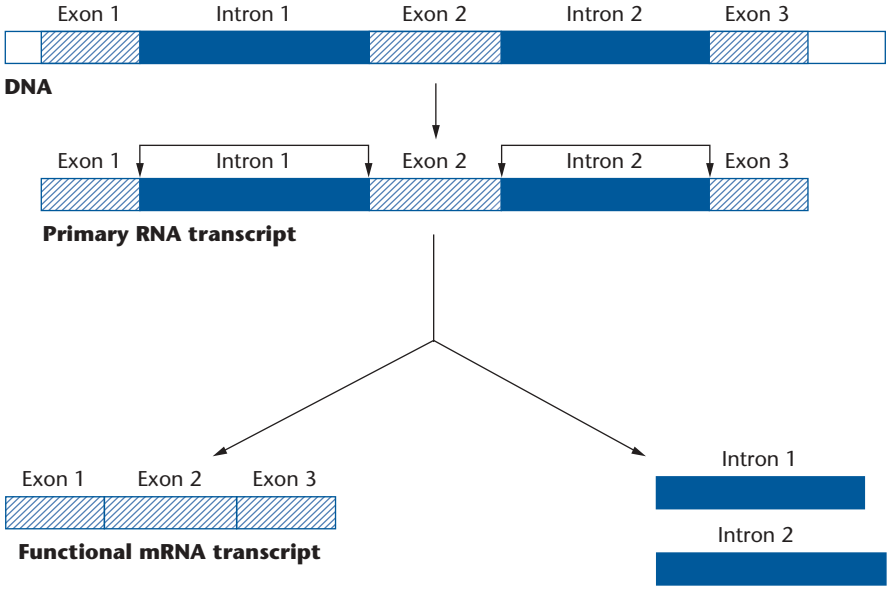


Figure 4.9 Splicing of a eukaryotic primary RNA transcript. The bracketing arrows mark the sites that are spliced together after the removal of the intervening DNA regions. In this example, introns 1 and 2 are spliced out of the primary transcript, and exons 1, 2, and 3 are spliced together to form a functional mRNA.

the termination signal sequences follow it. The DNA segment that precedes a gene is called the 5′ flanking (upstream) region, and the segment following a gene is the 3′ flanking (downstream) region.

From a molecular perspective, a gene is a specific nucleotide sequence that is transcribed into RNA. Structural genes encode proteins. The functional transcription product of a structural gene is an mRNA. In eukaryotic organisms, most structural genes consist of a set of coding regions (exons) separated by noncoding regions (introns, intervening sequences). After an entire eukaryotic structural gene is transcribed, the introns are enzymatically cut out of this primary transcript, and the exons are linked (spliced) together in the correct order to form a functional mRNA (Figure 4.9). Exons tend to be 150 to 200 bases in length, and introns can vary from as few as 40 to more than 10,000 nucleotides. A small number of structural genes lack introns. In addition, an mRNA may undergo alternative splicing events. For example, in one kind of tissue, all the exons of the primary transcript may be spliced together to form a functional mRNA. In another tissue, the initial transcript may undergo a different pattern of exon splicing; an exon is skipped during the process of intron removal, producing a novel functional mRNA. The exon-skipping mechanism generates different gene products (isoforms) in different tissues from the same structural gene (Figure 4.10). At least, 75% of human genes with multiple exons undergo alternative splicing.

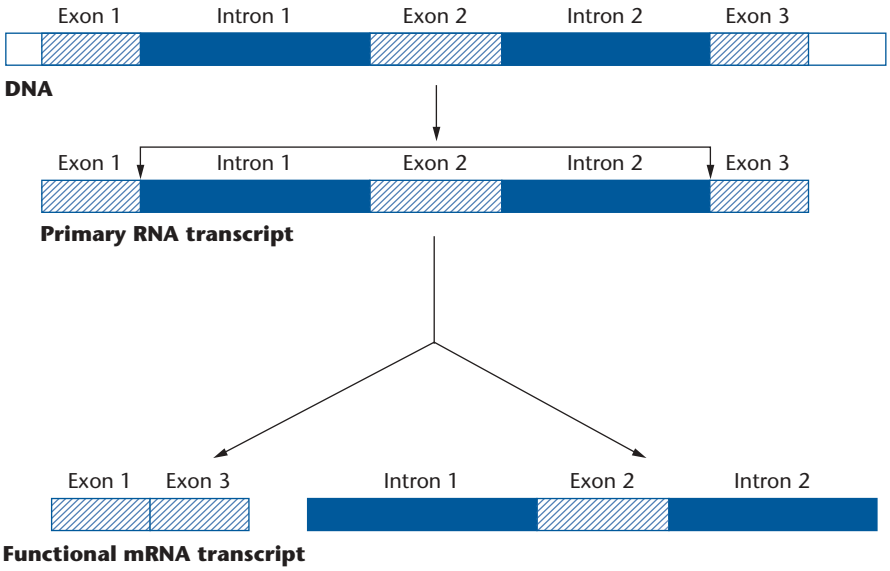


Figure 4.10 Alternative splicing of a eukaryotic primary RNA transcript. The bracketing arrows mark the sites that are spliced together after the removal of the intervening DNA region. In this example, exon 2 flanked by introns 1 and 2 is spliced out of the primary transcript, and exons 1 and 3 are spliced together to form a functional mRNA transcript.

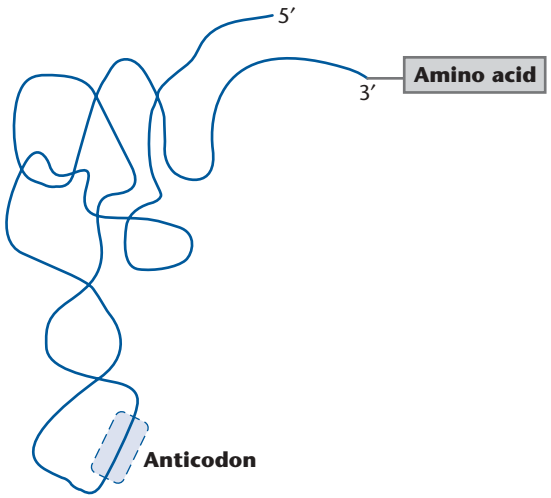


Figure 4.11 Conformation of a charged tRNA. The amino acid is attached to the 3′ end of the tRNA, and the location of the anticodon region is enclosed by the dashed lines.

In a metabolically active cell, approximately 3% to 5% of the cellular RNA is mRNA, 90% is rRNA, and about 4% is tRNA. Hundreds of different mRNAs can be in one cell. By contrast, there are four types of rRNA. Three of the rRNAs combine with a set of proteins to form a ribonucleoprotein complex called the large ribosomal subunit. The other rRNA combines with another set of proteins to form a small ribosomal subunit. In the cytoplasm of the cell, one large and one small ribosomal subunit combine to form a ribosome. The ribosome is the site of protein synthesis, and an active cell can have thousands of ribosomes.

There are approximately 50 different types of tRNA molecules in a cell that is actively synthesizing protein. The tRNAs range in length from about 75 to 93 nucleotides. Because of intrastrand complementary segments of nucleotides, tRNA molecules form a folded L-shaped structure (Figure 4.11). An amino acid is linked enzymatically by its carboxyl end to the 3′ end of a specific tRNA.

For example, the enzyme arginyl-tRNA synthetase adds the amino acid arginine to the $tRNA^{Arg}$ molecule. There is at least one tRNA for each of the 20 amino acids found in proteins. After the binding of a particular amino acid to its tRNA, the tRNA is said to be "charged." In another part of the tRNA molecule are three unpaired nucleotides, which together are called the anticodon sequence. This sequence plays a crucial role in the formation of the linear array of amino acids that constitute a protein.

Translation

The process of decoding the information content of an mRNA into a linear sequence of linked amino acids is called translation. Translation requires the interaction of mRNA, charged tRNAs, ribosomes, and a large number of proteins (factors) that facilitate the initiation, elongation, and termination of the polypeptide chain. In eukaryotic organisms, translation is initiated by the binding of a specific charged initiator tRNA, Met-$tRNA^{Met}$, and other factors to the small ribosomal subunit. No other charged tRNA can bind to a free small ribosomal subunit. Next, the 5′ end of an mRNA combines with the initiator tRNA–small ribosomal subunit complex, and the complex migrates along the mRNA until an AUG sequence (initiator codon) is encountered. Then, the UAC anticodon sequence of the initiator Met-$tRNA^{Met}$ base pairs with the AUG sequence of the mRNA, the migration stops, and the larger ribosomal subunit joins the complex (Figure 4.12).

The elongation process entails linking together by peptide bond formation the correct amino acid sequence encoded in the mRNA (Figure 4.13). The second set of three nucleotides (triplet, codon) in the mRNA that immediately follows the AUG codon dictates the anticodon sequence and, therefore, which charged tRNA will bind to the ribosome complex. Uncharged tRNAs do not bind efficiently to ribosomes. If the second nucleotide triplet in the mRNA is CUG, then the charged tRNA with the anticodon sequence GAC will bind. This charged tRNA carries the amino acid leucine. Once in place, a peptide bond is formed between the carboxyl group of the methionine and the amino group of the leucine. The leucine remains bound to its tRNA. Peptide bond formation is catalyzed by enzymatic activity associated with the large ribosomal subunit. The formation of the peptide bond "uncharges" the initiator tRNA, because the bond between the carboxyl group of methionine and its tRNA is cleaved to make the carboxyl group available for peptide bond formation. The uncharged tRNA is ejected from the ribosomal complex. The methionine-leucine-$tRNA^{Leu}$-mRNA combination shifts (translocates) along the ribosome, and, as a consequence, the next codon of the mRNA is available for binding by a charged tRNA with the appropriate anticodon sequence. If the third codon is UUU, then the charged tRNA with an AAA anticodon will bind. In this case, the tRNA with an AAA anticodon carries the amino acid phenylalanine. Once in place, the linkage between the carboxyl group of leucine and its tRNA is broken, and a peptide bond forms between the carboxyl group of the leucine and the amino group of the phenylalanine. After ejection of the uncharged $tRNA^{Leu}$, the "peptidyl" $tRNA^{Phe}$ in combination with the methonine-leucine-phenylanine amino acid sequence and the mRNA is trans-

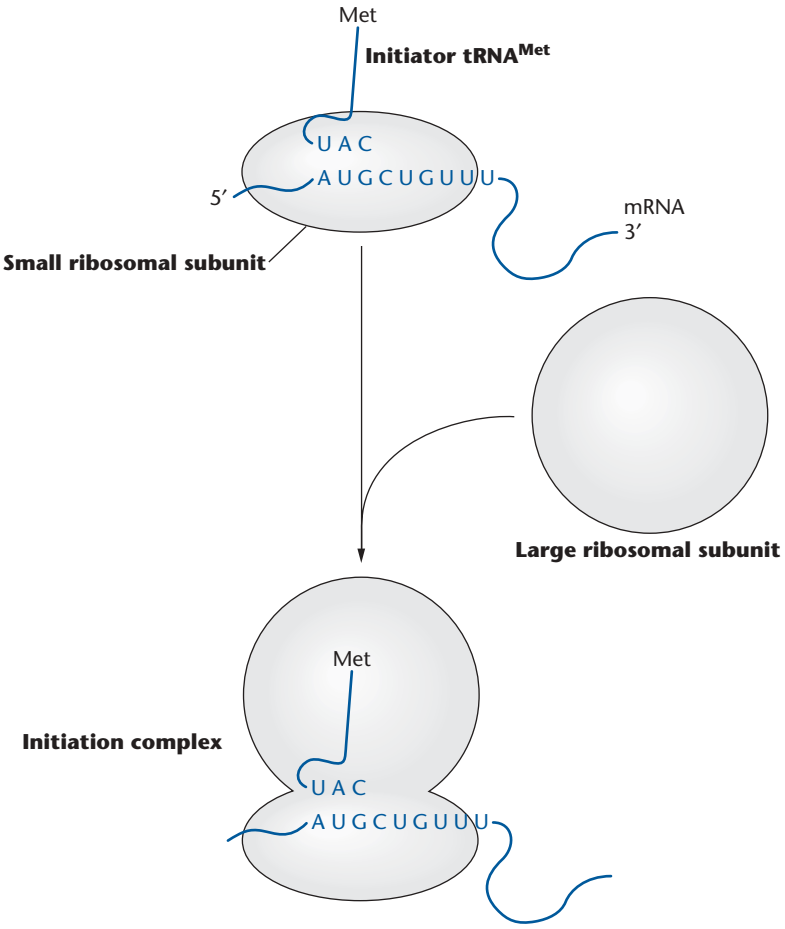


Figure 4.12 Schematic representation of the initiation of translation in eukaryotes. The initiator tRNA (Met-tRNA^{Met}) binds to a small ribosomal subunit, and then the mRNA moves along the complex until the anticodon (UAC) of the initiator tRNA base pairs with the start codon (AUG) of the mRNA. The large ribosomal subunit combines with the mRNA-initiator tRNA-small ribosomal subunit complex to form the initiation complex.

located to the peptidyl site, that is, the site that is occupied with the linked amino acids attached to a tRNA, thereby clearing the aminoacyl site (A site) and making the next codon available for binding by the appropriately charged tRNA.

The succession of operations including binding of a charged tRNA by means of anticodon:codon pairing, peptide bond formation, ejection of an uncharged tRNA, and translocation, continues until all the amino acids encoded by the mRNA are linked together. The ribosome moves along the mRNA in a 5′ to 3′ direction at a rate of about 15 amino acids per second. When the 5′ end of the mRNA is free of a ribosome, it can combine with another initiation complex. In this way, a single mRNA can be translated simultaneously by a number of ribosomes, with each ribosome producing a polypeptide chain.

The elongation process continues until a UAA, UAG, or UGA codon is encountered (Figure 4.14). There are no naturally occurring tRNAs with anticodons that are complementary to UAA, UAG, or UGA (stop codons, termination codons). However, a protein (termination factor, release factor) recognizes a stop codon and binds to the ribosome. After binding of a termination factor, the bond between the last tRNA, which has the complete chain of amino acids linked to it, and its amino acid is broken. This cleavage results in the release of the uncharged tRNA, the complete protein, and the mRNA.

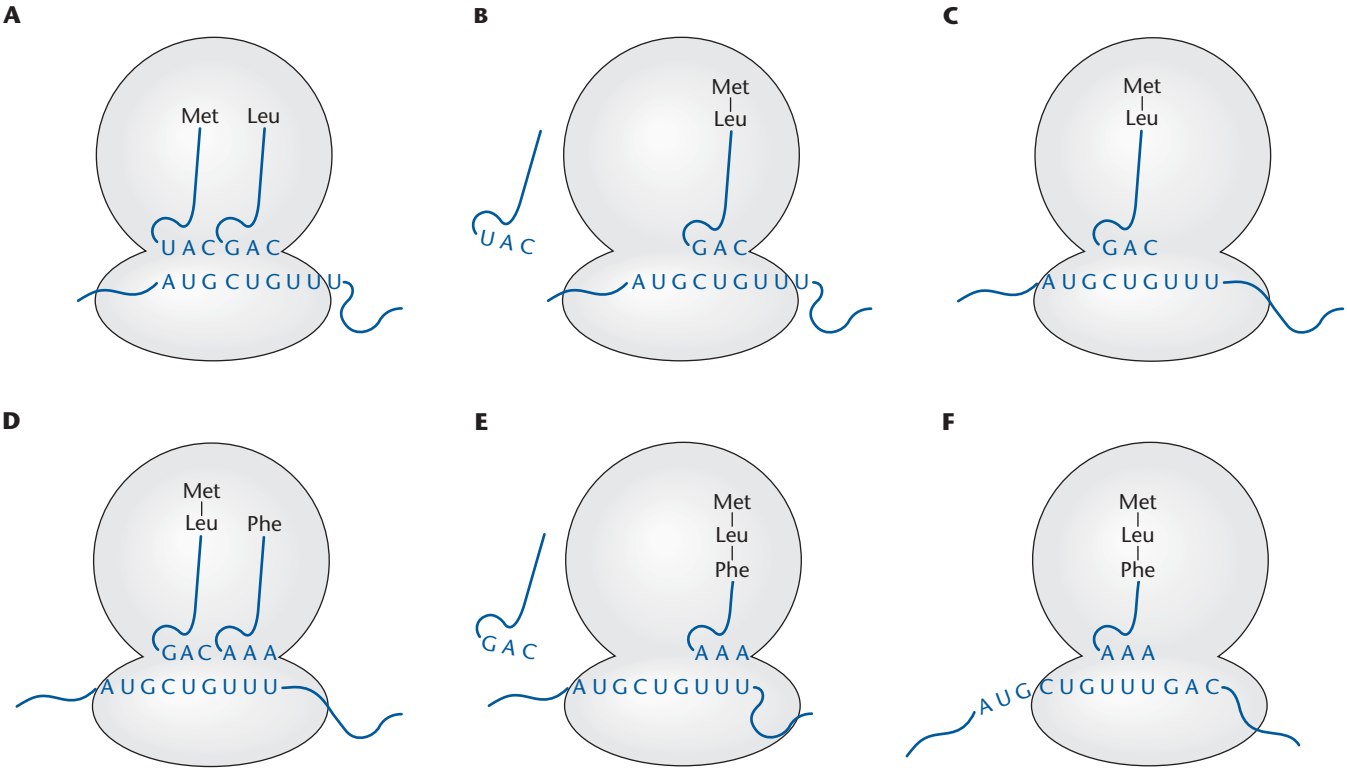


Figure 4.13 Schematic representation of the elongation phase of translation. (A) The second codon (CUG) base pairs with the anticodon (GAC) of Leu-tRNA^{Leu}. (B) The methionine of the initiator tRNA is joined to the leucine of the Leu-tRNA^{Leu} by a peptide bond, and the uncharged initiator tRNA is ejected from the ribosome. (C) Translocation of the peptidyl-tRNA to the peptidyl site from the aminoacyl site, which opens the aminoacyl site for the next codon (UUU). (D) The third codon (UUU) in the mRNA base pairs with the anticodon (AAA) of Phe-tRNA^{Phe}. (E) The leucine of the peptidyl-tRNA is joined to the phenylalanine of Phe-tRNA^{Phe} by a peptide bond, and the uncharged tRNA^{Leu} is ejected from the ribosome. (F) Translocation of the peptidyl-tRNA to the peptidyl site from the aminoacyl site, which opens the aminoacyl site for the next codon and codon:anticodon interaction.

In addition, the ribosomal subunits separate from each other. The components of the translation machinery can be used again. However, depending on its length and other parameters, an mRNA may remain intact for a few minutes or, in some cases, for several hours. The lengths of human structural genes, including both exons and introns, range from a few thousand base pairs to more than 2 Mb in some instances. The total coding region, that is, exons only, of a gene can be from 0.5 to about 15 kb in length. A coding region of 1 kb gives rise to a protein with 333 amino acids.

After translation, a protein may be modified in various ways. In some proteins, the methionine at the N-terminus is cleaved off, leaving the second encoded amino acid as the N-terminal moiety. In other cases, the protein is selectively cleaved at specific sites to make smaller protein chains with discrete functions. In other instances, phosphorus, lipids, carbohydrates, or other chemical groups are added enzymatically to specific amino acids to produce modified proteins that can carry out certain cellular activities.

The complete genetic code consists of 64 codons. Three are reserved for stops and one (AUG) for initiation (Table 4.2). When a methionine residue occurs internally in a protein, the codon AUG is recognized by another Met-tRNA^{Met} and not the initiator tRNA. There is one codon (UGG) for the amino acid tryptophan. For the rest of the amino acids found in proteins, there are at least two and sometimes six codons. For example, there are six codons (UUA, UUG, CUU, CUC, CUA, and CUG) for the amino acid leucine. Different codons are used to different extents in different organisms. Of the four codons for glycine, GGA is used approximately 26% of the time by human structural

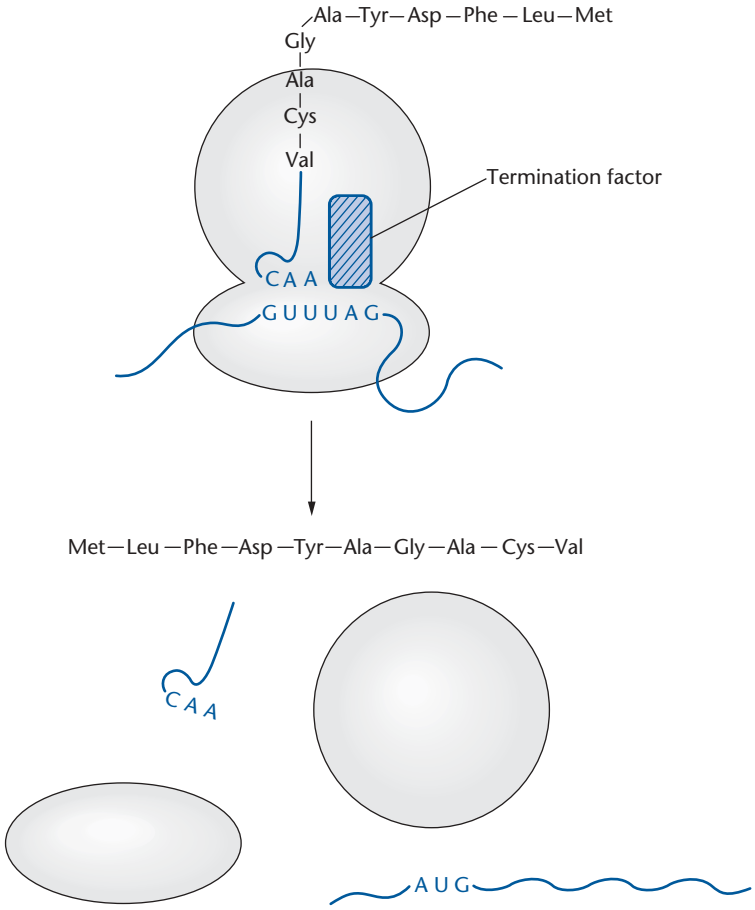


Figure 4.14 Schematic representation of the termination of translation. The stop codon (UAG) interacts with a termination factor that leads to the termination of translation. The last tRNA is cleaved from the peptide chain and ejected. The mRNA and the finished peptide are released. The ribosomes are prepared for recycling by a ribosome-releasing factor.

Table 4.2 The genetic code and human codon usage.

Codon	Amino acid	Frequency of use in humans
GGG	Glycine	0.23
GGA	Glycine	0.26
GGU	Glycine	0.18
GGC	Glycine	0.33
GAG	Glutamic acid	0.59
GAA	Glutamic acid	0.41
GAU	Aspartic acid	0.44
GAC	Aspartic acid	0.56
GUG	Valine	0.48
GUA	Valine	0.10
GUU	Valine	0.17
GUC	Valine	0.25
GCG	Alanine	0.10
GCA	Alanine	0.22
GCU	Alanine	0.28
GCC	Alanine	0.40
AAG	Lysine	0.60
AAA	Lysine	0.40
AAU	Asparagine	0.44
AAC	Asparagine	0.56
AUG	Methionine	1.00
AUA	Isoleucine	0.14

Table 4.2 The genetic code and human codon usage (*continued*)

Codon	Amino acid	Frequency of use in humans
AUU	Isoleucine	0.35
AUC	Isoleucine	0.51
ACG	Threonine	0.12
ACA	Threonine	0.27
ACU	Threonine	0.23
ACC	Threonine	0.38
UGG	Tryptophan	1.00
UGU	Cysteine	0.42
UGC	Cysteine	0.58
UGA	Stop	0.61
UAG	Stop	0.17
UAA	Stop	0.22
UAU	Tyrosine	0.42
UAC	Tyrosine	0.58
UUU	Phenylalanine	0.43
UUC	Phenylalanine	0.57
UCG	Serine	0.06
UCA	Serine	0.15
UCU	Serine	0.17
UCC	Serine	0.23
AGU	Serine	0.14
AGC	Serine	0.25
CGG	Arginine	0.19
CGA	Arginine	0.10
CGU	Arginine	0.09
CGC	Arginine	0.19
AGG	Arginine	0.22
AGA	Arginine	0.21
CAG	Glutamine	0.73
CAA	Glutamine	0.27
CAU	Histidine	0.41
CAC	Histidine	0.59
CUG	Leucine	0.43
CUA	Leucine	0.07
CUU	Leucine	0.12
CUC	Leucine	0.20
UUG	Leucine	0.12
UUA	Leucine	0.06
CCG	Proline	0.11
CCA	Proline	0.27
CCU	Proline	0.29
CCC	Proline	0.33

genes and about 9% of the time by the protein-coding genes of the bacterium *Escherichia coli*. The stop codons are also used to different extents in different organisms. In humans, the frequency of usage of UAA, UAG, and UGA is 0.22, 0.17, and 0.61, respectively, whereas in *E. coli*, it is 0.62, 0.09, and 0.30, respectively. Notwithstanding the differences in codon usage, the genetic code, with a few exceptions, is the same in all organisms.

Regulation of mRNA Transcription

Generally, humans and other organisms are what they are biologically because of their proteins. Normal development from a fertilized egg to a functional

multicellular organism, cell growth, response to environmental stresses, and maintenance of cellular activities after embryonic development all depend on proteins synthesized at correct times and within appropriate cell types. Most active cells transcribe a common (basal) set of structural genes for maintaining routine (household) cellular functions. In addition, in some cells specific structural genes are exclusively transcribed (activated, expressed) to give the tissue or organ its unique properties. For example, the genes that encode the α- and β-subunits of adult hemoglobin are transcribed only in the cells that develop into red blood cells. The numbers of cell-specific mRNA transcripts range from a few sequences in some cells to dozens of different mRNAs in, for example, brain and heart cells. The ability of cells to turn on (activate) or turn off (repress) transcription of particular structural genes is essential for establishing and maintaining cell specificity. Moreover, the selective prevention of transcription is an effective way to conserve cellular energy.

There are a number of diverse, highly specific processes that either activate or repress the transcription of various structural genes. In general, the control of transcription in eukaryotes is mediated by proteins collectively classified as transcription factors. Many transcription factors bind directly to DNA sequences that are frequently less than 10 bp in length. The naming of protein-binding sites is often idiosyncratic. However, for the most part, they are called boxes, DNA modules, initiator elements, or response elements. A frequently occurring response element does not necessarily have the exact same nucleotide sequence in each instance but contains a subset of invariant nucleotides. In these cases, the name of the response element is often based on the highly conserved sequence motif of one of the strands, for example, a TATA box. In addition to DNA-protein interactions, protein-protein associations are also important for regulating transcription. Response elements usually lie upstream from the starting point of transcription of a structural gene. At the DNA level, the nucleotide pair with the base that is complementary to the first nucleotide of an mRNA transcript is specified as the +1 bp. The adjacent nucleotide pair upstream from the +1 bp is the –1 bp. The location of the 25th base pair upstream from the +1 bp is –25 bp. Although several structural genes have their own specific collections of response elements, a representative structural gene might have a promoter sequence (TATA box, Hogness box) consisting of eight nucleotides, including a TATA sequence, a CCAAT sequence ("cat" box), and a sequence of repeated GC nucleotides (GC box), that occur at about –25 bp, –75 bp, and –90 bp, respectively (Figure 4.15).

The first step in the initiation of transcription of a number of structural genes with a TATA promoter is the binding of the transcription factor IID (TFIID, TBP, TATA-binding protein), a complex of at least 14 proteins, to an available TATA sequence. Subsequently, other transcription factors bind to TFIID and to the DNA adjacent to the TATA box. Then, RNA polymerase

Figure 4.15 The promoter and initiator elements of some eukaryotic structural genes. The negative numbers designate the location in the DNA of nucleotide pairs that lie upstream from the site of initiation (+1) of transcription. The right-angled arrow indicates the site of initiation and direction of transcription.

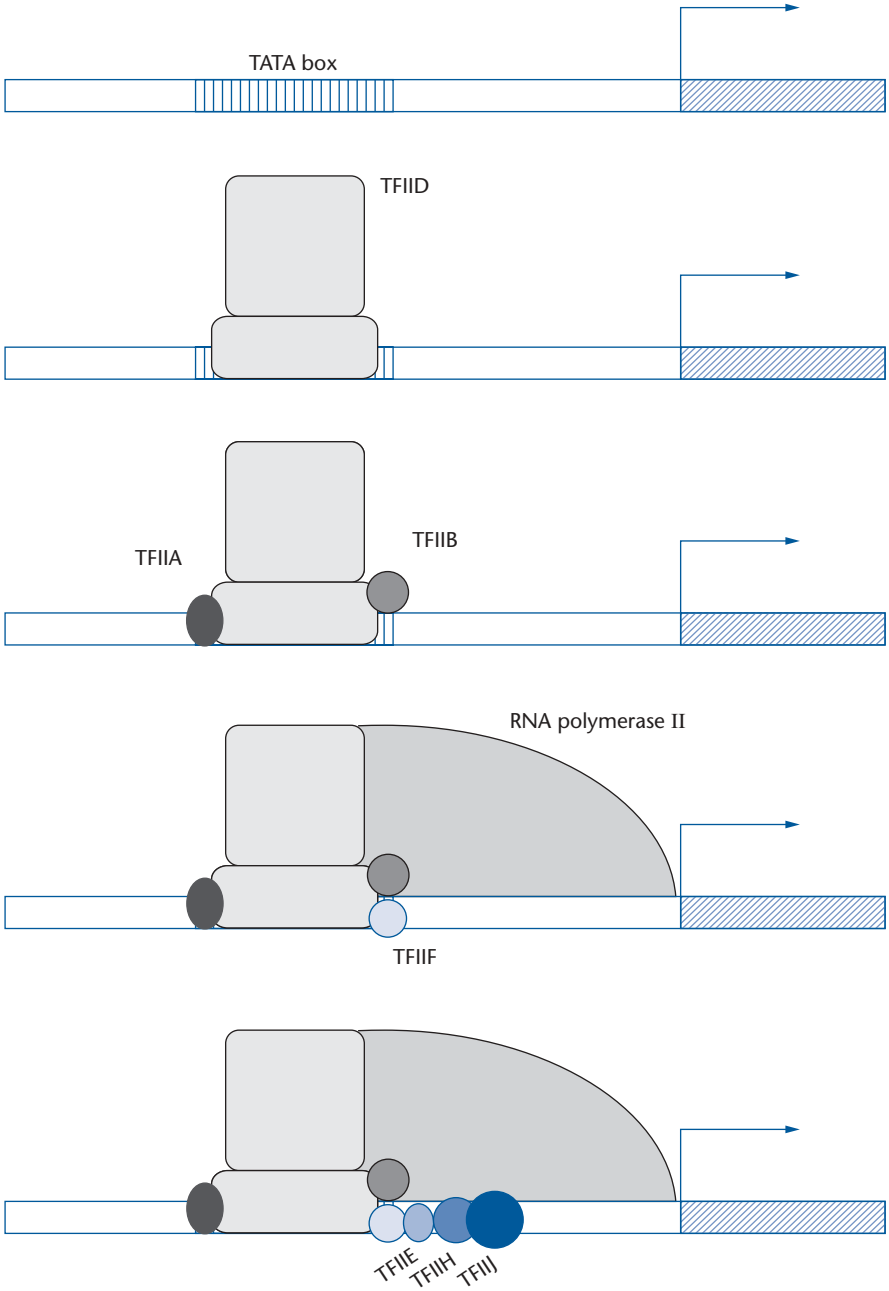


Figure 4.16 Formation of an RNA polymerase II-transcription initiation complex at a TATA box. Transcription factor TFIID binds to a TATA box and, in sequence, other transcription factors and RNA polymerase II bind to form a protein aggregate that is responsible for initiating transcription. The right-angled arrow designates the site of initiation and the direction of transcription.

II, which is oriented toward the structural gene, binds to the transcription complex. With the aid of additional transcription factors, transcription is initiated at the correct starting point (Figure 4.16). Clearly, if a TATA sequence is deleted or grossly altered, then transcription of the structural gene does not occur. Transcription factors specific for the CCAAT and GC response elements have been identified, but the mechanism by which these DNA-protein interactions affect the efficiency of transcription when they are 75 or more base pairs from the site of initiation of transcription is not known. In addition, "enhancer" sequences, which are gene specific and regulate transcription, are

located hundreds or even thousands of base pairs from the +1 bp. Folding, looping, or bending of the chromosomal DNA may bring DNA regions, far apart in the elongated state, close to one another. Also, transcription factors that bind to certain enhancers or response elements may form a chain of proteins and create bridges from one DNA site to another.

Some repressed (nonexpressed) structural genes are activated by a cascade of events triggered by a specific extracellular signal, such as temperature increase or the presence of a hormone. For example, a hormone released into the circulatory system comes into contact with a specific cell type with a receptor that binds the hormone and facilitates its entry into the cell. Once inside the cell, the hormone interacts with a cellular protein and changes the configuration of the protein. In this altered state, the protein now is able to enter the nucleus, where it binds to an exclusive response element that initiates transcription of the target gene.

Some proteins bind to response elements and prevent transcription. For example, a class of more than 30 vertebrate genes are actively transcribed in

from the HUMAN GENETICS files

Hemoglobinopathies and Thalassemias: An Abundance of Mutations

Inherited abnormalities of hemoglobin chains are the result of a variety of mutations. Hemoglobin (Hb) is a four-chain (tetrameric) oxygen-carrying protein of red blood cells (erythrocytes). The major postnatal hemoglobin, which makes up about 97% of the total hemoglobin of the red blood cell, consists of two alpha (α) and two beta (β) chains, that is, $\alpha_2\beta_2$. α-Globin chains are synthesized at a high level before and after birth. β-chain production is initiated 4 to 6 weeks after conception, but becomes prevalent about 12 to 18 weeks after birth. There are other globin chains, such as gamma (γ), delta (δ), epsilon (ε), and zeta (ζ). Of these, γ-globin chains combine with α-globin chains to form an $\alpha_2\gamma_2$ molecule, which is the major hemoglobin present during late embryonic stages, throughout fetal development, and for the first 12 to 18 weeks after birth. The ε- and ζ-globin chains are only synthesized during early embryonic development. δ-Globin chains combine with α-globin chains to form a minor hemoglobin called Hb A_2 that is present during the late stages of fetal development and throughout the life of an individual.

The designation of various hemoglobins, including variants, started with letters. For example, Hb A is the major adult hemoglobin; Hb F, the major fetal hemoglobin ($\alpha_2\gamma_2$); and Hb S, the hemoglobin found associated with sickle cell anemia. However, as the number of documented cases of hemoglobin variants mounted and the same letter was inadvertently given to different abnormal hemoglobins, researchers started to name them after the place of discovery, whether it was a region, city, hospital, or laboratory, for example, Hb Manitoba, Hb Bologna, and Hb Beth Israel. In some instances, idiosyncratic names were adopted, for example, Hb Abraham Lincoln and Hb Aida.

Because the α-globin gene is naturally duplicated and each of these two closely linked genes is functional and encodes the same product, there are two α-globin gene symbols, *HBA1* and *HBA2*. The gene symbol for the β-globin gene is *HBB*. To be more informative, the specific hemoglobin gene with a mutation, the position in the protein chain, and the nature of the amino acid substitution are sometimes given along with the designation of the hemoglobin variant. For example, Hb S (*HBB*, GLU6VAL), Hb Miyano (*HBA1*, THR41SER), and Hb Kurdistan (*HBA2*, ASP47TYR) are representative of this particular naming system. There are about 189 allelic variants for *HBA1*, about 34 for *HBA2*, and 276 for *HBB*.

Approximately 95% of all of hemoglobin gene mutations cause amino acid substitutions that lead to abnormal functioning of hemoglobin. Many of these missense mutations, regardless of whether they are *HBA1*, *HBA2*, or *HBB* gene mutations, alter the configuration of the assembled hemoglobin molecule, which either interferes with its ability to bind and/or release oxygen or changes its stability under normal conditions. These changes may cause a reduction in the number of red blood cells, lower the total concentration of hemoglobin, and decrease the volume percentage of red blood cells in the blood.

In general, lower than normal levels of hemoglobin produce anemia. The symptoms of anemia are heart palpitations, shortness of breath, and chronic fatigue. Severe anemia during fetal development can cause death in utero or shortly after birth. In adults, severe anemia is extremely debilitating and often fatal. Mild anemia can be treated with periodic blood transfusions. Other than anemia, some mutations may cause increased numbers of red cells (erythrocytosis) or an increased

nerve cells (neurons) and are turned off in nonneuronal cells. Each of these neuron-active genes has a 24-bp response element lying upstream of its +1 bp site. This DNA sequence is a neuron-restrictive silencer element (NRSE). In nonneuronal cells, a protein called neuronal-restrictive silencer factor (NRSF) is synthesized and binds to each NRSE to prevent transcription of each member of this set of genes. Conversely, NRSF is not produced by neuronal cells, and each gene with an NRSE is active.

On the whole, the regulation of transcription is a complex phenomenon. Some target genes have a number of different response elements, with each one capable of responding to a particular signal that is sufficient to initiate transcription. In these cases, the structural gene can be activated both in different cell types and by diverse signals at different times during the life cycle of an organism. There are also instances where a unique transcription factor regulates the expression of a single structural gene. As well, proteins interact with a protein(s) of the transcription complex to block transcription, either before initiation or during the elongation process.

concentration of reduced hemoglobin (cyanosis).

The term hemoglobinopathy is used to describe an abnormal phenotype that results from altered hemoglobin function, whereas thalassemia (Cooley anemia) refers to anemia that results from a reduction in the production of either an α- or a β-globin chain. This demarcation of disorders is not absolute, because there are mutations that both affect hemoglobin function and lower the level of production of a globin chain.

The thalassemia syndromes are often the result of globin gene nonsense, splice, and frameshift mutations. Some mutations produce longer than normal hemoglobin chains. For example, elongated α-globin chains arise from mutations of the stop codon (UAA), which is codon 142 of the *HBA1* gene. When mutations change the UAA codon to AAA, GAA, UCA, or CAA, amino acids lysine (K), glutamic acid (D), serine (S), or glutamine (N), respectively, are inserted into amino acid position 142, and the α-globin chain is extended by an additional 31 amino acids before another stop codon is encountered. Elongated α-globin chains are translated at a lower rate than normal chains. In another α-globin gene disorder, deletion of the last nucleotide pair of DNA codon 138 creates an altered reading frame that does not recognize UAA as a stop codon and extends the α-globin chain by five amino acids. There are also hemoglobin gene mutations that remove one or a few more intact codons to produce slightly shortened globin chains.

Depending on the globin chain that is diminished, thalassemias are designated α-thalassemia or β-thalassemia. Thalassemias are common in Africa, Mediterranean Europe (Greece, Italy, Cyprus), the Middle East, India, and Southeast Asia. In some regions as many as 20% of the population may be affected to some extent by thalassemia. Generally, α-thalassemias are the consequence of the loss of one or more of the four functional α-globin genes. Elimination of one α-globin gene, two α-globin genes from the same chromosome, or two α-globin genes, one from each homologous chromosome, causes a mild form of anemia. The anemia is moderate if an individual has only one α-globin gene. However, as one might deduce, death occurs in utero when a fetus has no α-globin genes.

In cases of β-thalassemia, the major fetal hemoglobin ($\alpha_2\gamma_2$) is unaffected, whereas the impact after birth depends on the extent of the output of β-chains. If no or very few β-chains are present, the α-globin chains clump, red blood cell production is blocked, and red blood cells are destroyed. In these cases, the fetal hemoglobin may persist and help perform some of the functions of the major adult hemoglobin. This fail-safe mechanism is not that effective, and severe anemia is often the result of extremely reduced levels of β-globin chains in red blood cells. The amount of β-globin chain production depends on the nature of the mutation and whether a patient is heterozygous or homozygous for the mutation. There are at least 100 different *HBB* mutations for β-thalassemia. Many of these are nonsense and frameshift mutations that cause protein truncation. Large deletions of the *HBB* gene are rare among cases of β-thalassemia. A number of β-thalassemia mutations occur close to splice sites for removing introns and joining exons, and probably cause exon skipping and other splicing errors. Regardless of how they arise, truncated proteins are unstable and susceptible to degradation by proteolytic enzymes. Mutations and structural alterations of the promoter region also lower the rate of transcription of the *HBB* gene.

In sum, hemoglobinopathies and thalassemias have provided important information about the many different ways that mutations can alter protein function and affect the cellular abundance of a protein because inherited anemias are rather common, hemoglobin is an abundant protein, and efficient methods are available for determining both the amino acid sequences of hemoglobin chains and the sequences of nucleotides of globin genes.

In addition, translation is regulated by various subsets of small noncoding RNAs (ncRNAs). For example, microRNAs (miRNAs) join with proteins that bind to specific mRNAs and prevent translation. Active miRNAs are about 21 to 26 nucleotides in length and are derived from ~70-base precursor molecules. On the other hand, small, double-stranded interfering RNAs (siRNAs) form ribonucleoprotein complexes that bind to mRNAs and direct their degradation. Some small RNAs combine directly with mRNAs by base complementarity and trigger enzymatic cleavage, whereas others, after base pairing with mRNAs, block translation. There may be more than 200 ncRNA genes in the human genome.

Generally, RNA is synthesized from only one of the two strands of DNA of a particular chromosome region. However, if both strands from the same segment were transcribed, then the RNAs would be complementary to each other. In other words, there would be sense and antisense RNAs. Theoretically, an antisense RNA would be able to base pair with a sense RNA and prevent translation of a specific protein. Interestingly, antisense transcripts have been observed in human cells that, presumably, act as modulators of gene expression.

Nucleotide Sequence Alteration: Mutation

The replication of DNA is not a perfect process, despite many safeguards against mistakes. Although infrequent, errors that affect one or more base pairs can occur. In addition, external agents, including various chemicals, ultraviolet light, radiation, and radioactive compounds can permanently alter the sequence of nucleotides of a DNA molecule. A change to the genetic material is called a mutation. An agent that induces mutations is a mutagen. In the absence of any evidence of a mutagenic effect, a naturally occurring mutation is considered to be spontaneous. The extent of a mutation can range from a change to a single base pair (point mutation) to the alteration of a large region of a chromosome (chromosome aberration). Mutations can occur anywhere in the total DNA of an organism. In humans, approximately 95% of DNA does not code for any gene products. As a result, many mutations have no effect on the phenotype, because they are located in regions of the genome that have no impact on cellular functions. By contrast, a mutation within an exon of a structural gene can alter the functioning of the gene product and cause a dramatic phenotypic change. Ultimately, understanding of the biological basis of a genetic disorder depends on characterizing the gene responsible for the condition and determining the consequences of mutations of that gene.

When gene mutations occur in cells that do not give rise to gametes, they are called somatic mutations and have no chance of being passed on from parent to offspring. On the other hand, mutations in the DNA of germ line cells (germ line mutations) may be transmitted by gametes to the next generation. A number of attempts have been made to calculate the rate of germ line gene mutation in humans. However, reliable estimates of mutation rates require very large sample sizes, which makes these studies somewhat arduous. In addition, results can be undermined by illegitimacy, misdiagnosis, incomplete penetrance, and other factors.

The mutation rate of a normal autosomal gene to a dominant allele that causes a clearly defined phenotype at birth can be determined by scoring the number of affected newborns from marriages where both parents have a normal phenotype. In an early study of achondroplasia (ACH), a form of short-limbed dwarfism that is the consequence of mutation of the fibroblast growth factor receptor gene 3 (*FGFR3*), 8 affected individuals out of 94,075 newborns were observed. Because newborns inherit two chromosomes, one from each parent, the frequency of new *FGFR3* mutations was $8/(2 \times 94{,}075)$ or 25×10^{-5} per gamete per generation. On the one hand, this value could be too high as a result of the inclusion of other closely related and possibly nonallelic conditions, such as Jeune asphyxiating thoracic dystrophy, thanatophoric dysplasia, and achondrogenesis, each of which easily could be mistaken for achondroplasia without a thorough clinical examination. Alternatively, the estimate might be too low if the diagnostic criteria overlooked mutations of the *FGFR3* locus that produce other phenotypes. When this study was repeated with more advanced diagnostic methods, the mutation rate for achondroplasia was estimated to be $\sim 2 \times 10^{-5}$ per gamete per generation. Overall, the mutation rates for 20 different autosomal dominant conditions range from 0.1 to 18×10^{-5} per gamete per generation. It is not clear why some gene loci, notwithstanding size, mutate much more frequently than others.

Large-scale studies were conducted on single amino acid changes in 36 different proteins to estimate the rate of base pair mutation. The underlying premise of such analyses is that a single amino acid change in a protein reflects a point mutation in a gene. The presence of an altered protein is determined by a change of its mobility in an electric field in comparison to that of the normal protein. Out of 1,226,097 tests for single amino acid changes ("gene tests"), four valid mutations were discovered. Thus, the mutation rate is 3.3×10^{-6}. The gene test takes into consideration only a changed amino acid in a fully formed protein. But other kinds of mutations, such as deletion of a base pair, can occur and go undetected by this particular gene test. As a result, the actual mutation rate is probably higher. After adjusting the gene test results, an overall rate of 1×10^{-5} per generation is considered reasonable. On the basis of this estimated rate, other parameters can be calculated. For example, if the human genome contains 25,000 genes, then there are 0.25 new mutations per structural gene in each egg or sperm ($1 \times 10^{-5} \times 25{,}000$). Each fertilized egg would carry 0.5 new gene mutations (2×0.25). If the average coding region of a structural gene is assumed to contain 1500 bp, then there would be an estimated 0.67×10^{-8} nucleotide changes per gene per generation [$(1 \times 10^{-5})/1500$]. For the entire human genome, which contains 3×10^{9} bp, the total estimated number of nucleotide changes would be about 20 in each gamete ($0.67 \times 10^{-8} \times 3 \times 10^{9}$) and 40 in each fertilized egg ($2 \times 0.67 \times 10^{-8} \times 3 \times 10^{9}$). Based on another type of analysis, there are about 1.6 detrimental protein-coding mutations per diploid genome per generation in humans.

Mutations of Structural Genes

A simple terminology describes the types of nucleotide substitutions that can occur at the DNA level. The bases guanine and adenine have the same funda-

mental chemical structures and fall into the class of compounds called purines. Similarly, the bases cytosine and thymidine are chemically related to each other and to other compounds called pyrimidines. Any substitution of a purine with a different purine (G↔A) or a pyrimidine with a different pyrimidine (T↔C) in a DNA molecule is a transition mutation. A transversion mutation is any substitution of a purine by a pyrimidine or vice versa (G↔T, A↔T, G↔C, A↔C). Transitions occur more frequently than transversions.

A base substitution within the coding region of a structural gene can change an mRNA codon and lead to the insertion of a different amino acid in the protein. Some confusion may arise when these kinds of mutations are described, because nucleotide changes of both DNA and RNA sequences must be considered. Here, the term codon is reserved for the three nucleotides in an mRNA that specify an amino acid (e.g., UUU) or a stop. The set of three nucleotides of a DNA sequence that act as the template for a codon is a "transcribed triplet" (e.g., AAA). The complementary DNA sequence to a transcribed triplet is a "coding triplet" (e.g., TTT). And a unit consisting of both a complementary transcribed and coding triplet is a DNA codon, for example, AAA/TTT. The consequences of mutation of a DNA codon depend on which nucleotide pair is changed, the nature of the substitution, the specificity of the new codon, and the relative location of the mutated codon in an mRNA, among other factors. In general, DNA codon mutations are classified as silent, neutral, missense, or nonsense.

A silent mutation occurs when there is a change of a DNA codon, but the amino acid that is inserted into the protein is not changed (Figure 4.17A). For many amino acids, there are multiple codons. In a number of these cases, the difference between the codons for a single amino acid lies in the third position, where U, G, C, or A can be present without altering the specificity of the codon. A silent mutation has no impact on the function of the protein.

A neutral mutation represents a nucleotide change at the DNA level that alters a codon so that another amino acid is incorporated into the protein with no apparent loss of function (Figure 4.17B). This type of change is tolerated if the substitution occurs in a part of the protein that is not important for its function or if the alternative amino acid has physicochemical properties similar to the original one, as when valine substitutes for leucine.

A base pair substitution producing a codon that specifies another amino acid is a missense mutation (Figure 4.17C). The severity of a missense mutation depends on the nature of the substituted amino acid and whether the original

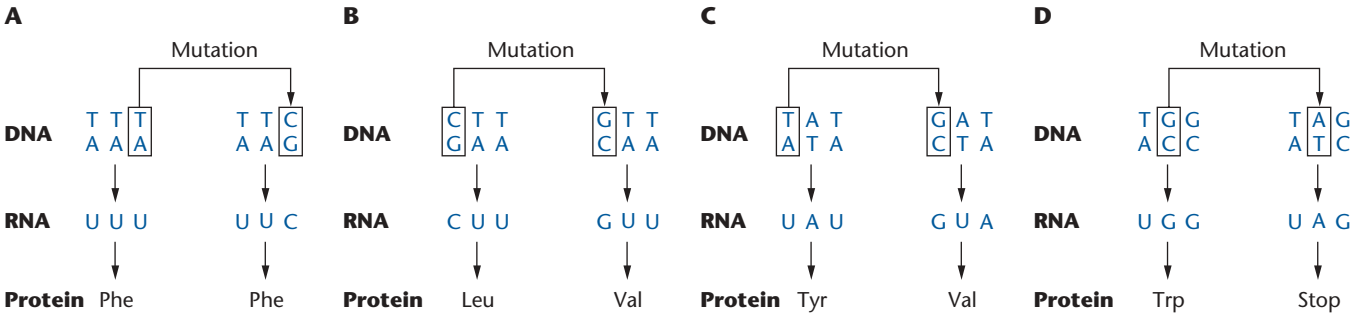


Figure 4.17 Consequences of base pair substitutions within the coding region of a structural gene. (A) Silent mutation. The base pair substitution in the DNA codon does not change the coding specificity. (B) Neutral mutation. The base pair substitution changes the amino acid specified by the DNA codon, but the replacement amino acid has physicochemical properties similar to the original one. (C) Missense mutation. The base pair substitution changes the amino acid specified by the DNA codon. (D) Nonsense mutation. Base pair substitution changes a DNA codon that codes for an amino acid into one that specifies a stop codon.

amino acid plays an essential role in the function of the protein. A neutral mutation is a missense mutation with no obvious consequences.

A nonsense mutation occurs when a nucleotide substitution changes a codon that specifies an amino acid into one that is a stop codon (Figure 4.17D). The presence of a stop codon within an mRNA causes an incomplete (truncated) protein to be produced. If a nonsense mutation is near the N-terminus, it is unlikely that a functional protein fragment will be synthesized. However, if it is near the C-terminus, an incomplete polypeptide chain might be produced with some limited activity.

When a base pair is either inserted into or deleted from a coding region of a structural gene, the sequence of codons following either of these mutation events can be changed so that the new codons are translated into a completely novel sequence of amino acids bearing absolutely no resemblance to the original protein (Figure 4.18). These types of changes are called frameshift mutations because the reading frame of the normal array of codons is shifted. Moreover, it is common in frameshift mutations for one of the "new" codons near the site of insertion or deletion to be a stop codon that causes the termination of translation (Figure 4.19). A frameshift mutation usually has a devastating effect on the function of a protein, because of either protein truncation or the addition of an aberrant string of amino acids. However, if a frameshift mutation occurs near the C-terminus of a protein, some limited capability might be retained.

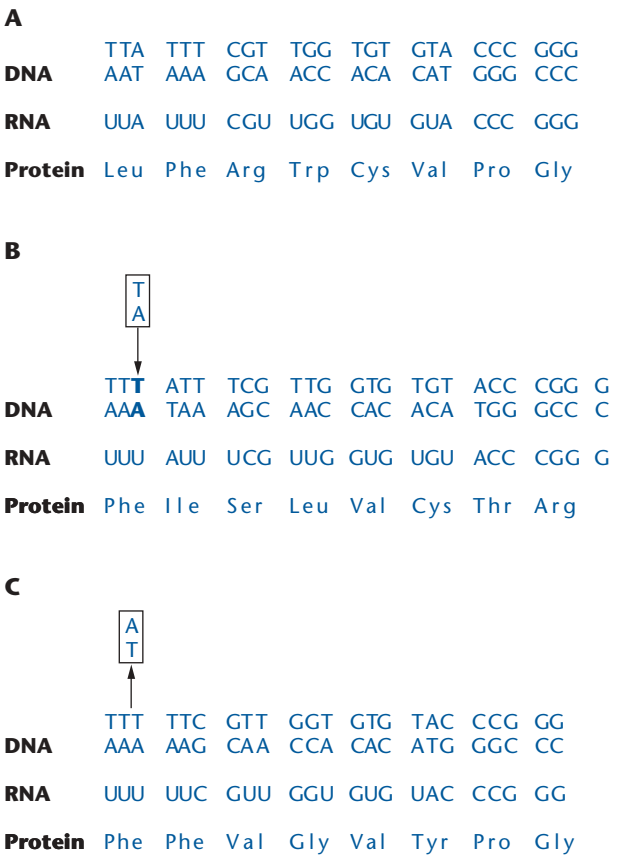


Figure 4.18 Consequences of frameshift mutations. (A) A portion of a coding region of a structural gene with the expected transcribed and translated sequences. (B) The insertion of a base pair (bold letters) after the second nucleotide site of the DNA sequence presented in A changes the reading frame. (C) The deletion of the base pair at the third nucleotide site of the DNA sequence presented in A changes the reading frame.

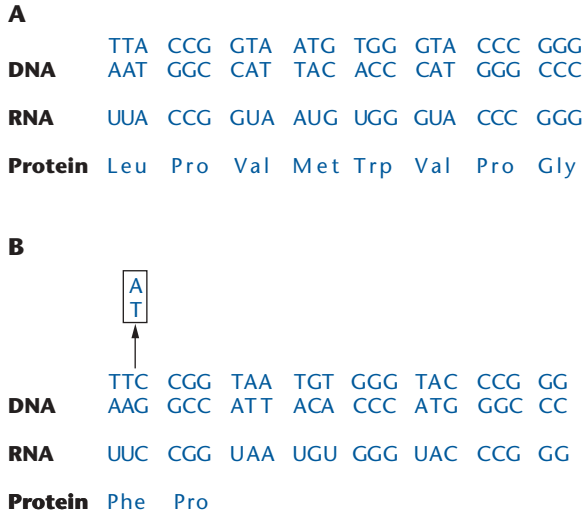


Figure 4.19 Protein truncation by frameshift mutation. (A) A portion of a coding region of a structural gene with the expected transcribed and translated sequences. (B) The deletion of the base pair at the third nucleotide site of the DNA sequence presented in A changes the reading frame, and the third DNA codon specifies a stop codon, namely, UAA.

The removal of introns from the primary RNA transcript depends on specific nucleotides at the boundaries of (a) the end of an exon and the beginning of an intron (5′ splice site) and (b) the end of an intron and the beginning of the next exon (3′ splice site). The splicing machinery joins nucleotides of a 5′ splice site to those of the next 3′ splice site. If the nucleotide sites or neighboring nucleotides of a splice site in the DNA are altered by mutation, then, after transcription, abnormal splicing occurs. Specifically, if a 3′ splice site is mutated, the splicing machinery will skip over this site and complete the splice at the next appropriate 3′ splice site. In this case, an exon flanked by two introns is removed from the primary RNA transcript, and the processed mRNA has a missing exon (Figure 4.20A). This aberrant form of splicing is called exon skipping. Similarly, if a 5′ splice site is modified, the splicing machinery will ignore this site in the primary RNA transcript, and an intron will be included as part of the processed mRNA (Figure 4.20B). In both cases, an alternative splicing event is also possible (Figure 4.20). However, when either a 3′ or 5′ splice site mutation occurs, regardless of the splicing event, none of the processed mRNA molecules has the correct nucleotide sequence for the translation of the authentic protein.

Mutation can also create a new splice site within an intron. For example, an additional 3′ splice site will cause part of an intron sequence to be included in the processed mRNA (Figure 4.21). In addition, an aberrant processed mRNA will be formed if a new 5′ splice site occurs within an intron. In both instances, the normal splice sites in some of the primary RNA molecules will also be processed, producing functional mRNAs. The inclusion of part of an intron in an mRNA would likely create a shift in the reading frame and generate a truncated protein. Finally, a mutation(s) within an exon can lead to single or multiple exon skipping. In some of these cases, the mutation may alter the folding of the primary RNA transcript and cause faulty splicing, and in others, the mutation may mask a nearby normal splice site and produce exon skipping. Of all the mutations in disease-causing genes, missense/nonsense mutations are about three and six times more frequent than deletions and splicing defects, respectively (Table 4.3).

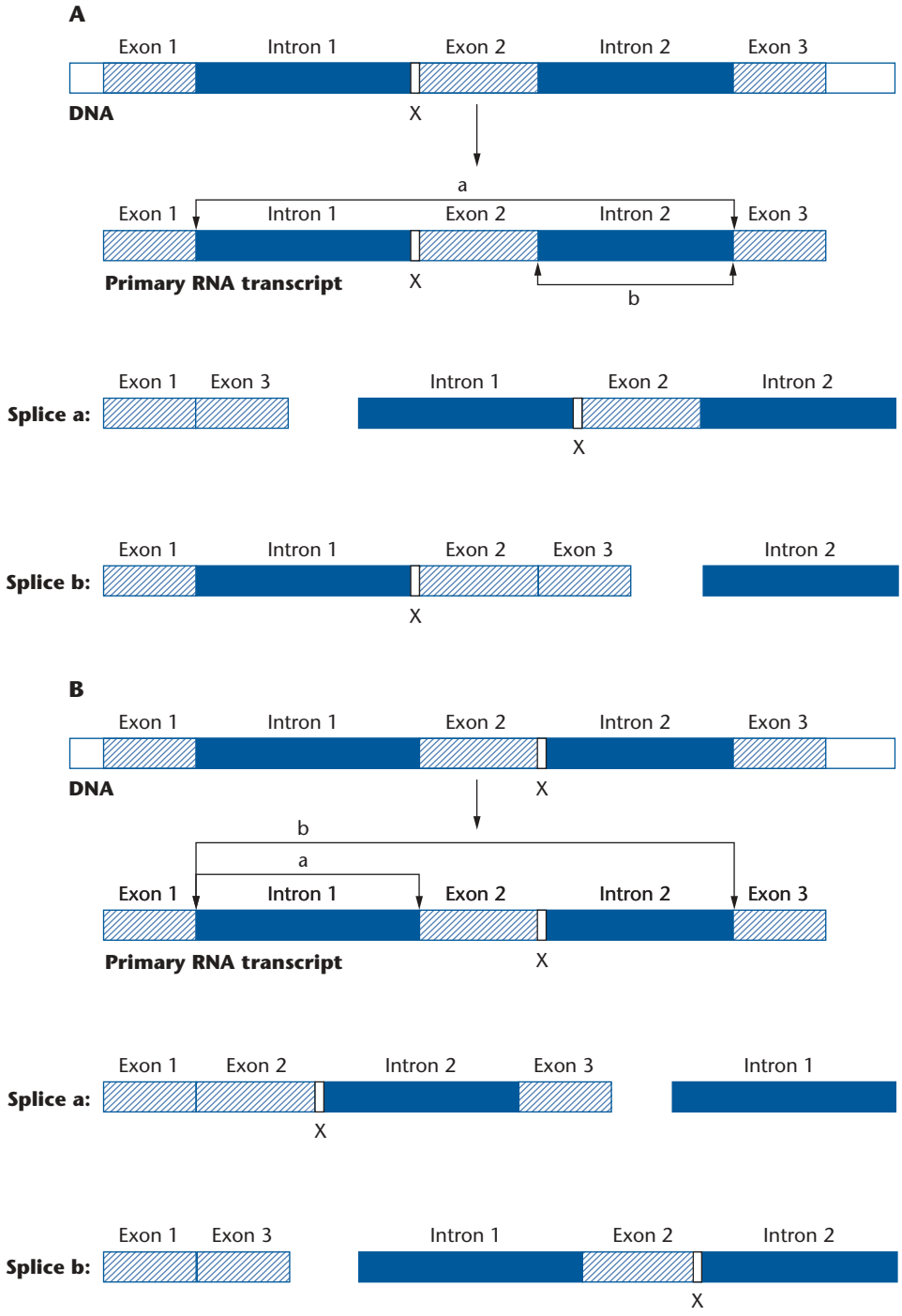


Figure 4.20 Consequences of mutated 3′ and 5′ splice sites on the processing of a primary RNA transcript. (A) The mutated 3′ splice site is designated by the X. The bracketing arrows (a, b) each mark adjacent sites that can be spliced together. In some primary transcripts, splice a removes intron 1-exon 2-intron 2 as a unit and joins exon 3 to exon 1 (splice a). In other primary transcripts, splice b removes intron 2 and joins exon 3 to exon 1-intron 1-exon 2 (splice b). (B) The mutated 5′ splice site is designated by the X. The bracketing arrows (a, b) each mark adjacent sites that can be spliced together. In some primary transcripts, splice a removes intron 1 and joins exon 2-intron 2-exon 3 to exon 1 (splice a). In other primary transcripts, splice b removes intron 1-exon 2-intron 2 and joins exon 3 to exon 1 (splice b).

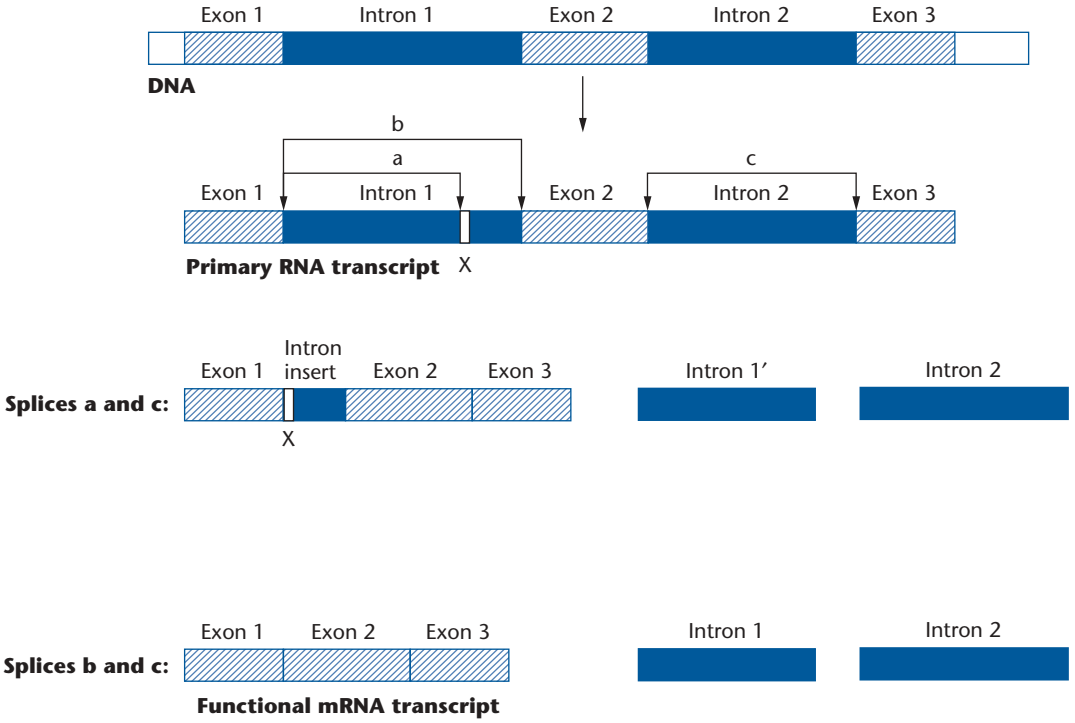


Figure 4.21 Consequences of a novel 3′ splice site within intron on processing of a primary RNA transcript. The mutated 3′ splice site is designated by the X. The bracketing arrows (a, b, c) each mark adjacent sites that can be spliced together. In some primary transcripts, splices a and c remove a truncated intron (intron 1′) and intron 2 and join the fragment that contains part of intron 1 (intron insert) and exon 2 to exon 1 and exon 3 to the end of exon 2 of the exon 1-intron insert-exon 2 component. In other primary transcripts, splices b and c remove introns 1 and 2 and join exon 2 to exon 1 and exon 3 to the end of the exon 1-exon 2 component to form a functional mRNA transcript.

Table 4.3 Frequencies of mutations in disease-causing genes.

Type of mutation	Frequency (%)
Complex rearrangements	1.8
Deletion	21.8
Insertion/duplication	6.8
Missense/nonsense	58.9
Regulatory	0.8
Repeat sequences	0.1
Splicing	9.8

From Botstein D, Risch N (2003). Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nat Genet* **33** Suppl:228–237.

Nomenclature for Mutations

Because of the availability of complete sequences of normal and mutated versions of genes and proteins, there was a need for a common and concise system to designate the site and nature of a mutation at both the DNA and protein levels. The sites of the amino acids of a protein are numbered consecutively from the initial methionine, which is designated as 1, to the C-terminal amino acid. By contrast, the numbering of the nucleotide sites of a structural gene is not as straightforward, because the first nucleotide of the first exon is seldom, if ever, the starting point of transcription (+1). There is the additional problem of distinguishing between the nucleotide sites of the coding regions of a structural gene and those of the introns. Generally, the nucleotide sites are numbered consecutively from the starting point of transcription (+1) to the end of the first exon. Sequential numbering is continued for the nucleotide sites of

the remaining exons of the gene. A nucleotide site of an intervening sequence (IVS, intron) is often given a combined designation consisting of the number of the last nucleotide site of the adjacent 5′ exon and a plus sign with an integer to indicate the downstream nucleotide position within the intron. For example, a nucleotide site designated as 100 + 7 represents the 7th nucleotide pair of the intron that follows nucleotide site 100 of the coding region. In some instances, the designation of an intron nucleotide site is specified by a minus sign with an integer representing the upstream nucleotide site. For example, 201 – 12 signifies the 12th nucleotide site upstream from nucleotide 201, which is the first nucleotide site of the adjacent 3′ exon.

A mutation in the coding region of a structural gene is often designated by both the specific nucleotide change and site of the mutation. For example, the notation A→T at 279 indicates that the adenine (A) at position 279 in the coding region of the normal gene is a thymidine (T) in the mutant form. The arrow shows the direction of the change. Only the nucleotides of the coding (sense) DNA strand are presented in this notation. The change(s) in the transcribed strand is obvious because of DNA base pair complementarity. In addition, the mutational basis for an allele at the DNA level can be used as part of the gene name. For example, with the gene symbol, *FGFR3*1138A* signifies that this allele of the fibroblast growth factor receptor 3 gene has an adenine residue at nucleotide site 1138.

The notation for specifying a missense mutation at the level of the protein includes three elements. First, the original amino acid is designated by its single-letter code. Second, the site of the affected amino acid is noted. Third, the replacement amino acid is represented by its single-letter code. For example, D89G signifies that a mutation led to the replacement of aspartic acid by glycine at amino acid site 89 in the protein. Alternatively, the three-letter amino acid code can be used, that is, Asp89Gly. A nonsense mutation is usually designated by an X. For example, R81X (Arg81X) signifies that the DNA codon in the normal protein that encodes an arginine residue at amino acid site 81 has mutated to a stop codon. In some instances, the symbol ter is used instead of X to represent a nonsense mutation, for example, G136ter (Gly136ter).

Frameshift mutations are often represented by the change to the DNA and not the protein. For example, 351delAT indicates that, in terms of the sense DNA strand, a deletion (del) occurred in the coding region of the gene that included both an adenine residue (A) at nucleotide site 351 and the adjacent downstream thymidine residue (T). And 106insT indicates that a thymidine residue was inserted into the sense DNA strand after nucleotide position 106 of the gene. If the nucleotide change is greater than two nucleotides, then numbers are used to describe the extent of the mutation. For example, 109del27 signifies that the next 27 bp downstream from nucleotide position 109 have been deleted.

A notation for splicing mutations includes the nucleotide change, its location, and the specific intron. For example, G→T + 5IVS20 signifies that a base change from a guanine to a thymine occurred at nucleotide position 5 of intron (IVS) 20 of the gene. Alternatively, the depiction 711 + 5 G→T indicates that the fifth nucleotide of the intron following nucleotide 711 of the coding region

is changed from G to T in the DNA sense strand. Many mutations can be represented with some form of simple code. However, a full description may be required for very complex changes.

Dominant Mutations and Genetic Disorders

By strict definition, recessiveness and dominance are properties of the phenotype, although it is common to refer to genes as recessive or dominant. Most mutations have a recessive effect. In individuals with one normal and one recessive allele, an adequate amount of functional gene product is synthesized and the phenotype is normal. In homozygous recessive individuals, insufficient functional gene product is produced, which, in turn, is responsible for an aberrant phenotype. However, there are a number of human genetic diseases that are the result of a single dominant allele. These dominant conditions raise an intriguing question. How does a dominant allele cause a harmful effect in the presence of a normal allele? In general, a mutation that alters, either through underproduction or overproduction, the amount of a gene product, when a specific quantity is needed for normal activity, can cause a dominant effect. In addition, dominant disorders occur when either a toxic gene product or a novel protein with an unusual mode of action is produced.

There are many ways that mutation can lower the amount of a gene product. A deletion, for example, can remove an entire allele (null allele) so that heterozygotes produce half the gene product, in comparison to the presence of two normal alleles. Missense, nonsense, and exon splicing mutations can reduce the quantity of a functional gene product. In addition, changes to response elements can decrease the transcription of an mRNA and, consequently, lower the level of gene product.

The active state of many proteins consists of multiple subunits from one or more loci. As well, many important cellular functions are performed by ensembles of proteins (protein complexes). As an example of dominance due to the interaction between normal and mutant polypeptides, consider a two-chain (dimeric) protein with the same polypeptide (homodimer), where the dimer, and not a single chain, is functional. If a missense or frameshift mutation occurs that abolishes the site of activity in a polypeptide but not its ability to combine with either the normal or variant polypeptide to form a dimer, then, in a heterozygote, each allele produces a protein chain. Because polypeptide chains join randomly, 25% of the dimers will be active and 75% will be inactive (Figure 4.22). In this example, the α2 polypeptide, the product of the *A*2* allele, is responsible for the dominant effect, because 25% activity is not adequate to

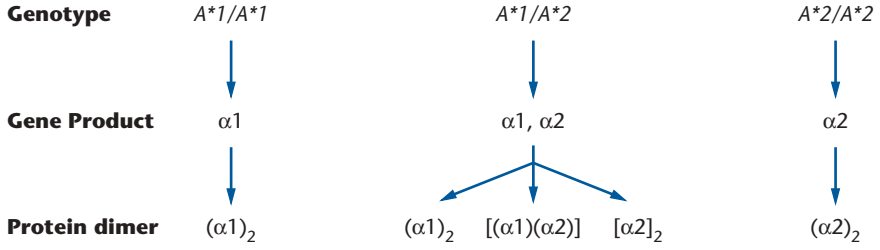


Figure 4.22 Example of the dominant negative effect of a mutant protein. An inactive dimer is formed when the α2 protein combines with itself, that is, $(\alpha 2)_2$, or with a α1 polypeptide, that is, [(α1)(α2)]. In heterozygotes (*A*1/A*2*), only the $(\alpha 1)_2$ dimers are active, whereas the [(α1)(α2)] and $(\alpha 2)_2$ dimers are not. In this case, the *A*2* allele has a dominant effect because the amount of active dimer falls below the threshold that is needed for the normal phenotype.

produce a normal phenotype. This example illustrates a dominant negative effect where, in a heterozygote, the combination of a mutant polypeptide from one allele and a normal polypeptide from the other allele forms an inactive protein. By extrapolation, one faulty component of a multiprotein complex may also give rise to a dominant condition.

Just as too little of a gene product can cause an abnormality, so can too much of gene product lead to a biological disorder. (To some, these conditions are reminiscent of Goldilocks and her predicament after illegally entering the home of the three bears and helping herself to some of their amenities!) Although a rare mutational event, extra copies of a gene will produce an excess of a gene product. In addition, mutation of a response element can lead to uncontrolled transcription, resulting in the overproduction of an mRNA and, consequently, to a surplus of a gene product.

Increased stability of either an mRNA or protein can cause, in contrast to the normal condition, an elevated amount of a functional protein. Normally, most mRNAs and proteins are degraded enzymatically within a few hours after synthesis. A mutation can change the sequence of noncoding nucleotides of an mRNA that is recognized by nucleic acid-degrading enzymes (nucleases) and prolong the lifetime of the mRNA, leading to an increased amount of the gene product. Similarly, a mutation of a codon that does not affect function but alters a proteolytic enzyme recognition site would make a protein less susceptible to breakdown and allow it to accumulate. Among other possibilities, an augmented amount of a normal protein could disrupt the timing of a developmental process or alter the developmental fate of a cell. If an enzyme is present in excess, then too much of an intermediate compound might be metabolized and create a deficiency that is responsible for an abnormal condition.

Another kind of dominant effect occurs when a mutant allele encodes a protein that produces a toxic effect. For example, a missense or frameshift mutation could cause a protein to assume a new conformation that causes abnormal, insoluble aggregates to form. These aggregates, not readily degraded by proteolytic enzymes, accumulate within cells and disrupt normal cellular functions. Finally, mutation events, especially chromosomal translocations, can, on rare occasions, produce a new gene product by combining parts of different genes to form a "hybrid" gene. The product of a hybrid gene would have a dominant effect if the new activity leads to cellular malfunction.

In a broad sense, dominant phenotypes result from either "loss of function" or "gain of function" mutations. A loss of function mutation often occurs when two normal alleles of a gene are required for normal activity and the presence of a mutant allele alters a critical threshold level. A gain of function mutation is the result of a mutated gene that produces a protein that obstructs a normal process.

key terms

adenine
anticodon
antisense RNA
aminoacyl site
codon
codon usage
complementary base pairs
cytosine
dominance
DNA polymerase
elongation
eukaryotes
exons
exon skipping
frameshift
guanine
initiation
introns
messenger RNA (mRNA)
methionine
missense mutation
noncoding RNA
nonsense mutation
peptide bond
peptidyl site
phosphodiester bonds
polynucleotide
reading frame
recessive
response element
ribosomal RNA (rRNA)
splice mutation
stop codon
TATA box
termination
termination factor
thymine
transcription
transfer RNA (tRNA)
transition
transversion
truncation

summary

The three fundamental properties of genetic material (deoxyribonucleic acid, DNA) are replication, encoding information for the production of proteins, and the potential to mutate. A DNA molecule has two polynucleotide strands that form an antiparallel double helix. The monomeric unit of a DNA strand is a nucleotide consisting of an organic base, deoxyribose sugar, and a phosphate group. The successive nucleotides of a DNA strand are linked by phosphodiester bonds, and the two strands of DNA are held together by specific complementary pairs of bases. The base adenine forms hydrogen bonds only with thymine, and guanine forms bonds only with cytosine. During replication, which is mediated by a number of different proteins including DNA polymerases, each existing DNA strand acts as a template for the production of a complementary strand. The base-pairing requirements ensure that each newly synthesized DNA strand has the correct nucleotide sequence.

Proteins are vital for the maintenance of all biological functions. A protein consists of a specific sequence of amino acids linked by peptide bonds. The sequence of amino acids for a protein is encoded by a sequence of DNA. The process of decoding genetic information is carried out by ribonucleic acid (RNA) molecules [messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA)], various enzymes, and protein factors. Generally, all RNA is transcribed from DNA. Sequences of DNA in combination with protein factors ensure that RNA synthesis (transcription) is initiated at a precise starting point, the appropriate strand is used as the template, and termination occurs at a specified nucleotide site. In eukaryotic organisms, most structural genes consist of coding regions (exons) separated by noncoding segments (introns). Primary transcripts contain both exons and introns. However, a processing system removes the introns and joins the exons, in the proper order, to form a functional mRNA. An mRNA carries the code for the sequence of amino acids of a protein.

Both tRNA and rRNA molecules play important roles in the biosynthesis of proteins (translation). There are more than 50 different tRNA molecules in a cell. Each tRNA has two significant features. First, 1 of the 20 different amino acids found in proteins is bound enzymatically to the 3′-end of a tRNA. The relationship between a tRNA and its amino acid is absolutely specific. Second, in another location of the tRNA molecule, a set of three contiguous nucleotides called the anticodon enables a tRNA to bind to a complementary set of three nucleotides (codon) of an mRNA during translation. Two major rRNA molecules, one small and one large, each combine with different groups of proteins to form a small and a large ribosomal subunit, respectively.

Translation in eukaryotes is initiated by the combining of a unique initiator tRNA, which carries the amino acid methionine, Met-tRNA^{Met}, with a small ribosomal subunit. Then, an mRNA is threaded through the "initiator tRNA-small ribosomal subunit" complex until the first AUG sequence in the mRNA pairs with the UAC sequence of the anticodon of the initiator tRNA. The large ribosomal subunit joins the initiator tRNA-small ribosomal subunit-mRNA combination to form a ribosome that is ready for the translation of the mRNA sequence.

After the formation of a ribosome, the next three nucleotides in the mRNA pair with the anticodon of a tRNA that carries its specific amino acid, for example, AA_2. The first amino acid, methionine, is cleaved from

the initiator tRNA and is joined to the second amino acid by a peptide bond. The "methionine-less" initiator tRNA is ejected from the ribosome, the ribosome complex shifts, and the peptidyl-tRNA (Met-AA_2-$tRNA^{AA2}$) occupies the site vacated by the ejected initiator tRNA. As a consequence of the shift (translocation), the next codon of the mRNA is available to pair with the appropriate anticodon of the tRNA that carries its specific amino acid, for example, AA_3. Then, AA_2 of the Met-AA_2-$tRNA^{AA2}$ combination is cleaved from its tRNA and joined to AA_3 with a peptide bond to give Met-AA_2-AA_3-$tRNA^{AA3}$. The "peptidyl-less" $tRNA^{AA2}$ is ejected from the ribosome; the ribosome complex shifts, and the remaining peptidyl-tRNA (Met-AA_2-AA_3-$tRNA^{AA3}$) occupies the site vacated by the previous peptidyl-tRNA. By repeating these steps, a protein with a specific sequence of amino acids is formed.

Translation is terminated when one of three codons (stop codons) is encountered on a ribosome. No normally occurring tRNA has an anticodon that is complementary to a stop codon. However, a termination factor(s) recognizes a stop codon, and, after this factor attaches to the ribosome, the bond between the last tRNA and the completed amino acid chain is cleaved, which causes the tRNA, mRNA, and completed protein to be released and the two ribosomal subunits to separate. The genetic code consists of 64 different codons. Of these, 61 code for amino acids and 3 represent stop codons. Because there are 20 amino acids in proteins, some of these amino acids have two or more codons.

A mutation is a change in one or more nucleotide pairs in DNA. Some single base pair substitutions in the coding region of a structural gene can change the amino acid specificity of a codon to another amino acid (missense mutation). Some missense mutations have no impact on the function of the gene product. For others, the contrary holds. A single base pair substitution can change an amino acid–specifying codon into a stop codon (nonsense mutation), which leads to the formation of a truncated protein. Mutations at splice sites cause faulty processing of primary transcripts, producing nonfunctional mRNA transcripts. The insertion or deletion of a nucleotide pair in the coding region of a structural gene can alter a reading frame of the codons and change the entire amino acid sequence downstream from the site of the mutation. In many instances, a change in a reading frame introduces a stop codon that causes premature termination of translation.

For some biological functions, a certain amount of gene product must be present to give a normal phenotype. In heterozygotes, an allele may be responsible, directly or indirectly, for the production of either too much or too little of a gene product, leading to an inherited dominant disorder. A dominant effect also occurs when a mutated gene produces a toxic protein.

References

Antonarakis, S. E. 1998. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat* **11**:1–3.

Baker, T. A. and S. P. Bell. 1998. Polymerases and the replisome: machines within machines. *Cell* **92**:295–305.

Beaudet, A. L. and L.-C. Tsui. 1993. A suggested nomenclature for designating mutations. *Hum Mutat* **2**:245–248.

Botstein, D. and N. Risch. 2003. Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nat Genet* **33** Suppl:228–237.

Buratowski, S. 1994. The basics of basal transcription by RNA polymerase II. *Cell* **77**:1–3.

Cartegni, L., S. L. Chew, and A. R. Krainer. 2002. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* **3**:285–298.

Dietz, H. C. et al. 1993. The skipping of constitutive exons in vivo induced by nonsense mutations. *Science* **259**:680–683.

Diffley, J. F. and K. Labib. 2002. The chromosome replication cycle. *J Cell Sci* **115**: 869–872.

Eddy, S. R. 2001. Non-coding RNA genes and the modern RNA world. *Nat Rev Genet* **2**:919–929.

Eyre-Walker, A. and P. D. Keightley. 1999. High genomic deleterious mutation rates in hominids. *Nature* **397**:344–347.

Fisher, C. W. et al. 1993. Occurrence of a 2-bp (AT) deletion allele and a nonsense (G-to-T) mutant allele at the E2 (DBT) locus of six patients with maple syrup urine disease: multiple-exon skipping as a secondary effect of the mutations. *Am J Hum Genet* **52**:414–424.

Johnson, J. M. et al. 2003. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* **302**:2141–2144.

Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J Biol Chem* **266**:19867–19870.

Kunkel, T. A. 2004. DNA replication fidelity. *J Biol Chem* **279**:16895–16898.

Lodish, H. et al. 2003. *Molecular Cell Biology.* 5th ed. W. H. Freeman and Co., New York.

Neel, J. V. et al. 1986. The rate with which spontaneous mutation alters the electrophoretic mobility of polypeptides. *Proc Natl Acad Sci USA* **83**:389–393.

Schoenherr, C. J. and D. J. Anderson. 1995. The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. *Science* **267**:1360–1363.

Schoenherr, C. J., A. J. Paquette, and D. J. Anderson. 1996. Identification of potential target genes for the neuron-restrictive silencer factor. *Proc Natl Acad Sci USA* **93**:9881–9886.

Schwarz, D. S. and P. D. Zamore. 2002. Why do miRNAs live in the miRNP? *Genes Dev* **16**:1025–1031.

Tate, W. P. and C. M. Brown. 1992. Translational termination: "stop" for protein synthesis or "pause" for regulation of gene expression. *Biochemistry* **31**:2443–2450.

Tjian, R. and T. Maniatis. 1994. Transcriptional activation: a complex puzzle with few easy pieces. *Cell* **77**:5–8.

Wilkie, A. O. 1994. The molecular basis of genetic dominance. *J Med Genet* **31**:89–98.

Yelin, R. et al. 2003. Widespread occurrence of antisense transcription in the human genome. *Nat Biotechnol* **21**: 379–386.

review questions

1. If the guanine content of a genome is 24%, what are the proportions of the other nucleotides in the DNA of this organism?
2. Discuss the basic features of DNA replication.
3. Compare and contrast DNA and RNA.
4. Describe the initiation of transcription of a eukaryotic gene.
5. What is a primary RNA transcript?
6. Describe the initiation of translation in a eukaryotic organism.
7. Describe the elongation phase of translation.
8. Deduce the most likely DNA coding sequence for the following human protein:

   MAGGTWYQLFPRKMWNDSTLHPFILPMNVAG
9. Determine the amino acid sequence encoded by the following mRNA:

   GCGAUCGACGAUGUUUCUAAAAGUAUCUCAUC
   GAAAUGAGGGUUCGUAAUAGCGACCCGGGCGG
10. What is an isoform?
11. What is a TATA box?
12. Describe, at least, two different modes of regulating gene expression.
13. What is a nonsense mutation?
14. What would be the consequences of a mutation of the anticodon of a tRNA?
15. With the DNA sequence

    ATGTTTTTCTATTGAAGCGGTGTGTAG
    TACAAAAAGATAACTTCGCCACACATC

    which strand is likely to be transcribed? Why? What are the RNA and protein sequences?
16. Show the consequences at the RNA and protein levels of a single nucleotide deletion of the first base pair of the second DNA codon of the DNA sequence in Question 15.
17. Show the consequences at the RNA and protein levels of a single nucleotide insertion between the second and third base pairs of the third DNA codon of the DNA sequence in Question 15.
18. Describe each of the following notations: V6L, Y201X, and G→C 3056.
19. What is exon skipping?
20. Explain various ways that mutant alleles give rise to a dominant phenotype.

chapter 5

Recombinant DNA Technology

Molecular biology and the allied technology of genetic engineering are second only to the computer in evoking the name "revolution" in the daily press.

I. BERNARD COHEN (b. 1914)

It is easy enough to say what we would do if we had a method to measure A or isolate B. But it is in inventing and applying these methods that our biggest problems often arise.

J. B. S. HALDANE (1892–1964)

RECOMBINANT DNA TECHNOLOGY, also called gene cloning, genetic engineering, or molecular cloning, is an umbrella term encompassing a number of protocols. In brief, the purpose of these experimental manipulations is to introduce a gene from one organism into a host cell, where it can be perpetuated and studied. More specifically, a typical recombinant DNA experiment often has the following characteristics (Figure 5.1). DNA is extracted from a source organism and cleaved enzymatically. Each enzymatically cut DNA fragment (insert DNA) is joined (ligated) to a specifically designed DNA entity (cloning vector), capable of replicating in a particular host cell, to form an intact recombinant DNA molecule (DNA construct). Each DNA construct is transferred into a host cell, where it is maintained. Usually, a host cell receives only one DNA construct. However, the efficiency of uptake of DNA constructs is very low. Consequently, the host cells that carry DNA constructs must be identified and separated (selected) from those that do not. Moreover, the host cell with a specified DNA insert must be recognized and isolated. Subsequently, the insert DNA (target DNA, cloned DNA) of a DNA construct can be characterized. In some cases, the insert DNA is expressed in the host cell and, if required, the protein encoded by a cloned gene can be harvested.

Since its inception in the early 1980s, recombinant DNA technology has been responsible for a dramatic upsurge in the understanding of the molecular basis of human genetic disorders. Either directly or indirectly, this technology has led to the routine ability to locate, isolate, and characterize both normal and mutant versions of genes that cause monogenic disorders. It has made possible the creation of high-density comprehensive genetic maps of each human

An Introduction to Human Molecular Genetics, Second Edition, by Jack J. Pasternak
ISBN 0-471-47426-6

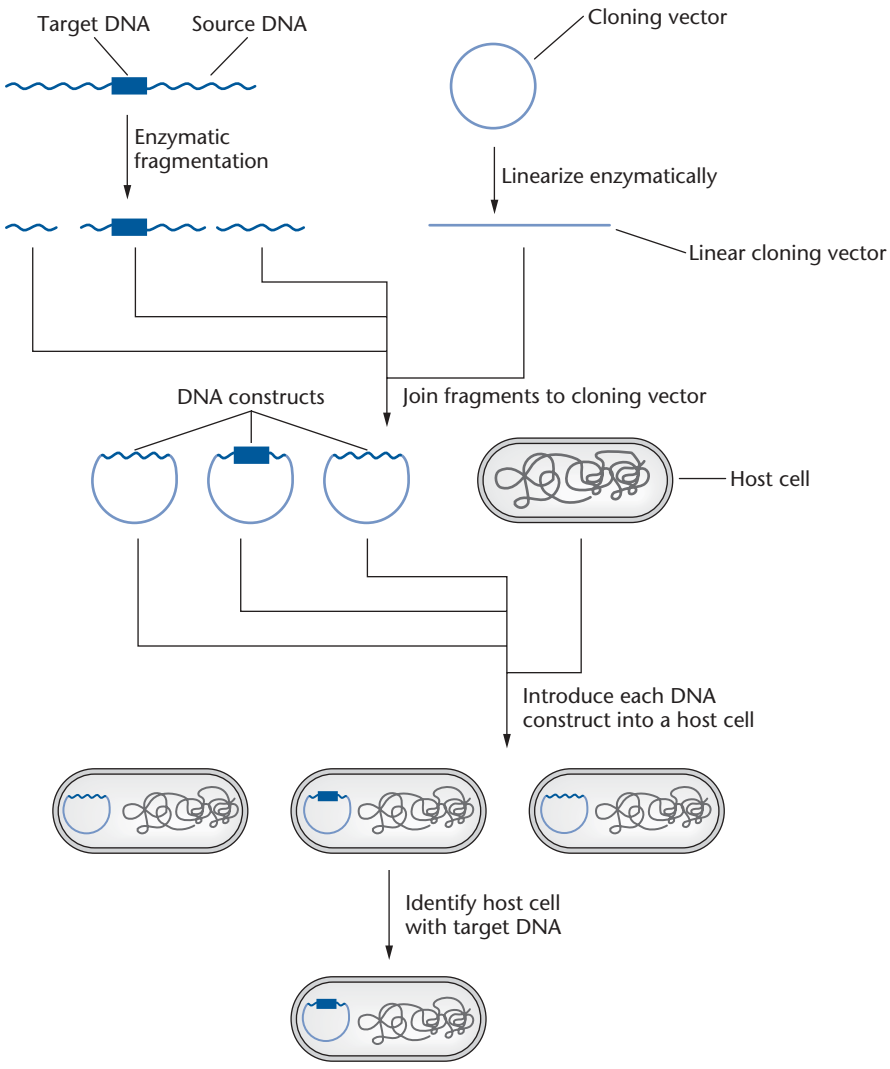


Figure 5.1 Schematic representation of DNA cloning. DNA from a source organism (source DNA) is cleaved with a restriction endonuclease and inserted into a cloning vector to form DNA constructs. Each DNA construct is introduced into a host cell. Cells carrying the target DNA construct are identified, isolated, and grown.

chromosome and facilitated the determination of the complete DNA sequence of the human genome. Recombinant DNA technology is behind the development of sensitive DNA tests for diagnosing specific genetic diseases and will assist in the formulation of gene-based therapies for various disorders.

Restriction Endonucleases

For molecular cloning, both the source DNA that contains the target sequence and the cloning vector must be consistently cut into discrete and reproducible fragments. Subjecting isolated chromosomal DNA either to passage through a small-bore needle or to sonication produces double-stranded pieces of DNA that may range from 0.3 to 5 kilobase pairs (kb) in length. Unfortunately, these simple procedures induce breaks randomly, so each time a DNA sample is treated a different set of fragments is generated. It was only after bacterial enzymes that cut DNA molecules internally at specific base pair sequences were

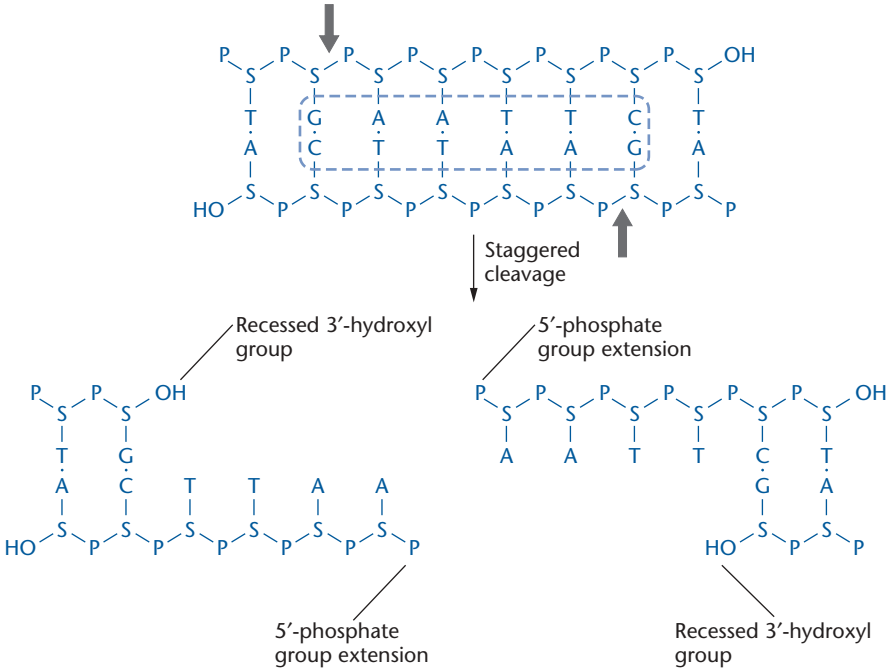


Figure 5.2 Symmetrical, staggered cleavage of a short fragment of DNA by the type II restriction endonuclease *Eco*RI. Bold arrows show the sites of cleavage in the DNA backbone. The letter S denotes the deoxyribose sugar; P, phosphate group; OH, hydroxyl group; A, adenine; C, cytosine; G, guanine; and T, thymine. The *Eco*RI recognition sequence is highlighted.

discovered that molecular cloning became feasible. These enzymes are called type II restriction endonucleases. Despite the occurrence of other kinds of restriction endonucleases, the type II restriction endonucleases are commonly called restriction endonucleases or, simply, restriction enzymes.

One of the first of these type II restriction endonucleases to be characterized was from the bacterium *Escherichia coli*, and it was designated *Eco*RI. This enzyme binds to a DNA region with a specific palindromic sequence. In other words, the two strands of the binding site are identical when either is read in the same polarity, that is, either the 5′ to 3′ or 3′ to 5′ direction. The *Eco*RI-recognition sequence consists of 6 base pairs (bp) and is cut between the guanine and adenine residues on each strand (Figure 5.2). *Eco*RI specifically cleaves the internucleotide bond between the oxygen of the 3′-carbon of the sugar of one nucleotide and the phosphate group attached to the 5′-carbon of the sugar of the adjacent nucleotide. The symmetrical staggered cleavage of DNA by *Eco*RI produces two single-stranded, complementary cut ends, each with extensions of four nucleotides. In this case, each single-stranded extension terminates with a 5′-phosphate and the 3′-hydroxyl of the opposite strand is recessed.

In addition to *Eco*RI, hundreds of other type II restriction endonucleases with more than 120 different recognition sites have been isolated from various bacteria. The naming protocol for these enzymes is the same as that for *Eco*RI; the genus is the capitalized letter and the first two letters of the species name are in lowercase letters. The strain designation is often omitted from the name, and roman numerals are used to designate the order of characterization of different restriction endonucleases from the same organism. For example, *Hpa*I and *Hpa*II are the first and second type II restriction endonucleases that were isolated from *Haemophilus parainfluenzae*.

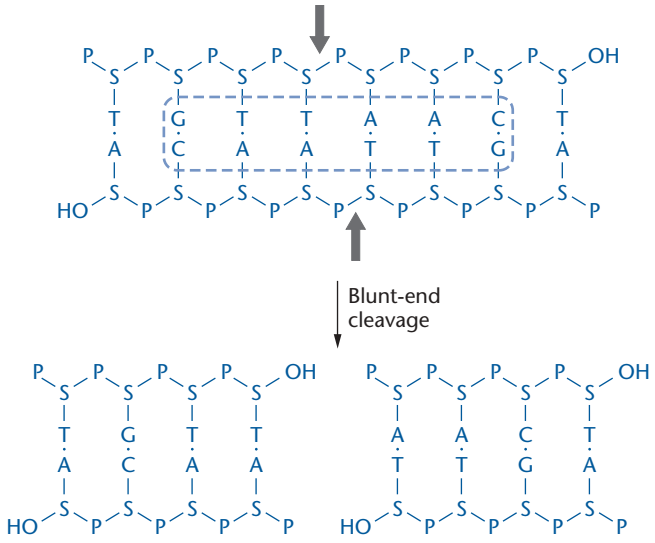


Figure 5.3 Blunt-end cleavage of a short fragment of DNA by the type II restriction endonuclease *Hind*II. Bold arrows show the sites of cleavage in the DNA backbone. The letter designations are the same as in Figure 5.2. The *Hind*II recognition sequence is highlighted.

Table 5.1 Recognition sequences of some type II restriction endonucleases.

Enzyme	Recognition site	Type of cut end
*Eco*RI	G↓A-A-T-T-C C-T-T-A-A↑G	5′-Phosphate extension
*Bam*HI	G↓G-A-T-C-C C-C-T-A-G↑G	5′-Phosphate extension
*Pst*I	C-T-G-C-A↓G G↑A-C-G-T-C	3′-Hydroxyl extension
*Sau*3AI	↓G-A-T-C C-T-A-G↑	5′-Phosphate extension
*Pvu*II	C-A-G↓C-T-G G-T-C↑G-A-C	Blunt end
*Hpa*I	G-T-T↓A-A-C C-A-A↑T-T-G	Blunt end
*Hae*III	G-G↓C-C C-C↑G-G	Blunt end
*Not*I	G↓C-G-G-C-C-G-C C-G-C-C-G-G-C↑G	5′-Phosphate extension

Arrows mark sites of phosphodiester cleavage.

The palindromic sequences where most type II restriction endonucleases bind and cut a DNA molecule are called recognition sites. Some restriction endonucleases digest (cleave) DNA, leaving 5′-phosphate extensions (protruding ends, sticky ends); some leave 3′-hydroxyl extensions; and some cut the backbone of both strands within a recognition site to produce blunt-ended (flush-ended) DNA molecules (Figure 5.3). The length of the recognition site for different enzymes can be four, five, six, eight, or more nucleotide pairs (Table 5.1). Of all restriction enzymes, six-cutters occur most often, followed by four-cutters. Because of the frequency with which their recognition sites occur in DNA, restriction endonucleases that cleave within sites of four (four-cutters) and six (six-cutters) nucleotide pairs are used for most of the common molecular cloning protocols. The importance of the type II restriction endonucleases for gene cloning cannot be overstated. When a DNA sample is treated with one

of these enzymes, the same set of fragments is always produced, assuming that all of the recognition sites are cleaved. In addition, ready access to a variety of restriction endonucleases adds versatility to gene cloning strategies.

Under natural conditions, bacteria use restriction endonucleases to cleave foreign DNA and have developed systems that protect their own DNA from being degraded. Most often, methylation of the cytosine residues of a restriction endonuclease site in the host DNA prevents restriction endonucleases from cutting at these sites but leaves the corresponding nonmethylated sites of foreign DNA vulnerable to attack. Because so many bacteria use restriction endonucleases as a defense mechanism, interesting fortuitous relationships frequently occur. In some instances, different phosphodiester bonds within the same recognition site are cleaved by restriction endonucleases from different organisms. For example, the restriction enzymes *Xma*I and *Sma*I both recognize the sequence $\begin{matrix}\text{CCCGGG}\\ \text{GGGCCC}\end{matrix}$, but *Xma*I cleaves after the 5′-cytosine in each strand and produces 5′-phosphate extensions whereas *Sma*I generates blunt ends by cleaving between the $\begin{matrix}\text{CG}\\ \text{GC}\end{matrix}$ base pair in the middle of the recognition site.

Restriction endonucleases that cut the same recognition site but at different locations are called isoschizomers. On the other hand, restriction endonucleases that produce the same nucleotide extensions but have different recognition sites are designated isocaudomers, for example, *Bam*HI and *Sau*3AI (Table 5.1). There are some restriction endonucleases from different organisms that have both the same recognition sequences and cleavage locations, for example, *Xho*I and *Pae*R7I. In some cases, a restriction endonuclease will only cleave a sequence if its cytosines are not methylated, whereas another restriction endonuclease will cut the same sequence if these cytosines are methylated. For example, *Hpa*II cleaves only nonmethylated $\begin{matrix}\text{CCGG}\\ \text{GGCC}\end{matrix}$ sites and *Msp*I, an isoschizomer of *Hpa*II, cuts this sequence regardless of cytosine methylation. This pair of restriction endonucleases are often used to determine the methylation status of genomic DNA. If a DNA molecule is not cut by *Hpa*II but is cut by *Msp*I, then the recognition site is methylated. If both restriction endonucleases cleave a DNA molecule, then the site(s) is not methylated.

Physical maps that designate the linear order of restriction endonuclease sites on a specific piece of DNA can be constructed by treating the DNA molecule singly with different restriction endonucleases and then with combinations of the same restriction endonucleases. The positions of the cleavage sites can be deduced from an analysis of fragment sizes, which are determined by agarose gel electrophoresis. By way of illustration, the fragment sizes produced by various digestions are shown in Figure 5.4A. It can be deduced that this piece of DNA has two *Bam*HI sites and two *Eco*RI sites in a specific order with a specified number of base pairs separating the sites. More specifically, the fragment sizes produced in each single digestion can be compared with those from double digestions to determine the positions of the restriction endonuclease sites from which a restriction endonuclease site map (restriction endonuclease map) is generated. In the example shown in Figure 5.4, the analysis goes as follows. Because each single digestion produces three fragments from a linear DNA molecule, the original piece of DNA must contain two sites for each of

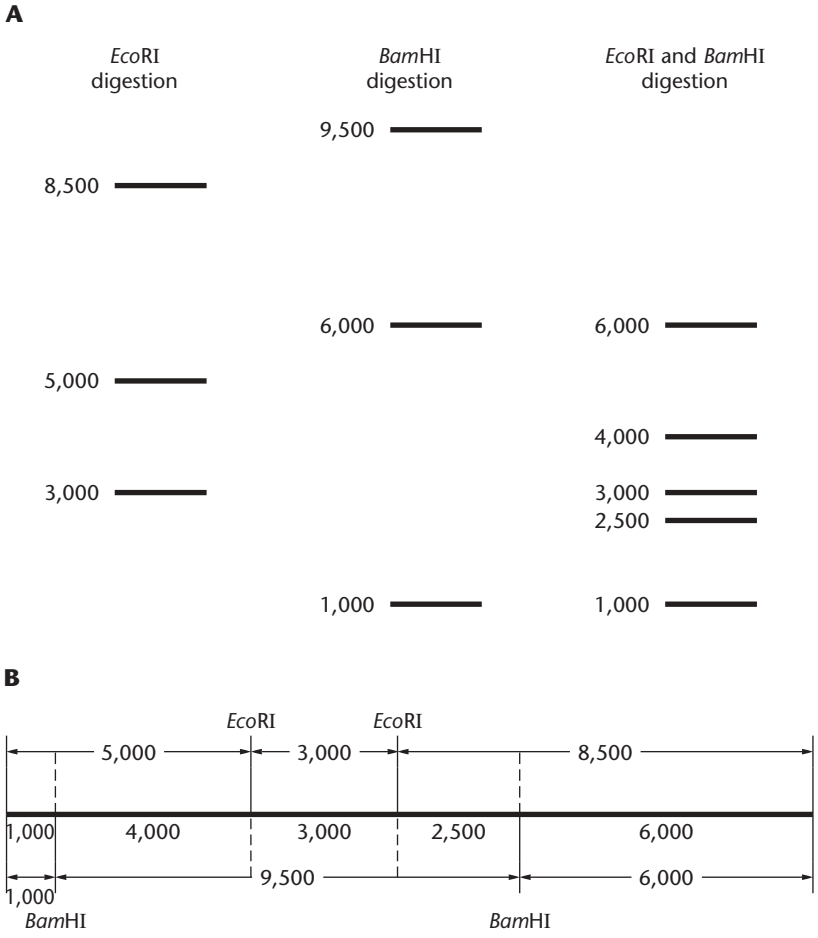


Figure 5.4 Physical mapping of restriction endonuclease sites. (A) Restriction endonuclease digestions and electrophoretic separation of cleaved fragments. A purified piece of DNA is cut with *Eco*RI and *Bam*HI separately (single digestions) and with both enzymes together (double digestion). The horizontal lines under each of the digestion conditions schematically represent the locations of DNA fragments (bands) in a gel after electrophoresis and staining of the DNA with ethidium bromide. The numbers denote the length of the digestion products (fragments) in base pairs (bp). (B) The restriction endonuclease map deduced from the results of the electrophoretic separations from the single and double digestions shown in (A).

the restriction endonucleases. The 3000-bp fragment that is produced by the single *Eco*RI digestion remains intact after the double digestion, whereas the 8500- and 5000-bp *Eco*RI fragments are cleaved. Therefore, the two *Eco*RI sites are 3000 bp apart with no intervening *Bam*HI site, and there is a single *Bam*HI site within each of the 8500- and 5000-bp *Eco*RI fragments. The 9500-bp fragment that is produced by the single *Bam*HI digestion is cleaved by *Eco*RI in the double digestion into three pieces (2500 + 3000 + 4000 = 9500 bp). Therefore, the two *Bam*HI sites lie 2500 and 4000 bp to either side of the *Eco*RI sites. Digestion with *Bam*HI cleaves the 8500-bp *Eco*RI fragment into 2500- and 6000-bp fragments, and one of the *Eco*RI sites is 2500 bp from a *Bam*HI site, so the 6000-bp region must include one of the ends of the original molecule. Using the same logic, we also note that digestion with *Bam*HI cuts the 5000-bp *Eco*RI fragment into 1000- and 4000-bp fragments and that one of the *Eco*RI sites is 4000 bp from a *Bam*HI site; therefore, the 1000-bp region must include the other end of the original molecule. In the final map (Figure 5.4B), the assigned locations of the restriction endonuclease sites are consistent with the fragment lengths that were observed in each of the digestion reactions.

For some restriction endonuclease mapping experiments, the sum of the fragments of some multiple digestions is less than the total length of the start-

ing DNA because the fortuitous locations of some sites produce fragments of the same size. Under these conditions, two different fragments with the same length that migrate to the same location in a gel after electrophoresis often stain more heavily than a band with one kind of fragment. This difference in staining intensity gives an indication that coincidental fragments have been produced by restriction endonuclease digestion. Generally, computer programs are used to configure restriction endonuclease maps for large DNA molecules with many single and multiple digestions. As well, for very large DNA molecules, specialized electrophoresis systems are used to separate the restriction endonuclease digestion products.

The resolution of fragments for restriction endonuclease mapping can be enhanced by labeling the pieces of DNA, usually at 5′ ends, with a radioactive compound or fluorescent dye and determining their lengths after electrophoretic separation with autoradiography or fluorography, respectively. A common 5′-end labeling procedure entails dephosphorylation of the 5′ ends of a linear DNA molecule with calf intestine alkaline phosphatase and the addition of radiolabeled γ-phosphate from ATP to the 5′-OH ends by T4 polynucleotide kinase. The labeled DNA fragments are separated from unincorporated label by column chromatography before gel electrophoresis. Parenthetically, recombinant DNA technology requires many different enzymes with various activities. Some of these are listed in Table 5.2.

Table 5.2 Some of the enzymes that are used for recombinant DNA technology.

Enzyme	Activity
Alkaline phosphatase	Removes 5′ phosphate groups of DNA molecules; bacterial alkaline phosphatase (BAP) is more stable but less active than calf intestinal alkaline phosphatase (CIP)
DNase I	Degrades double-stranded DNA by hydrolyzing internal phosphodiester linkages
E. coli exonuclease III	Sequentially removes nucleotides from 3′-OH ends of DNA molecules except from protruding 3′-OH termini
Klenow fragment	Proteolytic product of *E. coli* DNA polymerase I that has both polymerase and 3′-exonuclease activities and no 5′-exonuclease activity because fractionation of the digestion products removes the fragment with the 5′-exonuclease activity; a Klenow fragment with only DNA polymerase activity because of a mutation in the 3′-exonuclease sequence is also available
Mung bean nuclease	Single-strand DNA and RNA endonuclease
Nuclease Bal-31	Degrades both 3′ and 5′ ends of DNA without internal cleavages
Poly(A) polymerase	Adds AMP from ATP to the 3′ end of mRNA
Reverse transcriptase	Retroviral RNA-directed DNA polymerase
RNase H	Degrades the RNA strand of a DNA:RNA hybrid molecule
S1 nuclease	Degrades single-stranded DNA
T4 polynucleotide kinase	Catalyzes the transfer of the terminal (γ) phosphate from a nucleoside 5′-triphosphate to a 5′-hydroxyl group of a polynucleotide
T7 DNA polymerase	DNA polymerase and 3′-exonuclease activities
Taq DNA polymerase	Heat-stable DNA polymerase from *Thermus aquaticus*
β-Agarase I	Digests agarose; used to retrieve separated DNA molecules from agarose gels

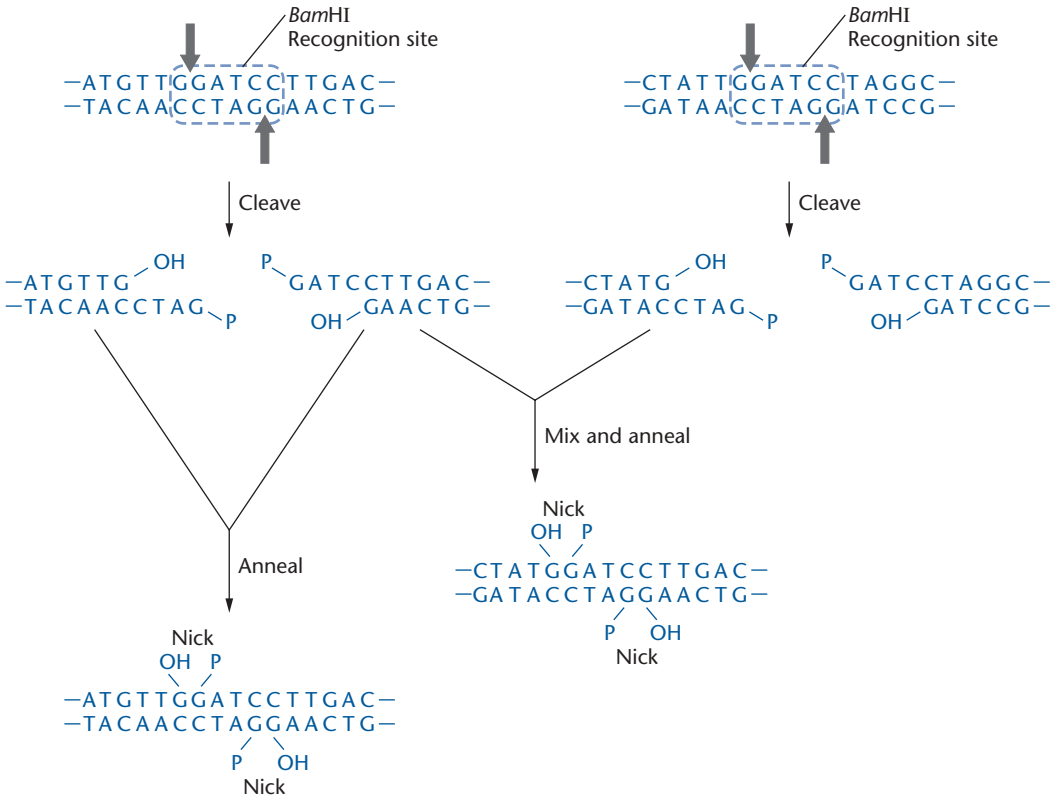


Figure 5.5 Base pairing of complementary extensions ("sticky ends") from different DNA molecules after cleavage with a type II restriction endonuclease. Two different DNA molecules are cut with the restriction endonuclease *Bam*HI and mixed under conditions that allow complementary extensions to base pair. The four fragments generated by the *Bam*HI digestion can join to one another to form any of six different DNA molecules. Only two of these possible combinations are shown here. A break in the phosphodiester in one strand of duplex DNA is called a nick. The hydrogen bonds of the four base pairs of the extensions between nicks on opposite strands are not sufficiently strong to hold DNA molecules together for long periods in solution. The letters A, C, G, and T represent deoxyribonucleotides.

Restriction endonuclease cleavage is also used in another way. When two different DNA samples are digested with the same restriction endonuclease that produces a staggered cut, that is, the same 5′ or 3′ extension or sticky end, and then mixed together, new DNA combinations (recombinant DNA molecules) are formed as a result of base pairing between the extension (overhang) regions (Figure 5.5). Molecular cloning is based on the formation of recombinant DNA molecules. However, restriction enzymes alone are not sufficient for molecular cloning. Other enzymes and manipulations are necessary. First, when the extended ends that are created by a restriction enzyme are aligned, the hydrogen bonds of the complementary bases are not strong enough to keep two DNA molecules together for an extended period of time. A means of forming an internucleotide linkage between the 3′-hydroxyl group and the 5′-phosphate group in the backbone at the two broken bond sites (nicks) is required. This problem is resolved by using the enzyme DNA ligase, usually from bacteriophage T4. This enzyme catalyzes the formation of phosphodiester bonds either at the ends of DNA strands that are held together by the base pairing of two extensions or between blunt ends that come in contact (Figure 5.6). The reaction conditions for DNA ligations depend on whether the DNA molecules have extensions or blunt ends. With protruding ends, the reaction is often carried out at low temperatures for long periods to ensure that the extensions remain base paired. Blunt-end ligations require ten to a hundred times more T4 DNA ligase than ligations of DNA molecules with extensions

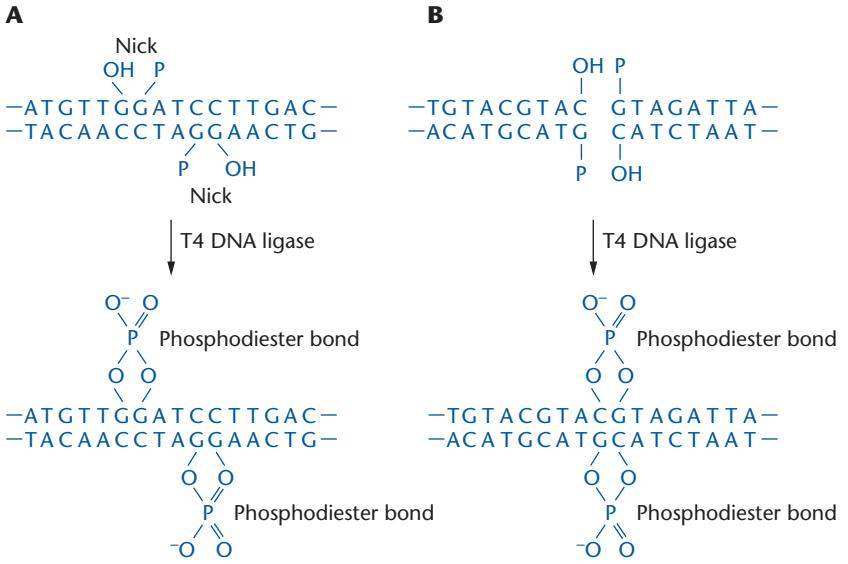


Figure 5.6 The use of T4 DNA ligase for DNA cloning experiments. The enzyme T4 DNA ligase forms phosphodiester bonds by joining 5′-phosphate and 3′-hydroxyl groups either at nicks in the backbone of duplex DNA or at the ends of two DNA molecules. (A) Ligation of sticky-ended DNA. (B) Ligation of blunt-ended DNA. The letters A, C, G, and T represent deoxyribonucleotides. Only the phosphate and hydroxyl groups of the nucleotides at the sites of nicks and blunt ends are shown.

and are conducted at room temperature because stable base pairing is not required.

Second, the ability to join different DNA molecules would not by itself be useful unless the recombinant DNA could be perpetuated in a host cell. Cloning vectors were developed for this purpose. One component of the ligated construct carries the information for maintaining the recombinant DNA in a host cell and the other a fragment of DNA from the source organism.

Third, recombinant DNA molecules must be introduced into host cells. Because this process in inherently inefficient, it is important to identify the small number of cells that carry recombinant DNA molecules. Fourth, because digestion of the source DNA with a restriction endonuclease produces a mixture of DNA molecules and a number of different DNA constructs are formed after ligation with a cloning vector, there has to be a way of detecting host cells that carry specific DNA inserts from the source organism.

Cloning Vectors

A myriad of cloning vectors have been constructed based on self-replicating extrachromosomal bacterial elements (plasmids), bacterial viruses [M13, lambda (λ), P1], combinations of plasmid and bacterial virus DNA sequences (cosmids, fosmids), and yeast and human DNA sequences including a site(s) for the initiation of DNA synthesis (origin of replication, origin) as well as centromere and telomere sequences for constructing artificial chromosomes. Many of these systems are used for cloning DNA fragments of specific lengths (Table 5.3). A bacterial artificial chromosome (BAC), for example, is derived from a stable plasmid that exists as a single copy in *E. coli* and is meant to carry large DNA inserts. Generally, except for some specialized systems, most vectors have an origin of replication that functions in *E. coli*. In addition to cloning for the size of the insert, a number of vector systems have been designed for special

Table 5.3 Insert capacities of cloning vector systems frequently used as resources for isolating human genes and characterizing the human genome.

Vector system	Host system	Insert capacity (kb)
Plasmid	*E. coli*	0.1–10
Bacteriophage λ	λ/*E. coli*	10–20
Cosmid	*E. coli*	35–45
Bacteriophage P1	*E. coli*	80–100
Bacterial artificial chromosome (BAC)	*E. coli*	50–300
P1-derived artificial chromosome (PAC)	*E. coli*	100–300
Yeast artificial chromosome (YAC)	Yeast	200–2000
Human artificial chromosome (HAC)	Cultured human cells	>2000

purposes such as transcription, translation, in vitro mutagenesis, or DNA sequencing of insert DNA.

Cloning vectors, regardless of their biological basis, have more or less the same fundamental attributes. First, they are maintained and replicated within a host cell. Second, they contain one or more single (unique) restriction endonuclease sites that provide a choice of possible insertion (cloning) sites. For example, digestion of the source and cloning vector DNAs with the same restriction endonuclease that recognizes a unique cloning site of the cloning vector DNA creates the opportunity for a fragment of the source DNA to be ligated into a specific site of the cloning vector. Moreover, after insertion, two cloning sites that are equivalent to the original restriction endonuclease site are formed on each side of the insert DNA. Thus, when a DNA construct is treated with the original cloning site restriction endonuclease, the insert DNA is released intact unless it contains any of these sites. Third, cloning vectors have one or more genes (selectable markers) that enable host cells with DNA constructs to be distinguished from cells that either do not carry a DNA construct or carry a cloning vector without an insert.

Plasmid Cloning Vector pUC19

The plasmid pUC19, which is maintained in the bacterium *E. coli*, is a general-purpose vector often used for cloning DNA fragments up to about 8 kb in length. This vector is well conceived and useful for illustrating the basic principles of vector-based cloning strategies. Generally, plasmid cloning vectors are denoted by a lowercase p, for plasmid, followed by an abbreviation that may be descriptive or anecdotal. The pUC19 vector is a circular double-stranded DNA molecule with 2686 bp containing an origin of replication that functions in *E. coli*; a regulatable segment of the β-galactosidase gene (*lacZ′*) derived from the lactose utilization operon of *E. coli*; a gene that confers resistance to the antibiotic ampicillin (Amp^r); a *lacI* gene that encodes a repressor protein and regulates the expression of the *lacZ′* gene, and a short sequence called a multiple cloning sequence with fourteen unique cloning sites, namely, *Eco*RI, *Sac*I, *Kpn*I, *Xma*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Hinc*II, *Acc*I, *Bsp*MI, *Pst*I, and *Hind*III (Figure 5.7).

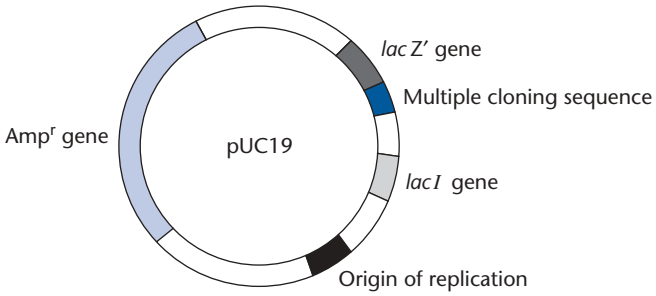


Figure 5.7 Genetic features of the plasmid cloning vector pUC19. The multiple cloning site (multiple cloning sequence) contains unique sites for the restriction endonucleases *Eco*RI, *Sac*I, *Kpn*I, *Xma*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Hinc*II, *Acc*I, *Pst*I, *Bsp*MI, *Sph*I, and *Hind*III and is used for the insertion of cloned DNA. The plasmid contains an ampicillin resistance gene (Amp^r), an origin of replication that functions in *E. coli*, and the *lacI* gene, which produces a repressor that blocks the transcription of *lacZ′* gene in the absence of the inducer IPTG. The complete sequence of pUC19 is 2686 base pairs.

The pUC19 cloning vector system is designed to distinguish host cells with DNA constructs from those that carry unmodified pUC19 by the color of cell colonies growing on agar culture plates. The rationale for identifying cells with intact pUC19 will be explained first and followed by a discussion of how cells with insert-pUC19 constructs are detected. When cells with unmodified pUC19 are grown in the presence of isopropylthiogalactoside (IPTG), an inducer of the lactose utilization operon (*lac* operon), the plasmid-borne *lacZ′* gene is transcribed and translated because the product of the *lacI* gene cannot bind to the promoter-operator region of the *lacZ′* gene and repress transcription. The lacZ′ protein by itself is enzymatically inactive, but after it combines with multiple units of a protein encoded by a sequence in the bacterial chromosome, an active β-galactosidase is formed. In pUC19, the multiple cloning sequence is incorporated into the *lacZ′* gene without interfering with the function of the lacZ′ protein. If the substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) is present in the growth medium, it is hydrolyzed to a blue product by active β-galactosidase. Under these conditions, colonies containing unmodified pUC19 appear blue. Otherwise, without X-gal in the medium, these colonies have their normal creamy (white) color.

To insert DNA into pUC19, DNA from a source organism is cut with one of the restriction endonucleases corresponding to a recognition site in the multiple cloning sequence and is mixed with pUC19 plasmid DNA that has been treated with the same restriction endonuclease. After ligation with T4 DNA ligase, the reaction mixture is introduced into host cells that synthesize the part of β-galactosidase that combines with the lacZ′ protein. Generally, the few cells that acquire DNA only take up one molecule. The DNA-treated cells are plated onto a medium that contains ampicillin, IPTG, and X-gal. Cells that do not take up either form of pUC19, that is, with or without an insert, are not able to grow in the presence of ampicillin. Cells with unmodified pUC19 (recircularized pUC19) grow with ampicillin in the medium and produce blue colonies because they form functional β-galactosidase. In contrast, host cells carrying an insert DNA-vector construct produce white colonies on the same medium. When DNA is inserted into the multiple cloning sequence, it usually disrupts the reading frame of the *lacZ′* gene and a nonfunctional lacZ′ protein is produced. In the absence of β-galactosidase, X-gal is not converted to the blue-colored compound, so the colonies, each one derived from a single cell, remain white.

The process of introduction of DNA into a host bacterial cell, that is, transformation, is extremely inefficient. Consequently, there is a need for selectable

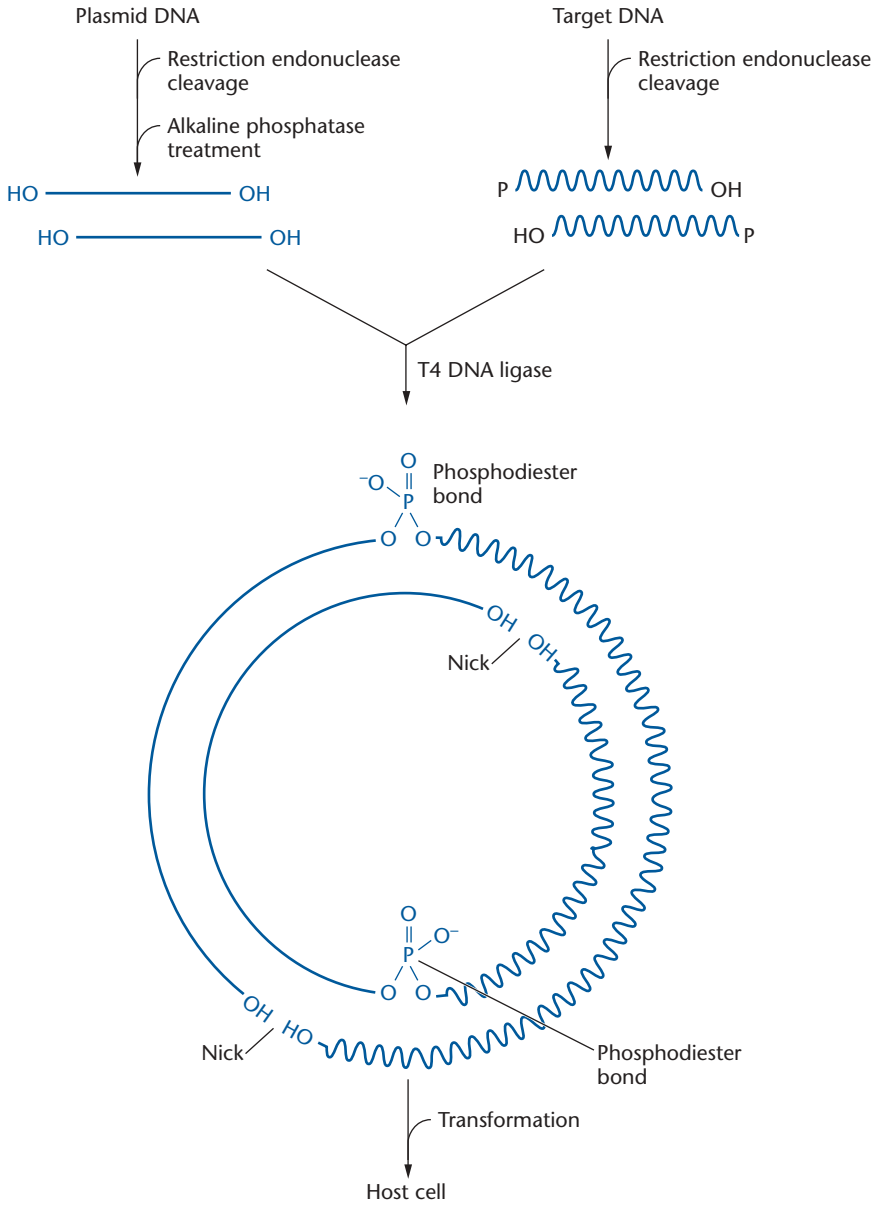


Figure 5.8 Cloning of DNA into a plasmid vector. After restriction endonuclease cleavage and alkaline phosphatase treatment, the plasmid DNA is ligated to the restriction endonuclease-digested target (source) DNA, and two of the four nicks are sealed. This molecular configuration is stable, and the two DNA molecules are covalently linked. After the introduction into a host cell, ensuing replication cycles produce new complete circular DNA molecules with no nicks. Linear plasmid DNA molecules without 5′-phosphate groups are not covalently circularized by T4 DNA ligase.

markers, such as the Amp^r and *lacZ′* genes, to distinguish those cells that carry DNA insert-vector constructs from cells that were not transformed and from those with plasmids that recircularized during the ligation reaction. The amount of recircularized plasmid DNA is lowered by treating the restriction endonuclease-cleaved plasmid DNA sample with alkaline phosphatase. This enzyme removes the 5′-phosphate groups from both ends of the linearized plasmid DNA, preventing recircularization by T4 DNA ligase. However, the two phosphate groups provided by the cleaved source DNA are sufficient to ligate source and plasmid DNA molecules (Figure 5.8). The alkaline phosphatase step is not completely efficient. Therefore, it is still necessary to identify unequivocally the cells that carry recircularized plasmids after

transformation. In the case of the pUC19 system, these cells produce blue colonies.

Screening DNA Constructs by DNA Hybridization

After the selection of host cells with DNA constructs, it is necessary to identify the colony(ies) with a specific DNA insert. A number of methods for this task rely on the formation of stable double-stranded DNA regions between a tester DNA sequence (DNA probe) and the insert sequence. The base pairing of complementary polynucleotide strands to form duplex DNA molecules in vitro is called DNA hybridization. All DNA hybridization procedures are based on the fact that certain treatments, such as heat or alkali, convert double-stranded DNA into single strands. For example, heating duplex DNA to ~100°C breaks the hydrogen bonds that hold the bases together (denaturation) but does not affect the phosphodiester bonds of the DNA backbone. If the heated solution is cooled rapidly, the DNA remains single stranded. If the temperature of a heated DNA solution is cooled slowly, the double-stranded helical conformation of DNA is reestablished (renaturation), as a result of base pairing of complementary nucleotides (Figure 5.9). The process of heating and slowly cooling double-stranded DNA is called annealing.

Because *E. coli* is the host cell for many cloning vectors and these cells are regularly grown on agar plates as discrete colonies, DNA hybridization assays have been devised to identify the colonies that carry DNA constructs with specified inserts. For example, a sample from each individual colony with a DNA construct is placed in a particular location on a support matrix, such as nitrocellulose or nylon. The cells of each sample then are lysed, and the released DNA is denatured and irreversibly bound to the matrix. Next, a denatured DNA probe, labeled with either a radioisotope or another tagging system, is incubated under hybridization conditions with immobilized single-stranded DNA samples. If the nucleotide sequence of the DNA probe is complementary to a nucleotide sequence in one of the samples, then base pairing (hybridization) occurs (Figure 5.10). Hybridization can be detected by autoradiography or other visualization procedures, depending on the nature of the

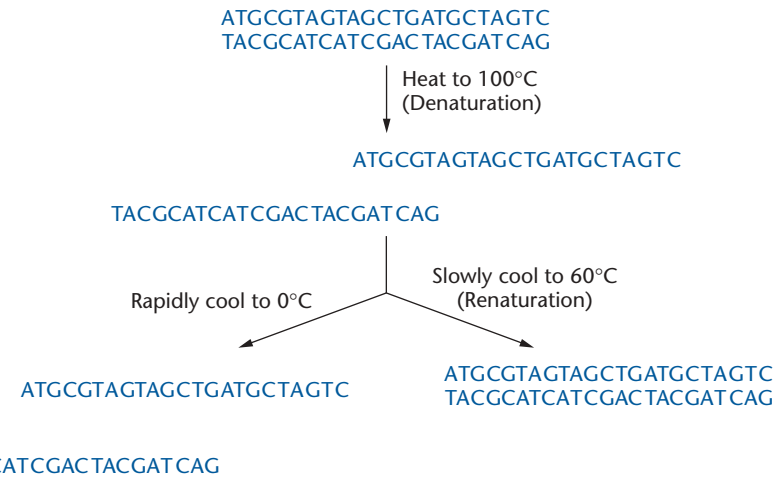


Figure 5.9 DNA denaturation and renaturation. When duplex DNA molecules are heated to 100°C, hydrogen bonds of the nucleotide pairs break and single strands are released. If the reaction mixture is rapidly cooled, the DNA remains single stranded. Alternatively, if the reaction mixture is slowly cooled to 60°C, nucleotide pairing occurs and duplex molecules are reconstituted.

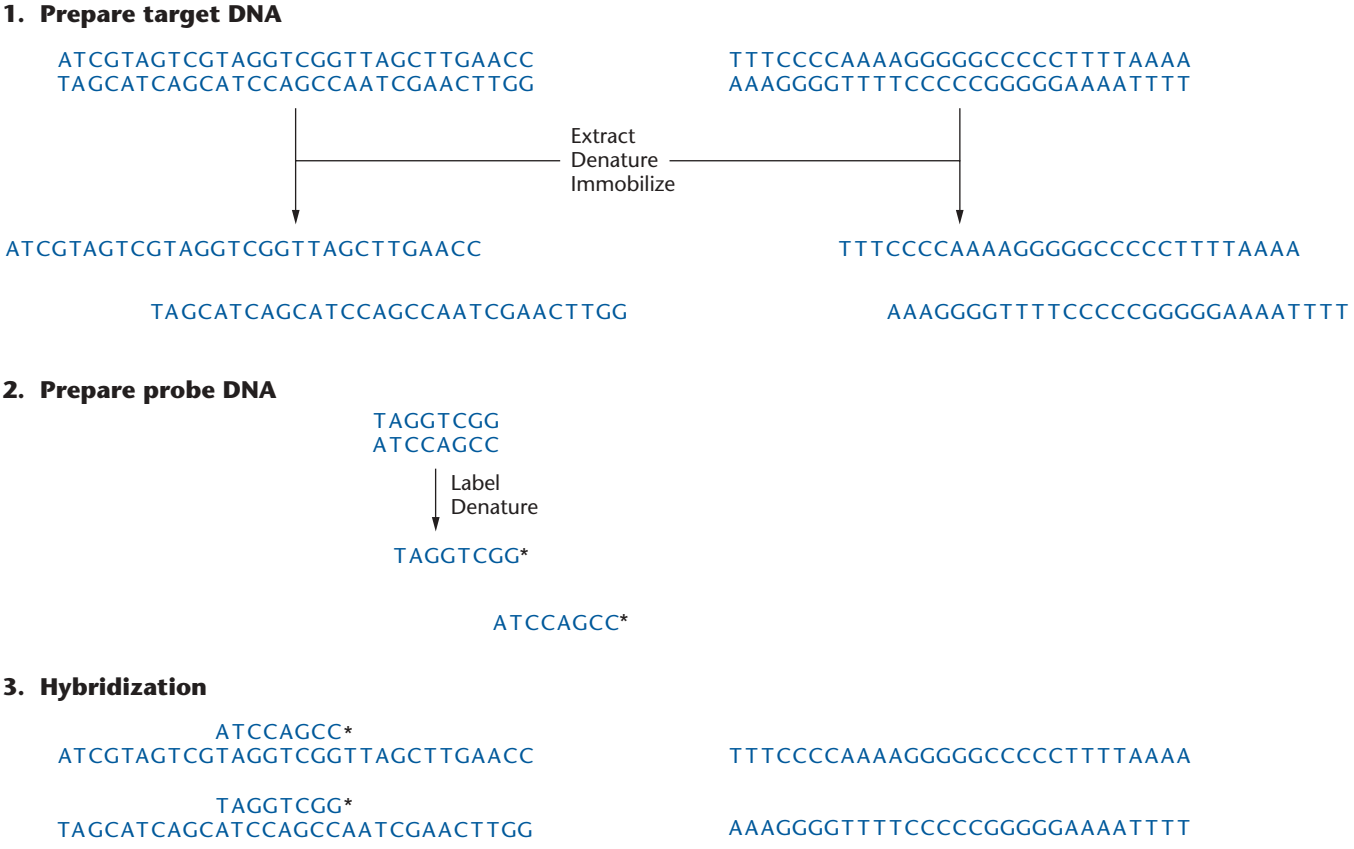


Figure 5.10 DNA hybridization. (1) The DNA of samples containing the putative target DNA is denatured and the single strands are kept apart, usually by binding them to a solid support, such as a nitrocellulose or nylon membrane. (2) The probe DNA is labeled, denatured, and mixed with the denatured putative target DNA under hybridization conditions. (3) The membrane is washed to remove unhybridized probe DNA, and the membrane(s) is assayed for the presence of the labeled tag. If the probe does not hybridize, then no label is detected. The stars denote the labeled tag (signal) of the probe DNA.

label incorporated into the probe DNA. If the nucleotide sequence of the probe does not base pair (bind) with a DNA sequence in a sample, then no hybridization occurs, and the assay gives a negative result. With the most commonly used hybridization condition, stable binding usually requires a greater than 80% match of complementary bases within a segment of 50 bases. Probes can range in length from 100 bases to more than 1000 kb. In most instances, DNA probes are derived from cloned DNA inserts that have been characterized with respect to chromosome location, coding information, or both.

DNA probes can be labeled in various ways. One strategy, called the random primer method, uses a mixture of synthetic random oligonucleotides (oligomers) containing all possible combinations of sequences of six nucleotides (hexamers). When these oligomers are mixed with a denatured probe, some will hybridize to complementary sequences of the probe DNA. The hybridized oligomers provide an available 3′-hydroxyl group for the initiation of DNA synthesis, and the nucleotide sequence of the probe is used as the template (Figure 5.11). In vitro DNA synthesis is initiated by the addition of four deoxyribonucleotides (dNTPs) and a portion of *E. coli* DNA polymerase I called the Klenow fragment. The Klenow fragment retains both DNA polymerase and 3′-exonuclease activities but lacks the 5′-exonuclease activity normally associated with *E. coli* DNA polymerase I. Exonucleases degrade nucleic sequences from the ends by removing one base at a time. The presence of 5′-exonuclease activity would destroy some of the newly synthesized DNA. The radioisotope ^{32}P in the α-phosphate position of one of the deoxyribonucleotides is often used

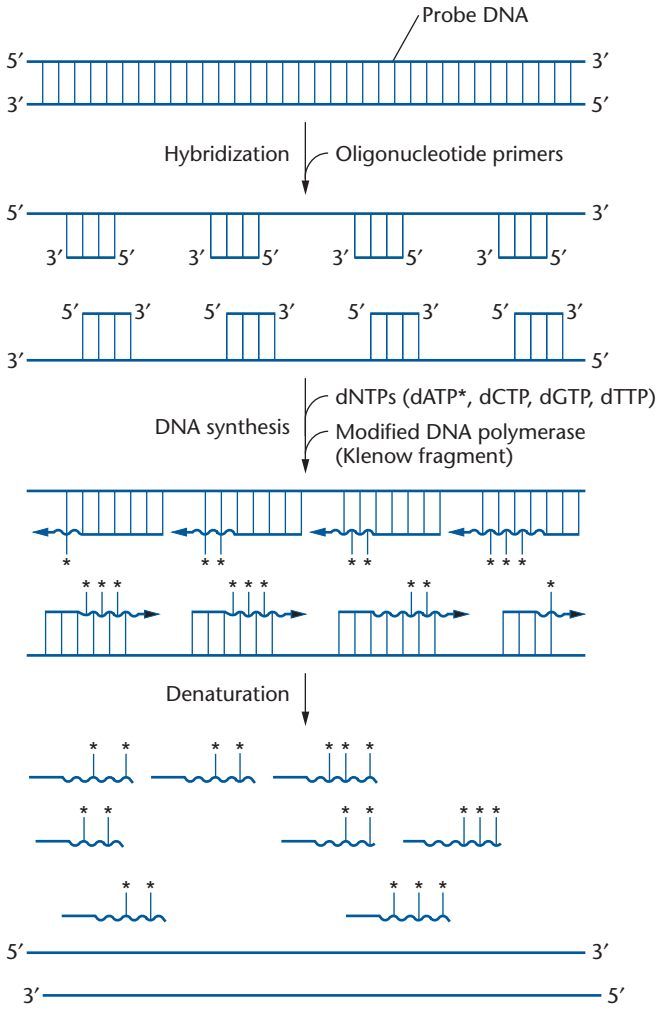


Figure 5.11 Production of labeled probe DNA by the random primer method. A duplex probe DNA sequence is denatured, and an oligonucleotide sample containing all possible sequences of 6 (or 14) nucleotides is added. It is statistically certain that some molecules in the oligonucleotide mixture will hybridize to the strands of the denatured probe DNA. The bound oligonucleotides provide primers for in vitro DNA synthesis. A modified form of *E. coli* DNA polymerase I (Klenow fragment), which lacks 5′-exonuclease activity, and the 4 deoxyribonucleotides, one of which is labeled with a tag (*), are added for DNA synthesis. All the growing DNA strands (wiggly arrows) are labeled. This sample, which consists of a number of separate tagged DNA molecules that collectively constitute most of the sequence of both template strands, is used to detect the complementary sequences in a hybridization reaction.

for the incorporation of radioactive phosphates into the backbone of newly synthesized strands. Alternatively, for nonisotopic detection of hybridization, which is safer than radiolabeling, biotin or other tags can be attached to one of four deoxyribonucleotides and incorporated during DNA synthesis or various fluorescent dye markers can be added to the ends of hybridization probes.

Autoradiography is used to visualize the presence of a radioactive or chemiluminescent signal from a labeled hybridized probe. Briefly, after the hybridization reaction, all nonhybridized probe is washed away and the support matrix is overlaid with X-ray film. The energy from the radioactivity or chemiluminescence reduces the grains in the emulsion of the X-ray film and is revealed as exposed regions (spots) when the film is developed. Because the samples on the matrix correspond to cultures from a master plate, the darkened spots indicate which clone(s) carries the target DNA sequence.

When biotin or other nonisotopic molecules are used to detect hybridization between a DNA probe and insert DNA, the protocols require an enzyme-conjugated intermediary compound that binds with a high affinity to the nucleotide tag (label). For example, streptavidin binds avidly to biotin. In other assays, an antibody is used that binds to the compound that is attached to an

incorporated deoxyribonucleotide. Depending on the assay, the conjugated enzyme can either convert a colorless substrate to a colored compound to be visualized directly, or it can produce a compound that emits light (chemiluminescence) that can be detected by autoradiography. In either case, a positive result is localized to a specific DNA sample on the matrix. Similarly, a fluorescent dye can be attached to streptavidin that in turn binds to a biotinylated probe. In this case, the dye is activated by a laser and the fluorescence is scored with a fluorometer. Hybridization probes that are labeled directly with a fluorescent dye are also used to detect cloned DNA sequences.

In Situ Hybridization

The ability to pinpoint a cloned human DNA sequence to a specific chromosome region is invaluable for genetic mapping studies. The most direct way to obtain this information is to use a technique called in situ hybridization. This procedure uses an insert as a labeled probe, mixes it with denatured metaphase chromosomes under hybridization conditions, and then visualizes the location of the label. Currently, fluorescent dyes are used routinely as tags instead of radioactivity. The commonly used acronym for this method is FISH (fluorescence in situ hybridization, fluorescent in situ hybridization).

FISH entails the following steps: Human cells are grown in culture and treated with a compound, for example, Colcemid, that stops the cell division cycle at metaphase. The metaphase-enriched cell population is applied to a microscope slide under conditions that separate (spread) the metaphase chromosomes of each cell from one another. The spread chromosomes are stained to produce a chromosome banding pattern and then photographed. Next, the DNA of the chromosomes is chemically denatured with 70% formamide. The denatured chromosomes remain attached to the slide. The probe, often labeled with biotin (biotinylated), is hybridized to the denatured chromosomes. After hybridization, the unbound probe is washed away. To detect where the probe hybridized, streptavidin or avidin, conjugated with a fluorescent dye, is added to the slide. This complex binds to the biotin of the hybridized probe. After the unbound streptavidin dye complexes are washed away, the slides are viewed with an epifluorescence microscope equipped for digital imaging. When excited by the appropriate wavelength, the bound dye fluoresces, and the signal can be seen and captured electronically. Counterstaining with a DNA-specific fluorochrome that emits a different color than the dye used for the probe enables the chromosomes to be visualized and highlights the signal from the probe. The chromosome location of the probe is assigned to the regions of homologous chromosomes by matching the sites of the fluorescent spots with the chromosome banding pattern of the photographed metaphase spread. Some protocols allow the hybridization signal and chromosome banding pattern to be viewed simultaneously.

Chemical Synthesis of DNA

The ability to easily, inexpensively, and rapidly synthesize a strand of DNA with a specified sequence of nucleotides has contributed significantly to the

methodologies of molecular cloning and characterization of DNA inserts. Machines (DNA synthesizers) that automate the chemical reactions for the synthesis of DNA have made the production of single-stranded oligonucleotides (<50 nucleotides) a commonplace process. DNA synthesizers consist of a set of valves and pumps programmed to introduce, in the correct order, specified nucleotides and reagents required for the coupling of each consecutive nucleotide to the growing chain. Chemical DNA synthesis does not follow the biological direction of DNA synthesis; rather, in the chemical process, each incoming nucleotide is coupled to the 5′-hydroxyl terminus of the growing chain. All reactions are carried out in succession in a single reaction column, and both the duration of each reaction and the washing steps are computer controlled. Currently, the phosphoramidite method of chemical DNA synthesis is the process of choice (Figure 5.12).

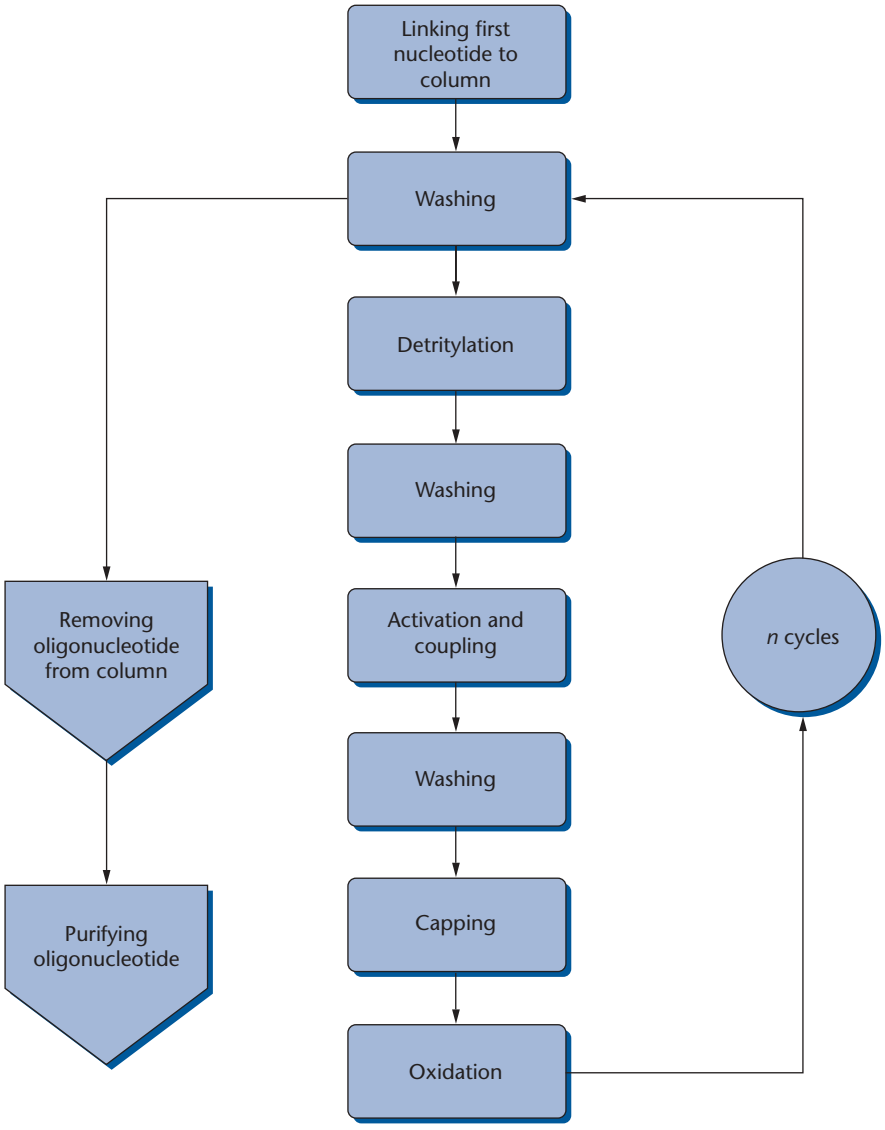


Figure 5.12 Flowchart for the chemical synthesis of DNA oligonucleotides. After n coupling reactions (cycles), a single-stranded DNA with $n + 1$ nucleotides is produced.

Briefly, the chemical synthesis of DNA occurs on a solid surface, namely, controlled-pore glass beads. The first step in this multistep process is the removal of a protecting group from the 5′-OH group of a bound nucleotide. Next, the phosphorus-containing group at the 3′-carbon of the incoming phosphoramidite, which also has a protected 5′-OH group, is activated. The activation step enables an internucleotide linkage to be formed between the 3′ end of an incoming nucleotide and the available 5′-OH end of a bound nucleotide. The 5′-OH ends of bound nucleotides that do not react with an activated phosphoramidite are capped to prevent them from participating in ensuing cycles. The internucleotide linkage, which is a phosphite triester bond, is converted to a more stable pentavalent phosphate triester bond. The cycles are repeated with successive phosphoramidites and continue until the complete oligonucleotide sequence is formed. After the last cycle, the amino-protecting groups are removed from the guanine, cytosine, and adenine residues. The oligonucleotides are cleaved from the solid supports and purified on the basis of size. Finally, a phosphate group is added either enzymatically or chemically to the 5′ end of each oligonucleotide to produce a bona fide DNA strand.

Chemically synthesized oligonucleotides have a number of uses. For example, double-stranded sequences, called linkers, can be created by synthesizing an oligomer, which is a single-stranded DNA sequence that base pairs to itself and contains a palindromic restriction endonuclease recognition site to help in the cloning of DNA. One of these short DNA duplexes can be blunt-end ligated to the ends of a target DNA molecule. When this molecule is cut with the appropriate restriction endonuclease, the single-stranded extensions (sticky ends) enable this DNA to be inserted into a cloning site of a vector (Figure 5.13). In addition, single-stranded oligonucleotide probes (20 to 40 bases) can be formulated by deducing the codons from the amino acid sequence of part of a protein. These oligonucleotides are used as hybridization probes to identify clones carrying a DNA insert with either part or all of a gene sequence. Undoubtedly the most extensive use of chemical DNA synthesis is for the production of single-stranded sequences (17 to 24 bases) used as primers for DNA sequencing reactions and polymerase chain reaction (PCR) protocols.

Sequencing DNA

In molecular terms, the definitive understanding of a DNA molecule is derived from knowledge of its nucleotide sequence. The function of a gene can often be deduced by comparing a determined, but unknown, sequence with sequences of genes with known functions. In addition, nucleotide sequence information is important for determining the presence of single base pair mutations. The most commonly used DNA sequencing procedure is the dideoxynucleotide method developed by F. Sanger (b. 1918), who received Nobel Prizes for inventing sequencing techniques for both protein and DNA.

A dideoxynucleotide is a human-made molecule that lacks a hydroxyl group at both the 2′- and 3′-carbons of the sugar moiety (Figure 5.14A). In contrast, the natural deoxyribonucleotide has a 3′-hydroxyl group on the sugar unit (Figure 5.14B). During DNA replication, an incoming natural nucleoside triphosphate, which is determined by base pairing to the nucleotide of the

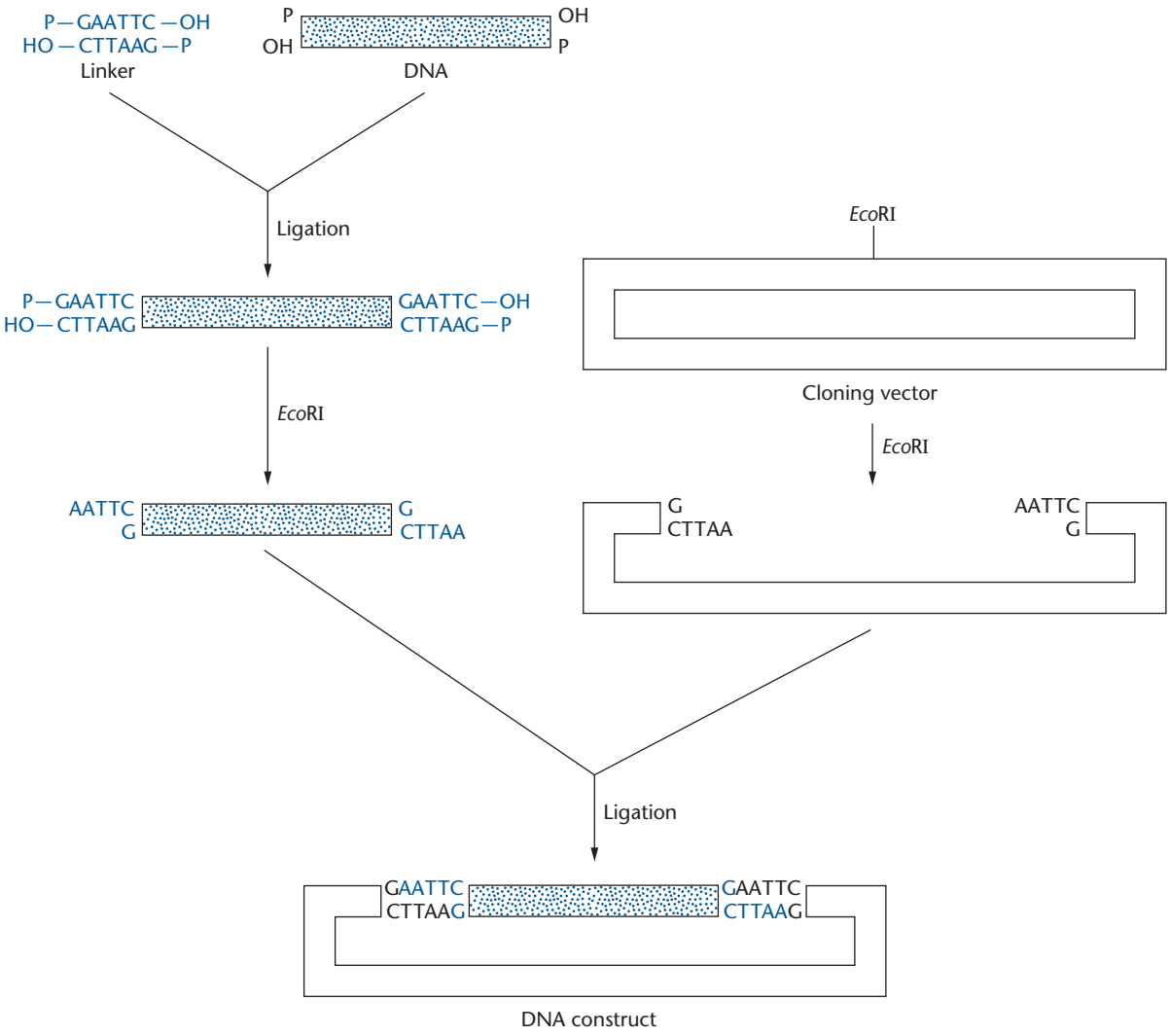


Figure 5.13 The use of a linker for DNA cloning. A sample of the chemically synthesized hexamer GAATTC is heated to 100°C and slowly cooled to produce a double-stranded *Eco*RI linker $\frac{\text{GAATTC}}{\text{CTTAAG}}$. The *Eco*RI linkers are ligated to the ends of the DNA fragments (stippled fill). The fragments with *Eco*RI extensions are cloned into an *Eco*RI site of cloning vector (open fill).

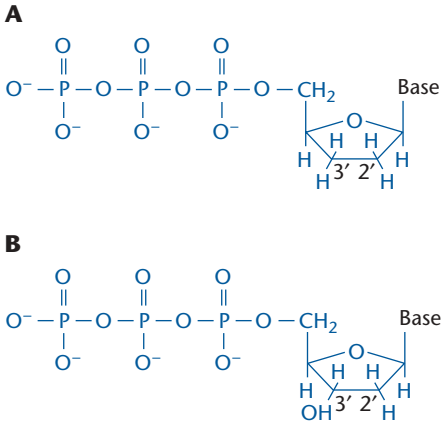


Figure 5.14 (A) Dideoxynucleotide. Its 2′ and 3′ carbons lack hydroxyl groups. (B) Deoxyribonucleotide.

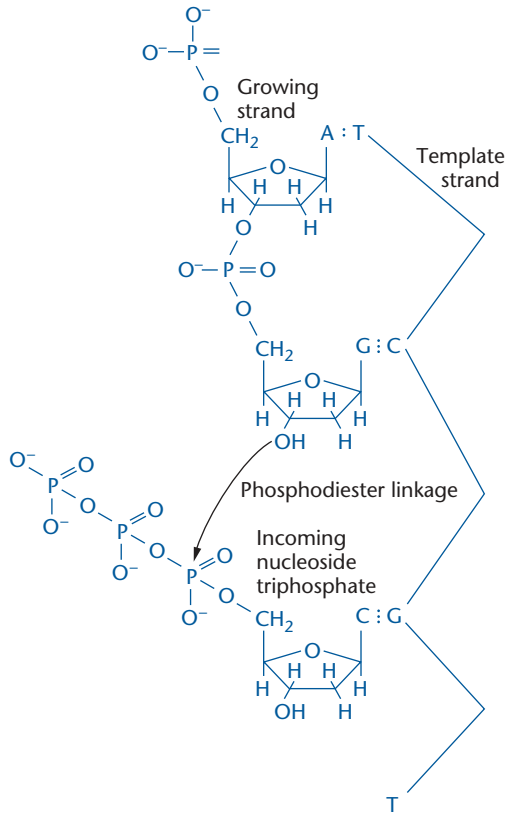


Figure 5.15 An incoming deoxyribonucleotide base pairs with the complementary nucleotide of the template strand. The internucleotide linkage occurs between the 3′-hydroxyl group of the last nucleotide of the growing strand and the α-phosphate of the incoming nucleotide.

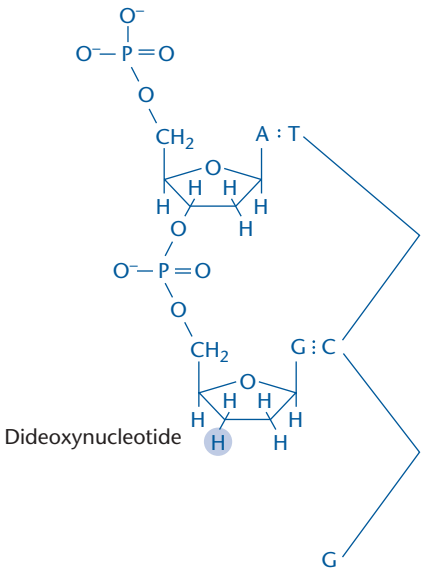
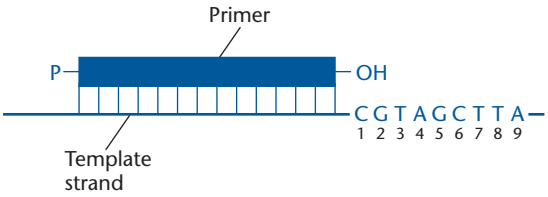


Figure 5.16 Dideoxynucleotide termination of DNA synthesis. Strand elongation is stopped by the addition of a dideoxynucleotide to the end of the growing strand. The internucleotide linkage between the last nucleotide, which is a dideoxynucleotide, and the next incoming nucleotide cannot be formed because there is no 3′-hydroxyl group (shaded hydrogen molecule) on the dideoxynucleotide sugar.

template strand, is linked by its 5′ α-phosphate group to the 3′-hydroxyl of the last nucleotide of the growing chain (Figure 5.15). However, if a dideoxynucleotide is incorporated at the end of the growing chain, DNA synthesis stops, because a phosphodiester bond cannot be formed with the next incoming nucleotide (Figure 5.16). The termination of DNA synthesis is the quintessential feature of the dideoxynucleotide DNA sequencing method, although other experimental steps must be carried out before a DNA sequence is determined.

The first step in the standard laboratory procedure for dideoxynucleotide DNA sequencing entails annealing a synthetic oligonucleotide (primer) to a predetermined segment of a strand of the cloning vector near the insertion site of the cloned DNA. A primer, which provides a 3′-hydroxyl group for the initiation of DNA synthesis, is usually from 17 to 24 bases long to ensure that it base pairs to a specific complementary sequence. Shorter primers might base pair to more than one sequence in the DNA construct, which would produce multiple initiation sites. Of course, the 3′-hydroxyl group of the primer "points" in the direction of the DNA to be sequenced.

The primed DNA sample is partitioned into four separate reaction tubes. Each tube contains four deoxyribonucleotides (dATP, dCTP, dGTP, and dTTP), one of which is radiolabeled; one of the four dideoxynucleotides (ddATP, ddCTP, ddGTP, or ddTTP); and DNA polymerase. The concentration of each dideoxynucleotide in each reaction tube is carefully adjusted to



Contents of reaction tube	Size of primer and extension	Primer and sequence of extension
ddATP + four dNTPs	Primer + 3	Primer – dGdCddA
	Primer + 7	Primer – dGdCdAdTdCdGddA
	Primer + 8	Primer – dGdCdAdTdCdGdAddA
ddCTP + four dNTPs	Primer + 2	Primer – dGddC
	Primer + 5	Primer – dGdCdAdTddC
ddGTP + four dNTPs	Primer + 1	Primer – ddG
	Primer + 6	Primer – dGdCdAdTdCddG
ddTTP + four dNTPs	Primer + 4	Primer – dGdCdAddT
	Primer + 9	Primer – dGdCdAdTdCdGdAdAddT

Figure 5.17 Primer extension during DNA synthesis in the presence of dideoxynucleotides. Each of the four reaction tubes after DNA synthesis contains a unique set of nucleotide extensions attached to the primer because the incorporation of a dideoxynucleotide (ddA, ddC, ddG, or ddT) into a growing strand terminates synthesis. A few full-length DNA molecules will be synthesized in each reaction tube.

ensure that it is incorporated into growing chains at every possible site and not just at the first occurrence of the complementary nucleotide of the template strand. As a consequence of this important feature, after enzymatic DNA synthesis, each reaction tube will contain a unique set of oligonucleotides, each with a primer sequence (Figure 5.17). DNA synthesis is often mediated by a modified form of bacteriophage T7 DNA polymerase.

The synthesis of DNA is stopped by the addition of formamide, which also prevents the strands from forming base pairs. The contents of each tube are loaded into a well of a polyacrylamide gel, and the DNA molecules are separated by electrophoresis. This separation procedure resolves pieces of DNA that differ in size by as little as a single nucleotide. An autoradiograph of the gel shows only the radiolabeled DNA fragments produced during the enzymatic DNA synthesis step, with each lane corresponding to a reaction tube that contained one of the four dideoxynucleotides (Figure 5.18).

The sequence of nucleotides of a segment of one strand of a cloned piece of DNA is determined by noting the order of the bands, as accurately as possible, from the bottom to the top of the autoradiograph. In the example shown in Figure 5.18, the first six bases of the sequenced DNA are AGCTGC. In this case, the fastest migrating band in the gel (the bottom-most radiolabeled fragment), which corresponds to the smallest DNA fragment, is in the ddATP lane. The next band is in the ddGTP lane, the next in the ddCTP lane, the next in the ddTTP lane, and so on. Between 250 and 350 bands can be resolved clearly on most autoradiographs. Usually, the primer sequence is positioned about 10 to 20 nucleotides away from the insertion site of the cloned DNA, so that a known sequence can be recognized at the start of reading of the autoradiograph, thereby identifying precisely the first nucleotide of the cloned DNA.

A number of strategies have been devised to obtain the complete sequence of a cloned piece of DNA. One approach is to create a detailed restriction

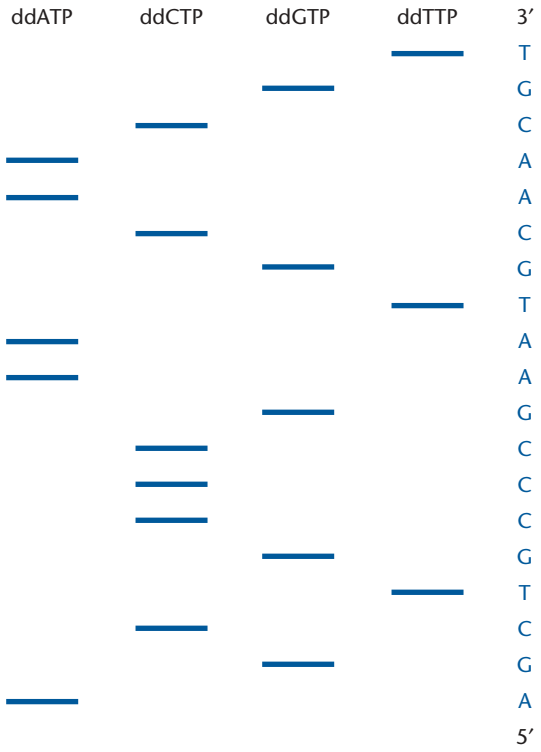


Figure 5.18 Simulated autoradiograph of a DNA sequencing gel. Each lane of the gel was loaded with the contents of one of the four reaction tubes that contained ddATP, ddCTP, ddGTP, or ddTTP. The bands of the autoradiograph are read from the bottom to the top to give the sequence of nucleotides. Results of the sequence determination are shown on the right.

endonuclease map of the insert. With this information, overlapping segments of 100 to 500 bp of the insert can be identified and each segment subcloned into a cloning vector and sequenced. To ensure that the correct sequence is obtained, with no ambiguity regarding the identity of a single nucleotide, both strands must be sequenced several times. Unfortunately, subcloning can be time consuming and, for very long inserts (>5000 bp), a large number of subclones must be generated and maintained. To overcome these inconveniences, a "primer walking" procedure is often used (Figure 5.19). The first 300 bases of an insert are sequenced with a primer that base pairs with one strand of the cloning vector near the insertion of the cloned DNA. On the basis of these sequence data, a second oligonucleotide primer is synthesized chemically and is complementary to a sequence of the insert DNA strand that is approximately 250 nucleotides away from the binding site of the initial primer. This second primer is used to determine the sequence of the next 300 nucleotides. In a similar manner, a third primer binding site is selected, another oligonucleotide is synthesized, and the sequence of the next 300 nucleotides is determined. This primer extension strategy proceeds until the complete cloned DNA is sequenced. The complementary strand of the insert can be sequenced in the same way, starting with a primer that hybridizes to the opposite strand of the cloning vector near the vector-insert junction at the other end of the cloned DNA.

False priming of DNA synthesis can give erroneous or ambiguous results. This situation arises when a primer binds to more than one site within the insert DNA. To prevent this problem, the primers for the "primer walking" method are at least 24 nucleotides long. In addition, the annealing conditions

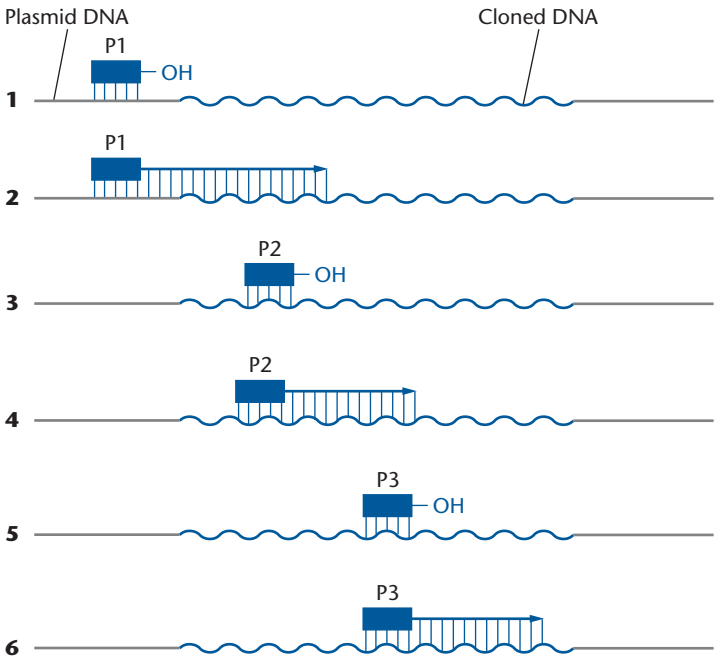


Figure 5.19 DNA sequencing by primer walking. (1) DNA sequencing is initiated with a primer (P1) that is complementary to a site on a plasmid near the site of insertion of the cloned DNA. (2) Based on the segment of the cloned DNA just sequenced, a second primer, complementary to a stretch of 24 nucleotides near the end of the segment, is synthesized. (3) The second primer (P2) is used to sequence the next few hundred nucleotides of the cloned DNA. (4) Based on the segment of the cloned DNA just sequenced, a third primer, complementary to a stretch of 24 nucleotides near the end of the segment, is synthesized. (5) The third primer (P3) is used to sequence the next few hundred nucleotides of the cloned DNA. (6) Based on the segment of the cloned DNA just sequenced, a fourth primer, complementary to a stretch of 24 nucleotides near the end of the segment is synthesized. The process of successively synthesizing and using new primers continues until the entire insert is sequenced.

are set so that the primer must have a complete base pair match for binding to occur. It is unlikely that there will be two exact sets of 24 nucleotides in the DNA of an insert.

For laboratories engaged in sequencing tens of thousands of base pairs of DNA on a regular basis, a number of automated procedures have been developed. Automated DNA sequencing minimizes manual manipulations and increases the rate of acquiring sequence data. These systems have been especially helpful in assembling vast amounts of nucleotide sequence data from prokaryotic and eukaryotic genomes. Currently, the dideoxynucleotide method forms the basis of automated DNA sequencing. Sequence analysis can be carried out with four different fluorescent dyes, one for each dideoxynucleotide reaction, or with the same fluorescent dye for each dideoxynucleotide in each reaction mixture. In some cases, the primer, not a dideoxynucleotide, is labeled with a fluorescent dye. With a four-fluorescent dye system, the samples at the completion of each reaction are pooled and the fragments are separated in a single lane of a polyacrylamide gel or polymer-filled capillary tube. This type of analysis is called "4-color, 1-lane" detection. Alternatively, with one fluorescent dye marker, each sample is run in a separate lane, namely, "1-color, 4-lane" detection.

Each fluorescent dye emits a narrow spectrum of light with a distinctive peak when it is struck by an argon ion laser beam. The beam scans a fixed location near the bottom of the electrophoretic matrix. As each successive labeled fragment passes through the beam, the excitation causes an emission with specific spectral features that is detected by a photomultiplier tube. The emission data are recorded and stored in a computer. After a run is completed, the succession of fluorescent signals is converted to nucleotide sequence information. For a "4-color, 1-lane" system, each fluorescent dye emits a different wavelength

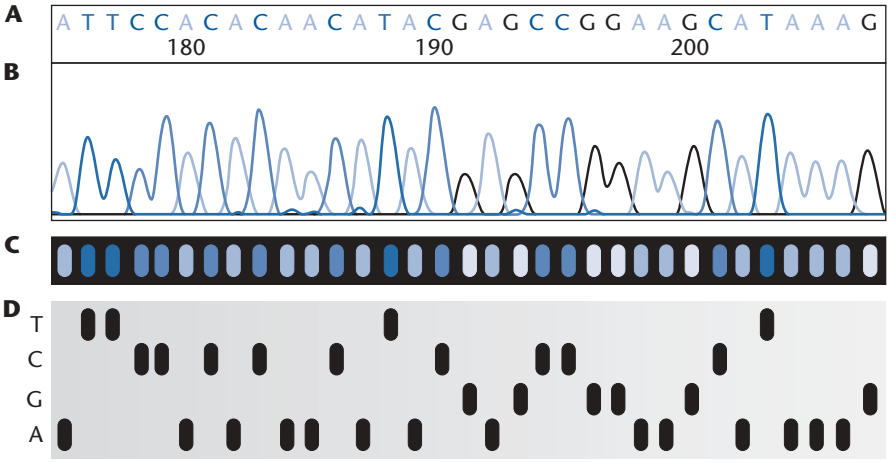


Figure 5.20 DNA sequencing systems. (A) A computer readout of the fluorescent signals from a "4-color, 1-lane" sequencing gel. (B) The numbers denotes nucleotide positions. (C) Electrophoretic separation of the fluorescence-labeled dideoxynucleotide fragments in a single lane that correspond to the nucleotide sequence shown in A. The colored dyes are usually red, green, blue and yellow. (D) An autoradiograph of a "1-label, 4-lane" sequencing gel for the nucleotide sequence shown in A. Courtesy of Applied Biosystems.

and the order of spectral responses in a single lane corresponds to the sequence of nucleotides (Figure 5.20). In other words, each dye represents a particular nucleotide. With a "1-color, 4-lane" system, the fluorescent signals from the dideoxynucleotide-terminated fragments are recorded in succession across the four lanes. In this case, the overall order of the fluorescent signals as a function of each lane corresponds to a nucleotide sequence. Parenthetically, DNA sequencing with radioactive label is equivalent to the "1-color, 4-lane" format (Figure 5.20).

Generally, automated DNA sequencing systems read with high accuracy about 500 bases per run and, under optimal conditions, one instrument can resolve about 20,000 bases per hour. The electrophoretic matrix for separating dideoxynucleotide-terminated products may be a slab gel or a liquid polymer in a capillary tube. Automated capillary DNA sequencing machines handle larger numbers of samples with faster separations than units that analyze slab gels.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a powerful, widely applied procedure using DNA amplification to generate large quantities of a specific DNA sequence in vitro. The most important constituent of the PCR is a pair of synthetic oligonucleotide primers (~20 nucleotides each) that are complementary to regions on opposite strands flanking the sequence of DNA that is targeted for amplification. After annealing to the sample DNA, the primers must have their 3′-hydroxyl ends oriented toward each other. The target DNA sequence can be from 100 to 35,000 bp in length. The second essential requirement for PCR is a thermostable DNA polymerase that can withstand heating to 95°C or higher without losing its activity.

A typical PCR process entails a number of cycles. Each cycle has three successive steps: DNA denaturation, DNA renaturation, and DNA synthesis (primer extension). The first step in the PCR amplification system is the thermal denaturation of the DNA sample, achieved by raising the temperature within a reaction tube to 95°C. In addition to the source DNA, the reaction

tube contains a vast molar excess of the two oligonucleotide primers, a thermostable DNA polymerase (e.g., *Taq* DNA polymerase, isolated from the bacterium *Thermus aquaticus*), and the four deoxyribonucleotides dATP, dCTP, dGTP, and dTTP. The temperature is maintained for about 1 min. For the second step, the temperature is slowly cooled to ~55°C. During this step, the primers base pair with their complementary sequences in the sample DNA. In the third step, the temperature is raised to ~75°C, which is optimal for the enzymatic functioning of *Taq* DNA polymerase. DNA synthesis is initiated at the 3′-hydroxyl end of each primer. Temperature changes and the duration of each step of a PCR cycle are programmable and computer controlled. The reaction tubes are placed in wells of a covered heating block. One cycle generally lasts from 3 to 5 min.

To understand how the PCR protocol succeeds in amplifying a discrete segment of DNA, it is important to keep in mind the location of each primer sequence and its complementary sequence within the strands synthesized during each cycle. During the synthesis phase of the first cycle, the newly synthesized DNA attached to each primer is extended beyond the end point of the sequence complementary to the second primer (Figure 5.21). These new strands form "long templates" that will be used in the next cycle.

During the second cycle, the original DNA strands and the new strands that were synthesized during the first cycle (long templates) are denatured and then hybridized with available primer sequences. A second round of DNA synthesis produces long templates from the original strands and short templates from the long templates. The short templates have a primer sequence at one end and a sequence complementary to the second primer at the other end (Figure 5.22).

During the third cycle, short templates, long templates, and original strands all hybridize with primer sequences and are replicated (Figure 5.23). In subsequent cycles, the short templates preferentially accumulate, and by the thirtieth cycle these strands are approximately one million times more frequent than either the original or long template strands (Figure 5.24).

The PCR protocol has had an inestimable impact on all aspects of the study of human molecular genetics. It is used in diverse ways for innumerable purposes. Some of these applications are described in later sections of this chapter and a number of others in ensuing chapters.

Human-Rodent Somatic Cell Hybrids

Multicellular organisms are inherently complex. By establishing permanent in vitro cell lines from human somatic tissues, such as fibroblasts (connective tissue cells), lymphoblasts (immature white blood cells), cancer cells, and other cell types, the molecular features of the cell division cycle, gene expression, and malignancy can be studied more readily. Cells in culture act as hosts for mammalian-based DNA insert-vector constructs and are routinely used to grow and study viruses.

Some genetic features of homozygous recessive metabolic disorders have been studied with cultured cells that display the mutant phenotype. A complementation test can be used to determine whether mutant sites from nonallelic genes are responsible for the same phenotype in unrelated affected

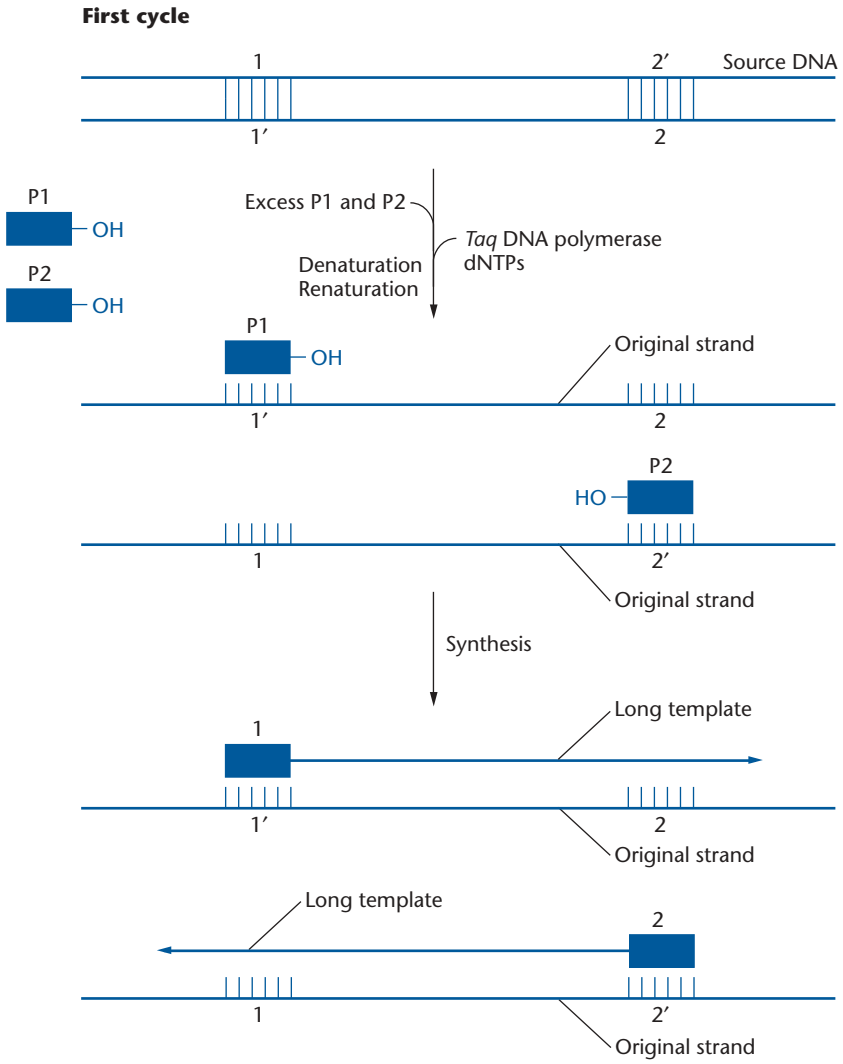


Figure 5.21 First cycle of the polymerase chain reaction. The target DNA lies between sequences 1′ and 2 on one strand and between 1 and 2′ on the other strand. The sequences of the two primers (P1, P2) are complementary to sequences 1′ and 2′, respectively. An excess of the two primer sequences, *Taq* DNA polymerase, and the four deoxyribonucleotides (dNTPs) are mixed with the sample DNA and heated to 95°C to denature the DNA, and then the mixture is slowly cooled to 55°C for renaturation. During the renaturation step, the primers base pair with the complementary sequences of the original strands. The temperature is raised to 75°C, and DNA synthesis commences from the 3′-hydroxyl ends of the primer sequences and continues past the regions of the DNA strands that are complementary to the other primer sequence. The products of this reaction are two long strands of DNA (long templates), which serve as templates in the second cycle of the PCR.

individuals. For this test, cultured cells from two different people with the same inherited disorder that affects cells in culture are fused. Each cell fusion, induced chemically with polyethylene glycol, creates a single cell with one nucleus containing both sets of chromosomes. All possible combinations of cell fusions will occur during treatment with polyethylene glycol. In other words, cells of each cell line will fuse with each other as well as cells from the different cell lines fusing with each other. However, if some of the lines of fused cells have a normal phenotype, then each of the original cell lines has mutations at two different loci that affect different steps of the same metabolic process. In other words, after cell fusion each original genome supplies the gene product that the other cell line was missing. In this case, the mutants of the original cell lines are said to belong to different complementation groups. Alternatively, if all of the lines of fused cells from two mutant cell lines from different individuals maintain the mutant phenotype, then both of the original cell lines carry mutations within the same gene and belong to the same complementation group.

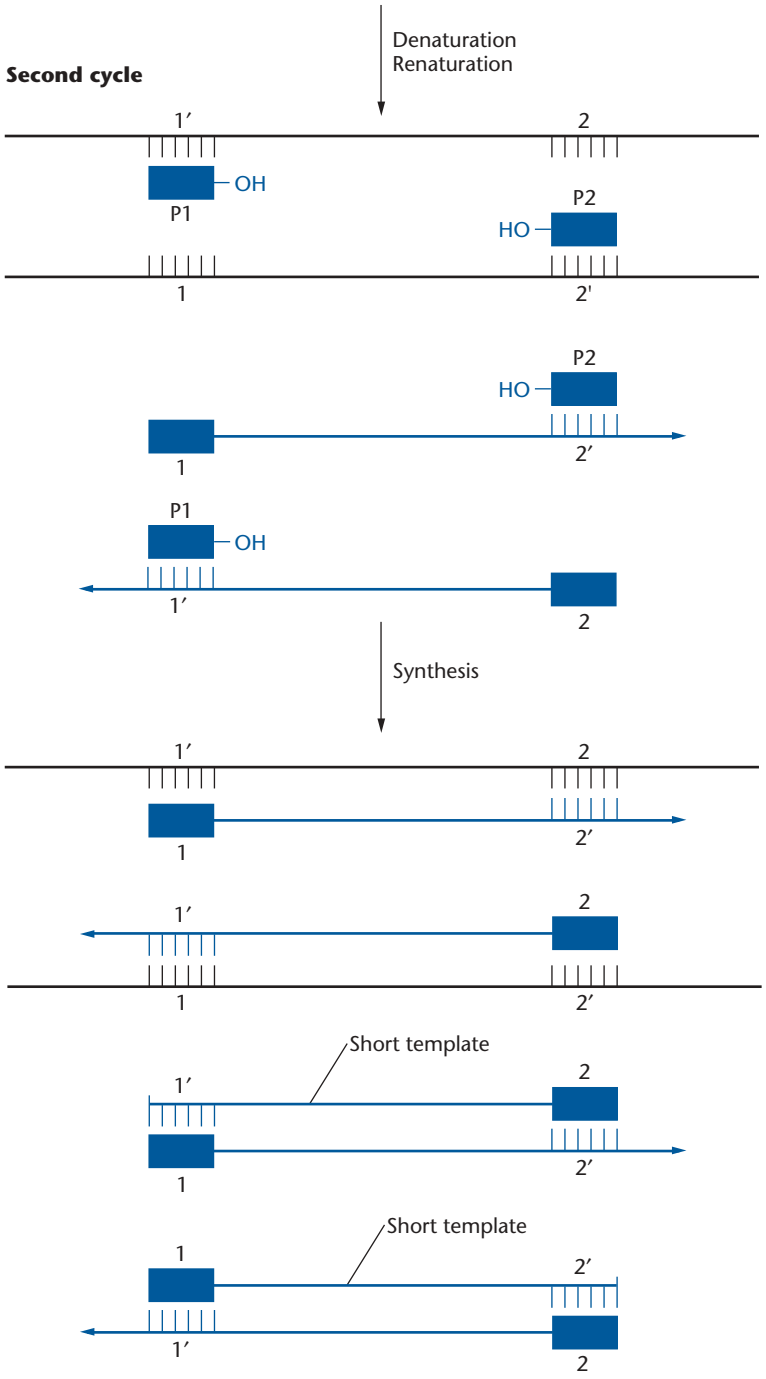


Figure 5.22 Second cycle of the polymerase chain reaction. After denaturation and renaturation of the mixture from the first PCR cycle, the primer sequences hybridize to complementary regions in both the original strands and the long template strands. The DNA synthesis step produces more long templates from the original strands and short templates from the long template strands. A short template has a primer sequence at one end and the complementary sequence to the other primer at the other end.

Interestingly, after human and rodent (mouse, hamster, or rat) cells are fused and grown in culture, most of the human chromosomes are preferentially and randomly lost, whereas the full complement of rodent chromosomes is maintained. Eventually, in many of these lines, a stable state is reached in culture, with one or a few single human chromosomes maintained permanently. The identification of specific retained human chromosomes is straightforward, because none of the rodent chromosomes, especially mouse chromosomes, has

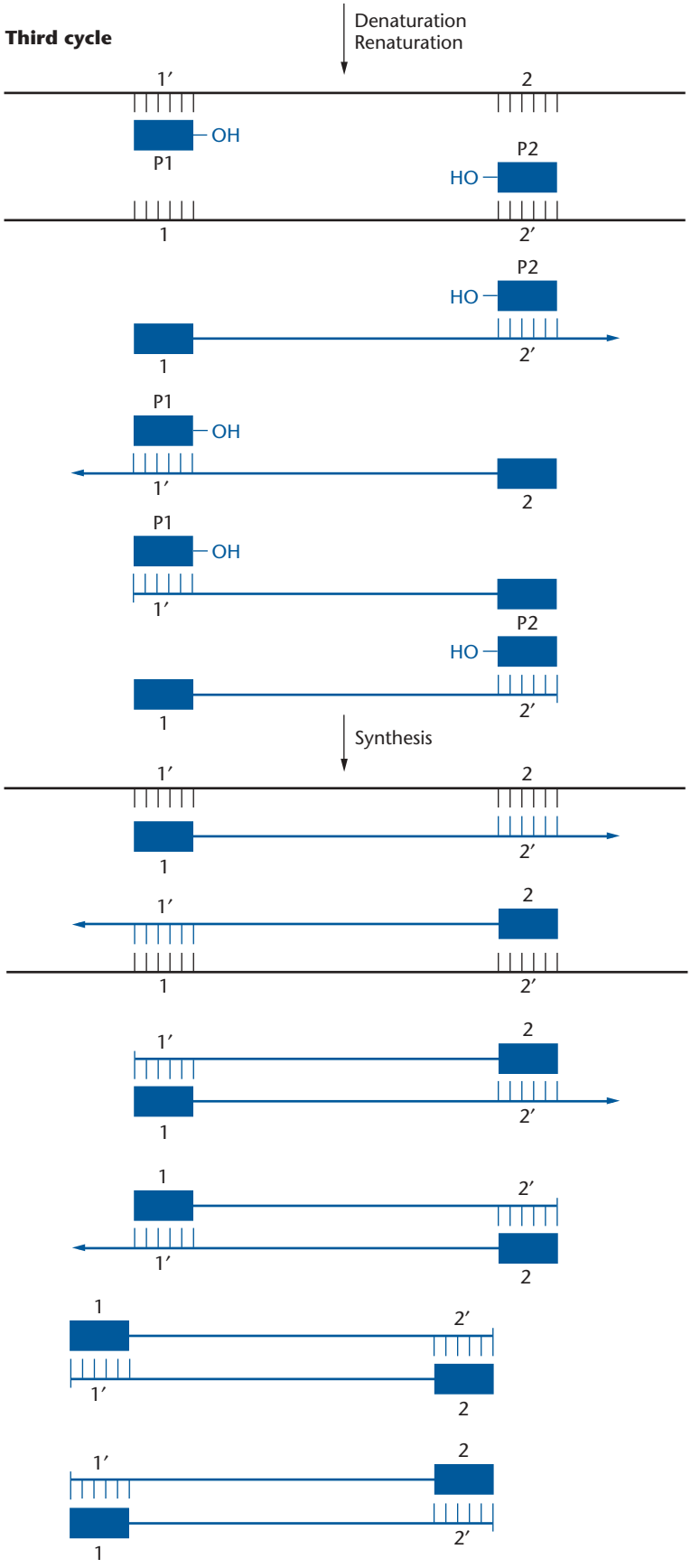


Figure 5.23 Third cycle of the polymerase chain reaction. During the renaturation step of the third cycle, the primer sequences hybridize to complementary regions of the original, long template, and short template strands. In vitro DNA synthesis produces more long template strands from the original strands and more short template strands from both the long template strands and the short template strands. During the ensuing PCR cycles, the short templates accumulate exponentially.

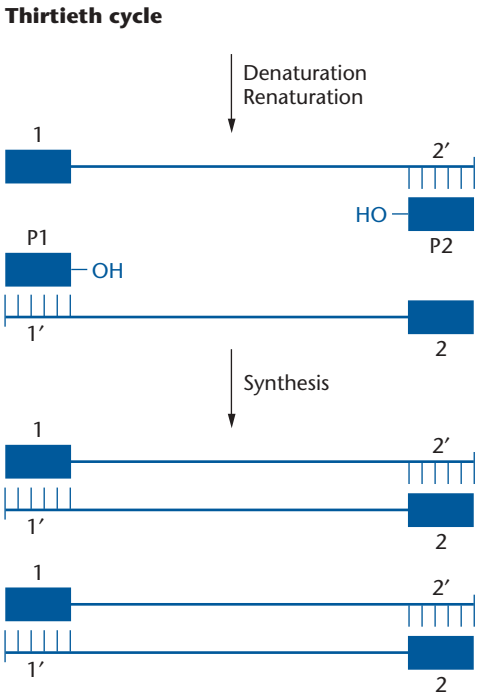


Figure 5.24 Thirtieth cycle of the polymerase chain reaction. By the thirtieth cycle, the population of DNA molecules in a reaction tube consists mainly of short template strands, which can be further amplified during additional cycles, if necessary.

the same banding pattern as any human chromosome. Human-rodent cell fusions and other interspecies cell combinations are called somatic cell hybrids. During a human-rodent fusion experiment, not only will human and rodent cells fuse, but rodent-rodent cell fusions, human-human cell fusions, and nonfused rodent and human cells will be present in the sample. However, various protocols are available for selecting only the fused human-rodent cells.

Somatic cell hybrids that carry a single human chromosome (monochromosomal cell hybrids) are an important resource for human molecular genetic studies. For example, monochromosomal somatic cell hybrids for each of the 22 autosomes and the X and Y chromosomes are used to determine the chromosome location of a cloned piece of DNA (DNA insert). To make this determination, DNA is extracted from each monochromosomal somatic cell hybrid line, digested with *Eco*RI, denatured, and fractionated in separate lanes of an agarose gel by electrophoresis. Then, the DNA is transferred from the gel by blotting to a matrix for hybridization with a labeled DNA insert. This routinely used transfer procedure is called Southern DNA blotting. A positive hybridization signal, corresponding to a particular lane of the gel, identifies the chromosome with the nucleotide sequence that is complementary to the DNA insert. Clearly, if the DNA insert contains a sequence that is complementary to rodent DNA, then all of the samples will produce a positive signal. A FISH analysis with each monochromosomal somatic cell hybrid line can also be used for localizing a DNA insert to a particular chromosome.

After a DNA insert has been assigned to a specific chromosome, it is important to establish where the insert sequence is located within the chromosome. This objective can be met by using a panel of somatic cell hybrid lines that each carry a chromosome with a different internal deletion. To be effective, the

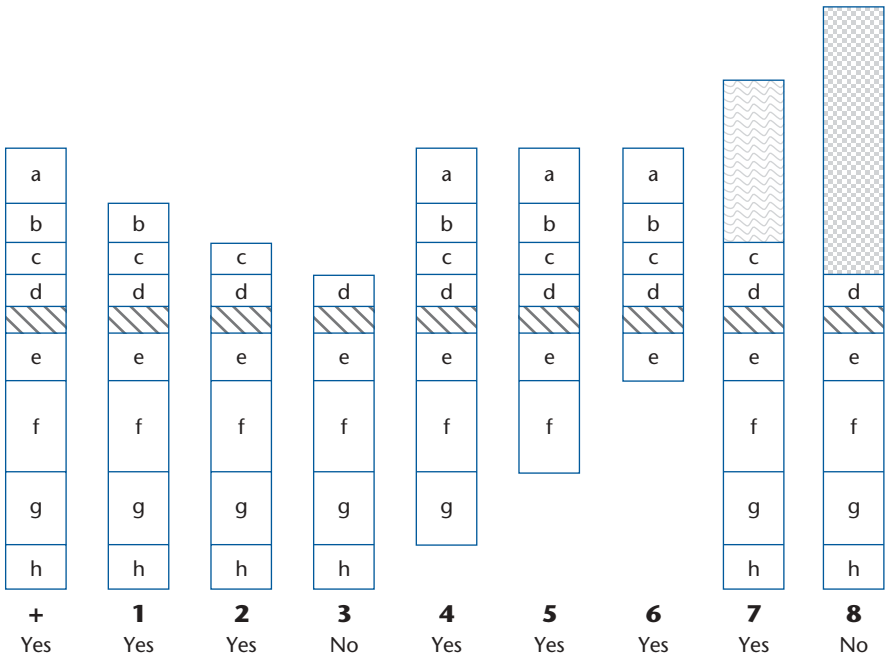


Figure 5.25 Somatic cell hybrid mapping panel. The plus sign (+) denotes the whole chromosome from a monochromosomal somatic cell hybrid line. The numbers represent the members of a somatic cell hybrid panel with chromosome-specific deletions (1 to 6) and translocations (7 and 8). The letters (a to h) mark chromosome intervals. The downward diagonals highlight the centromere of each chromosome. The fill-in patterns of chromosomes 7 and 8 depict translocated material from different chromosomes. The word "Yes" represents a positive response between a DNA insert used as a probe and the DNA sample from a somatic cell hybrid. The word "No" means that no hybridization occurred between the probe and the DNA from a somatic cell hybrid. The hybridization results with somatic cell hybrid lines 1, 2, 4, 5, and 6 establish that the sequence represented by the probe is not in interval a, b, h, g, or f, because positive results are observed in the absence of each of these intervals. The hybridization results with somatic cell hybrid line 7 exclude intervals a and b as possible locations of the probe DNA. Intervals d and e are also excluded, because no hybridization occurred between the probe and DNA from somatic cell hybrid lines 3 and 8, which have both of these intervals. By deduction, the sequence represented by the probe lies within chromosome interval c.

retained regions of the deleted chromosomes of a panel should overlap one another and the complete set of deletions should span the entire length of each chromosome arm (Figure 5.25). Somatic cell hybrids with chromosome-specific deletions are created by fusing various human cell lines that have deleted chromosomes with rodent cells and isolating those somatic cell hybrid lines that maintain a single deleted human chromosome. In addition, deleted chromosomes can be generated by treating monochromosomal somatic cell hybrid lines with X rays, which cause chromosome deletions. Individual cell lines established from irradiated cells are screened with chromosome band staining to identify the somatic cell hybrids with deleted chromosomes. Because the location and extent of each deletion is a chance occurrence, the ends of the deletions are used to partition either a whole chromosome or a chromosome arm into arbitrary intervals.

On the basis of the results of hybridization between a DNA insert and the DNA from each deleted chromosome of a somatic cell hybrid panel, it is possible to pinpoint the DNA probe to a specific chromosome interval. Briefly, this analysis is based on the rationale that when a probe does not hybridize to a deleted chromosome, then the DNA probe lies within the limits spanned by the deleted segment. Consequently, with a panel of overlapping chromosome deletions, it is possible to localize the DNA probe to a particular interval on a chromosome (Figure 5.25). An even finer localization of a DNA probe can be achieved with a panel of chromosomes with deletions confined to a specific chromosome region. In addition, somatic cell hybrid lines with translocated chromosomes can also be used for mapping DNA inserts to specific chromosome locations. In these instances, the occurrence of a hybridization signal directly relegates the DNA probe to a specific portion of a chromosome.

Human DNA Libraries

Genomic Libraries

As part of the goal of isolating human genes, DNA from the whole genome, single chromosomes, or regions of chromosomes is severed into clonable fragments that are inserted into a suitable vector and maintained in host cells. A population of host cells carrying many different DNA constructs from a single source of DNA is called a library. Because of the size of the human genome, large-insert cloning systems, especially BACs, are used to construct genomic and other chromosome-based libraries.

It is important that a genomic library contain all or nearly all the sequences of the source DNA. Consequently, the DNA used for these libraries is usually prepared by a partial restriction endonuclease digestion. Simply put, a sample of clonable DNA molecules of all possible lengths is obtained by treating a DNA sample with a restriction endonuclease, either for a short duration or with a small amount of enzyme (Figure 5.26). In some cases, a partially digested DNA sample is enriched for large pieces within a defined size range. For example, yeast artificial chromosome (YAC) libraries are constructed with DNA molecules from partial digestions that are 200 kb or more in length.

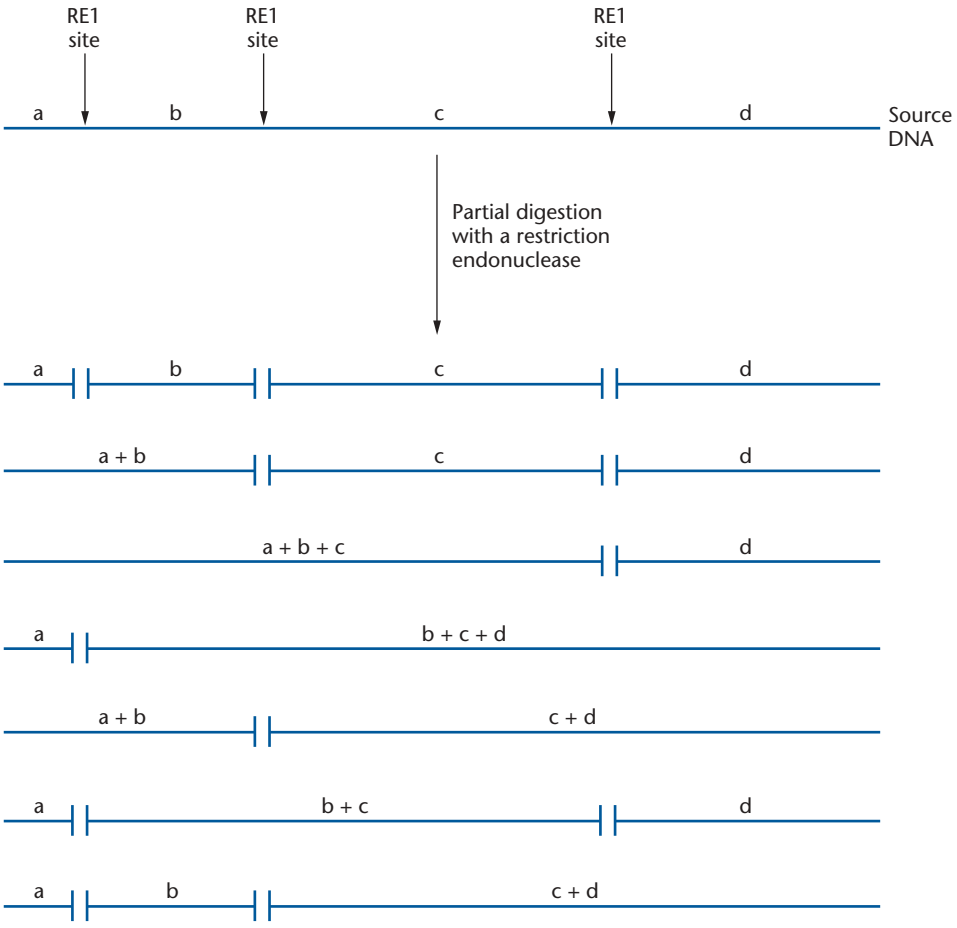


Figure 5.26 Partial digestion of DNA with a type II restriction endonuclease. Partial digestions are performed by varying either the length of time or the amount of the enzyme used for the digestion. Here, a genome is represented as a single DNA molecule with three restriction endonuclease sites (RE1). The desired outcome is a population of fragments derived from all possible combinations of restriction endonuclease cleavages.

A vector system that carries large inserts (>100 kb) is helpful for the analysis of complex eukaryotic genomes. For example, very-large-insert vector systems are indispensable for generating complete physical maps of eukaryotic genomes. There is also a high probability that a single clone will carry one or more intact genes. Moreover, in contrast to a small-insert library, there are fewer clones to maintain with a large-insert genomic library.

The uptake of high-molecular-weight DNA can be induced by subjecting bacteria to a high-voltage electric field. This procedure is called electroporation, a term that is a contraction of the descriptive phrase "electric field-mediated membrane permeabilization." The experimental protocols for electroporation are formulated for the species and size of the cloning vector. For *E. coli*, the cells (~50 μl) and a BAC-insert DNA construct are placed in a chamber fitted with electrodes and a single pulse of approximately 25 microfarads, 2.5 kilovolts, and 100 ohms is administered for about 2.4 milliseconds (ms). This treatment yields transformation efficiencies of about 10^6 transformants per microgram of DNA.

A low-copy-number *E. coli* plasmid vector that is based on the P1 bacteriophage cloning system has been devised for cloning DNA molecules that are from 100 to 300 kb in length. The DNA insert-vector constructs of this system are called bacteriophage P1-derived artificial chromosomes (PACs). Similarly, the F plasmid (F-factor replicon, sex plasmid, fertility plasmid) of *E. coli*, which is present at one to two copies per cell, has been used along with the *lacZ′* selection system of the pUC vectors to construct an extremely stable cloning vector that carries DNA inserts from 50 to 300 kb, with an average insert length of about 150 kb. The F-plasmid-based DNA insert-vector constructs, which are used extensively, are called bacterial artificial chromosomes (BACs).

To establish a genomic library that is as complete as possible, the sum of the DNA lengths of all of the members (clones) of the library must be greater than the total amount of DNA of the genome of the source organism. For example, a BAC human genomic library of 110,000 clones with an average insert size of about 150 kb represents 5 genome equivalents, that is, $\frac{110{,}000 \times 150}{3.3 \times 10^6} = 5$. A comparable coverage of the human genome with a cosmid cloning vector carrying about 40 kb DNA inserts would require a library of 800,000 clones. As a rule of thumb, 15-fold redundant coverage is used for initial BAC genome libraries. In other words, researchers prefer to start with about 15 clones for each chromosome region. Eventually, as discussed in Chapter 6, the library is reduced to a set of minimally overlapping clones (contigs, tiled clones) that cover the entire genome. For humans, about 25,000 to 30,000 BAC clones are sufficient for this purpose.

The maintenance and subculturing of the individual clones of a genomic library are time-consuming tasks. However, automated equipment that dispenses each colony of a library into a well of a multiwell culture plate has made library management easier and enhanced the reliability of the transfer of clones. With a multiwell format, each clone has a specific designation (address) that corresponds to a particular row and column of a certain culture plate. In other words, the clones of the library are distributed into defined arrays, and the organization of each array is readily and accurately perpetuated. Parentheti-

cally, because a DNA library is such an important resource, all the testing of clones is conducted with replica sets of the master plates.

After a library is created, DNA hybridization is often used to identify and isolate a clone(s) carrying a particular nucleotide sequence. Obviously, with tens of thousands of clones in a typical human genomic library, conducting a hybridization test with each clone is exceptionally arduous. Fortunately, the number of these tests can be minimized by the simple strategy of forming pools of clones. Here, for convenience, the benefits of pooling will be illustrated with an unrealistically small library consisting of only 125 clones. In this case, the library is maintained in five 25-well culture plates, each with five rows (A–E) and five columns (1–5). Thus, if this library were to be screened one clone at a time, it would require 125 hybridization assays. By comparison, in the real world, a P1-based human genomic library that occupies 1500 96-well plates would require 144,000 individual DNA hybridization tests.

In our example, the first pooling entails combining all the wells of each plate and dispensing each of these pools into a well of a fresh culture plate. Hybridization assays can be carried out in various ways. Generally, after pooled cells are grown, they can be removed from a well and lysed. Then, the DNA is extracted, denatured, and bound to a supporting matrix. For the second pooling, the wells of each row of each plate are combined, and each pooled row is transferred to a single well of a new culture plate. Each of the five wells from the first pooling is screened with a DNA probe (Figure 5.27). If, for example, the pool of clones from plate 2 gives a positive signal (Figure 5.27B), then only the five wells of the pooled rows from plate 2 need to be screened (Figure 5.27C). If the well with the pool of the wells from row C of plate 2 gives a positive signal, then the five wells of row C of a replicate plate of master plate 2 are assayed to identify the clone that hybridizes with the probe (Figure 5.27A). Thus, instead of screening 125 clones, only 15 assays are required to pinpoint a particular clone. More complex pooling strategies with elaborate geometries have been used for the screening of very large libraries.

Chromosome DNA Libraries

In addition to genomic libraries, whole chromosome and chromosome region-specific libraries are useful resources for isolating genes, genetic mapping, and determining interchromosomal abnormalities. Whole chromosome libraries are assembled with DNA from single chromosomes isolated by flow cytometry from either human or human-rodent hybrid cell lines.

Individual whole human chromosomes can be isolated by using fluorescence-activated flow cytometry. A flow cytometer is an apparatus that determines both physical and chemical features of particles, cells, or chromosomes as they pass in single file by a measuring device. In addition, by electronically presetting the measurement criteria for a particular entity in a mixture, all items with the same features are identified and diverted into a single collecting tube. This capability to selectively purify specified objects is called flow sorting. Many human chromosomes can be separated and collected in this way. Briefly, human cell lines are treated with an agent that blocks mitosis. The mitotic chromosomes are isolated and treated with Hoechst 33258 and chromomycin A3, DNA-specific dyes. Hoechst 33258 binds to chromosome regions rich in

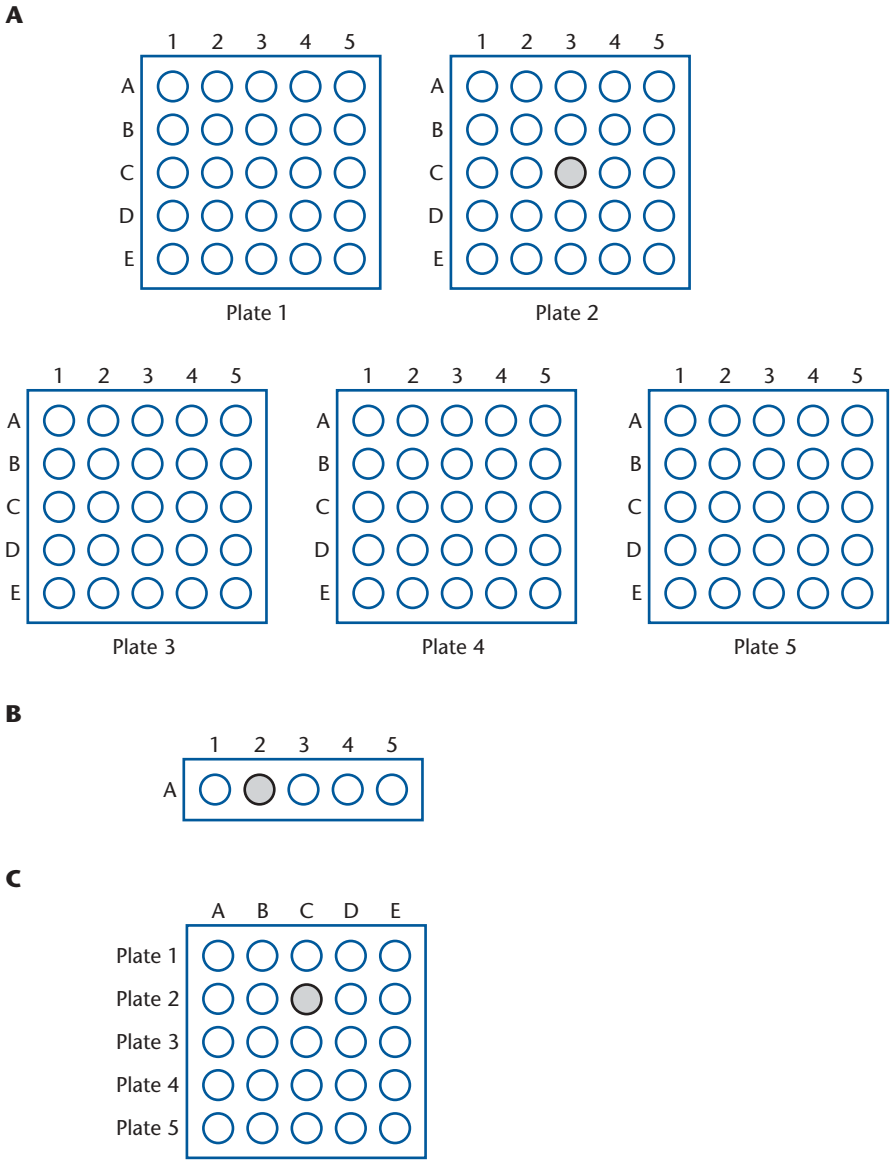


Figure 5.27 The use of pooling clones of a library to minimize the number of DNA hybridization tests. (A) The clones of a library are arrayed in a set of plates. In this example, the clones are dispensed into five 25-well plates. Each clone has a unique address, for example, plate 2, row C, column 3. (B) All the clones of each plate are pooled and placed in a single well (wells A-1 to A-5). A positive result (shaded area) with the hybridization probe to DNA from well A-2 indicates that a clone that carries DNA that is complementary to the probe is located in one of the wells of plate 2 of the arrayed library. (C) The wells from each row of each original plate are pooled and placed in a single well. Based on the results in B, only the wells of each pooled row from plate 2 need to be screened with the hybridization probe. The positive result (shaded area) with DNA from the plate 2-C well indicates that a clone that carries DNA that is complementary to the probe is located in row C of plate 2 of the arrayed library. As depicted in A, after screening each clone of row C of plate 2, well C-3 of plate 2 (shaded area) contains the clone with DNA that is complementary to the hybridization probe.

AT base pairs, and chromomycin A3 binds to GC-rich regions. The doubly stained mitotic chromosomes are carried sequentially in a liquid stream by a high-speed flow sorter through two laser beams. One of the lasers is set to excite Hoechst 33258 and the other to excite chromomycin A3. The range and extent of the combined fluorescent emissions produces a unique profile (peak position) for each human chromosome, except for chromosomes 9 to 12. It is not possible to separate the chromosomes of this group because they have similar proportions of AT- and GC-rich regions.

Sorting occurs when a computer-stored chromosome profile matches the combined response elicited by the dual lasers. When the two profiles correspond, the designated chromosome is deflected into a collecting tube. Two different chromosomes can be collected separately during a single fluorescence-

activation sorting experiment. The signals from clumped chromosomes, fragments, chromosomes 9 to 12, nonmatching chromosomes, and cell debris are distinguished and diverted into a waste container. Pure samples of chromosomes 9 to 12 can be obtained by flow sorting each of these chromosomes from monochromosomal human-hamster hybrid cell lines. None of the hamster chromosomes produces a combined emission peak that is equivalent to any of these human chromosomes.

The extracted DNA from a sample of flow-sorted chromosomes is digested with a restriction endonuclease and cloned into an appropriate site of a cloning vector. A major limitation of chromosome flow sorting is the quantity of a purified chromosome that can be obtained with reasonable experimental conditions. For example, thousands of chromosomes are required to form a cosmid-based library. To date, cosmid (~40-kb insert) and lambda (~15-kb insert) chromosome-specific libraries have been constructed. It has not been feasible to collect enough chromosomes to make large-insert, chromosome-specific libraries. Chromosome-specific libraries with small inserts (~200 to 1000 bp) are readily constructed with plasmid vectors from a small number of flow-sorted chromosomes by linker-adaptor PCR (ligation-adaptor PCR).

Briefly, the linker-adaptor PCR protocol entails treating DNA from a flow-sorted chromosome sample with a restriction endonuclease to produce protruding 5′ (or 3′) ends and ligating these fragments with a synthetic double-stranded oligonucleotide (adaptor-linker sequence) that has a 5′ (or 3′) extension complementary to the 5′ (or 3′) extension of the DNA fragments. A single oligonucleotide sequence (20 bases), complementary to one of the strands of the linker-adaptor sequence, is used as the primer for the PCR. Because the adaptor-linker sequences at the ends of the DNA fragments are oriented in opposite directions, the 3′-hydroxyl ends of a single primer sequence face each other, which enables the intervening DNA to be amplified. After amplification, the DNA sample is treated with the same restriction endonuclease used to cleave the chromosomal DNA. This treatment creates large amounts of DNA fragments with 5′ (or 3′) extensions at both ends that are used for cloning into the corresponding restriction endonuclease site of a plasmid vector (Figure 5.28).

Small-insert (~1500 bp), chromosome-specific libraries can be used in a number of different ways. First, they provide probes for isolating large-insert (>40 kb) clones from BAC, P1, PAC, and YAC libraries. Second, they are a potential resource for probes used in gene mapping (see Chapter 6). Third, as an ensemble, they are an excellent source of probes for FISH to identify chromosome abnormalities in prenatal and cancer cells.

Monochromosomal cell hybrid lines are also used to construct large-insert, chromosome-specific libraries. High-molecular-weight DNA is prepared from a monochromosmal somatic cell hybrid line, partially digested with a restriction endonuclease, and ligated into the corresponding restriction endonuclease site of a cloning vector that can carry a large insert. After transformation and screening, each clone with a DNA insert is placed into a well of a set of culture plates. The problem at this stage of the protocol is to distinguish the clones that carry only human DNA inserts. To this end, a DNA sample from each well is transferred to a filter. The DNA on the filters is hybridized with a labeled

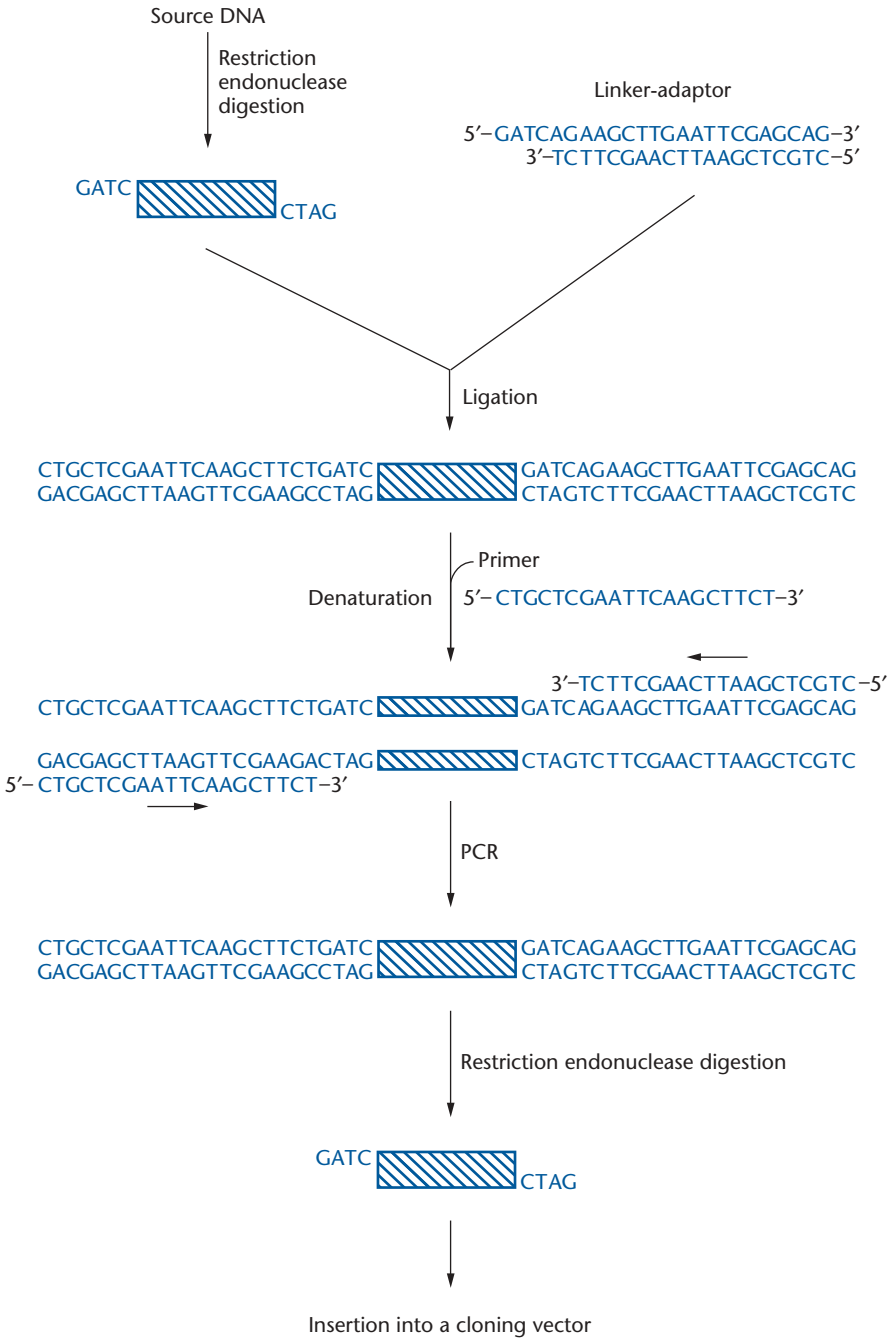


Figure 5.28 Linker-adaptor PCR. The source DNA is cut with a restriction endonuclease. The fragments are ligated to a linker-adaptor that has an extension complementary to the protrusion created by the restriction endonuclease that was used to cut the source DNA. PCR is carried out with a primer (20 bases) that is complementary to the linker-adaptor sequence. A horizontal arrow indicates the direction of DNA synthesis from the 3′-hydroxyl group of the primer. The amplified products are treated with the same restriction endonuclease that was used to cleave the source DNA and inserted into this restriction endonuclease site of the cloning vector.

Alu sequence probe. Briefly, *Alu* is the term for a family of about 500,000 non-coding, human-specific DNA sequences, each about 300 bp and often with a single *Alu*I restriction endonuclease site near the middle of the sequence. *Alu* sequences tend to be closely spaced in the human genome and occur about every 100 to 5000 base pairs. Hybridization signals represent the clones that comprise the human chromosome-specific library. Clones with negative responses are likely derived from the rodent genome and are discarded.

from the HUMAN GENETICS files

Multicolor Karyotyping: Coloring Chromosomes

G-banded human karyotypes have been extremely useful for determining chromosome abnormalities, although the analysis of metaphase spreads requires considerable expertise. In addition, the variation in the uptake of stain by bands from one preparation to another and the similarities between clusters of bands on different chromosomes makes it difficult to automate these analyses. However, the advent of fluorescent in situ hybridization (FISH), PCR, chromosome-specific libraries, and an expanded set of fluorescent dyes made it possible to color individual metaphase chromosomes. By using chromosome-specific paints rather than banding patterns, it should be easier to determine chromosome alterations and to automate karyotypic analyses.

Initially, the technique of "painting" a chromosome a specific color used a flow-sorted chromosome clone bank as the source of chromosome-specific probes. Clones were either labeled directly by the incorporation of a nucleotide with a fluorescent dye during the labeling reaction or, indirectly, by the incorporation of nucleotides that carried an affinity group. After the hybridization reaction, fluorescence was visualized directly by fluorescent microscopy, or the slides were treated with a fluorescent dye conjugate that bound to the affinity group of the probe and then were examined for fluorescence. To ensure that the chromosome-specific probes did not hybridize to repeated sequences, denatured metaphase chromosomes were hybridized with an unlabeled DNA sample containing only repetitive DNA added to the probe mixture prior to hybridization.

A PCR protocol has replaced flow-sorted chromosome libraries as the source of chromosome-painting probes. Specifically, the chemical synthesis of the sequence 5′-CCGACTCGAGNNNN NATGTGG-3′, where N means that any of the four nucleotides are incorporated at these sites, produces a family of 21-mers. With this family of oligonucleotides, PCR amplification will occur at about 10^6 sites scattered throughout the human genome, or approximately 21,000 sites per chromosome. This type of PCR system is called degenerative oligonucleotide primer-PCR (DOP-PCR). Thus, with either a flow-sorted chromosome preparation or a microdissected chromosome region as the template DNA, DOP-PCR produces a large pool of probes from either an entire chromosome or parts of it. This PCR method eliminates the need to maintain clones and gives a high yield of probes for a specific chromosome.

Since there are only about 7 or 8 manufactured fluorescent dyes suitable for DNA labeling that can be discriminated spectrally from each other, only a small number of the 24 human chromosomes (i.e., 22 autosomes, X, Y) can be painted with a different color in a sample. If 24 different fluorescent dyes could be differentiated spectrally at the same time, then each chromosome could be identified by its distinctive hue. Moreover, chromosome interchanges could be spotted readily on the basis of unexpected color juxtapositions. In comparison to searching a G-banded karyotype for the origins of displaced groups of bands, it should be easier to identify chromosome translocations and other interchromosomal changes among multicolored chromosomes. Unfortunately, organic chemists have not created 24 different fluorescent dyes that can be used for coloring each human chromosome. However, some molecular cytogeneticists reasoned that hybridizing a chromosome with sets of chromosome-specific probes, each labeled with a different fluorescent dye, might produce a combined fluorescence emission spectrum unlike each fluorescent dye when it is used singly. Theoretically, with 5 fluorescent dyes, there are 31 ($2^n - 1$; $n = 5$) possible combinations. In other words, hypothetically, there are enough combinations with just five fluorescent dyes to produce a distinct spectral emission for each chromosome. After testing various combinations of labeled chromosome-specific probes, 24-chromosome color protocols with 5 fluorescent dyes were formulated. Later, for enhanced resolution, this set was increased to 7 fluorescent dyes. The scheme in the table shows one example of the different combinations of labeled chromosome probes that produces a unique fluorescence response from each chromosome.

Spectral differences among various combinations of fluorescent dyes are subtle, and detection requires specialized equipment and software. The problem of deciphering spectral dissimilarities has been handled two ways. One approach uses a set of 7 different filters that each transmits the emission from one of the fluorescent dyes. The equipment captures the image of each chromosome, and, after 7 separate readings of the painted chromosomes, each with a different filter, a computer program combines the spectral data for each chromosome (spectral signature) and assigns it a color. The color designated by the computer is not necessarily the real color, but one that reflects the composite spectral information of each chromosome and facilitates recognition of one chromosome from another. This way of discerning each of the 24 chromosomes from a single metaphase spread was dubbed combinatorial multi-fluor FISH (M-FISH, mFISH, multi-color FISH).

An alternative method for resolving spectral differences, which uses the same kind of chromosome labeling protocol as the M-FISH strategy, is called multicolor spectral karyotyping (SKY). In this case, the entire spectrum emitted from each chromosome is determined, and each chromosome is assigned a pseudocolor. Briefly, a charge-coupled device camera captures the images of the metaphase chromosomes, the emissions are passed through an interferometer, the data are processed into spectral information, and a color is allocated to each chromosome. Generally, the lower limit of resolution of spectral karyotyping is about 1000 kb DNA. Aberrations that involve smaller chromosome segments are not visualized by spectral karyotyping.

continued

More than 500,000 karyotypic analyses are conducted annually in the United States and Canada to determine the chromosome status of fetuses, newborns, and cancer cells. Multicolor karyotyping will make it easier to determine loss or gain of chromosomes (aneuploidy) and complex structural rearrangements, such as translocations, between nonhomologous chromosomes. For example, in a clinical setting, since cancer cells have distinctive chromosome abnormalities, multicolor karyotyping can be used to determine whether a therapeutic regimen has been effective in lowering a patient's population of cancer cells.

In its current format, 24-chromosome painting protocols do not readily identify intrachromosomal rearrangements such as inversions, small duplications, and small deletions. However, multicolor FISH with labeled probes directed to specific regions within chromosomes, often derived from microdissected chromosome regions, has been developed to overcome this limitation. Various multicolor banding techniques using either G-band-specific or region-specific probes have detected inversions, insertions, and deletions. In addition, another FISH technique called comparative genomic hybridization (CGH) is used to determine variations in DNA copy numbers in tumor cells. (See Molecular Genetics of Cancer Syndromes chapter for information about CGH.)

Table Scheme for distinct fluorescent dye labeling of each human metaphase chromosome with chromosome-specific probes (x) tagged with one or more of seven different fluorescent dyes (A to G).

	Chromosomes																						
Dye	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**X**	**Y**
A	x					x				x			x			x							x	
B	x			x	x		x											x	x		x			
C			x			x		x			x				x			x		x				
D			x	x					x	x							x					x		x
E		x										x	x		x		x				x			
F		x					x		x		x			x		x								
G					x			x				x		x								x	x	

From Azofeifa J, Fauth C, Kraus J, Maierhofer C, Langer S, Bolzer A, Reichman J, Schuffenhauer S, Speicher MR (2000): An optimized probe set for the detection of small interchromosomal aberrations by use of 24-color FISH. *Am J Hum Genet* **66**:1684–1688.

Region-Specific Chromosome Libraries

Chromosome region-specific libraries can be very helpful for gene mapping and isolation of a gene that has been localized to a specific chromosome region. To a great extent, the strategies for generating these libraries are the same as those for creating chromosome-specific libraries.

One approach for obtaining a regionally defined segment of chromosomal DNA is to use glass microneedles to remove the specified chromosome region from a banded chromosome preparation. The microdissected regions from about 20 to 30 metaphase chromosomes are combined. Then, in a very small volume, the DNA is extracted, treated with a restriction endonuclease, ligated to a linker-adaptor oligonucleotide, amplified by PCR, and cloned into a plasmid cloning vector.

Alternatively, a computer-controlled apparatus can be programmed to recognize any specified banded chromosome region and automatically destroy ("burn out") all the other chromosome DNA of a metaphase chromosome spread. Enough amplified DNA to create a representative region-specific library can be generated by using a modified form of linker-adaptor PCR with the remaining chromosome regions of the homologous chromosomes from a single metaphase chromosome spread.

Constructing a cDNA Library

Human structural genes are generally complex. Exons, which on the average are approximately 150 bp in length, alternate with introns, which have different lengths and, in some cases, can extend for thousands of base pairs. It is important to have DNA inserts of the complete coding regions of human genes for studies of gene expression and other research purposes. A number of protocols have been devised for the in vitro synthesis of double-stranded DNA copies (cDNA, complementary DNA) from mRNA molecules.

A functional eukaryotic messenger RNA (mRNA), after the excision of introns, has a G cap at the 5′ end and, usually, a string of up to 200 adenine residues [poly(A) tail] at its 3′ end. The poly(A) tail provides the means for separating the mRNA fraction of a tissue from the ribosomal RNA (rRNA) and transfer RNA (tRNA). Short chains of 15 thymidine residues [oligo(dT), dT_{15}] are attached to cellulose beads, and the oligo(dT)-cellulose beads are packed into a column. Extracted cellular eukaryotic RNA is passed through an oligo(dT)-cellulose column, and the poly(A) tails of the mRNA molecules bind by base pairing to the oligo(dT) chains. The tRNA and rRNA molecules, which lack poly(A) tails, pass through the column. The mRNA is removed (eluted) from the column by treatment with a buffer that breaks the A:T hydrogen bonds, thereby releasing the base-paired mRNAs.

Before the mRNA molecules are cloned into a vector, they must be converted to double-stranded DNA. This synthesis is accomplished by using, in succession, two different kinds of nucleic acid polymerases. Reverse transcriptase synthesizes the first DNA strand, and the Klenow fragment of DNA polymerase I synthesizes the second one (Figure 5.29). After the mRNA fraction is purified, short, unattached sequences of oligo(dT) molecules are added to the sample, along with the enzyme reverse transcriptase and the four dNTPs (dATP, dTTP, dGTP, and dCTP). An oligo(dT) molecule base pairs with the adenine residues of the poly(A) tail of a mRNA and provides an available 3′-hydroxyl group to prime the synthesis of a DNA strand.

Reverse transcriptase, which is produced by certain RNA viruses (retroviruses), uses an RNA strand as a template while directing deoxyribonucleotides into the growing chain. Thus, when an A, G, C, or U nucleotide of the template RNA strand is encountered, the complementary deoxyribonucleotide, namely, T, C, G, or A, is incorporated into the growing DNA strand. Before reverse transcriptase synthesis ceases, the DNA strand usually turns back on itself and a few nucleotides are added (Figure 5.29) to form a hairpin loop.

The second DNA strand is synthesized by the addition of the Klenow fragment of *E. coli* DNA polymerase I, which uses the first DNA strand as a template and adds deoxyribonucleotides to the growing strand, starting from the 3′-hydroxyl group at the end of the hairpin loop. After the reaction is complete, the sample is treated with the enzyme RNase H, which degrades the mRNA molecules, and with S1 nuclease, which opens the hairpin loops and degrades single-stranded DNA extensions. At the end of the procedure, the sample contains a mixture of partial and complete (full length) double-stranded complementary DNA (cDNA) copies of the more prevalent mRNAs in the original sample. Subsequently, a cDNA population is cloned by blunt-end

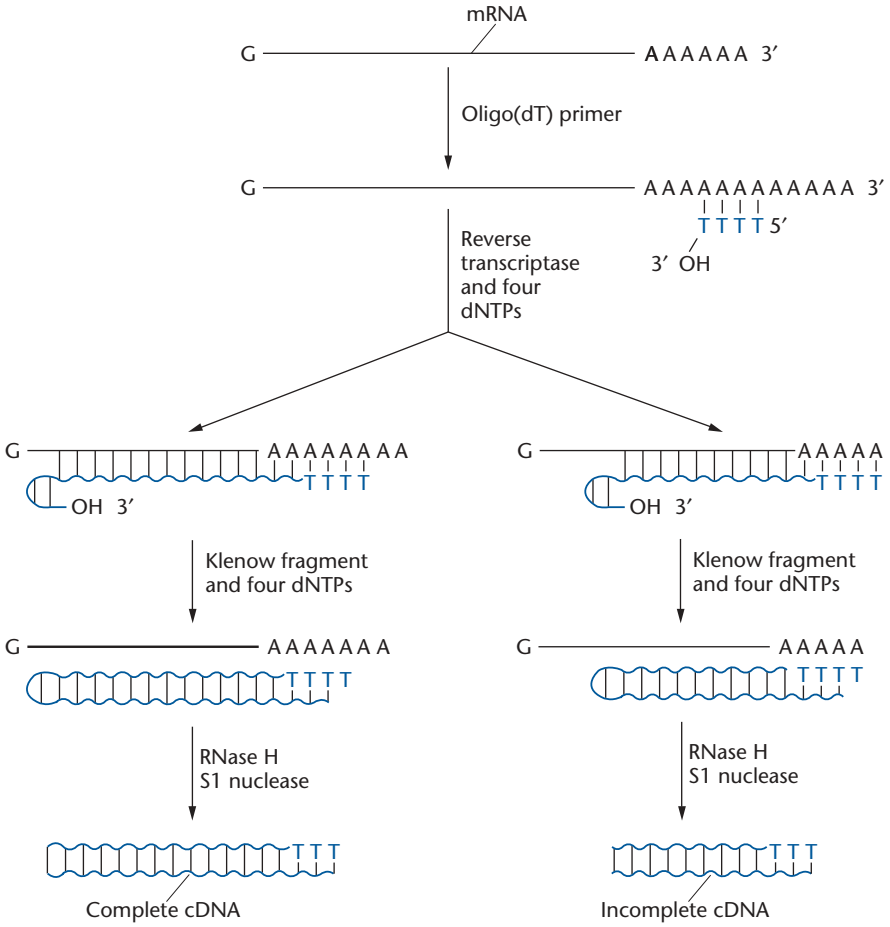


Figure 5.29 Synthesis of cDNA. Oligo(dT) primer is added to a purified mRNA preparation, and reverse transcriptase with the four dNTPs is used for the production of DNA from the RNA template. Reverse transcriptase does not always produce full-length cDNA copies of the RNA from every template molecule (lower right). The looping back of the growing DNA strand produced by the reverse transcriptase provides a 3′-hydroxyl group for the Klenow fragment to complete the synthesis of the second strand with the first DNA strand acting as the template. After synthesis of the second strand is completed, the mRNA is hydrolyzed with the enzyme RNase H and the DNA is treated with S1 nuclease to produce blunt-ended linear molecules without hairpin loops.

ligation or other joining mechanisms into a plasmid cloning vector to form a cDNA library.

As noted, the standard cDNA synthesis protocol produces both complete (full length) and incomplete molecules. Unfortunately, much time and effort is spent on identifying clones of a cDNA library with full-length sequences. Various strategies have been devised to overcome this inconvenience. With one multistep procedure, full-length, first-strand cDNAs are captured and they alone are used as templates for the synthesis of second strands (Figure 5.30). Briefly, the primer for first-strand DNA synthesis is bipartite with a poly(dT) sequence at the 3′ end of an oligonucleotide joined to a recognition site for a restriction endonuclease. This dual function nucleic acid sequence is called a primer-adaptor. The disaccharide trehalose is added to the reverse transcriptase reaction to stabilize the enzyme and allow DNA synthesis to proceed at a high temperature. Secondary structure (intrastrand pairing) of the mRNAs is disrupted by high temperature, and the likelihood that complete molecules will be synthesized is increased. In addition, one of the four deoxyribonucleotides in the reverse transcriptase reaction mixture is 5-methyl-dCTP that is incorporated into the growing strand. The presence of methyl groups in one strand (hemimethylation) of double-stranded DNA protects the DNA from being

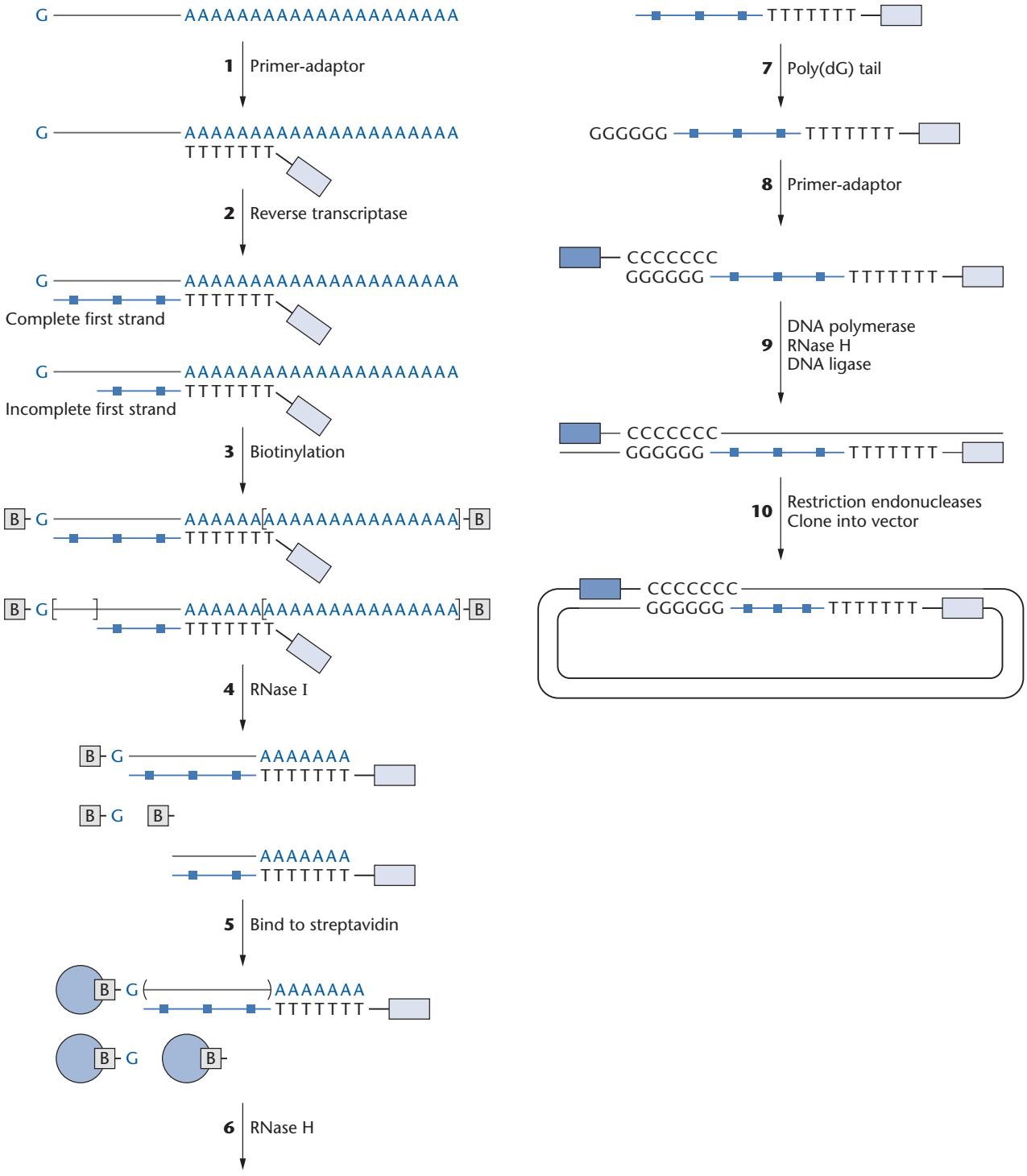


Figure 5.30 Schematic representation of a method for selecting and cloning full-length cDNA molecules. (1) Purified messenger RNA is mixed with a combined oligo(dT)-restriction endonuclease site oligonucleotide (primer-adaptor). (2) Reverse transcriptase synthesizes the first cDNA strand with 5-methyl-dCTP (blue boxes) as one of the four dNTPs. Both incomplete and complete DNA strands are synthesized. (3) Biotin (B) is attached to both ends of the mRNA molecules. RNase I-susceptible regions are marked by square brackets. (4) Single-stranded segments of RNA are degraded by RNase I. (5) Biotinylated molecules bind to streptavidin-coated magnetic beads. After RNase I treatment, full-length RNA:DNA hybrids are biotinylated, and therefore they bind to streptavidin. Incomplete cDNAs are not biotinylated and do not bind to streptavidin. The RNase H-susceptible region of a full-length RNA:DNA hybrid is marked by curved brackets. (6) RNase H treatment degrades the RNA of streptavidin-bound RNA:DNA hybrids and releases full-length, first-strand cDNA molecules. (7) A poly(dG) tail is added to the 3′-hydroxyl end of the first cDNA strand. (8) An oligo(dC)-restriction endonuclease site oligonucleotide (primer-adaptor) pairs with the oligo(dG) tail and provides a 3′-hydroxyl group for synthesis of the second DNA strand by DNA polymerase. (9) During the synthesis of the second cDNA strand by DNA polymerase none of the dNTPs are methylated; RNase H removes any remaining base-paired RNA; and DNA ligase joins DNA segments that were synthesized internally from bits of mRNA that escaped degradation. The oligo(dC) primer-adapter sequence acts as a template for DNA synthesis from the 3′-hydroxyl group at the end of the poly(dG) tail. (10) The final full-length cDNAs are cut with two restriction endonucleases, one for each end, and cloned into a vector that has complementary extensions. Hemimethylation protects the cDNA from cleavage by the restriction endonucleases that are used for cloning because these enzymes cannot cut methylated restriction endonuclease sites.

cleaved by certain restriction endonucleases. This DNA modification is important for the final step of the procedure.

After the first strand is synthesized, biotin is chemically attached to the ribose sugars of the cap nucleotide and the 3′ ends of the mRNA molecules. Deoxyribose is not biotinylated under these conditions. Next, the hybrid RNA : DNA molecules are treated with RNase I. This enzyme cleaves single-stranded RNA. It does not attack RNA that is base paired with DNA or DNA strands. As a result, both the 5′ single-stranded regions of the mRNAs with incomplete cDNAs and the nonpaired poly(A) tails of the mRNA molecules are degraded. The mRNA strands of completely synthesized cDNA strands are not affected by this enzyme. The sample is then mixed with streptavidin-coated magnetic beads. Biotin has a high affinity for streptavidin. After RNase I treatment, the only biotinylated RNA : DNA hybrid molecules that remain are those with a biotinylated cap. In other words, only full-length cDNAs are captured. A magnet is used to separate out the beads from solution. Next, the RNA of the streptavidin-bound RNA : DNA hybrids are hydrolyzed with RNase H, which cuts base-paired RNA and releases the full-length cDNA strands into solution.

Because the sequences at the 3′ ends of the first-strand cDNAs are not known, a string of guanine nucleotides is added to the 3′-hydroxyl ends to provide a complementary sequence for a DNA primer that initiates the synthesis of second cDNA strands. The enzyme terminal deoxynucleotidyl transferase adds deoxyribonucleotides (dNTPs) sequentially by phosphodiester bond formation to the 3′-hydroxyl end of a polynucleotide. If only one type of dNTP is present in the terminal transferase reaction mixture, a homopolymeric tail is formed. After poly(dG) tailing, the poly(dC) segment of second primer-adaptor base pairs with the poly(dG) tail and provides an available 3′-hydroxyl group for second-strand cDNA synthesis. The adaptor portion of this oligonucleotide contains the sequence for another restriction endonuclease site. Second-strand cDNA synthesis is carried out at high temperatures with thermostabilized DNA polymerase, RNase H, and DNA ligase. As with the synthesis of the first cDNA strand, the high temperature diminishes intrastrand folding and increases the efficiency of synthesis of a full-length strand. The dCTP used for second-strand synthesis is not methylated. RNase H removes any RNA that escaped the previous treatment, and DNA ligase joins segments that were primed by remaining bits of RNA. The final product is a full-length, double-stranded cDNA that is furnished with nonmethylated restriction endonuclease recognition sites at both ends. These sites are cleaved with the appropriate restriction endonucleases and cloned into a vector that has complementary extensions. Hemimethylation protects a cDNA from cleavage if it contains the same restriction endonuclease sites that are used for cloning and, of course, these restriction endonucleases do not cut at methylated sites.

key terms

annealing
bacterial artificial chromosome
biotin
cDNA
chromosome painting
cloning vector
denaturation
dideoxynucleotide
DNA library
DNA polymerase I
DNA probe
DNA sequencing
exons
flow cytometry
fluorescence in situ hybridization
genomic library
Klenow fragment
linker-adaptor PCR
M-FISH
monochromosomal hybrid cell line
oligonucleotide
phosphodiester bonds
plasmid
polymerase chain reaction (PCR)
pUC19
renaturation
restriction endonuclease
reverse transcriptase
RNase H
Southern DNA blotting
spectral karyotyping
streptavidin
somatic hybrid cell panel
T4 DNA ligase
thermostable DNA polymerase
yeast artificial chromosome

summary

Recombinant DNA technology has had a profound impact on all aspects of the biological and medical sciences. The ability to identify and characterize both normal and mutant genes has enabled investigators to determine the molecular basis of many inherited disorders. However, the cloning of DNA is no simple matter. It requires the use of a number of enzymes in vitro and many different laboratory protocols. Of these enzymes, type II restriction endonucleases, which cleave duplex DNA at specific recognition sites, are essential. Many type II restriction endonucleases produce DNA molecules with short complementary nucleotide extensions at the ends of opposite strands. This feature is extremely useful for the cloning of DNA.

Generally, as part of any molecular study of a human disorder, genomic DNA is partially digested with a restriction endonuclease. Theoretically, this limited digestion produces all possible lengths of DNA fragments. A cloning vector, which facilitates the perpetuation of individual genomic fragments in a host cell, is cleaved at a single site by the same restriction endonuclease that is used to cut the genomic DNA. Cloning vectors have been designed for various functions. Many are used to carry DNA fragments within a specified range of lengths. For example, plasmids, cosmids, bacterial artificial chromosomes (BACs), and yeast artificial chromosomes (YACs) accommodate DNA inserts that are approximately 5, 45, 150, and 800 kb, respectively. After the restriction endonuclease-treated genomic and cloning vector DNA molecules are mixed, some of these molecules are joined transiently by base pair formation between complementary extensions. The enzyme T4 DNA ligase is used to catalyze the formation of phosphodiester bonds that covalently link these two molecules together to create a DNA construct. The DNA from the ligation mixture is introduced into host cells. Strategies are available that identify specifically cells that carry a DNA construct. The collection of cells with DNA constructs from a partially digested genomic DNA sample is called a genomic DNA library.

A DNA hybridization assay is used often to identify those cells of a library that carry a DNA insert of interest. In principle, DNA hybridization techniques consist of mixing a denatured (single stranded), labeled DNA (DNA probe) with denatured DNA of the samples that are being tested and then scoring, as a consequence of nucleotide complementarity, the binding of the labeled DNA probe to the DNA of a particular sample. Generally, a DNA probe is a cloned piece of DNA with known chromosome location, function, or both.

An insert DNA sequence can be assigned to a chromosome location by labeling it with a fluorescent dye, mixing it with denatured mitotic metaphase chromosomes on a microscope slide under hybridization conditions, and, after removing the unbound DNA probe, determining the site of fluorescence. This procedure is called fluorescence in situ hybridization (FISH).

In addition to the various procedures directly related to DNA cloning, a number of other techniques are important for molecular studies of genes, including the chemical synthesis of DNA, DNA sequencing, the polymerase chain reaction (PCR), and the formation of

continued

monochromosomal and subchromosome somatic hybrid cell (somatic cell hybrid) lines. Single-stranded DNA molecules with 50 or fewer nucleotides are efficiently produced chemically by computer-controlled DNA synthesizers. These DNA oligomers can be used as probes for screening DNA libraries, linkers and adaptors that facilitate the cloning of DNA, and primers for DNA sequencing or PCR amplification.

The dideoxynucleotide procedure is used routinely for determining the nucleotide sequence of a cloned insert. This technique depends on the in vitro incorporation of a dideoxynucleotide into a growing DNA strand. This nucleotide terminates DNA synthesis, because it does not have a 3′-hydroxyl group. In a typical sequencing experiment, four DNA synthesis reactions, each with a different dideoxynucleotide, are carried out simultaneously. The products of these reactions are separated electrophoretically in individual lanes, and the pattern of separation of the synthesized radiolabeled fragments is used to determine the nucleotide sequence of the growing strand. For large-scale sequencing projects, each dideoxynucleotide is labeled with a different fluorescent dye and the bases are read off sequentially during separation by capillary or polyacrylamide gel electrophoresis.

Discrete segments of DNA can be amplified a millionfold with the PCR. A typical PCR entails 30 or more successive cycles, with each cycle consisting of denaturation, renaturation, and in vitro DNA synthesis steps. The DNA synthesis step depends on a temperature-resistant DNA polymerase and a pair of DNA primers that hybridize to opposite strands of a DNA molecule and have their 3′-hydroxyl ends oriented toward each other. The distance between a pair of PCR primers can be from 100 bp to more than 25 kb. The denaturation temperature is 95°C, which breaks the hydrogen bonds of duplex DNA but not the internucleotide phosphodiester bonds. During the renaturation step, the primer sequences, which are present in excess, hybridize to the DNA molecules of the sample in the first cycle and to complementary primer sequences of synthesized DNA molecules in subsequent cycles. During the DNA synthesis step, a new strand grows from the 3′-hydroxyl group of each primer sequence. By the thirtieth cycle, the amplified DNA molecules have one of the primer sequences at one end and the complementary sequence to the second primer at the other end.

Somatic rodent and human tissue culture cells are fused to create a hybrid cell. During the growth of a hybrid cell, the human chromosomes are progressively lost until one or a few are maintained stably while all of the rodent chromosomes are retained. A hybridization test between a labeled DNA insert and DNA from a monochromosomal somatic hybrid cell line for each human chromosome establishes the chromosome location of the insert. A somatic hybrid cell panel, consisting of cell lines that carry discrete portions of a particular chromosome, can be used to pinpoint a DNA insert to a specific chromosome region.

In addition to genomic DNA libraries, chromosome-specific and chromosome region-specific DNA libraries are useful resources for isolating genes. Individual chromosomes, isolated by flow-sorting cytometry from human cell lines or monochromosomal somatic hybrid cells, are the source of DNA for creating chromosome-specific DNA libraries. The DNA obtained from a particular chromosome region, either by microdissection of metaphase chromosomes with glass needles or by laser beam destruction of all of the chromosomal material of a metaphase spread except for a defined chromosome region, can be amplified with linker-adaptor PCR and cloned into a vector to form a chromosome region-specific library.

The cloning of a complete duplex DNA copy (cDNA) synthesized from an mRNA molecule is a useful way to perpetuate all the exons of a gene. The first strand of a cDNA molecule is synthesized by reverse transcriptase, which uses the RNA sequence as a template. The second DNA strand is produced by DNA polymerase I, using the first DNA strand as the template. Strategies have been devised to ensure that only full-length cDNA molecues are cloned. Generally, cDNA libraries are formed with purified mRNA preparations from specific cell types, tissues, or organs.

References

Alphey, L. 1997. *DNA Sequencing: from Experimental Methods to Bioinformatics*. BIOS Scientific Publishers Ltd., New York.

Azofeifa, J. et al. 2000. An optimized probe set for the detection of small interchromosomal aberrations by use of 24-color FISH. *Am J Hum Genet* **66**:1684–1688.

Best Laboratory Web Protocols. http://www.orbigen.com/commerce/misc/protocols.jsp.

Carninci, P. and Y. Hayashizaki. 1999. High-efficiency full-length cDNA cloning. *Methods Enzymol* **303**:19–44.

Cold Spring Harbor Laboratory. Web Resources Protocols. http://nucleus.cshl.org/CSHLlib/webres/protocols.htm.

Erlich, H. A., D. Gelfand, and J. J. Sninsky. 1991. Recent advances in the polymerase chain reaction. *Science* **252**:1643–1651.

Fan, Y.-S. et al. 2000. Sensitivity of multiple color spectral karyotyping in detecting small interchromosomal rearrangements. *Genet Test* **4**:9–14.

Frengen, E. et al. 1999. A modular, positive selection bacterial artificial chromosome vector with multiple cloning sites. *Genomics* **58**:250–253.

Glick, B. R. and J. J. Pasternak. 2003. *Molecular Biotechnology: Principles and Applications of Recombinant DNA*. 3rd ed. ASM Press, Washington, DC.

Ioannou, P. A. et al. 1994. A new bacteriophage P1-derived vector for the propagation of large DNA fragments. *Nat Genet* **6**:84–89.

Joos, S. et al. 1994. Mapping and chromosome analysis: the potential of fluorescence in situ hybridization. *J Biotech* **35**:135–153.

Kakazu, N. et al. 2003. A new chromosome banding technique, spectral color banding (SCAN), for full characterization of chromosomal abnormalities. *Genes Chromosomes Cancer* **37**:412–416.

Kim, U.-J. et al. 1996. Construction and characterization of a human bacterial artificial chromosome library. *Genomics* **34**:213–218.

Krzywinski, M. et al. 2004. A set of BAC clones spanning the human genome. *Nucleic Acids Res* **32**:3651–3660.

Maierhofer, C. et al. 2002. Multicolor FISH in two and three dimensions for clastogenic analyses. *Mutagenesis* **17**:523–527.

Monaco, A. P. and Z. Larin. 1994. YACs, BACs, PACs and MACs: artificial chromosomes as research tools. *Trends Biotech* **12**:280–286.

Ried, T. 2004. Cytogenetics—in color and digitized. *N Engl J Med* **350**:1597–1600.

Roberts, R. J. et al. 2003. REBASE: restriction enzymes and methyltransferases. *Nucleic Acids Res* **31**:418–420. Website: http://rebase.neb.com/rebase/rebase.html.

Saiki, R. K. et al. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**:487–491.

Sambrook, J. and D. Russell. 2001. *Molecular Cloning: a Laboratory Manual*. 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**:5463–5467.

Schröck, E. and H. Padilla-Nash. 2000. Spectral karyotyping and multicolor fluorescence in situ hybridization reveal new tumor-specific chromosomal aberrations. *Semin Hematol* **37**:334–347.

Shepherd, N. S. et al. 1994. Preparation and screening of an arrayed human genomic library generated with the P1 cloning system. *Proc Natl Acad Sci USA* **91**:2629–2633.

Shizuya, H. et al. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc Natl Acad Sci USA* **89**:8794–8797.

Sun, T.-Q., D. A. Fenstermacher, and J.-M. H. Vos. 1994. Human artificial episomal chromosomes for cloning large DNA fragments in human cells. *Nat Genet* **8**:33–41.

Van Dilla, M. A. and L. L. Deaven. 1990. Construction of gene libraries for each human chromosome. *Cytometry* **11**:208–218.

Veldman, T. et al. 1997. Hidden chromosome abnormalities in haematological malignancies detected by multicolour spectral karyotyping. *Nat Genet* **15**:406–410.

review questions

1. Why are restriction endonucleases, T4 DNA ligase, and alkaline phosphatase important for DNA cloning?
2. Outline, with specific examples, the major characteristics of a well-designed cloning vector.
3. Describe how the pUC19 system distinguishes among cells that are not transformed, carry intact pUC19 without an insert, and have pUC19-DNA insert constructs.
4. Construct a restriction enzyme map based on the fragment sizes (bp) that were determined by agarose gel electrophoresis after treatment with the restriction endonucleases *Kpn*I and *Pst*I separately and together:

Fragment size (bp)		
***Kpn*I**	***Pst*I**	***Kpn*I and *Pst*I**
7000	8500	6500
5000	4800	4200
2000	1300	2000
1000	400	800
		600
		500
		400

5. How is *E. coli* transformed with plasmids carrying large DNA inserts?

continued

6. What is a partial restriction endonuclease digest? Why is it used to form a genomic library? What is a genomic library?
7. Why are large-DNA insert cloning systems important for studies of human genetic disorders?
8. Describe the main features of the dideoxynucleotide method of DNA sequencing.
9. Compare "4-color, 1-lane" and "1-color, 4-lane" DNA sequencing.
10. What is a DNA primer? Enumerate the various procedures that require a DNA primer(s).
11. What is PCR? How does it work?
12. What are the chromosome locations of probes A to D based on the hybridization results (+, hybridization; –, no hybridization) with the somatic cell hybrid mapping panel in Figure 5.25?

	1	2	3	4	5	6	7	8
Probe A	+	+	+	+	+	–	+	+
Probe B	+	+	+	–	–	–	+	+
Probe C	+	–	–	+	+	+	–	–
Probe D	–	–	–	–	–	–	+	–

13. In the absence of somatic cell hybrid panels, how is a cloned piece of DNA assigned to a specific chromosome region?
14. What are "genome equivalents"?
15. Describe chromosome flow-sorting cytometry.
16. How are chromosome region-specific (band-specific) libraries assembled?
17. What is one of the uses of cloned chromosome region-specific DNA?
18. What is SKY?
19. What is a cDNA library ?
20. How is DNA biotinylated? What are some of the uses of biotinylated DNA?

chapter 6

Genetic and Physical Mapping of the Human Genome

The best way to reach any destination is with a good map.

ANONYMOUS

Maps are . . . an invention of such vast use to mankind, that there is scarce anything for which the World is more indebted to the studies of ingenious men, than this describing of maps.

WILLIAM ALINGHAM (written 1703)

A MAP IS A GRAPHIC REPRESENTATION that provides information about the location of sites and the spacing between them. Both genetic and physical maps can be constructed for human chromosomes. A genetic map (linkage map, meiotic map) shows the order of sites derived from meiotic recombination frequencies. The distance between sites is measured in centimorgans (cM). The formation of extensive human linkage maps requires both alleles that occur frequently at many different loci on every chromosome and the ability to identify readily the presence of a specific allele. On the other hand, a physical map of a chromosome is a set of ordered DNA clones that cover the complete chromosome. In practice, these clones overlap each other to form a contiguous array, which is called a contig. Various laboratory procedures assist in assembling physical maps. A physical map provides a valuable resource for generating the ultimate physical map: the complete DNA sequence of a chromosome. A genetic map is essential for determining the chromosome locations of disease-carrying genes, and a physical map is required for isolating them.

Genetic Mapping of Human Chromosomes

Genetic Polymorphism

Discovery of linkage between the ABO blood group locus (*ABO*) and nail-patella syndrome (*NPS*) loci was possible for two reasons. First, the presence of each major *ABO* allele can be determined accurately with a simple laboratory test. As a result, the genotypes of all relevant parents and offspring are

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known. Second, each *ABO* allele occurs with a high frequency in the population at large. In Great Britain, where the *ABO-NPS* linkage study was initiated, the frequencies of *ABO*O*, *ABO*A*, and *ABO*B* alleles are about 0.66, 0.28, and 0.06, respectively. High frequencies of two or more alleles at one locus increases the likelihood that parents will have different genotypes, making a linkage analysis feasible.

The term "frequency of an allele" denotes the proportion of a particular allele to the total number of alleles of a locus in a population. For example, for a locus with two alleles (*A1*, *A2*) in a population of 13,000 people, with 3800 individuals who are *A1A1*, 6400 who are *A1A2*, and 2800 who are *A2A2*, the frequency of allele *A1* is 0.54, that is, $\frac{(2\times 3800)+6400}{2\times 13,000}$. The frequency of allele *A2* is 0.46, that is, $\frac{(2\times 2800)+6400}{2\times 13,000}$ or $1.00 - 0.54 = 0.46$. (More information about alleles in populations is presented in Chapter 9 on Human Population Genetics.) For most loci, one allele is considerably more frequent, for example, 0.999, than the other allele(s) that have a frequency of 0.001 or less. In a large population, if the frequency of one allele at a locus is 0.999 and the other is 0.001, then the vast majority (99.8%) of the individuals would be homozygous for the more frequent allele, about 0.198% would be heterozygous, and only 0.001% would be homozygous for the less frequent allele. Under these circumstances, it is virtually impossible to observe either segregation of the alleles of this locus or linkage with another locus, because most matings would be between homozygotes for the more frequent allele. If, on the other hand, the frequencies of two alleles at a locus were 0.99 and 0.01, then about 2% of the population would be heterozygous. In contrast to the previous example, the chance of observing segregation or linkage is increased, because there are more heterozygotes at this locus in the population. And, if two alleles were equally frequent, that is, with frequencies of 0.5, then half the population would be heterozygotes at this locus. In other words, human genetic analysis depends on loci with frequently occurring alleles (Table 6.1).

When two or more alleles of a locus occur with a frequency of 0.01 or greater in a population, a genetic polymorphism exists, and the locus is said to be

Table 6.1 Frequencies of alleles and genotypes in a large, random-mating population.

Allelic frequency		Genotypic frequency		
A1	*A2*	*A1A1*	*A1A2*	*A2A2*
1.0	0	1.0	0	0
0.999	0.001	0.998001	0.001998	0.000001
0.99	0.01	0.9801	0.0198	0.0001
0.90	0.10	0.81	0.18	0.01
0.75	0.25	0.5625	0.3750	0.0625
0.50	0.50	0.25	0.50	0.25
0.25	0.75	0.0625	0.3750	0.5625
0.10	0.90	0.01	0.18	0.81
0.01	0.99	0.0001	0.0198	0.9801
0.001	0.999	0.000001	0.001998	0.998001
0	1.0	0	0	1.0

The shaded area includes the range of genetic polymorphisms for loci with two alleles.

polymorphic (Table 6.1). Genetic mapping can be conducted only with large numbers of readily identified polymorphic loci. Because genetic polymorphisms similar to the alleles of the *ABO* locus are rare, extensive mapping could not be carried out until strategies were devised to detect many polymorphic sites scattered throughout the genome.

Restriction Fragment Length Polymorphism

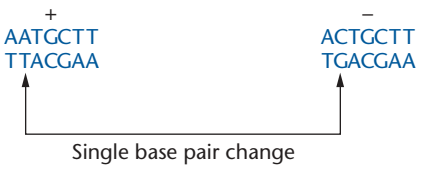
At the DNA level, a single base difference between two homologous genes is sufficient to create alleles. In many instances, a single base pair change can cause a gene product to differ drastically from the normal product. There also should be a large number of single base pair differences within genes that have no effect on gene products, and others that occur, without any biological consequences, in segments of the DNA that do not code for proteins. Such innocuous base pair differences should be dispersed throughout every chromosome and represent a reservoir of potential polymorphic sites (marker loci, genetic markers) that could be used for genetic mapping. The problem, then, is how to detect these polymorphic sites.

In 1980, D. Botstein (b. 1942), R. L. White (b. 1943), M. H. Skolnick (b. 1946), and R. W. Davis (b. 1941) developed the theoretical framework for identifying polymorphic single base pairs and using them as marker loci to map human chromosomes. Briefly, the rationale for this methodology is as follows: Restriction endonucleases cleave DNA at specific recognition sites. When a single base pair change occurs within a recognition site, the sequence is no longer cut by the restriction endonuclease, whereas an intact site on another chromosome can be cleaved (Figure 6.1A). In other words, one allelic form has a functional recognition site for a particular restriction endonuclease and the other does not. As a consequence of this allelic difference, DNA fragments of different sizes are produced when the DNA is digested with a specific restriction endonuclease. And DNA hybridization with a single-copy DNA probe can be used to determine DNA fragment lengths and, thereby, reveal the presence or absence of the polymorphic restriction endonuclease site.

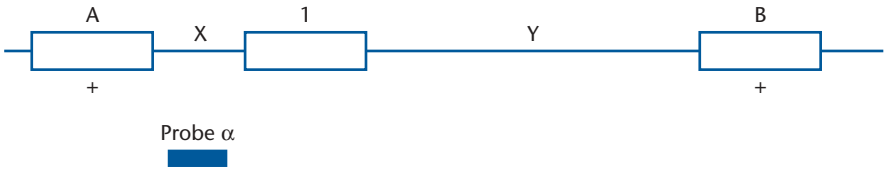
As an example, assume that a portion of a chromosome has three restriction endonuclease sites recognized by the restriction endonuclease *Hind*III (Figure 6.1B). In addition, assume that in all individuals this chromosome has a functional *Hind*III recognition site at both sites A and B; that is, within the population there are no other alleles for these sites. In other words, these sites are not polymorphic. However, for site 1, there is a high frequency of a single base pair change that makes this site resistant to cleavage by *Hind*III. Thus, in a population, two possibilities exist for a single chromosome with respect to this site. It can either be cleaved (+) or not cut (–).

If the distances between the sites are different, with each less than about 20 kb, and if a single-copy DNA probe hybridizes to the DNA between site A and site 1 (Figure 6.1B), then two possibilities can be identified by Southern DNA blotting after separating *Hind*III-treated DNA by agarose gel electrophoresis. If site 1 is cut, then two fragments are produced, and the probe hybridizes to the DNA fragment from site A to site 1. If site 1 is not cut, then the probe hybridizes to the DNA fragment from site A to site B.

A



B



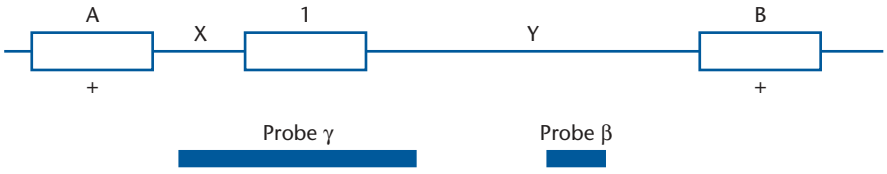
Probe α

Site 1	Fragments produced	Fragments detected
+	X,Y	X
–	(X + Y)	(X + Y)

C

Genotypes	Fragments produced	Fragments detected
+/+	X,Y	X
+/–	X,Y,(X + Y)	X,(X + Y)
–/–	(X + Y)	(X + Y)

D



Probe β

Site 1	Fragments produced	Fragments detected
+	X,Y	Y
–	(X + Y)	(X + Y)

Probe γ

Site 1	Fragments produced	Fragments detected
+	X,Y	X,Y
–	(X + Y)	(X + Y)

Figure 6.1 Use of restriction endonuclease sites as genetic markers. (A) A single base pair change in a restriction endonuclease recognition site prevents cleavage. Functional (presence) and nonfunctional (absence) restriction endonuclease sites are shown by plus (+) and minus (–) signs, respectively. (B) A segment of a single chromosome with three restriction endonuclease sites (A, 1, and B) that are recognized by the same restriction endonuclease. The distance between sites A and 1 is X, between sites 1 and B is Y, and between sites A and B is X + Y. In all DNA samples, sites A and B are functional (both +), whereas site 1 can be either a functional (+) or nonfunctional (–) cleavage site. With a functional site 1 (+), DNA fragments X and Y are produced after treating the DNA with the restriction endonuclease. With a nonfunctional site 1 (–), a single DNA fragment (X + Y) is produced after treating the DNA with the restriction endonuclease. Probe α, after Southern blot analysis, will detect fragment X when site 1 is functional (+) and fragment X + Y when it is nonfunctional (–). (C) For each of the possible genotypes at site 1 (+/+, +/–, and –/–), the fragments that are produced after treating with the restriction endonuclease and those that are detected by probe α after Southern blot analysis are presented. (D) The fragments produced from a single chromosome after treating with the restriction endonuclease and the fragments detected by DNA hybridization with either probe β or probe γ.

The actual analysis of DNA samples from a group of individuals is slightly more complicated, because chromosomes occur as pairs (Figure 6.1C). However, each genotype (+/+, +/–, and –/–) produces a distinctive pattern of fragments after hybridization with the probe. In addition, probes that hybridize to different regions between sites A and B are also effective in distinguishing the presence or absence of the recognition site at site 1 (Figure 6.1D). The pattern of DNA fragments that is the result of the presence and/or absence of a mutated restriction endonuclease site occurring frequently in a population is called a restriction fragment length polymorphism (RFLP). Polymorphic restriction endonuclease sites are marker loci for a chromosome.

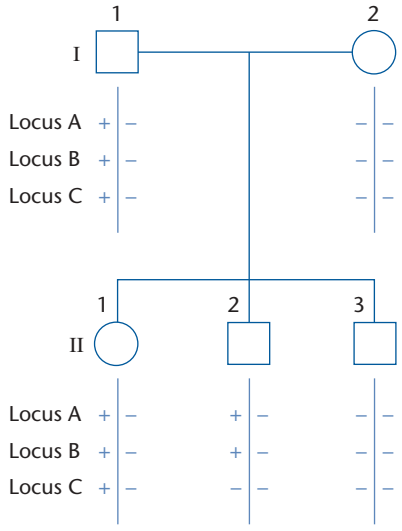
The genetic status of an RFLP locus (or loci) on a single chromosome is called the haplotype. When a single site is examined, there are two possible haplotypes (+ or –); with two different sites on the same chromosome, there are four haplotypes (++, +–, –+, and ––); and with n loci there are 2^n haplotypes. RFLP loci are inherited in a strictly Mendelian manner, and the inheritance of an RFLP locus can be traced within a pedigree. The determination of the alleles of an RFLP locus in an individual is called haplotyping (genotyping, DNA typing). When two or more linked RFLP loci are followed in a pedigree, it is possible to determine the occurrence of a recombination event.

As shown in Figure 6.2, the father (I-1) is heterozygous for three different RFLP loci on the same chromosome and the mother (I-2) is triply homozygous and lacks the restriction endonuclease site of each locus on both chromosomes. The genetic status of the chromosome inherited from the father can be evaluated in each offspring by genotyping. In this case, offspring II-2 received from his father a chromosome that had undergone a crossover event; the other offspring inherited noncrossover chromosomes from their father.

During the 1980s, the identification of thousands of RFLP loci expanded the number of alleles that were used for genetic studies. In practice, cloned single-copy DNA segments were tested with DNA taken from a number of individuals and treated with different restriction endonucleases to determine whether an RFLP was present. In situ hybridization with a DNA probe that recognizes an RFLP locus identified the chromosome location of the locus. A standardized nomenclature was necessary to organize the thousands of RFLP loci. For example, the notation D21S18 represents a locus identified by a DNA probe (D) that hybridizes to chromosome 21 (21), to a single (S)-copy sequence, and was registered as isolate number 18 (18) by the DNA Committee of the International System for Human Linkage Maps (ISLM). Polymorphic marker sites falling within a known gene are often designated by the name of that gene. For example, the marker ADH represents a polymorphic locus within the alcohol dehydrogenase (*ADH1*) gene. The designation of RFLP alleles is not standardized. Some laboratories number the alleles (1, 2, etc.); others use the length (kb) of the fragment created by the presence or absence of a site(s) (8 kb, 12 kb, 4 kb, etc.).

Although RFLPs have provided a useful set of loci for genetic studies, additional studies revealed that these loci are not distributed uniformly throughout every chromosome. Moreover, the maintenance of the probes as clones is not very convenient, and the need to haplotype many individuals from a number of pedigrees by Southern DNA blotting is cumbersome. Fortunately, other

Figure 6.2 Detection of segregation and recombination of restriction fragment length polymorphism loci in a pedigree. The plus (+) and minus (–) signs represent functional and nonfunctional alleles at three restriction fragment length polymorphism loci (A, B, and C) on the same chromosome. The genetic constitution of I-1 is based on his parents' genotypes, which were +++/+++ and –––/––– (not shown). Haplotyping of the offspring indicates that II-2 received a recombinant chromosome from I-1 and a nonrecombinant chromosome from I-2. Offspring II-1 and II-3 have noncrossover chromosomes from both parents. The vertical line separating each set of restriction fragment length polymorphism alleles under the symbol for each member of the pedigree depicts homologous chromosomes.



CACACACACACACACACACACACACACACACACACACACACACACA
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGT

Figure 6.3 A dinucleotide short tandem repeat DNA sequence. This (CA).(GT) sequence has 24 repeating dinucleotides, and its designation is $(CA)_{24}$.

polymorphic loci, consisting of simple repeating units of two, three, or four nucleotide pairs (short tandem repeats, STRs) occur in large numbers (>100,000) throughout the human genome and can be scored readily with the polymerase chain reaction (PCR).

Short Tandem Repeat Polymorphism

The dinucleotide repeat CA/GT [(CA).(GT); see, e.g., Figure 6.3] occurs about 100,000 times throughout the human genome. Overall, these blocks consist of repeating CA/GT units ranging in length from 2 to 40 units, with any one block at a particular chromosome location retaining more or less the same number of repeating units. A convenient notation for describing a CA/GT repeat is (CA)*n*, where *n* represents the number of CA repeats. In addition to CA dinucleotide repeats, other dinucleotide repeats, as well as tri- and tetranucleotide repeats, are also found scattered throughout the human genome. Collectively, these simple multicopy repetitive sequences are called short tandem repeats (STRs, microsatellites).

The first step leading to the identification of polymorphic STR loci entails screening a small-insert (~1000 bp) human genomic library with the appropriate hybridization oligonucleotide probe. For example, a 15-CA unit oligonucleotide is used for isolating cloned $(CA)_n$ repeats. The insert of each positive clone is sequenced to determine the length of the CA/GT repeat and the sequences that flank the tandem repeat. Fluorescence in situ hybridization (FISH), using probes with sequences on either side of the repeated (CA).(GT) block, determine whether these flanking sequences represent single-copy DNA. If the flanking sequences occur only once in the genome, then a pair of primers, directed to amplify the CA repeat and to correspond to the flanking sequences, are synthesized. Next, PCRs are run with DNA samples from a large number of individuals. The PCR products are analyzed with polyacrylamide gel electrophoresis. If the amplified DNA is the same length in all DNA samples for a pair of primers, then that repeat is not polymorphic (Figure 6.4A). Alternatively, if there are different PCR products, then an STR polymorphism is indicated (Figure 6.4B). The different PCR products represent differences in the lengths of the CA repeats at a locus. In other words, these different CA repeats are alleles (Figure 6.5). Allelic differences are quite common among the CA repeats, with frequencies often greater than 0.20 for the less frequent allele. In addition, the number of CA repeats of an allele is a stable characteristic and is, for the most part, maintained from one generation to the next. Generally, the primer pairs for short tandem repeat polymorphisms (STRPs) amplify DNA of 200 bp, which is readily observed after polyacrylamide gel electrophoresis.

The naming convention for STRP loci is the same as for RFLP loci. To date, thousands of STRP loci have been identified and registered. The names of STRP primers are often idiosyncratic. Many STRP loci have been characterized by French researchers working with the financial support of the Association Française contre les Myopathies (Muscular Dystrophy Association of

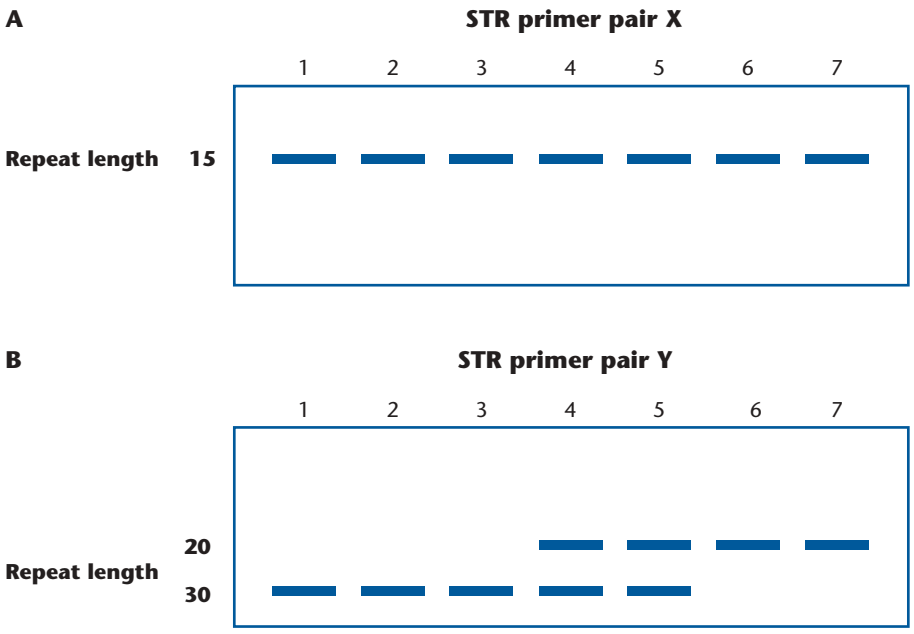


Figure 6.4 DNA typing of short tandem repeat loci. (A) The DNA from seven different individuals is amplified by PCR with a pair of primers (X) that flank a (CA).(GT) repeat. In all cases (lanes 1 to 7), the PCR product (solid rectangles) has the same size after polyacrylamide gel electrophoresis. If this result is observed with DNA from additional individuals, there is only one short tandem repeat allele at this locus. (B) Same as in A, except a different primer pair (Y) for another short tandem repeat locus was tested. The occurrence of two different PCR products (solid rectangles) indicates that there are two short tandem repeat (STR) alleles at this locus. Lanes 1, 2, and 3 contain amplified DNA from individuals who are homozygous for one STR allele; lanes 4 and 5 contain amplified DNA from heterozygotes who carry two different STR alleles; and lanes 6 and 7 contain amplified DNA from individuals who are homozygous for the other STR allele. The amplified fragments are larger than the repeat length. The lengths of the STR repeats are determined initially by DNA sequencing, but all that is required for genotyping is a difference in the size of PCR fragments after gel electrophoresis.

France), and, in recognition, many STRP markers have the prefix AFM. STRP loci have superseded RFLP loci as the choice for human gene mapping studies. In contrast to an RFLP probe, which must be maintained in a vector, purified, and labeled, only the sequence information for a pair of STRP primers, which can be stored in a computer database, is required.

Mapping of a Genetic Disease Locus to a Chromosome Location

Pedigree analysis, except for X-linked inheritance, does not reveal the chromosome location of the locus of a disease gene. However, the availability of RFLP and STRP probes that identify polymorphic loci throughout the genome makes it possible to use the LOD score method to test for linkage between a polymorphic and a disease locus. This approach works best with a genetic disease that is clinically unambiguous; with a large, single multigeneration pedigree or many small families in which the mode of inheritance is unequivocal; and with a reasonable estimate of the extent of penetrance.

In general, a linkage study entails collecting blood cells from members of several two- and three-generation families or from individuals of a large multi-

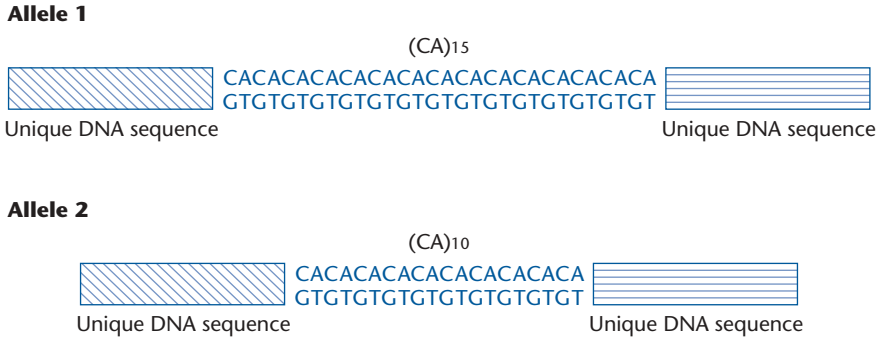


Figure 6.5 An example of two STR alleles. One allele (allele 1) has a $(CA)_{15}$ repeat, and the other (allele 2) has a $(CA)_{10}$ repeat. The DNA segments that flank the repeated DNA sequences of both alleles have the same single-copy (unique) sequences.

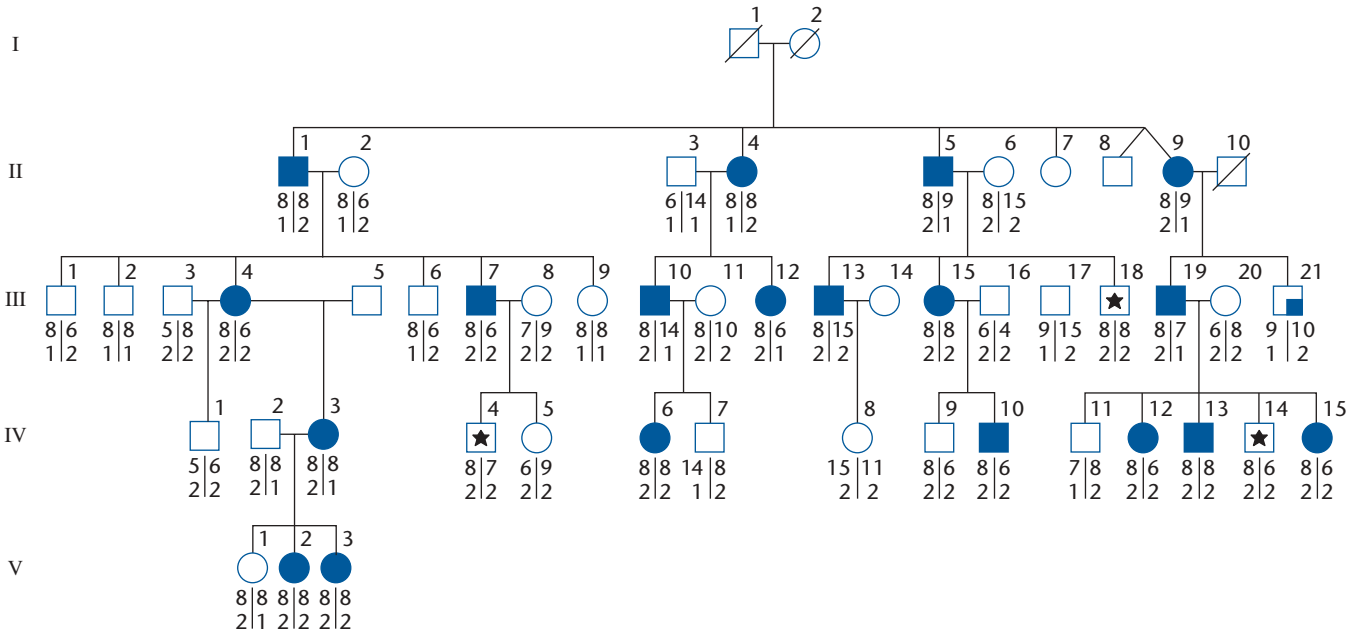


Figure 6.6 Haplotype analysis of two chromosome 20 polymorphic loci in a large kindred with benign familial neonatal convulsions. The haplotypes of the two polymorphic sites are represented by a number in which the allele of one locus is written above the allele of the second locus. The haplotypes of the two chromosomes of an individual are separated by a vertical line. The pedigree symbols with a diagonal slash denote deceased individuals. Solid symbols (■, ●) mark individuals affected by the disease. The symbol (III-21) with a filled-in quadrant represents a case where the individual has a related phenotype but one that differs clinically from other affected members of the family. Individuals II-8 and II-9 are nonidentical (fraternal) twins. The stars mark cases of possible incomplete penetrance (III-18, IV-4, and IV-14). The (8,2) allelic constitution in the affected members of the family cosegregates with the disease, and these individuals carry a chromosome that is a copy of one from a common ancestor. (Pedigree adapted from Leppert M et al. (1989). Benign familial neonatal convulsions linked to genetic markers on chromosome 20. *Nature* **337**:647–648.

generation family with a specific genetic disorder. Today most studies operate under ethical guidelines that require participants to sign official informed consent forms. Once collected, the blood cells often are cultured and become a source of DNA for future procedures, eliminating the need to draw blood repeatedly from the same subjects. The DNA from each individual is genotyped for a number of different polymorphic markers. In some studies, more than 250 polymorphic markers (probes), representing sites from all parts of all autosomes, are used. A two-point (two-locus) LOD score is calculated for each polymorphic locus and the site of the genetic disease from all informative parent-offspring combinations. A Z value of +3.00 or more supports linkage, and one of –2.00 or less excludes linkage.

A large multigeneration pedigree of individuals with benign familial neonatal convulsions (BFNC) was studied to determine the chromosome location of the disease (Figure 6.6). This condition is characterized by episodes of violent and uncontrolled jerking of the face, body, arms, and legs within the first six months of life. In about 90% of cases, these symptoms disappear by the first birthday. The seizures have no apparent effect on subsequent neurological or intellectual functioning. BFNC is a rare disorder, inherited as an autosomal dominant trait with high penetrance, and can be readily defined clinically.

Of all of the polymorphic markers tested, two probes (D20S20 and D20S19) showed linkage with the *BFNC* locus within the pedigree under study (Table 6.2). (The *BFNC* locus is now called *KCNQ2*; however, for the discussion in this section, the original terminology will be used.) The alleles for the D20S20 and D20S19 loci of each genotyped member of the pedigree in Figure 6.6 are shown as stacked numbers, with the vertical line separating the allelic constitution of each chromosome. The upper numbers are for the D20S19 locus and the lower numbers for the D20S20 locus.

Table 6.2 Pairwise LOD scores between a benign familial neonatal convulsion locus and two chromosome 20 polymorphic loci.

	Recombination fraction (θ)					
Locus	**0.00**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
D20S20	3.12	3.05	2.88	2.32	1.57	0.69
D20S19	2.87	2.92	2.83	2.36	1.63	0.76

Adapted from Leppert M et al. (1989). Benign familial neonatal convulsions linked to genetic markers on chromosome 20. *Nature* **337**:647–648.

In this kindred, the D20S19 probe distinguishes among 10 different alleles of an RFLP locus, and the D20S20 probe identifies 2 alleles of its locus. For some RFLP loci, a number of restriction endonuclease recognition sites may be clustered in a small segment (~20 kb), which collectively are considered as part of the same locus. Four closely spaced restriction endonuclease sites could give rise to 16 different fragment lengths identified by a single probe. Similarly, an STRP locus can have more than 2 alleles. In these cases, the lengths of the repeated nucleotide units differ from each other.

The (8,2) haplotype in the BFNC kindred cosegregated with the disease in many instances. It was expected that individuals III-18, IV-4, and IV-14, each of whom received an (8,2) chromosome from an affected parent, would also be affected, because in this family the *BFNC* locus is on this specific chromosome. These exceptions probably represent incomplete penetrance. Although unlikely in this case, this kind of anomaly could be the result of either misdiagnosis or the lack of onset of the disease with studies of other traits.

In some instances in the BFNC family, when an offspring of an affected parent has an (8,2) chromosome but does not have the disease (e.g., IV-7 and IV-11), it is possible to track the origin of the (8,2) chromosome in these cases. For example, individual IV-7 inherited a (14,1) chromosome from the affected parent and did not inherit the (8,2) chromosome with the defective *BFNC* gene. By contrast, the genotypes of individuals IV-9 and V-1 are not readily explained, because they may have inherited an (8,2) chromosome from outside the family that carries the normal *BFNC* gene, or they could represent cases of incomplete penetrance. It should be stressed that, in other families with BFNC, polymorphic alleles other than (8,2) of the D20S19 and D20S20 sites will show linkage with the *BFNC* locus. The linkage of the (8,2) alleles and the dominant form of the *BFNC* gene in this pedigree has no biological significance. The (8,2) alleles have nothing to do with the BFNC phenotype. They merely happened to be on the same chromosome as a *BFNC*D* allele. And the chromosome with these alleles happened to be present in the common ancestor who passed it on to his/her offspring which, eventually, gave rise to this particular pedigree. In principle, loci—not alleles—are linked.

On the basis of the two-point LOD scores, the D20S19 and D20S20 loci are approximately 4 and possibly less than 1 cM, respectively, from the *BFNC* locus. These values are rough approximations, but they place the polymorphic loci from 1 to 4 million base pairs from the *BFNC* locus. Generally, the resolution of a two-point LOD score is 1 to 2 cM for very closely linked loci ($\theta = 0.00$). Because the D20S19 and D20S20 loci map to 20q13.2–13.3, then

the *BFNC* locus must be near or within this chromosome region. Further analyses since the original linkage study placed the *KCNQ2* locus at 20q13.33.

Pairwise linkage analysis with strongly positive LOD scores is not sufficient for determining the order of a set of mutually linked loci. Multipoint linkage analysis, which is discussed later in this chapter, must be performed with a number of polymorphic loci occurring near and around the disease gene.

When the DNA of individuals from kindreds with members affected by an inherited trait are screened with a large number of polymorphic DNA markers, evidence for linkage is usually obtained. In some instances, such a genome screen (genome scan) does not yield a highly positive LOD score. There are a number of possibilities that could account for such an outcome. A trivial explanation might be that the initial set of polymorphic markers did not contain, by chance, a marker identifying a locus linked to the disease gene. Although a researcher has no way of knowing whether this is indeed the case, additional markers, if available, may be tested.

On the other hand, if several closely spaced markers from a chromosome region are used, and no linkage is observed, then the region, with a degree of caution, can be excluded from additional screening. For example, pairwise LOD scores among 20 families with alcoholic individuals and a number of polymorphic DNA markers for chromosome 4q failed to detect any linkage (Table 6.3). In this case, it was concluded that a susceptibility gene for alcoholism was not within a substantial portion of 4q, including 4q13.1 to 4q32.

However, the failure to observe linkage could be due to genetic heterogeneity. When a data set for a linkage study is based on a number of different families, nonallelic mutant genes may be responsible for the same phenotype and may confound the calculated LOD scores. In this context, families from different continents, but with the same apparent phenotype, occasionally do not show linkage to the same polymorphic loci. This result is a good indicator of genetic heterogeneity. Also, linkage results from a single large pedigree do

Table 6.3 Pairwise LOD scores between an alcoholism susceptibility locus and chromosome 4q loci.

	Recombination frequency (θ)						
Locus	**0.00**	**0.001**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**
INP10	–5.56	–5.44	–4.63	–2.75	–1.61	–0.56	–0.16
ADH	–4.01	–3.93	–3.36	–2.00	–1.14	–0.35	–0.06
EGF	–5.16	–5.07	–4.33	–2.58	–1.55	–0.58	–0.18
D4S191	–3.90	–3.82	–3.14	–1.62	–0.83	–0.18	0.00
D4S194	–10.02	–9.37	–6.80	–3.54	–1.88	–0.57	–0.12
D4S175	–6.62	–6.44	–5.27	–2.69	–1.25	–0.11	0.13
D4S192	–5.62	–5.48	–4.52	–2.42	–1.24	–0.25	0.02
MNS	–6.12	–5.95	–4.85	–2.60	–1.36	–0.35	–0.06
MLR	–6.38	–6.12	–4.90	–2.84	–1.70	–0.65	–0.22
FG	–3.78	–3.57	–2.71	–1.34	–0.57	–0.06	0.17
A101	–9.05	–8.75	–7.19	–4.29	–2.57	–0.94	–0.29
D4S243	–6.37	–6.08	–4.68	–2.31	–1.01	–0.01	0.20

The data are derived from 20 different families. Analysis is based on a recessive mode of inheritance. Markers that do not have a D4S designation represent polymorphic loci occurring within known genes. Adapted from Neiswanger K et al. (1995). Exclusion of linkage between alcoholism and the MNS blood group region on chromosome 4q in multiplex families. *Am J Med Genet* **60**:72–79.

not, of course, preclude genetic heterogeneity. For example, a second *BFNC* locus was found at 8q24 in other families.

Errors during genotyping or data entry can lead to false LOD scores. Error checking to ensure that the genotypes of offspring are consistent with the genotypes of the parents has been computerized and is run routinely before a linkage analysis. In some instances, genotyping must be repeated to make sure that the initial results were correct. Notwithstanding these possible difficulties, the availability of DNA markers to identify polymorphic loci has led to the mapping of the chromosome locations of scores of genes. The establishment of linkage with one or more polymorphic loci is the first step leading to the identification, isolation, and characterization of a disease gene.

Multilocus Mapping of Human Chromosomes

The availability of thousands of widely dispersed polymorphic markers has made it possible to determine both the order of loci and the map distances between sites on each chromosome. A linkage map is an important resource for positioning a disease gene. And, after the map position of the disease gene is fixed, the process of isolating and characterizing the gene can be initiated. Human genetic mapping relies on families with living grandparents and very large sibships. The genotypes of the grandparents delineate the genetic phase of each parent. Large sibships also increase the likelihood that a recombination(s) will be observed. A group of 65 families consisting, in most instances, of three generations with an average sibship size of 8.5 has been assembled for genetic mapping projects. This reference panel of families actually consists of lymphoblastoid cell lines for most of the individuals. The cell lines provide a readily available source of DNA that can be used for genotyping polymorphic loci. In this way, the family members are not continually bothered, and there is a standardized source of genotyped information for all mapping projects. The Centre d'Etude du Polymorphisme Humain (CEPH) in Paris, now the Fondation Jean Dausset-CEPH, is the research institution that established the reference panel of families often called the CEPH family panel. An example of one of the CEPH families is shown in Figure 6.7. Fondation Jean Dausset-CEPH and other agencies maintain the CEPH families' cell lines, which are available to investigators around the world.

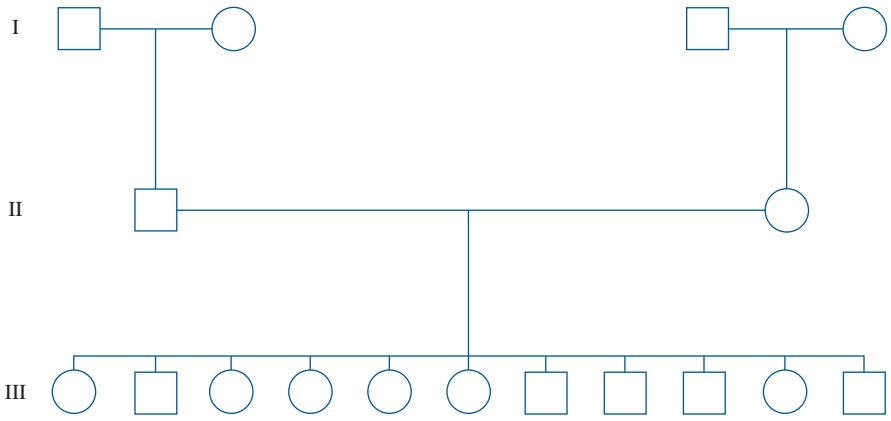


Figure 6.7 An example of the structure of a CEPH family. This pedigree is CEPH family K1331.

The construction of a human chromosome genetic (linkage) map is not a straightforward task. It is virtually impossible to formulate the correct linear order of loci and accurate map distances working solely from pairwise LOD scores and recombination frequencies (θ). The problem is compounded as the number of loci to be mapped increases. For N loci, there are $N!/2$ possible orders. For example, with 10 loci there are 1,814,4000 possible orders. Although some orders are obviously invalid based on a visual inspection of the data, there are a large number of likely orders. Several computer programs expedite the ordering of loci and the determination of map distances when many loci are being examined. Even so, genetic mapping is extremely time consuming and arduous. The main objective is to find the order of loci that is best supported by the recombination data. A common strategy is to determine the most likely order for a few linked loci at a time and then to combine these "best" orders to generate a statistically supported linkage map with all the loci being considered. The criterion for acceptance that one locus is next to another is a log-likelihood (LOD) score of +3.00 or greater.

Map construction is a stepwise process. First, several polymorphic markers for a single chromosome are assembled. Next, DNA samples from a group of CEPH families are genotyped for each polymorphic marker. Because of the structure of the CEPH families, it is not necessary to do DNA typing on the entire panel. The quality of a map is not enhanced enough to justify the extra workload of analyzing additional families. Often 15 and occasionally 40 families are used. Approximately 10,000 assays are required to genotype 40 CEPH families with 20 polymorphic markers. The genotype for each locus for each individual is entered into a database. At this stage, the database is checked for errors. Computer programs look for cases of impossible parent-offspring genotypes that would represent mistakes during data entry or, possibly, genotyping errors. In some instances, genotyping is carried out a second time to verify the original results. Errors lead to incorrect locus ordering and to inflated map distances. If possible, errors are filtered out of the analysis. All pairwise LOD scores and recombination fractions (θ) are determined from the genotyped CEPH families.

Some computer programs use the LOD score values, and others use recombination fractions to construct a genetic map. A computer program for multilocus linkage mapping, nicknamed CRIMAP, is used by a number of laboratories. The strategy is to identify two strongly linked loci in each chromosome arm and to use these loci as a foundation for adding, sequentially, other loci that are very likely to be linked to each other. For example, the pair of foundation loci for 16p and 16q has LOD scores of 29.71 ($\theta = 0.18$) and 83.89 ($\theta = 0.07$), respectively. Additional widely spaced loci are added to the map and ordered relative to the foundation loci, creating a map with loci that are 20 cM apart that can be used as a framework for the introduction of more loci. Loci are retained as part of a map, in most studies, if the possibility of a particular placement is 1000 : 1 or greater. After the incremental building up of a map for each chromosome arm, markers that flank the centromere are introduced into the program to complete the map for the entire chromosome. At this point in the mapping process, the data supporting the final map are examined for closely spaced (≥30 cM) double recombinants. Generally, more than

half of these double recombinants are artifacts and represent genotyping errors. If a detected error cannot be corrected by repeating the DNA typing, then the marker is removed from the map and the database. By convention, the order of loci and map distances for a linkage map are tabulated from the end of the shorter chromosome arm (pter). Because of the difference in recombination frequency between males and females, a genotyped CEPH family database can be used to generate female-specific, male-specific, and sex-average linkage maps (Figure 6.8). The female- and male-specific maps are based on recombination data for females and males, respectively. The sex-average map is formulated without regard to sex. Generally, a female-specific map has a greater distance (cM) than a male-specific map, because of the increased frequency of recombination in females.

Human chromosome linkage maps have been updated as additional polymorphic loci were identified. With more sites, a map becomes more dense, and the distances between loci decrease. By 1994, a set of CEPH families had been genotyped for approximately 6000 polymorphic markers, and the multilocus mapping analysis had placed approximately 1000 loci that were, on average, about 4 cM apart over the entire genome. With the accumulation of STRPs, comprehensive microsatellite-based genetic linkage maps were generated in the following years that made the 1994 map out of date. Generally, the microsatellite maps have an average spacing of 0.5 cM throughout the entire genome (Table 6.4). Genetic maps are based on recombination frequencies derived from meiotic events and therefore represent statistical approximations. Inevitably, errors occur during map construction that have misleading consequences such as mapping a disease-causing gene to the wrong chromosome. For this reason, human genetic maps are reevaluated and misspecifications, out-of-order loci, and false chromosome locations are corrected.

Inserting a Disease Gene into a Linkage Map

Genotyped individuals from pedigrees with a specific trait are the source of data for placing a disease locus within a group of mapped polymorphic loci. The polymorphic marker(s) that produces a very high LOD score with the disease-causing locus and other polymorphic sites that are closely linked to this marker are used to formulate a multilocus linkage map. Several computational algorithms have been devised for this type of analysis. One of the more commonly used programs tests each region on a fixed map as a likely location for the disease-causing locus. For example, a set of three ordered linked loci, that is, 1, 2, and 3, with the recombination values shown in parentheses, can be represented as 1 – (0.05) – 2 – (0.10) – 3. The disease locus (x) could precede locus 1 (x – 1 – 2 – 3), fall between loci 1 and 2 (1 – x – 2 – 3), fall between loci 2 and 3 (1 – 2 – x – 3), or follow locus 3 (1 – 2 – 3 – x). Because, theoretically, the disease locus can be anywhere within a region, the computer program divides each region into equal intervals, usually five in number, which creates four possible positions for gene x. In this case, there is a possible site for the disease locus at each arbitrary boundary within a region. Each of these possible positions has a different genetic distance from the adjacent polymorphic locus.

The location of the disease gene that best agrees with the observed data is determined by using the same principle as a two-point LOD score linkage

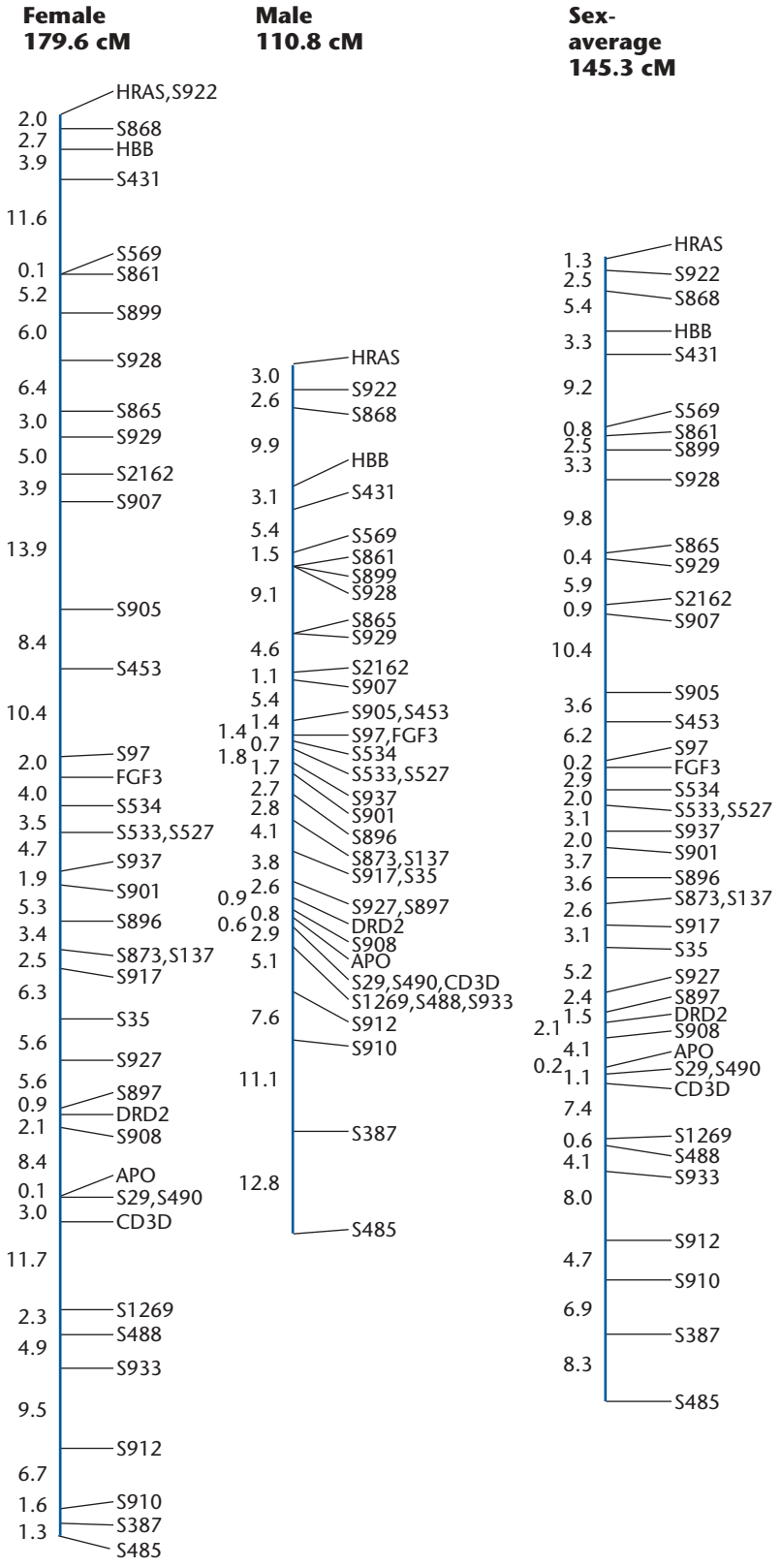


Figure 6.8 Female-specific, male-specific, and sex-average linkage maps of chromosome 11. The D11 prefix has been removed from the numbered loci on the right of each map. The loci designated with letters represent polymorphic loci within known genes. The numbers on the left of each map between sites represent centiMorgans. Adapted from Litt et al. (1995). The CEPH consortium linkage map of human chromosome 11. *Genomics* **27**:101–112.

Table 6.4 Data from comprehensive human genetic map (2004).

Chromosome	Number of markers	Distance spanned (cM)	Average spacing (cM)
1	667	276.40	0.42
2	635	266.79	0.42
3	548	221.83	0.40
4	456	204.74	0.45
5	479	205.69	0.43
6	465	189.60	0.41
7	428	187.09	0.43
8	399	166.08	0.42
9	298	160.01	0.54
10	420	179.12	0.42
11	398	152.45	0.38
12	391	171.96	0.44
13	250	129.52	0.52
14	251	124.81	0.48
15	203	133.61	0.66
16	266	130.00	0.49
17	297	137.99	0.46
18	218	121.65	0.56
19	218	109.73	0.51
20	220	98.63	0.45
21	114	78.48	0.66
22	103	70.60	0.69
X	261	190.67	0.73
Total	7985	3707	0.49

From Nievergelt CM et al. (2004). Large-scale integration of human genetic and physical maps. *Genome Res* **14**:1199–1205. The genetic map was formulated by Kong A et al. (2002). A high-resolution recombination map of the human genome. *Nat Genet* **31**:241–247.

analysis. For a multipoint linkage analysis, gene x, which is on the same chromosome as the linked set of polymorphic loci, can be linked to one of these loci or not linked to any of them. In this case, the multipoint LOD score is the ratio of the logarithm ($\log_{10}$) of the likelihood that a specific order of linked loci, including the disease locus with specified map distances between each locus, to the likelihood that the disease locus is not linked to any of the fixed loci. A LOD score is determined for each possible order with its specific map distances. For example, the likelihood ratio for locus x, positioned midway between loci 1 and 2, is

$$\log_{10} \frac{L(1-(0.025)-x-(0.025)-2-(0.10)-3)}{L(x-(0.5)-1-(0.05)-2-(0.10)-3)}$$

where L is the likelihood for a specific order, and the distance 0.5 in the denominator provides for the case of nonlinkage between locus x and the other loci. With four positions where locus x may be situated in each of the four possible regions, the computer program uses the observed data and determines 16 LOD scores and the map distances between loci.

The disease locus is placed in the region that shows the highest LOD score greater than +3.00. The results from a multilocus linkage analysis can be presented graphically by plotting the LOD score for each position in each region

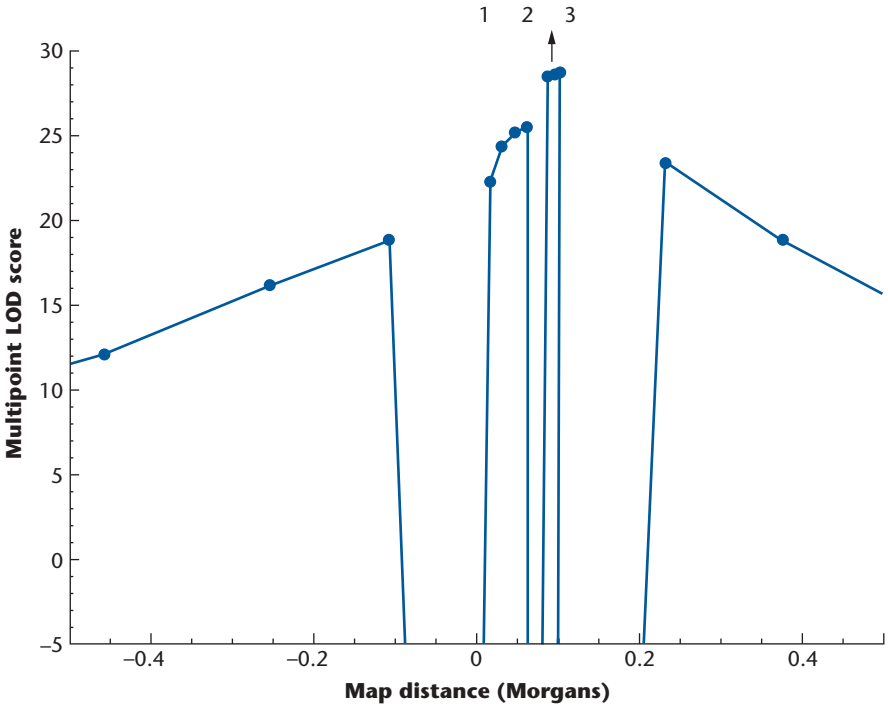


Figure 6.9 Graphic representation of the results of a multipoint LOD score analysis. The numbers 1, 2, and 3 indicate the location and order of three polymorphic loci. The disease-causing locus falls between loci 2 and 3 (arrow). The solid symbols denote the LOD scores at various positions within each possible interval. For convenience of presentation, some low LOD scores (≤–5.00) and those that are both less than the maximum LOD score and occur at the ends of the map have been omitted.

against the calculated map distances (Figure 6.9). The first (leftmost) locus of the ordered set of loci is arbitrarily designated as map position zero (0). In Figure 6.9, the highest LOD score (+28.88) falls within the region between loci 2 and 3, which means that this is the likely location of the disease gene. For convenience, some LOD scores, for example, negative infinity and some lower-than-maximum LOD scores at map distances beyond the conventional limits of the axes, are omitted from a multipoint LOD score graph.

Homozygosity Mapping

Rare recessive traits are difficult to map by conventional two-point linkage analysis with polymorphic markers because the families are small, which makes scoring recombinants difficult. However, by examining the extent of homozygosity of ordered polymorphic loci among the offspring of first-cousin, second-cousin, and other instances of marriages between close relatives (consanguineous marriages, inbreeding), it is possible to deduce the chromosome location of a disease gene. The principle underlying the homozygosity mapping strategy is that the alleles responsible for a homozygous recessive trait in an inbred individual are copies of an allele present in a common recent ancestor (Figure 6.10). With a high probability, the loci flanking the disease site should also be homozygous. The homozygosity that occurs as a result of consanguineous marriages is called either homozygosity-by-descent (HBD) or allelic identity-by-descent (IBD). Conversely, the two chromosomes that carry recessive alleles in a homozygous recessive offspring of a nonconsanguineous marriage are unlikely to share the same set of flanking alleles because each of these chromosomes comes from completely unrelated lineages. In this case, the

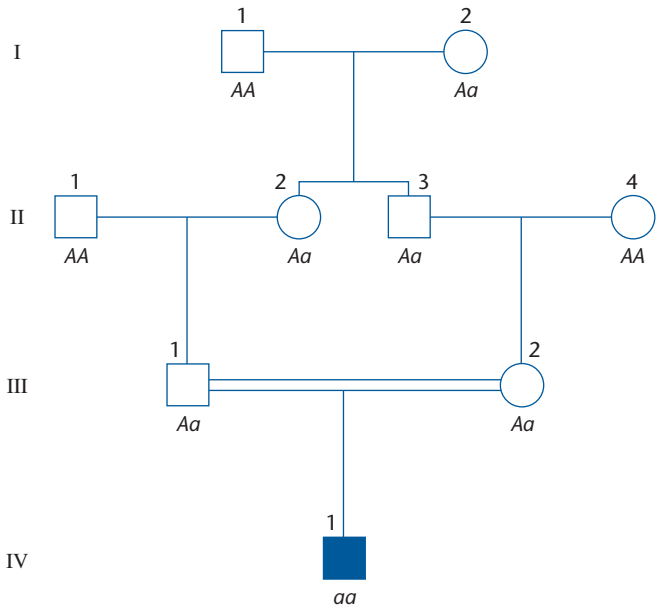


Figure 6.10 Consanguinity and homozygosity. Homozygosity is a consequence of consanguineous marriages. In this example, two first cousins (III-1 and III-2), who each carry a copy of a recessive gene (*a*) that was present in a common ancestor (I-2), have a child (IV-1) who is homozygous recessive (*aa*) for a rare trait (solid symbol).

original mutations could have occurred in different chromosomes with completely different sets of adjacent alleles or, if the mutation arose in a common chromosome, recombination events in previous generations would be sufficient to scramble the allelic content of these chromosomes. Regardless of the origin, the genetic contents of the chromosomes would be dissimilar.

To determine homozygosity-by-descent, a few ($n \leq 10$) consanguineous families with the same homozygous recessive trait are genotyped with a large set of polymorphic markers that cover each chromosome. The occurrence of a cluster of homozygous loci among the affected offspring indicates with considerable likelihood that the disease gene is within this region (Figure 6.11). And, based on the minimum extent of homozygosity shared among all the affected individuals, the region containing the disease gene can be delimited. A decrease in the length of the region with homozygosity in different families is the result of crossover events in previous generations. Two-point linkage analysis can be carried out to determine which locus (loci) of the set showing homozygosity is closely linked to the disease locus. And, if feasible, multipoint linkage analysis can be used to pinpoint the location of the disease locus.

Figure 6.11 Homozygosity mapping. Contiguous homozygous loci in 9 affected offspring (α1–ε2) from 5 consanguineous families (α–ε) for 10 ordered markers (A–J) are shown as double-numbered columns. The sets of numbers represent pairs of alleles. The dashes represent heterozygous loci. The minimum region of common homozygosity among the affected offspring is boxed. In this example, the disease-causing locus falls between loci C and G.

		Affected offspring								
		α1 α2	β1	β2	δ2	γ1	γ2	ε1	ε2
Markers	A	33 33	33	33	–	–	–	–	–
	B	11 11	11	11	–	–	–	–	–
	C	22 22	11	11	22	–	–	11	11
	D	11 11	22	22	11	11	11	22	22
	E	11 11	22	22	22	22	22	33	33
	F	33 33	33	33	44	33	33	22	22
	G	11 11	–	–	11	11	11	–	–
	H	22 22	–	–	–	22	22	–	–
	I	33 33	–	–	–	–	–	–	–
	J	22 22	–	–	–	–	–	–	–

Linkage Disequilibrium Mapping

Linkage disequilibrium (LD) is defined as the nonrandom association between two alleles at two different loci on the same chromosome. When a gene mutation first occurs, it forms part of a unique haplotype (Figure 6.12). However, this particular linkage relationship dissipates in successive generations, as recombination events scramble the alleles of the loci surrounding the disease gene. After many generations, the disease gene and only those very closely linked loci from the original chromosome remain together. These close linkages represent examples of linkage disequilibrium, and, in these instances, the

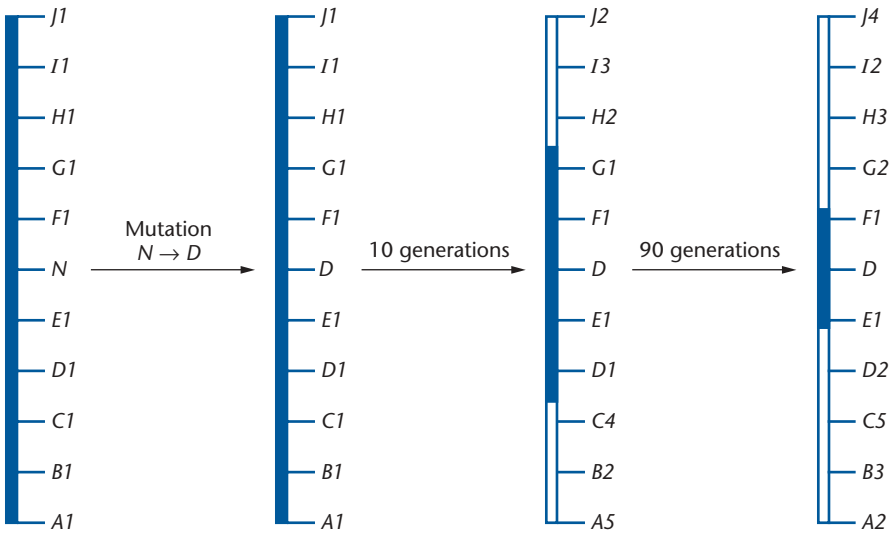


Figure 6.12 Linkage disequilibrium. The breakdown of linkage disequilibrium by recombination events over the span of 100 successive generations is depicted schematically. A normal allele (*N*) mutates to a disease-causing allele (*D*). Over many generations, recombination replaces the alleles of the original chromosome (solid segment) with other alleles. Eventually, only the original alleles closest to the disease-causing gene are retained.

distance between loci is often less than 1 to 2 cM, which is the lower limit that can be resolved by conventional genetic linkage studies.

Linkage disequilibrium is an extremely useful phenomenon for mapping genes. First, detection of linkage disequilibrium between linked loci produces a finer-scale map than can be constructed by genetic linkage. Second, the sites that show linkage disequilibrium are often within 0.10 cM or 100 kb of a disease gene, in comparison to 1 cM or 10^3 kb for sites that are genetically mapped. The closer the distance between a disease gene and a marker locus, the easier it is to discover and isolate the disease gene.

The best source of subjects for linkage disequilibrium mapping comes from affected families from a geographically isolated population. In these instances, most of the individuals of the current population under study can trace their ancestry back approximately 250 or more years (≥20 generations) to one or a few individuals who founded the population. The prevalence of a genetic condition(s) is the result of an allele for a disease gene from a common ancestor. This allele is passed on through the generations and accumulates so that it occurs with a high frequency in the present-day population (founder population effect). As a consequence, both spouses in many of the marriages within a founder population are heterozygous for a recessive disease gene, which, in turn, means they will have a 25% chance of having an affected offspring. Although uncommon, a dominant allele for a nonlethal condition also can accumulate in a founder population. Regardless of the genetic basis of a disorder, the high incidence of affected offspring in a founder population is not the result of inbreeding among individuals of recent generations.

Linkage disequilibrium mapping is carried out after genetic linkage between a polymorphic locus and the disease gene is determined. Then, members of families with a genetic disease within a founder population are haplotyped with a number of additional polymorphic markers on the same chromosome, and statistical tests are run to determine which loci are in linkage disequilibrium with the disease gene. The genetic distance (θ) between a marker locus and the

disease gene that are in linkage disequilibrium is calculated from the following equation:

$$P_{excess} = \frac{P_{affected} - P_{normal}}{(1 - P_{normal})} = (1 - \mu g q^{-1})(1 - \theta)^{g}$$

where θ is the recombination fraction between the marker and disease loci; μ is the mutation rate for the disease gene; g is the number of generations since the common ancestor; q is the worldwide frequency of the disease allele; P_{normal} is the proportion of the marker allele in normal ("nondisease") chromosomes; $P_{affected}$ is the proportion of the marker allele in chromosomes with the disease gene; and P_{excess}, which is a measure of disequilibrium, is the fraction of the excess occurrence of a chromosome with the disease gene and a marker allele in comparison to the chromosome with the nondisease gene and the marker allele. When the value of μ for a specific disease gene is not known, the typical rate for a human gene mutation (1×10^{-6}) is used. For many rare recessive genetic disorders, q is 0.001. A value for the number of generations (g), which is a difficult parameter to determine accurately, is deduced with the help of genealogical records and historical archives.

In one linkage disequilibrium study of Finnish families with an autosomal recessive disorder producing short stature and abnormalities of the joints (diastrophic dysplasia, DTD), the distance between the disease gene and the closest allele was ~0.065 cM or ~65 kb. This distance was confirmed when a single cloned piece of DNA contained both sites that were 60 kb apart. Despite this congruence, in most cases the genetic distances generated by linkage disequilibrium mapping may be underestimates, because some of the assumptions used for the calculation of θ may not be accurate. As a rule of thumb, linkage disequilibrium-based θ values represent approximations. This apparent shortcoming does not materially undermine the use of linkage disequilibrium mapping, because loci are identified closer to a disease gene than can be obtained by genetic linkage mapping. In addition, a multipoint linkage disequilibrium analysis based on LOD scores can be used to fix the location of the disease gene among the marker loci.

The number of genetic diseases that can be mapped by linkage disequilibrium analysis is limited to the conditions that occur in founder populations. To date, isolated populations in Finland, Japan, the United States, Italy, Canada, Costa Rica, the Middle East, and other parts of the world meet the criteria for linkage disequilibrium mapping. More than 50 different genetic diseases have been found in these populations. At a minimum, linkage disequilibrium mapping has facilitated the isolation of these genes. In addition, linkage disequilibrium can be used to construct detailed region-specific chromosome maps on a finer scale than can be generated by genetic linkage mapping.

Radiation Hybrid Mapping

Radiation hybrid (RH) mapping does not require the assembly of pedigrees or family member genotyping. Instead, it depends on somatic cell techniques and on using PCR-based probes to screen a number of cell lines that carry various

pieces (fragments) of human DNA. A monochromosomal hybrid or whole human genome cell line can be used for building radiation hybrid maps. The cells of the cell line (donor cells) are treated with a dose of ionizing radiation from X or gamma (γ) rays that will kill the cells. Both X and γ rays bombard living matter with particles that induce ion formation. The ions have damaging effects, such as disrupting membranes, inactivating enzymes, and fragmenting chromosomes. The standard unit of biologically absorbed energy from ionizing radiation is called a rad (radiation absorbed dose), which is equivalent to 0.01 joule of energy per kilogram of tissue (100 ergs per gram of tissue). Generally, a dose of 3000 rads is lethal to cells growing in culture. The larger the dose in rads, the greater the damage and the smaller the DNA fragments produced.

The most crucial aspects of the RH mapping protocol are the rescue and perpetuation of radiation-induced human DNA fragments. To accomplish these ends, the irradiated cells are fused with nonirradiated rodent cells (recipient cells). Irradiated cells that fuse to each other and those that do not fuse will die from radiation damage when cultured further. Recipient cells that fuse to each other or remain unfused do not have a selectable marker that is present in the irradiated cell line and will not grow in fusion culture medium. Consequently, only donor-recipient fused cells carrying the selectable marker proliferate. After the fusion of irradiated cells with nonirradiated rodent cells, the human DNA fragments are incorporated into rodent chromosomes. The surviving fused cells are cultured en masse before single cell lines are established. These single cell lines are called radiation hybrids. Many radiation hybrids carry one or more human DNA fragments as part of the host-cell chromosomes. These chromosomes function properly during the cell division cycle and are stably maintained. A panel of 100 or more radiation hybrids is required for effective RH mapping.

Radiation hybrid mapping entails extracting DNA from a subcultured sample of each radiation hybrid of a panel and screening this DNA with a number of chromosome- or region-specific PCR-based probes. Because of their availability, many of these probes recognize polymorphic sites, but this is not required for RH mapping. Thus, PCR primers that amplify unique DNA sequences that are monomorphic (not polymorphic) can also be used to screen a radiation hybrid panel. Monomorphic PCR-identifiable chromosome-specific sites are called sequence-tagged sites (STSs). The presence or absence of a PCR-identified site of each probe for each member of a radiation panel is recorded (Table 6.5). Any radiation hybrid that does not show amplification of a sequence for any of the probes is removed from the panel.

The theoretical basis of RH mapping closely resembles the rationale for meiotic mapping. The closer two sites are to one another on a chromosome, the more likely they will be retained on a radiation-induced DNA fragment. With meiotic mapping, the closer two sites are to one another on a chromosome, the more likely they will not be separated by recombination. The data used for RH mapping are based on the pattern of the presence (+) or absence (–) of each marker (retention pattern, signature) for each member of the RH panel. Methods have been devised to determine both the order of sites and the distance between them with a retention pattern.

Table 6.5 Retention data for radiation hybrid mapping.

Radiation hybrid panel	Markers						
	A	B	C	D	E	F	G
1	+	−	−	+	−	−	+
2	+	+	−	−	+	+	−
3	−	−	+	+	−	+	−
4	+	−	+	+	+	−	−
5	−	−	+	−	−	−	−
6	+	+	−	−	−	+	−
7	−	−	+	+	−	−	+
8	−	+	−	−	−	−	−
9	+	+	−	+	+	−	+
10	−	−	−	−	−	+	−

The numbers and letters represent radiation hybrids and PCR-based markers, respectively. The plus (+) and minus (−) signs indicate the presence or absence of a marker site in a radiation hybrid.

The analysis takes into consideration whether a radiation hybrid retains a DNA fragment with two sites or whether it carries two sites on different fragments. The basic assumptions of RH mapping are: radiation-induced breakage between two sites is independent of marker retention, and the retention of a fragment with one marker is independent of any other fragment. On the basis of these criteria, the frequency of breakage (θ) is equivalent to

$$\frac{(A^+B^-)+(A^-B^+)}{T(R_A+R_B-2R_AR_B)}$$

where (A^+B^-) is the number of radiation hybrids in the panel that retain marker A but not marker B; (A^-B^+) is the number of radiation hybrids in the panel that retain marker B but not marker A; T is the total number of radiation hybrids (RH clones) in the panel; R_A is the fraction of all radiation hybrids in the panel that have marker A; and R_B is the fraction of all of the radiation hybrids in the panel that retain marker B. The frequency of breakage is represented by theta (θ), which, notwithstanding the possibility of confusion, is the same symbol used to denote recombination frequency. Unlike meiotic recombination, the complete range of θ values for radiation breakage frequency extends from 0 to 1. A value of zero signifies that two marker sites are never separated by a particular dose of radiation, and therefore are extremely closely linked. When θ equals 1, then the markers are always separated by a specific dose of radiation; that is, they are not linked.

The likelihood ($L\theta$) of obtaining the observed retention pattern for a pair of markers is

$$[(1-\theta)R_{AB}+\theta R_AR_B]^{A^+B^+}[\theta R_A(1-R_B)]^{A^+B^-}[\theta(1-R_A)-R_B]^{A^-B^+}$$
$$\times[(1-\theta)(1-R_{AB})+\theta(1-R_A)(1-R_B)]^{A^-B^-}$$

where R_{AB} is the fraction of all radiation hybrids that retain both markers and (A^-B^-) is the observed number of radiation hybrids that lack both markers A and B. The remaining terms are the same as those used in the equation for

determining the frequency of breakage. A LOD score can be calculated from the relationship $\log\left[\frac{L\theta}{L\theta(\theta=1)}\right]$, where $L\theta$ represents the likelihood for a particular retention pattern and $L\theta\,(\theta = 1)$ denotes the likelihood that this is expected if the two markers are always separated by radiation-induced breakage. A LOD score of +3.00 or greater indicates that two markers are linked.

The distance (D) between two markers on an RH map is $-\ln(1 - \theta)$. The units of distance are called centiRays (cR), and 100 cR equals 1 Ray. The dose of radiation used to create a specific RH panel must be noted when D values are cited, because fragment size varies more or less inversely to the amount of irradiation. For example, a distance of 1 cR_{8000} represents a 1% occurrence of radiation-induced breakage between two markers with a dose of 8000 rads.

Methods for multipoint mapping of radiation hybrid data depend on either the pairwise LOD scores or the distance between two marker sites. One commonly used computer package (RHMAP) evaluates the most likely order of sites in two ways. First, an order is generated by calculating the minimum number of breaks that would account for the retention patterns among the radiation hybrids of the panel. Second, a maximum likelihood method determines the best order of marker sites from the retention data. In practice, these very different statistical approaches often produce the same order of marker sites. Overall, RH mapping panels are a convenient way of ordering polymorphic markers, sequence-tagged sequences, and short cDNA fragments (expressed sequence tags, ESTs) within a genome. For example, 33,627 STSs were ordered, at an average spacing of 94 kb, to a 50,000-rad human genome RH mapping panel that has an average fragment size of 800 kb.

Genotyping Single-Nucleotide Polymorphisms

Polymorphic marker systems are necessary for genetic linkage and association (allele sharing) studies, determination of disease genes by linkage disequilibrium, and other applications, such as identifying the loss of alleles from a chromosome(s) in tumor cells. With the emergence of PCR and the discovery of the highly polymorphic nature of dinucleotide repeats, the first-generation polymorphic markers, restriction fragment length polymorphisms (RFLPs), were replaced by short tandem repeat polymorphisms (STRPs). However, the average spacing between STRPs is approximately 5×10^5 bp, which puts a limit on their usefulness. As a result of advances in genome technologies, a third generation of polymorphic markers, single-nucleotide polymorphisms (SNPs), are being developed. An SNP represents a site at which two different nucleotide pairs occur at a frequency of 1% or greater. Preliminary estimates place an SNP every 500 to 1000 bp in the human genome. These sites are stable and widely distributed among all chromosomes, and the frequency of the more prevalent allele is about 70%. A frequency of 30% or greater for the less common allele of an SNP is sufficient for linkage analysis and other studies. Generally, SNPs consist of only two alleles, which in genetic terms means that they are biallelic. From a technical perspective, SNP assay systems are generally straightforward, inexpensive, and adaptable to automation.

Various strategies have been used to discover SNPs. Initially, differences within sequence-tagged sites (STSs) from different individuals revealed candidate SNP sites. More recently, detailed analysis of overlapping DNA sequences derived from the Human Genome Project provided huge numbers of potential SNPs. Additional studies are required to establish the extent of the polymorphism; whether the flanking sequences act as effective primer regions; and whether a site is a reliable DNA marker. Currently, public and private databases contain millions of possible SNP sites. Sets of evenly spaced, good-quality SNPs that can be used for genotyping have been assembled from these resources.

Over two dozen techniques have been devised for detecting a single-nucleotide difference between DNA molecules. Many of these were originally developed as diagnostic tools for determining the presence of known disease-causing gene mutations. Despite the large number of assay systems, many are based on the same underlying principles. In broad terms, the four major types of allele discrimination systems rely on hybridization, primer extension, oligonucleotide ligation, or nuclease cleavage that use various detection platforms such as gel electrophoresis, microarrays, mass spectrometry, and/or fluorescence plate readers (Table 6.6).

Hybridization assays depend on the differential stability of an oligonucleotide that either base pairs entirely with a target sequence that contains a known SNP site or is mismatched only at the SNP site. Mismatched DNA molecules, even at a single base pair, are not as thermostable as those that are fully complementary, that is, matched.

The molecular beacon strategy is one of the approaches that use hybridization to detect SNPs (Figure 6.13). A molecular beacon probe is a specially designed oligonucleotide for a specific SNP site. It contains a nucleotide that is complementary to one of the nucleotides of a SNP site and is flanked by short sequences that are complementary to DNA on either side of the targeted SNP nucleotide. In addition, there are self-complementary sequences on either side of the middle hybridizing sequence that do not bind to chromosome DNA sequences. At normal temperature, this combination of sequences forms a hairpin loop (Figure 6.13A). The features that facilitate detection of a molecular beacon probe are a fluorescent dye at one end of the oligonucleotide and, at the other end, another molecule (quencher) that, because of its close proximity to the fluorescent dye, absorbs the fluorescence emission when the fluorescent dye is activated.

Table 6.6 Assay systems and detection platforms for single-nucleotide polymorphisms.

	Detection platform			
Allele assay system	**Gel electrophoresis**	**Microarray**	**Mass spectrometry**	**Fluorescence plate reader**
Hybridization	+	+	+	+
Primer extension	+	+	+	+
Oligonucleotide ligation	+	+	–	+
Enzymatic cleavage	+	–	+	+

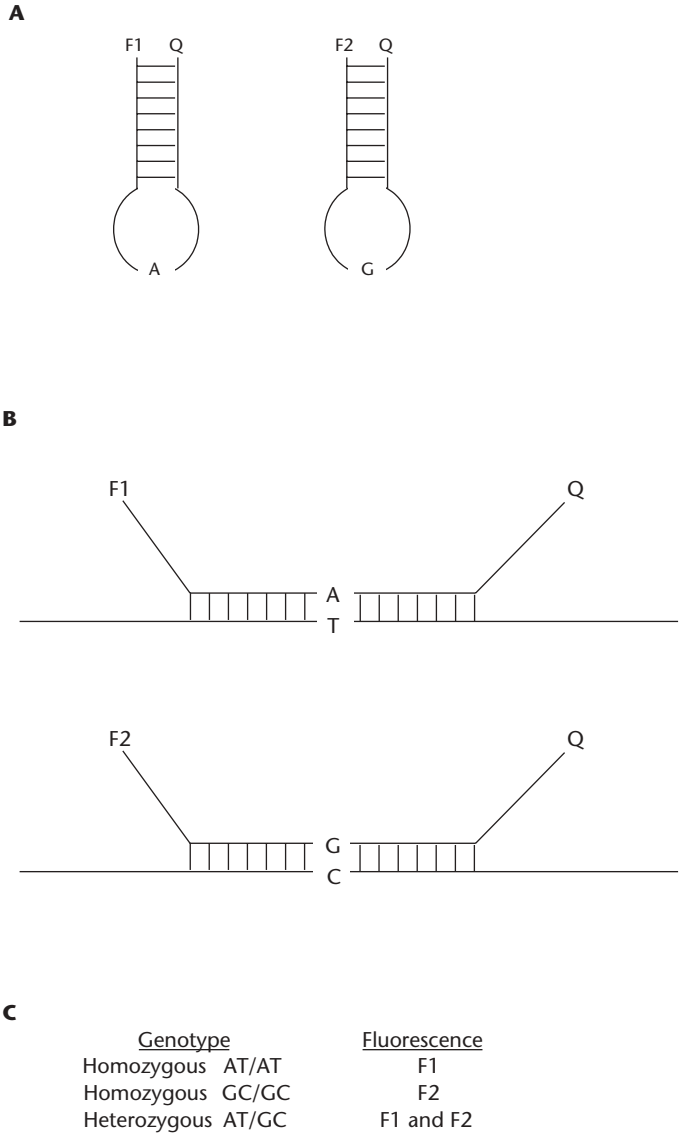
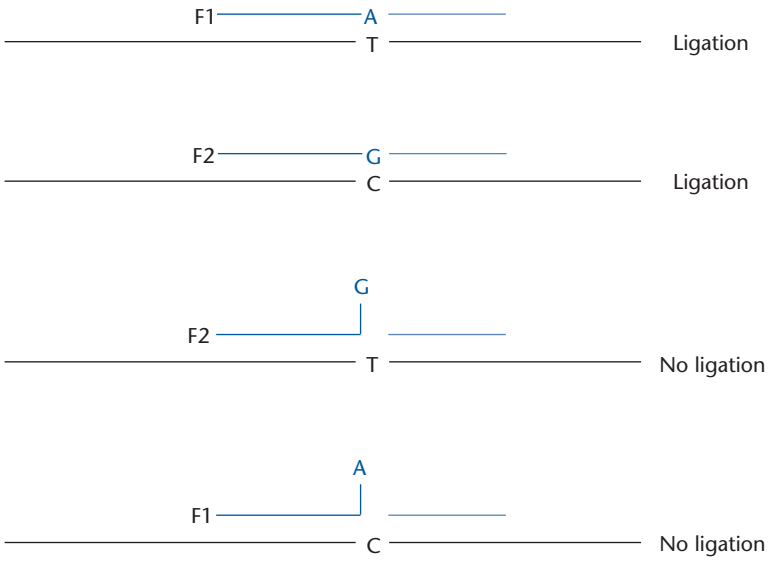


Figure 6.13 Molecular beacon assay. (A) Hairpin loop oligonucleotides (molecular beacons) tagged with fluorescent dyes (F1, F2) and quencher (Q) molecules. The loop of each molecular beacon probe contains a nucleotide (e.g., A or G) that is complementary to a nucleotide of a SNP allele and nucleotides that flank the SNP site. (B) Hybridization of molecular beacon probes to target DNA containing the SNP alleles. When the hairpin configuration is opened up by hybridization, fluorescent emission is not quenched. (C) Fluorescence as a function of genotypes.

In principle, a molecular beam probe is mixed with the target DNA, heated to a high temperature, and then brought to a specified temperature that allows a completely matched probe to remain hybridized and one that is mismatched at the SNP site to be released into the medium, where it forms a hairpin loop. With a hybridized probe, the fluorescent dye and quencher molecules are sufficiently far apart that fluorescence can be recorded, whereas the fluorescent dye of a molecular beacon probe that is in a hairpin configuration is quenched.

Specifically, to determine genotypes, two molecular beacon probes, one for each allele, for example, A and G, and each with a different fluorescent dye, are mixed with the target DNA. After hybridization, wells of a plate reader with DNA from homozygotes for one allele fluoresce one color, homozygotes for the other allele fluoresce the other color, and heterozygotes produce a combined fluorescence (Figure 6.13C).

A



B

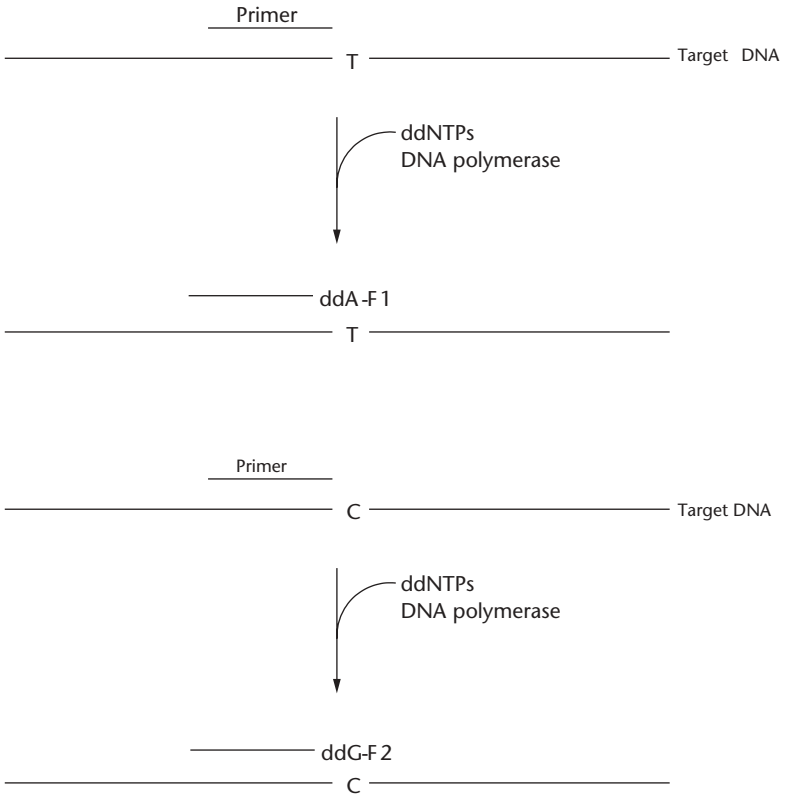
Genotype	Fluorescence of ligated product
Homozygous AT/AT	F1
Homozygous GC/GC	F2
Heterozygous AT/GC	F1 and F2

Figure 6.14 Oligonucleotide ligation assay. (A) Discriminating oligonucleotides (blue) each with a different fluorescent dye (F1, F2) and a nucleotide (A or G) at the 3′ end that is complementary to one of the nucleotides of a binary SNP site. The common oligonucleotide is shown as a wavy line. An upward nucleotide denotes a mismatch. The results of the ligation reactions are noted. (B) Fluorescence as a function of genotypes.

Oligonucleotide ligation assays (OLAs) entail binding amplified target DNA with two oligonucleotides. The upstream (discriminating) oligonucleotide is complementary to the DNA preceding the SNP site, and its 3′ nucleotide is complementary to one of the nucleotides of one of the SNP alleles. The second (common) oligonucleotide is complementary to the sequence downstream from the SNP site, with its 5′ nucleotide being complementary to the nucleotide immediately adjacent to the nucleotide at the SNP site (Figure 6.14). If the discriminating oligonucleotide matches the SNP site, then the two oligonucleotides can be joined by DNA ligase to form an intact DNA strand. If there is a mismatch at the SNP site, then ligation does not occur and the oligonucleotides are not joined.

Analysis of OLAs can be carried out in various ways. For example, two discriminating oligonucleotides, one for one allele and the other for the second allele, are each tagged with a different fluorescent dye (Figure 6.14). After a reaction is run with the two discriminating oligonucleotides and a common oligonucleotide, the ligated product(s) is separated from nonligated oligonucleotides. The two homozygotes can be distinguished because one will produce a fluorescence emission for one dye and the other a fluorescence emission for the second dye. The fluorescence from the DNA of heterozygotes will be a combination of the two dyes.

For primer extension strategies, an oligonucleotide primer is designed to be complementary to the DNA region preceding a SNP site with its 3′ end imme-



B

Genotype	Fluorescence after removal of unincorporated ddNTPs
Homozygous AT/AT	F1
Homozygous GC/GC	F2
Heterozygous AT/GC	F1 and F2

Figure 6.15 Primer extension assay. (A) The primer sequence is complementary to the target DNA with the 3′ end adjacent to the SNP site. DNA polymerase directs the incorporation of a fluorescent dye-tagged (F1 or F2) dideoxynucleotide that is complementary to the nucleotide at the SNP site. (B) Fluorescence as a function of genotypes.

diately adjacent to the SNP nucleotide (Figure 6.15). The primer is added to amplified target DNA with a DNA polymerase and dideoxynucleotides. The nucleotide at the SNP site determines which dideoxynucleotide is added to the primer. The reaction ceases after the addition of a single nucleotide. Determination of the added nucleotide reveals the polymorphic base pair at the SNP site. If DNA is derived from a homozygote, then only one type of nucleotide is added to the primer. The DNA from heterozygotes will have two different nucleotides added to the primer. Genotypes can be ascertained by labeling the two dideoxynucleotides that are expected to be added to the primer with different fluorescent dyes and then analyzing the emission spectra (Figure 6.15B).

Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectroscopy is another detection platform. Briefly, with this apparatus, DNA molecules are ionized with a short laser pulse and separated by mass. A single analysis is completed in seconds. Because each dideoxynucleotide has a

different mass, the extension of a primer can be determined from the difference in mass between unextended and extended primers. For example, if an unextended primer has a mass of 3607.4 and the extended primer is 3904.6, then the difference is 297.2, which corresponds to ddA. Thus, for one type of homozygote, the extended primer will have a particular dideoxynucleotide and the other primer another dideoxynucleotide. Two different extended primers in a sample identifies heterozygotes. MALDI-TOF mass spectroscopy is an efficient and accurate method of genotyping large numbers of individuals rapidly.

The *Taq*Man™ (*Taq*man) assay is one of the nuclear cleavage schemes that is frequently used to identify single-nucleotide differences among DNA molecules (Figure 6.16). Two oligonucleotides are required for the assay. One is

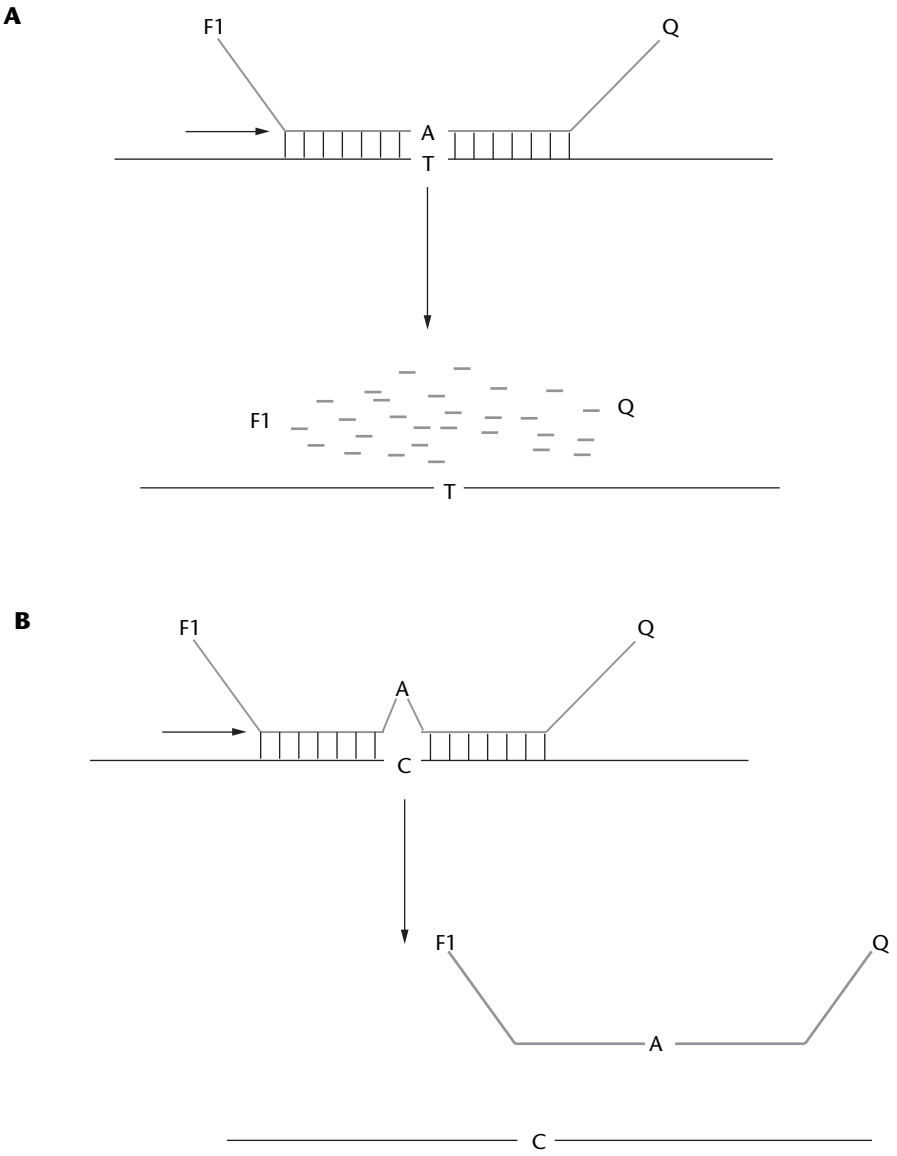
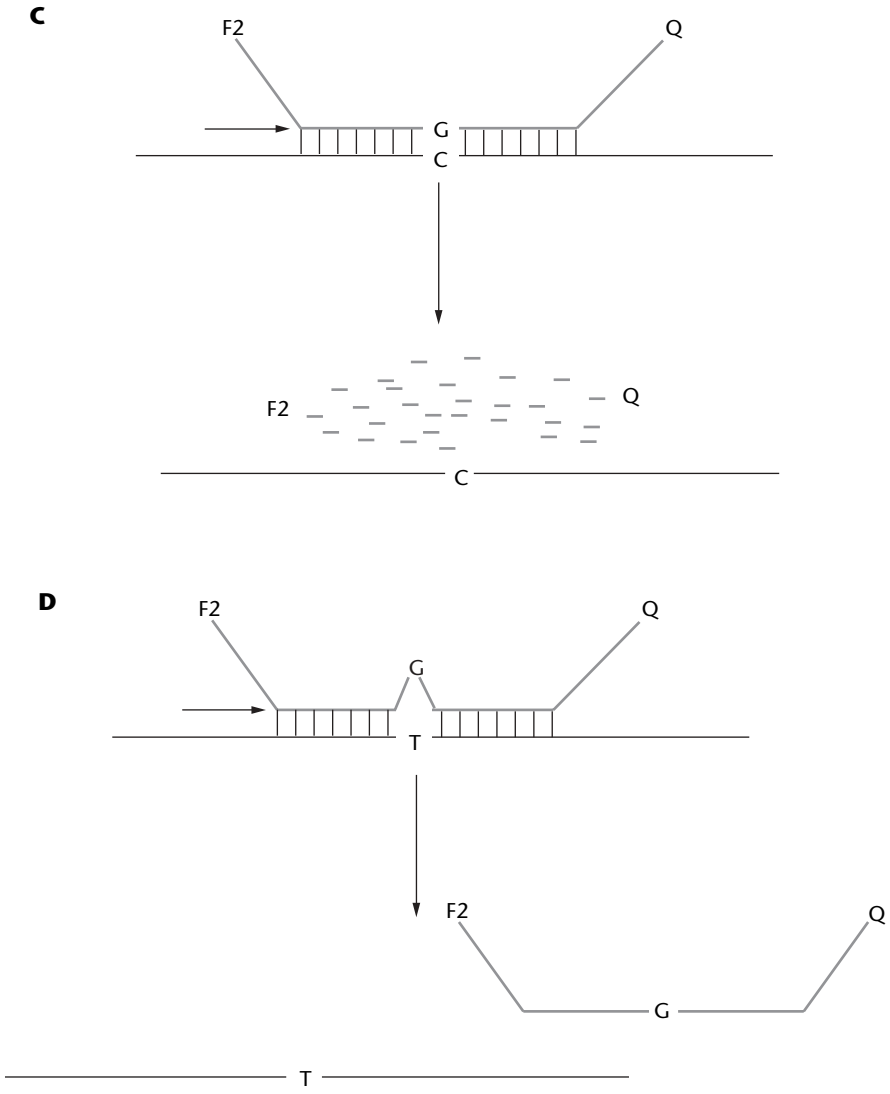


Figure 6.16 *Taq*Man™ assay. (A) Matched SNP-specific probe tagged with a fluorescent dye (F1) at the 5′ end and a quencher (Q) molecule at its 3′ end. The matched probe is degraded by the 5′-exonuclease activity of the DNA polymerase (horizontal arrow), and, as a result, fluorescent emissions are not quenched. (B) Mismatched probe is released intact from the target DNA, and fluorescent emissions are quenched. (C and D) Same as A and B, respectively, except that the probe is designed for the second allele at the SNP site. (E) Status of the probes and fluorescence response as a function of genotypes.



E

Genotype		A-Probe	G-Probe	Fluorescence F1	F2
Homozygous	AT/AT	Degraded	Intact	+	−
Homozygous	GC/GC	Intact	Degraded	−	+
Heterozygous	AT/GC	Degraded	Degraded	+	+

Figure 6.16 *continued*

complementary to the DNA sequence that covers the target SNP site and includes a nucleotide that is complementary to one of the alleles of the SNP site. The second oligonucleotide is the same as the first one except that it contains a nucleotide that is the complementary to a nucleotide of the other allele at the SNP site. A different fluorescent dye is added to the 5′ end of each oligonucleotide and a quencher to each 3′ end. The oligonucleotides are mixed with the DNA sample, and PCR is initiated. During the extension phase of PCR, the 5′ exonuclease activity of the DNA polymerase degrades perfectly

matched oligonucleotides, whereas mismatched oligonucleotides are displaced intact from the target DNA. By the time the PCR is terminated, all the fluorophore from the matching oligonucleotide is separated from its quencher because of oligonucleotide degradation, whereas the fluorescent dye of the mismatched oligonucleotide remains in close proximity to its quencher. Fluorescence from a single dye denotes a homozygous genotype, and the specific emission defines the particular homozygote. When the DNA is from a heterozygote, both oligonucleotides are degraded and, consequently, a combined fluorescence is produced.

No single assay and detection system has emerged as best for genotyping SNPs. The minimal requirements for large-scale SNP genotyping are low cost, speed, and accuracy. As well, the capability of genotyping many sites at the same time (multiplexing) is an important feature. Methods that meet these criteria are being sought. Preliminary SNP-based genetic maps at about 4 cM spacing have shown that the approach is feasible.

Physical Mapping of the Human Genome

Genetic and radiation hybrid maps are linear representations of a set of marker sites. In these cases, the distance between sites is based on either the frequency of recombination (cM) or the probability of coretention of two sites by a radiation hybrid (cR). The relative units of distances can be converted into approximate physical distances in base pairs. By contrast, a physical map records the actual distance (bp) between sites. In addition, a complete chromosome- or region-specific physical map provides direct access to the DNA, which, in turn, facilitates the isolation and characterization of genes and systematic DNA sequencing.

The construction of a physical map depends on retrieving, from a genomic DNA library, clones with overlapping segments. On the basis of the overlaps and other positional information, a continuum of clones can be established for a chromosome region, a whole chromosome, or the entire genome. Sets of contiguously ordered clones (contigs) have been generated from human YAC, BAC, PAC, P1, and cosmid libraries by various procedures. Large-insert cloning systems are preferred for human physical maps because fewer clones are required to cover the entire genome. Initially, in the 1990s, YACs were considered essential for constructing a human physical map. Despite considerable effort and some success, too many YAC clones contained combined DNA from different chromosomes, that is, chimeric DNA. Chimeric clones confound contig formation, and too much additional labor is required to identify them. BAC clones, with about 150-kb inserts, are stable during growth, easy to maintain, and not as chimeric. Consequently, BAC human DNA libraries became the source of cloned DNA for assembling the human physical map.

Assembling Contigs from BAC Libraries

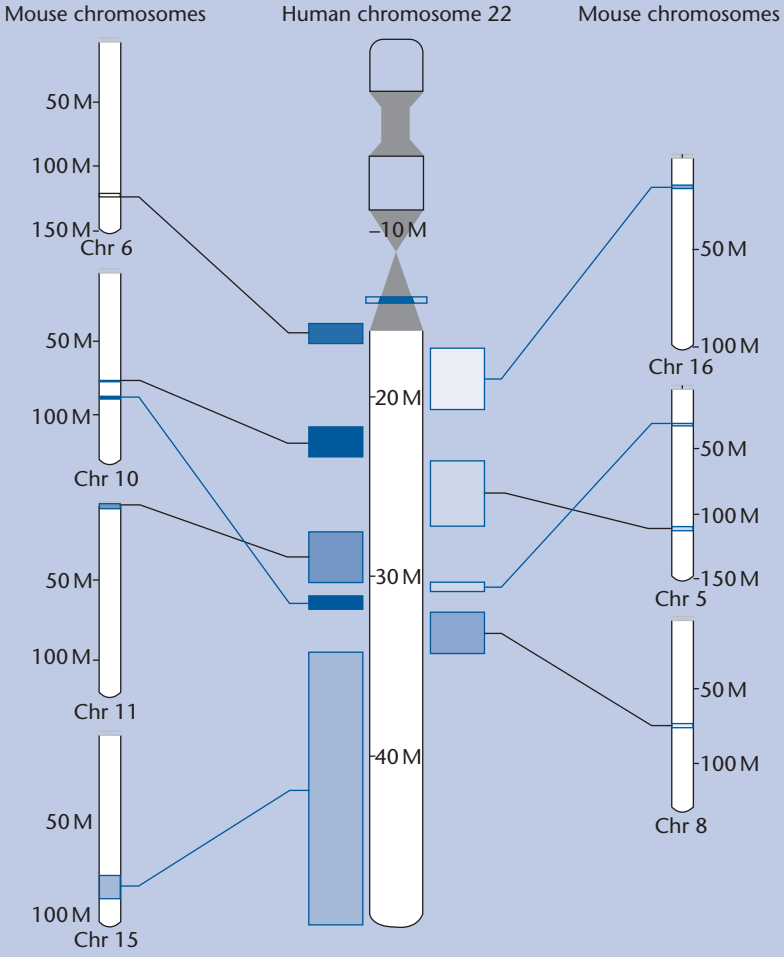
DNA fingerprinting and STS-content mapping are routinely used to construct large-insert contigs. The current commonly used human physical map started by the DNA fingerprinting of more than 415,000 BAC clones. The primary purpose of this massive project was to prepare a set of clones for

from the HUMAN GENETICS files

Comparative Genetic Maps

Shared chromosome segments across species are revealed by comparing genetic and physical maps. For example, sections of the long arm of human chromosome 22 (22q) correspond to regions from mouse chromosomes 5, 6, 8, 10, 11, 15, and 16 (Figure A). The array of conserved chromosome segments between two species is called a synteny map. Originally, the term synteny referred to set of loci on the same chromosome. More recently, it is used to describe a conserved assemblage of orthologous genes between different species. Orthologs are the same or closely related (homologous) genes or the corresponding proteins in different species, for example, myoglobins. Paralogs are homologous genes or proteins within the same species. Synteny maps that display human chromosomes with the corresponding segments from the chromosomes of other organisms can be viewed online at http://www.ensembl.org or http://www.sanger.ac.uk/HGP/.

Figure A Synteny between the long arm of human chromosome 22 and segments from mouse chromosomes. Connecting lines mark the shared DNA regions between mouse chromosomes and human 22q. M represents the distance from the end of the short arm (p) of a chromosome in megabases. Obtained from http://www.ensembl.org/Homo_sapiens/syntenyview?otherspecies=Mus_musculus&chr=22&x=35&y=11.



Syntenic relationships with human chromosomes have been determined for organisms that range from primates to microbes. The greater the evolutionary distance between two species, the more likely chromosome shuffling has occurred and the smaller the conserved chromosome regions. Comparative genetic mapping between the freshwater pufferfish, *Tetraodon nigraviridis*, and humans has shown that, although there has been scrambling by inversions, many of the genes of the human X chromosome are homologous with gene clusters on the pufferfish chromosomes 1, 2, and 7. The split between fish and what eventually became the mammalian lineage occurred about 400×10^6 years ago. Moreover, the X chromosome likely became a sex chromosome about 300×10^6 years ago after divergence of birds and mammals. Thus, conserved gene arrays existed on the X chromosome before its function as a sex chromosome. Other aspects of human chromosome evolution have been discovered by examining syntenic relationships. For example, among placental mammals, the ancestral form of human chromosome 1 (Hsa1) probably existed as a single entity and, over time, in different lineages, Hsa1 split into smaller chromosomes or parts of it were incorporated into other chromosomes. These observations negate the view that the largest human chromosome arose by the fusion of two chromosomes, at least during the evolution of placental mammals. In addition to providing insights into chromosome formation and the retention of gene clusters during evolution, comparative genetic mapping facilitates identification of genes, intron-exon boundaries, and regulatory elements.

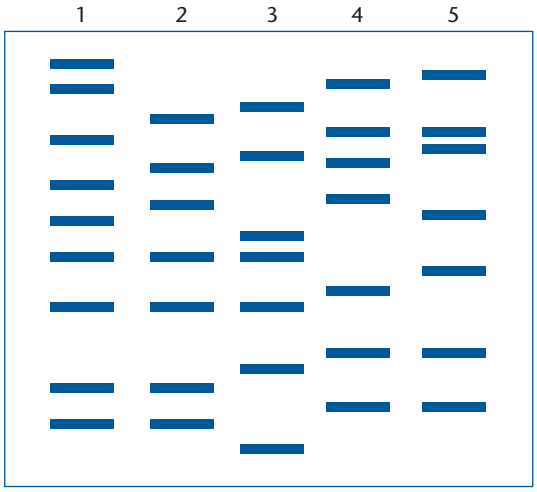


Figure 6.17 DNA fingerprinting. The DNA of five clones (1–5) is digested with a restriction endonuclease, separated by gel electrophoresis, and stained with a DNA-specific dye. Each lane (1–5) shows the pattern of DNA bands (solid rectangles) for a clone, namely, its fingerprint. In this case, clones 1, 2, and 3 share bands and probably overlap one another, and clones 4 and 5, which do not overlap clones 1, 2, and 3, overlap each other.

sequencing the human genome. Briefly, the DNA of each clone was digested with a restriction endonuclease, separated by agarose gel electrophoresis, and stained. The bands from each clone are scanned, recorded, and compared with the other banding patterns for matches. The distinctive pattern of DNA fragments from each clone is equivalent to a fingerprint; hence, the term DNA fingerprint (Figure 6.17). Much of the matching of the bands is computerized. However, manual inspection is required to identify redundant and nonoverlapping clones and to resolve inconsistencies that were not caught by the computer program. At this stage, because the chromosome origins of the members of the partially assembled contigs are not known, STSs and/or fluorescent in situ hybridization (FISH) with inserts are used to tie the clones to specific chromosome locations.

Sequence-tagged site content mapping has become an important method for constructing large-insert physical maps. As noted previously, an STS is a short single-copy segment of DNA (~100–300 bp) that can be detected specifically with a unique pair of PCR primers. Large numbers of STSs are required to confirm that a contig is continuous over a long span of a chromosome. Generally, the assembly of a high-resolution, continuous whole chromosome contig requires an STS site every 50 to 100 kb. Consequently, physical mapping of a chromosome that consists of about 160 Mb DNA would require 1500 to 3000 STSs. At least 30,000 STSs are needed for high-resolution physical mapping of the complete human genome.

Various strategies are used to generate STSs. For example, DNA from a flow-sorted human chromosome preparation is treated with a restriction endonuclease and cloned into a small-insert (<1000 bp) vector. Clones are selected at random, and the inserts are sequenced. Clones with inserts less than 100 bp and those that contain sequences from human repetitive DNA elements are discarded. The presence of repetitive DNA is determined by computer-based nucleotide matching of the insert sequence with all known human repetitive DNA sequences. Next, the primer sequences for each usable clone are selected.

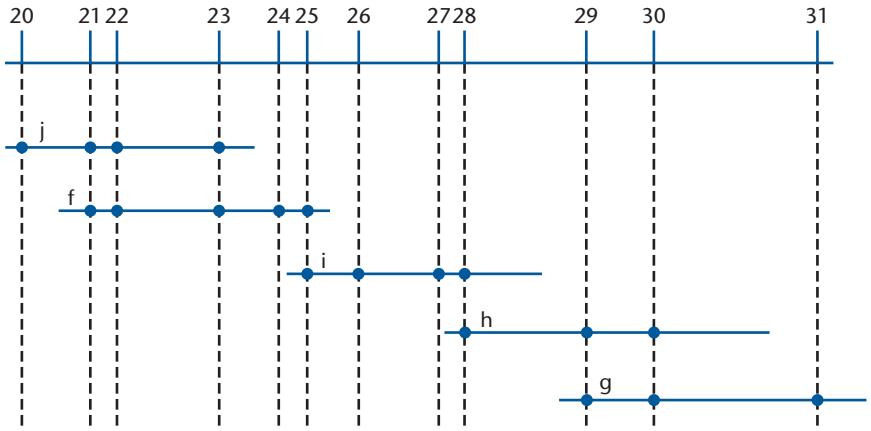


Figure 6.18 Sequence-tagged site-content mapping with ordered STSs. The numbers on the uppermost horizontal line are mapped STSs (20–31). The dots represent the STS content of each clone (f–j). Overlapping clones can be determined readily, because the STSs are ordered. The vertical dashed lines provide visual guides joining each mapped STS with the corresponding STS in one or more of the clones of the contig.

The criteria for choosing PCR primers for each insert include, among other features, a length of at least 20 nucleotides, no internal secondary structure, and no complementarity between the two primers of one set. After the primers are synthesized, a potential STS is tested for PCR amplification, with human DNA as the template. The presence of a PCR product is determined by agarose gel electrophoresis, hybridization, or a colorimetric assay. The chromosome specificity of human genome-specific STSs can be verified by using a DNA sample from the appropriate monochromosomal hybrid cell line. Region specificity is determined by PCR amplification assays with a chromosome deletion hybrid cell panel for the target chromosome. In addition, DNA from monochromosomal cell hybrid lines carrying other chromosomes can be assayed for PCR amplification to ensure that each STS is chromosome specific. The order of STSs can be determined with an RH mapping panel. Thus, by screening the STS content of each BAC clone, overlapping clones can be ordered and the chromosome region that is spanned by each contig is identified (Figure 6.18). After a prodigious effort, a whole-genome BAC map that consists of 1246 contigs and about 26,000 clones was assembled.

Integration of Cytogenetic, Genetic, and Physical Maps

The human genome can be mapped in a number of ways including cytogenetic maps that subdivide each chromosome into banding patterns, genetic maps that depict the relative position of DNA markers resulting from the frequency of meiotic recombination events, physical maps that consist of sets of ordered DNA clones, and sequence maps that represent the entire nucleotide sequence of each chromosome. (Aspects of the human genome DNA sequence are discussed in later chapters.) Because the units of distance and other features are quite different for the various kinds of chromosome maps, it is difficult to formulate a combined (integrated) map. However, by establishing cross-references, computer-stored information based on different maps can be accessed and displayed. For example, with NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606), a myriad

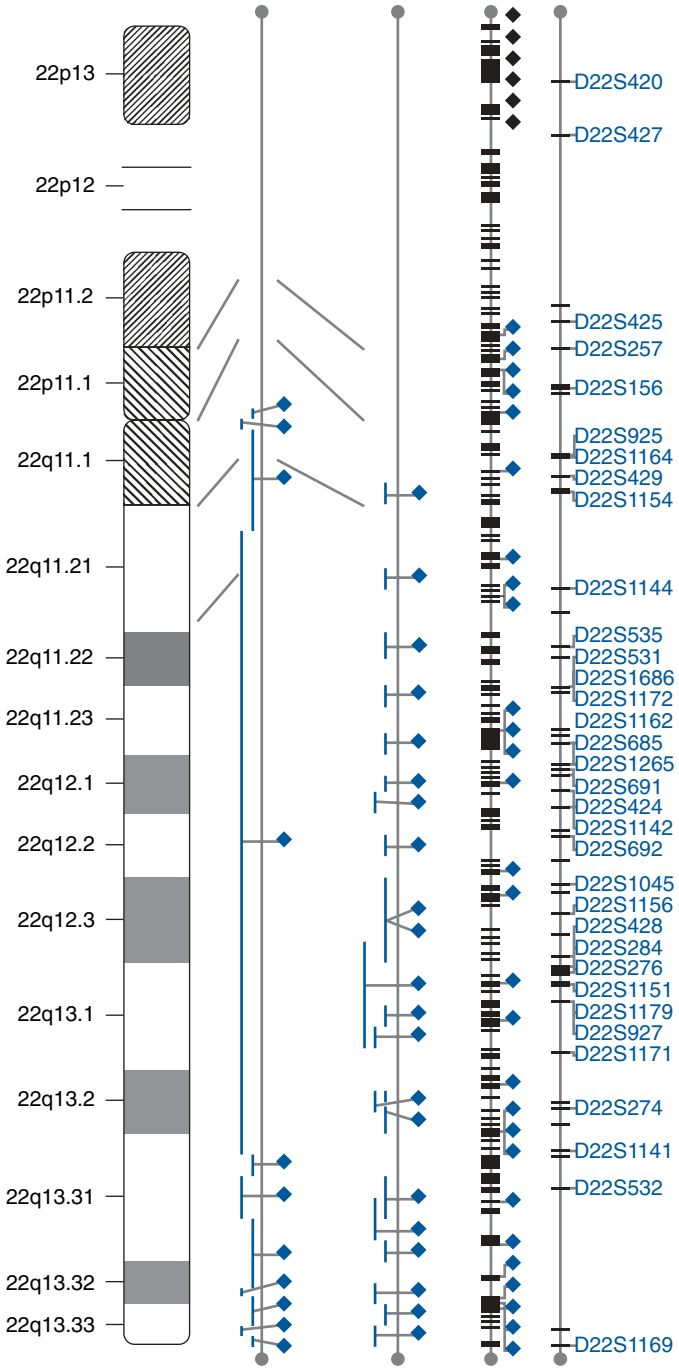


Figure 6.19 Genetic and physical maps of chromosome 22. Ideogram, contigs, FISH clones, Stanford TNG radiation hybrid map, and deCODE genetic linkage map are displayed from left to right. Created with NCBI Map Viewer at http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606.

of mapped features of each chromosome such as banding pattern (ideogram), contigs, FISH clones, genetic maps and RH maps can be visualized (Figure 6.19). More importantly, details about individual features such as probes and clones are also available.

key terms

allele frequency
BAC
centiRay (cR)
CEPH family panel
chimeric
consanguinity
contigs
cosmid
cytogenetic map
D22S1169
DNA fingerprinting
DNA marker
DNA probe
exclusion mapping
FISH clone
genetic mapping
genetic polymorphism
genotyping
haplotype
homozygosity mapping
homozygosity-by-descent
identity-by-descent
library
linkage disequilibrium
LOD
MALDI-TOF
molecular beacon
multilocus mapping
oligonucleotide ligation assay
physical mapping
primer extension
rad
radiation hybrid mapping
restriction fragment length polymorphism
sequence-tagged site (STS)
sex-average map
short tandem repeat polymorphism
single-copy DNA
single-nucleotide polymorphism
STS-content mapping
synteny
***Taq*Man**
YAC

summary

Thorough genetic mapping of human chromosomes requires large numbers of loci with high allelic frequencies that are easily identified and scattered throughout the genome. Initially, loci that showed restriction fragment length polymorphisms were used to map gene sites. However, short tandem repeat polymorphisms, which are readily scored by the polymerase chain reaction, became the preferred tool for mapping studies. DNA samples of individuals from either large pedigrees or a number of small pedigrees with members who have a well-defined genetic disease are tested with large sets of DNA markers to determine which polymorphic loci are linked to the disease-causing gene. Genetic maps are constructed with DNA samples from a panel of three-generation families that have a large number of offspring in the third generation (CEPH families). Multilocus linkage produces a more precise placement of a disease-causing gene within a mapped chromosome region.

The location of a disease-causing gene can often be determined by a chromosome segment with minimally shared homozygosity among affected relatives of consanguineous marriages (homozygosity mapping) and among individuals who share a common ancestor and live in an isloated population that was founded 10 or more generations ago (linkage disequilibrium mapping). Chromosome maps are also formed by determining the frequency of coretention of loci within DNA fragments of an irradiated human chromosome that are rescued by fusion with nonirradiated rodent cells (radiation hybrid mapping).

Single-nucleotide polymorphisms (SNPs) are abundant in the human genome and are being developed as the next generation of polymorphic markers. A number of assays such as molecular beacon, oligonucleotide ligation, primer extension, MALDI-TOF, and others are available for rapid SNP genotyping.

Physical maps that consist of sets of overlapping clones (contigs) covering the entire genome are important for cloning disease genes. DNA fingerprinting determines the shared regions among the clones. In addition, sequence-tagged sites (STSs) provide mapping landmarks for ordering the clones and forming contigs. Map Viewer, an Internet resource, provides access to a vast amount of genomic data including a variety of genetic and physical maps.

References

Boehnke, M., K. Lange, and D. R. Cox. 1991. Statistical methods for multipoint radiation hybrid mapping. *Am J Hum Genet* **49**:1174–1188.

Botstein, D. et al. 1980. Construction of a genetic map in man using restriction fragment length polymorphisms. *Am J Hum Genet* **32**:314–331.

Broman, K. W. et al. 1998. Comprehensive human genetic maps: individual and sex-specific variation in recombination. *Am J Hum Genet* **63**:861–869.

Chumakov, I. M. et al. 1995. A YAC contig map of the human genome. *Nature* **377** (Supplement):175–297.

Murray, J. C. et al. 1994. A comprehensive human linkage map with centimorgan density. *Science* **265**:2049–2054.

Curtis, D. and H. Gurling 1992. A procedure for combining two-point lod scores into a summary multipoint map. *Hum Hered* **43**:173–185.

Dausset, J. et al. 1990. Centre d'Etude du Polymorphisme Humain (CEPH): Collaborative genetic mapping of the human genome. *Genomics* **6**:575–577.

de Pontbriand, A. et al. 2002. Synteny comparison between apes and human using

fine-mapping of the genome. *Genomics* **80**:395–401.

Dib, C. et al. 1996. A comprehensive genetic map of the human genome based on 5264 microsatellites. *Nature* **380**:152–154.

Dubrovsky, J. et al. 1995. Sets of short tandem repeat polymorphisms for efficient linkage screening of the human genome. *Hum Mol Genet* **4**:449–452.

Green, E. D. and P. Green. 1991. Sequence-tagged site (STS) content mapping of human chromosomes: theoretical considerations and early experiences. *PCR Methods Appl* **1**:77–90.

Grützner, F. et al. 2002. Four-hundred million years of conserved synteny of human Xp and Xq genes on three *Tetraodon* chromosomes. *Genome Res* **12**:1316–1322.

Gyapay, G. et al. 1994. The 1993–1994 Genethon human genetic linkage map. *Nat Genet* **7**:246–287.

Gyapay, G. et al. 1996. A radiation hybrid map of the human genome. *Hum Mol Genet* **5**:339–346.

Hästbacka, J. et al. 1992. Linkage disequilibrium mapping in isolated founder populations: diastrophic dysplasia in Finland. *Nat Genet* **2**:204–211.

Hudson, T. J. et al. 1995. An STS-based map of the human genome. *Science* **270**: 1945–1954.

Kong, A. et al. 2002. A high-resolution recombination map of the human genome. *Nat Genet* **31**:241–247.

Lander, E. S. and D. Botstein. 1987. Homozygosity mapping: A way to map human recessive traits with the DNA of inbred children. *Science* **236**:1567–1570.

Lander, E. S. and P. Green. 1987. Construction of multilocus genetic linkage maps in humans. *Proc Natl Acad Sci USA* **84**:2363–2367.

Lathrop, G. M. and J.-M. Lalouel. 1988. Efficient computations in multilocus linkage analysis. *Am J Hum Genet* **42**:498–505.

Leppert, M. et al. 1989. Benign familial neonatal convulsions linked to genetic markers on chromosome 20. *Nature* **337**: 647–648.

Li, L., C. J. Stoeckert, Jr., and D. S. Roos. 2003. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res* **13**:2178–2189.

McPherson, J. D. et al. 2001. A physical map of the human genome. *Nature* **409**: 934–941.

Murphy, W. J. et al. 2003. The origin of human chromosome 1 and its homologs in placental mammals. *Genome Res* **13**:1880–1888.

Neiswanger, K., B. Kaplan, and S. Y. Hill. 1995. Exclusion of linkage between alcoholism and the MNS blood group region on chromosome 4q in multiplex families. *Am J Med Genet* **60**:72–79.

Nievergelt, C. M. et al. 2004. Large-scale integration of human genetic and physical maps. *Genome Res* **14**:1199–1205.

Olivier, M. et al. 2001. A high-resolution radiation hybrid map of the human genome draft sequence. *Science* **291**:1298–1302.

Terwilliger, J. D. and J. Ott. 1994. *Handbook of Human Genetic Linkage*. Johns Hopkins University Press, Baltimore.

Weber, J. L. and P. E. May. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am J Hum Genet* **44**:388–396.

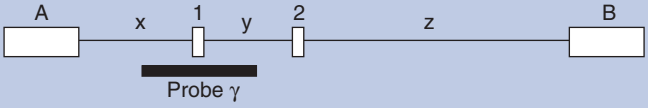
Weeks, D. E. 1991. Human linkage analysis: Strategies for locus ordering. In: Adolph, KW (ed.) *Advanced Techniques in Chromosome Research*. Marcel Dekker, New York, pp. 297–330.

review questions

1. What are the allelic frequencies in the following populations? Are the loci in these populations polymorphic?

Population	*A1A1*	*A1A2*	*A2A2*
A	600	300	100
B	400	200	400
C	0	1000	0
D	10	800	190
E	990	5	5

2. In the figure below, sites A and B are always cut by restriction endonuclease 1 (RE1) and sites 1 and 2 are polymorphic RE1 recognition sites. With probe γ, what fragments are detected when the genotypes are ++/++, +–/++, ––/+–, and –+/–+?



3. Individuals from three moderately sized families with the same disorder were screened with polymorphic markers, and LOD scores were determined. Which marker(s) is linked to the disease-causing locus?

Family	D7S472	D7S641	D7S2508	D7S2458	D7S496
A	–1.79	–0.29	1.76	1.36	0.68
B	1.28	–0.69	0.76	2.54	–0.39
C	–0.43	1.24	–0.39	0.69	1.12

4. All the members of two very large families with the same phenotype were screened with a set of polymorphic markers. LOD scores for one of the markers are shown below. Explain these results.

	$\theta =$ 0.05	0.10	0.15	0.20	0.25	0.30	0.35
Family A	3.96	4.35	5.65	4.45	3.25	2.98	1.54
Family B	–2.91	–1.69	0.79	–0.25	–1.34	0.78	1.12

continued

5. Account for the phenotypes of IV-6, IV-7, and V-1 in Figure 6.6.
6. How are STRPs used to map the chromosome location of a genetic disease locus?
7. What is exclusion mapping?
8. What is the CEPH panel of families?
9. Describe how multilocus chromosome linkage maps are constructed.
10. Describe how a disease gene locus is inserted into a multilocus linkage map.
11. Why do male and female human linkage maps differ?
12. What is "homozygosity-by-descent"?
13. What are the principal features of linkage disequilibrium mapping?
14. Describe how an RH map is formulated.
15. What is a SNP? Describe one assay for genotyping SNPs. How does this assay detect heterozygotes?
16. What is a DNA fingerprint? How is this technique used to assemble a physical map?
17. What is a contig? Describe STS-content mapping.
18. How are physical maps used?
19. What is synteny?
20. What is the "ultimate" genetic map? Why?

chapter 7

Discovering Human Disease Genes

If there are obstacles, the shortest line between two points may be the crooked line.
BERTOLT BRECHT (1898–1956)

So easy it seemed
Once found which yet unfound most would have thought impossible.
JOHN MILTON (1608–1674)

A THOROUGH UNDERSTANDING OF THE RELATIONSHIP between a phenotype, whether it is normal or abnormal, and its corresponding genotype depends ultimately on the ability to isolate (clone) and characterize an individual gene(s). The DNA sequence of a gene reveals the domains of the encoded protein, how mutations disrupt its function, and the extent to which mutations in different exons are responsible for a disease. With a cloned gene, experiments can be devised that determine protein : protein interactions and how various mutant gene products undermine normal processes. In addition, diagnostic tests for specific gene mutations can be developed from the DNA sequences of normal and mutated genes. And knowledge of gene function may lead to the formulation of therapeutic treatment for an inherited disease.

In February 2001, the initial draft of the nucleotide sequence of humans was released. With this landmark event, the task of identifying and isolating human disease-causing genes became considerably easier. Previously, gene discovery was, for the most part, an arduous task that did not follow a precisely defined set of experimental protocols, but rather entailed a repertoire of tools and resources that were implemented as the circumstances dictated. In the 1990s, on average, it took three or more years to isolate a gene. Today, seldom more than a year is required.

By itself, the human genome sequence is a vast source of information. As well, there are considerable amounts of important ancillary details concerning genetic, physical, and cytogenetic maps; location of known genes and transcribed regions; base pair composition; single-nucleotide polymorphisms; repeated sequences; as well as many other features. These data have been assembled into databases and can be viewed online at http://www.genome.ucsc.edu or http://www.ensembl.org. Moreover, exceptionally rapid sequence matching

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programs are available for pinpointing the chromosome location of a DNA or protein sequence. Computer access to the human DNA sequence has streamlined the process of gene discovery.

In general, the two primary strategies for discovering human disease genes are called functional/candidate and positional-candidate gene cloning. A critical feature of gene discovery is proving that an isolated sequence is the actual disease-causing gene. The process of gene authentication is based on detecting whether there are nucleotide changes in the genes of affected individuals that are not present in unaffected individuals.

Cloning Human Disease Genes

Functional/Candidate Gene Cloning

A functional/candidate gene cloning project starts with either a known protein that is responsible for an inherited disorder or a protein that is considered a likely candidate based on the symptoms and biochemistry of the disease. The key steps of this approach are outlined in Figure 7.1. Briefly, the amino acid sequence of the protein is used to deduce the possible coding sequence of the corresponding gene. The human genome sequence database is queried with this

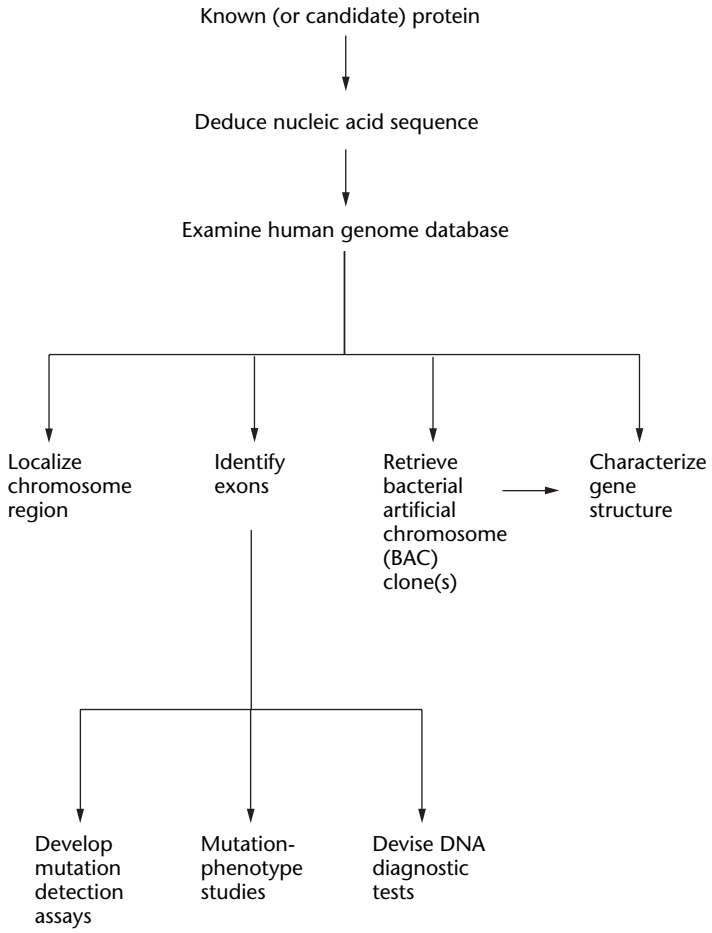


Figure 7.1 Functional/candidate gene cloning. The flow diagram highlights some of the steps that lead to the isolation and identification of a gene when the sequence of the gene product is known.

sequence to determine the corresponding exons. On the basis of the identified exon or intron sequences, PCR probes are synthesized for a mutation detection assay. Once it is established that mutations of the gene cause the disease under study, the BAC clone(s) that carries the gene can be obtained and used to characterize the structure of the gene. In addition, after authentication, gene cloning, and isolation of a full-length cDNA, various studies designed to determine when and where the protein is active, when the gene is expressed, and the mode of action of the protein can be undertaken. (See Chapter 8 on Bioinformatics: Genomics, Functional Genomics, and Proteomics for more information about determining gene function.)

Positional-Candidate Gene Cloning

The positional-candidate gene cloning approach is mandated when nothing is known about the gene product that is responsible for a genetic disease and there are no likely candidate genes (Figure 7.2). In these instances, the disease gene is mapped to a chromosome location with polymorphic markers. Once the chromosome location is narrowed down, the human genome database is consulted for the genes within this region. From this list of genes, a likely candidate(s) is selected and a mutation detection assay is run with PCR probes based on the gene sequence derived from the database. Once the disease-causing gene has been identified, a number of studies of the gene and the encoded protein can conducted to determine the molecular basis the inherited disorder. If the gene is not identified, then researchers are faced with the unenviable task of running mutation detection assays for more genes within the mapped region until the right one is discovered.

Detection of Mutations in Human Genes

Routinely, after a putative disease-causing gene has been cloned and sequenced, it is screened for a nucleotide change involving one or a few base pairs. The underlying principle of a mutation detection assay is that the nucleotide sequence of the gene in affected individuals will differ from the sequence content of the same gene in individuals with a normal phenotype. A number of simple and inexpensive assays, such as single-strand conformation polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis (HA), chemical mismatch cleavage (CMC), and the protein truncation test (PTT) have been developed to detect mutations.

Single-Strand Conformation Polymorphism Analysis

Single-strand conformation polymorphism analysis is one of the most widely used procedures for detecting gene mutations. Some or, when feasible, all of the exons of a cloned gene are amplified individually by PCR from the DNA of affected and unaffected individuals. Each pair of primers is determined from sequences that flank each exon or from the terminal ends of each exon. In addition, DNA sequence data from genomic clones can be used to develop PCR-based assays to search for mutations in the 5′ upstream region adjacent to the first exon, in the 3′ downstream region adjacent to the last exon of a gene, and across the splice junctions of exons and introns. Before implementation of the

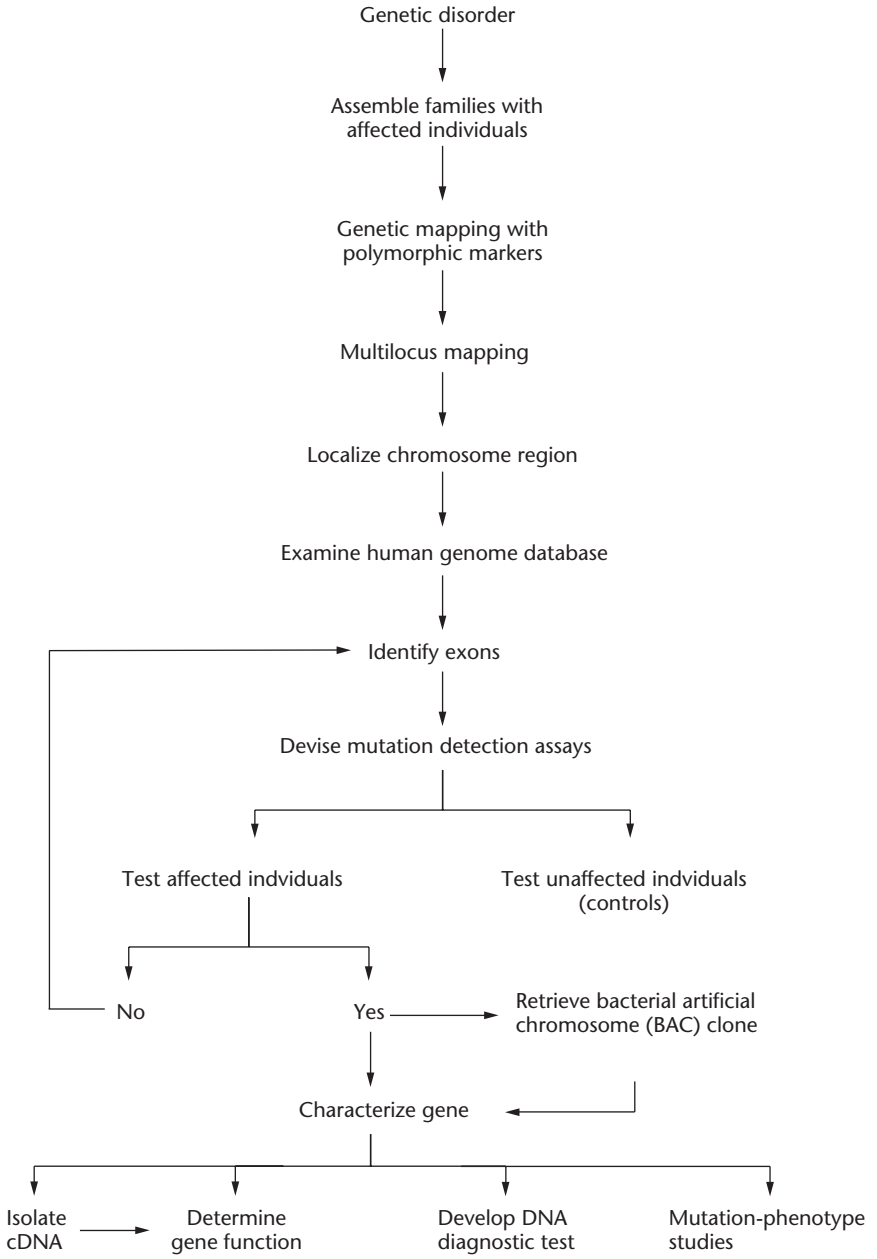


Figure 7.2 Positional-candidate gene cloning. The flow diagram highlights some of the steps that lead to the isolation and identification of a disease-causing gene when its product is unknown. Genetic mapping localizes the disease-causing gene to a specific chromosome region. Exon sequences in the vicinity of the chromosome site are obtained from a human genome database, and mutation detection assays are devised. After the disease-causing gene is substantiated, it can be characterized and its function determined.

assay, each pair of primers is tested with whole human DNA to ensure that they amplify only single copy regions. In other words, each pair of primers produces a single band. For optimal results with the SSCP, each primer pair should amplify about 200 bp of DNA.

After PCR amplification of DNA samples from affected and unaffected individuals, the products of each reaction are denatured, rapidly cooled, and electrophoresed. Each denatured single-stranded DNA molecule assumes a three-dimensional conformation that is dependent on its primary nucleotide sequence. The conformation is the consequence of intrastrand base pairing and the formation of other bonds. Because of base complementarity, the two

single strands of a double-stranded DNA molecule have different nucleotide sequences, and each has a different three-dimensional conformation. The different conformations have different rates of migration in a gel during electrophoresis. Hence, two bands can be visualized by DNA-specific staining, autoradiography, if the strands were radiolabeled during PCR amplification, or fluorescence with fluorescent dye markers.

If two DNA molecules from different sources representing the same segment of a gene differ by a single nucleotide pair, then the conformations of the two single DNA strands from one source are very likely to be different from those of the two strands from the other source. In other words, each of the four strands will migrate during electrophoresis at its own distinctive rate. With side-by-side comparisons between DNA from affected and unaffected individuals, the differences in the migration of single-stranded DNA molecules are easily detected (Figure 7.3). The SSCP localizes a nucleotide alteration to a specific region or exon of a gene. The nature of the mutational difference is not revealed by the SSCP. This information can be obtained by DNA sequencing. The SSCP can detect about 90% of the single base pair mutations in PCR products that are 200 bp or less.

Denaturing Gradient Gel Electrophoresis

For a DGGE assay, intact PCR products of the exons and other regions of a gene from the DNA of affected and unaffected individuals are electrophoresed individually in a gel through an increasing concentration gradient of DNA denaturants, such as urea or formamide. As a double-stranded DNA molecule migrates through the gradient, denaturation is initiated at a concentration capable of breaking hydrogen bonds between complementary bases. For example, regions with a large number of A:T base pairs will begin to separate at a lower concentration of denaturants than regions with many G:C base pairs. This differential denaturation separates segments of strands of a DNA molecule, altering its conformation and retarding its migration through the gel. A single base pair alteration within a DNA molecule can change the point in the gradient when it begins to denature and modify its rate of migration in the gel (Figure 7.4). To ensure that differentially denatured DNA molecules can be resolved readily by DGGE, one of each set of primers has a string of 40 GC units (GC clamp) attached to its 5′ end. After the PCR, this provides the product with a stretch of 40 G:C base pairs that will not denature at the highest concentration of denaturants in the gradient. The DGGE method can detect more than 95% of the single-base differences in PCR products that are 600 bp or less in length. The bands in the gel are visualized by DNA staining, autoradiography of labeled DNA molecules, or fluorescence with fluorescent dye markers.

Heteroduplex Analysis

A double-stranded DNA molecule with one or a few mismatched nucleotide pairs is called heteroduplex DNA (Figure 7.5). A DNA molecule without nucleotide mismatches is homoduplex DNA. In principle, a single nucleotide mismatch in a DNA molecule of 300 bp is sufficient to alter its electrophoretic mobility in comparison to homoduplex DNA molecules. A nucleotide mis-

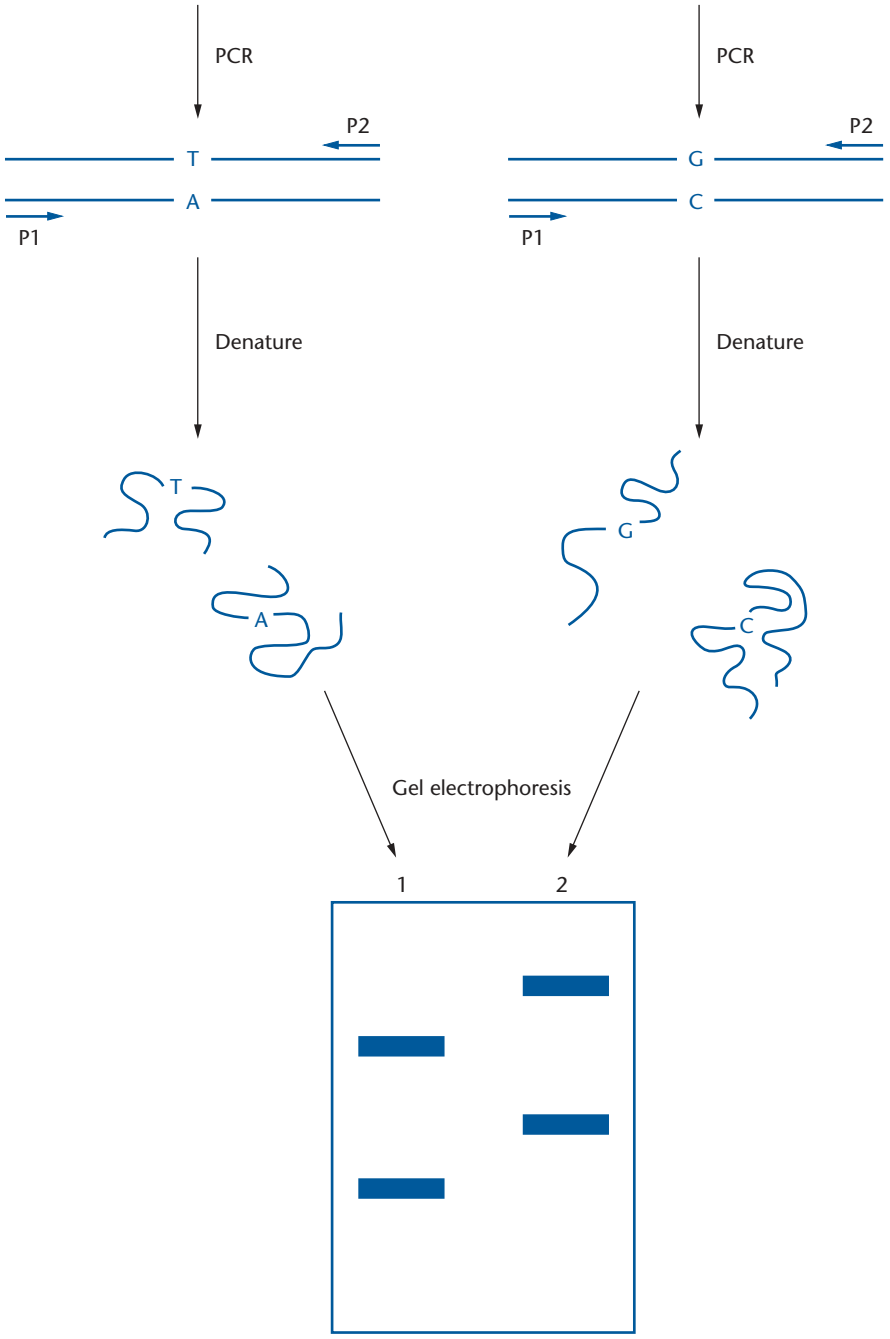


Figure 7.3 Single-strand conformation polymorphism analysis. Samples of DNA from two different sources that differ by a single nucleotide pair (A:T vs. G:C) are amplified by the polymerase chain reaction (PCR) with the same primers (P1 and P2). The PCR products are denatured and run in individual lanes (1, 2) of a nondenaturing gel. The distance that a single-stranded DNA molecule migrates into the gel depends on its conformation. A single nucleotide pair difference between DNA strands is often sufficient to produce different single-strand conformations.

match causes a bulge that distorts the conformation of the DNA molecule and retards its migration through a gel.

For heteroduplex analysis testing, DNA samples from an affected and a nonaffected individual are combined and amplified by PCR, with pairs of primers for the exons and flanking regions of a cloned putative disease gene. If an amplified DNA segment from the different DNA samples has a nucleotide difference, heteroduplex DNA molecules will form during the renaturation step after PCR amplification.

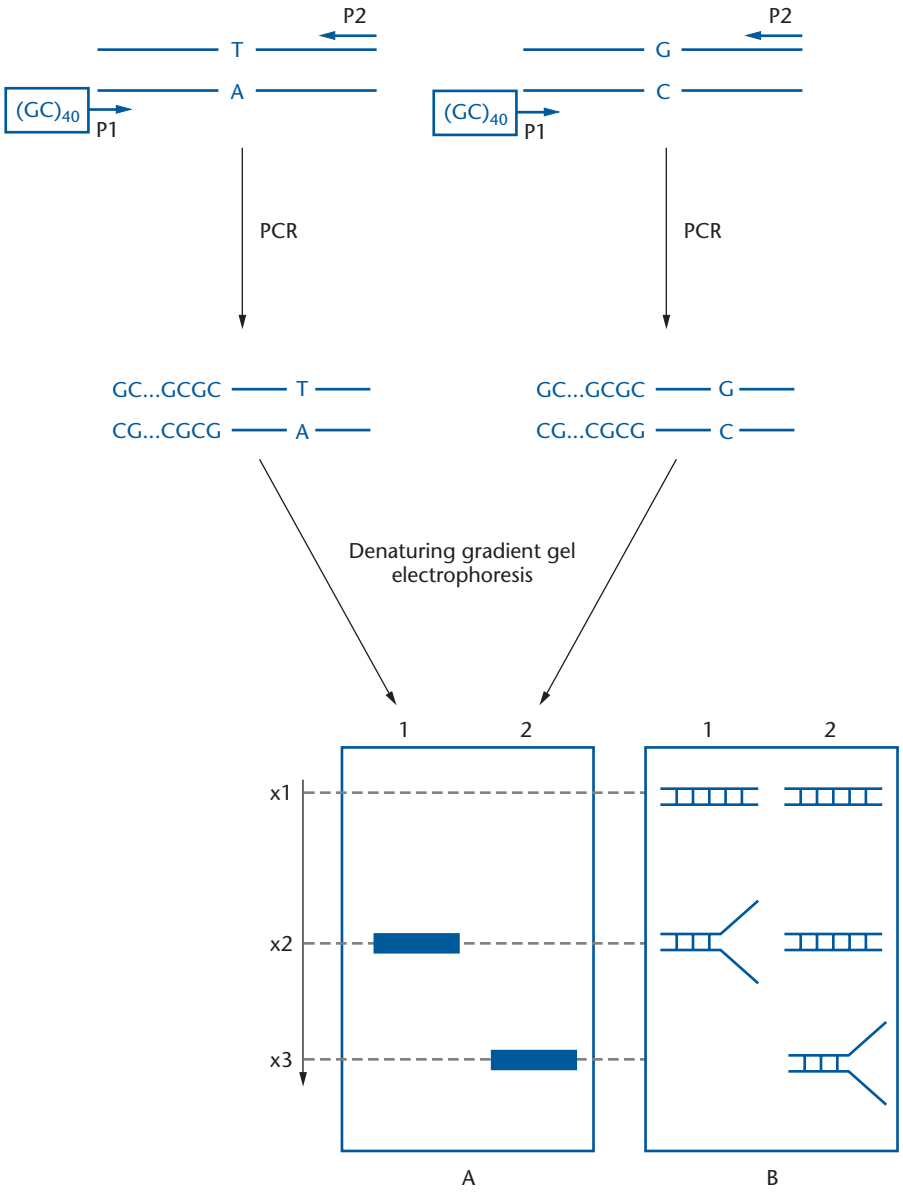
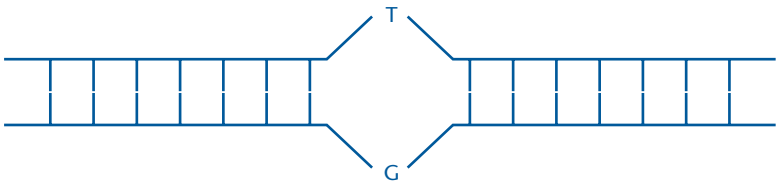


Figure 7.4 Denaturing gradient gel electrophoresis. Samples of DNA from two different sources that differ by a single nucleotide pair (A:T versus G:C) are amplified by the polymerase chain reaction (PCR) with the same primers (P1 and P2). One of the primers (P1) has a GC tail of 40 nucleotides [$(GC)_{40}$]. The PCR products are electrophoresed in individual lanes of a denaturing gradient gel (box A). The downward-pointing arrow to the left of box A represents the increasing concentration of DNA denaturants in the gel with ×1, ×2, and ×3 indicating various concentrations in the gradient. The extent of migration of the PCR products into the gel are depicted by the solid rectangles in box A. In box B, the "railroad track" DNA denotes intact duplex DNA molecules and the Y-shaped DNA molecules represent partially denatured DNA molecules at various points (×1, ×2, and ×3) during electrophoresis in the denaturing gradient of the gel.

After PCR, each amplified sample is heated to 95°C and slowly cooled to maximize heteroduplex DNA formation. Then, each DNA sample is electrophoresed in a lane of a special gel (HydroLink®; MDE™) that enhances the difference in mobility between homoduplex and heteroduplex DNA molecules. Under these conditions, both of the homoduplex molecules will migrate to the same extent, because they have the same conformation and length. The heteroduplex DNA molecules will migrate more slowly through a gel, and, often, both will migrate at the same rate to form a single band (Figure 7.6). The bands with heteroduplex and homoduplex DNA molecules can be visualized by DNA staining, autoradiography, if the PCR products are radiolabeled, or fluorescence with fluorescent dye markers. Heteroduplex analysis can detect

A



B

	A	C	G	T
A	mismatch	mismatch	mismatch	match
C	mismatch	mismatch	match	mismatch
G	mismatch	match	mismatch	mismatch
T	match	mismatch	mismatch	mismatch

Figure 7.5 Heteroduplex DNA. (A) A heteroduplex DNA molecule that is formed by a thymidine (T)-guanosine (G) mismatch. (B) All possible single nucleotide mismatches and matches between the deoxyribonucleotides (A, C, G, and T) of duplex DNA.

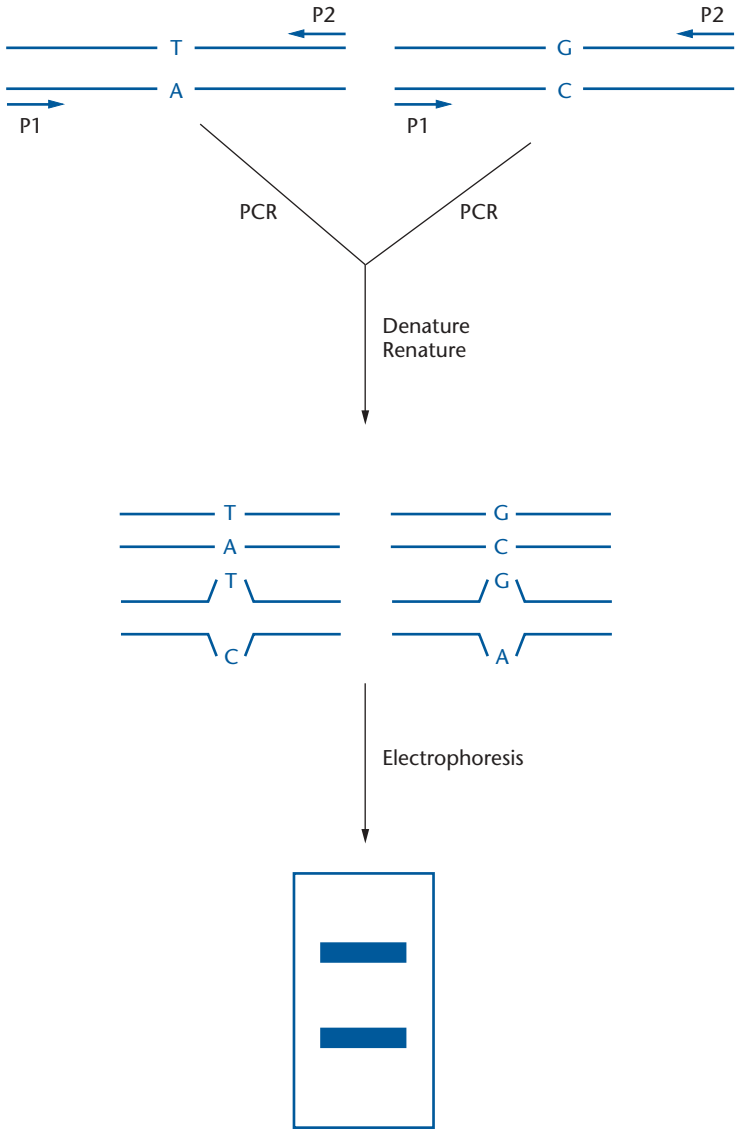


Figure 7.6 Heteroduplex analysis. Samples of DNA from two different sources that differ by a single nucleotide pair (A:T vs. G:C) are amplified by the polymerase chain reaction (PCR) with the same primers (P1 and P2). The PCR products are combined, denatured, and renatured. Homoduplex and heteroduplex DNA molecules are formed. Gel electrophoresis resolves heteroduplex (upper solid rectangle) from homoduplex (lower solid rectangle) DNA molecules.

from the HUMAN GENETICS files

Rapid Detection of Unknown Mutations: Capillary Electrophoresis

Quite often with positional-candidate gene cloning, a large number of candidate genes must be screened for mutations before the disease-causing gene is discovered. Theoretically, 50 candidate genes, each with 10 exons, and 25 samples from both affected and unaffected individuals could require as many as 25,000 mutation detection assays, without considering replications. Currently, most large-scale mutation detection systems rely on slab gel electrophoresis to resolve either single- or double-stranded DNA molecules. However, this technique is slow and time-consuming. To overcome these drawbacks, researchers have been examining other DNA separation systems, in particular, attention has focused on capillary electrophoresis (CE) and more specifically on multiple capillary instruments.

Briefly, for CE, DNA is separated in thin fused silica tubes (capillaries) that have an inside diameter of about 75 µm and a length from 30 to 80 cm. A capillary tube is filled with a low-viscosity sieving matrix, and the DNA sample (about 15 µl) is injected under pressure. A CE run with DNA is about 30 minutes at 15 kV. By comparison, the duration of slab gel electrophoresis for single-strand conformation polymorphism (SSCP) analysis is about 16 h. The DNA bands are either read by ultraviolet absorption or laser-induced fluorescence if the DNA is labeled with fluorescent dye markers as the molecules pass through the end of the capillary. The output data are stored and analyzed. Another significant advantage of CE is that the process can be automated. Currently, the maximum capacity for a commercial capillary electrophoresis instrument is 384 capillaries.

Single-strand conformation polymorphism and heteroduplex mutation detection assays have been adapted for capillary electrophoresis. In one test experiment, CE-SSCP detected 98% of the mutations. In addition, for the DGGE method with CE, a temperature gradient replaces the chemical gradient. Generally, under optimal conditions, a 16-capillary CE instrument analyzes about 654 SSCP samples in 24 h. Although a straight-line extrapolation may not be appropriate, a machine with 384 capillaries ought to perform more than 10,000 analyses in a 24-h period if the software is fast enough to process the data from each sample.

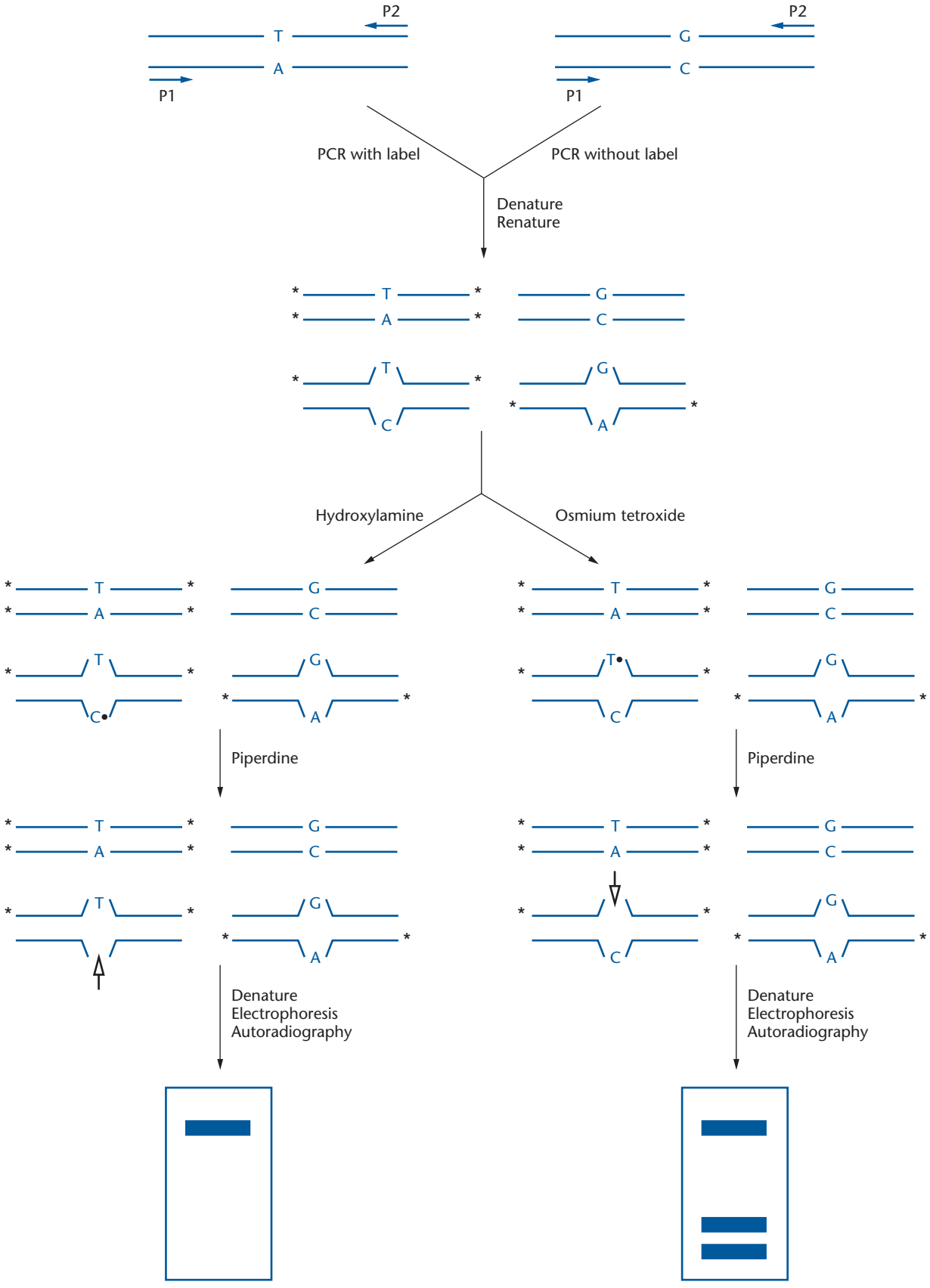
In addition to CE, other analytical procedures including microcapillary electrophoresis, DNA microarrays, and mass spectrometry are being examined for detecting unknown mutations. Certainly in the area of human molecular genetics, inventive techniques are devised as needs arise.

more than 95% of the single nucleotide mismatches in DNA fragments of 300 bp or less in length.

Chemical Mismatch Cleavage

The chemical mismatch cleavage (CMC) mutation detection assay is a variant of heteroduplex analysis. Samples of DNA from affected and unaffected individuals are amplified by PCR with pairs of primers for the exons and flanking regions of a cloned putative disease gene. The PCR product in one of the DNA samples and not the other is labeled during amplification. For the CMC assay, the PCR products from each reaction are mixed, denatured, and slowly cooled. If there is a nucleotide difference, heteroduplex DNA molecules will form.

A renatured sample is divided into two aliquots. One aliquot is treated with hydroxylamine and the other with osmium tetroxide. The hydroxylamine treatment modifies mismatched cytosine residues but not those that are matched. The osmium tetroxide treatment selectively modifies mismatched thymidine units, whereas matched thymidine residues survive unscathed. Next, both samples are treated with piperidine, which creates a nick in the DNA strand by cleaving a modified cytosine or thymidine residue. The DNA of each sample is separated by denaturing gel electrophoresis. If cleavage has occurred in a strand, then two fragments will be produced that are separated by gel electrophoresis. Strands that do not contain a modified cytosine or thymidine residue remain intact (Figure 7.7). Thus, cleavage products denote a mismatch,


P2
T
A
P1
P2
G
C
P1
PCR with label
PCR without label
Denature
Renature
Hydroxylamine
Osmium tetroxide
Piperdine
Piperdine
Denature
Electrophoresis
Autoradiography
Denature
Electrophoresis
Autoradiography

which signifies a nucleotide difference between the DNA samples. The pattern of DNA bands can be visualized by autoradiography or a chemiluminescent detection assay. The CMC assay detects more than 95% of single mismatched nucleotides in pieces of DNA up to 1700 bp in length.

In summary, DNA from affected individuals is usually screened for mutations with SSCP. Both the DGGE and HA assays are easy to implement, and the selection of assay often depends on the preferences of scientists in the laboratory setting. The CMC assay, which requires the toxic chemicals hydroxylamine and osmium tetroxide, is not recommended for mutation studies of a large number of DNA samples. Other DNA separation techniques such as capillary electrophoresis and high-performance liquid chromatography have been used for various assays. A mutation detection assay, for the most part, delimits the location of a mutation to an exon or flanking region. The actual nucleotide alteration is determined by DNA sequencing.

Direct DNA Sequencing

The location and nature of a mutation are defined unequivocally by DNA sequencing. Once a mutation is detected with a PCR-based assay, the segment with a mutation and the segment without this mutation can each be sequenced directly. Subcloning of these segments into a DNA sequencing vector is not required. In practice, after the initial set of PCR cycles of the segment to be sequenced, the product is amplified again, with the amount of one primer being 100 times or more than the other. This asymmetric PCR produces an abundance of one DNA strand that provides the template for the dideoxynucleotide termination procedure. Alternatively, during the initial PCR, one of the primers is biotinylated. After PCR, the end product is denatured, and the biotinylated strand is captured with bound streptavidin and used as the template for DNA sequencing.

Protein Truncation Test

The protein truncation test (PTT; in vitro synthesized protein assay, IVSP) detects nonsense mutations, out-of-frame deletions and insertions, in-frame deletions greater than 25 bp, and splice-site alterations that skip an exon greater

Figure 7.7 Chemical mismatch cleavage. Samples of DNA from two different sources that differ by a single nucleotide pair (A : T vs. G : C) are amplified by the polymerase chain reaction (PCR) with the same primers (P1 and P2). One of the PCR products is labeled (*) during the amplification process. The PCR products are combined, denatured, and renatured. Homoduplex and heteroduplex DNA molecules are formed. The renatured sample is divided into two aliquots. One aliquot is treated with hydroxylamine, which modifies mismatched cytosine residues (C•). The other aliquot is treated with osmium tetroxide, which modifies mismatched thymidine residues (T•). Both aliquots are treated with piperidine, which cleaves a DNA strand at a modified nucleotide (open arrows). The aliquots are denatured and electrophoresed under denaturing conditions. Autoradiography distinguishes intact labeled strands from labeled fragments. In this example, the autoradiograph of the denatured osmium tetroxide-piperidine-treated aliquot indicates that a heteroduplex DNA molecule was formed between the combined PCR products. The uppermost solid rectangle represents full-length labeled DNA strands, and the solid rectangles at the bottom of the gel denote labeled DNA fragments. The strand of heteroduplex DNA cleaved by the hydroxylamine-piperidine treatment is not labeled, and therefore no labeled DNA fragments are produced.

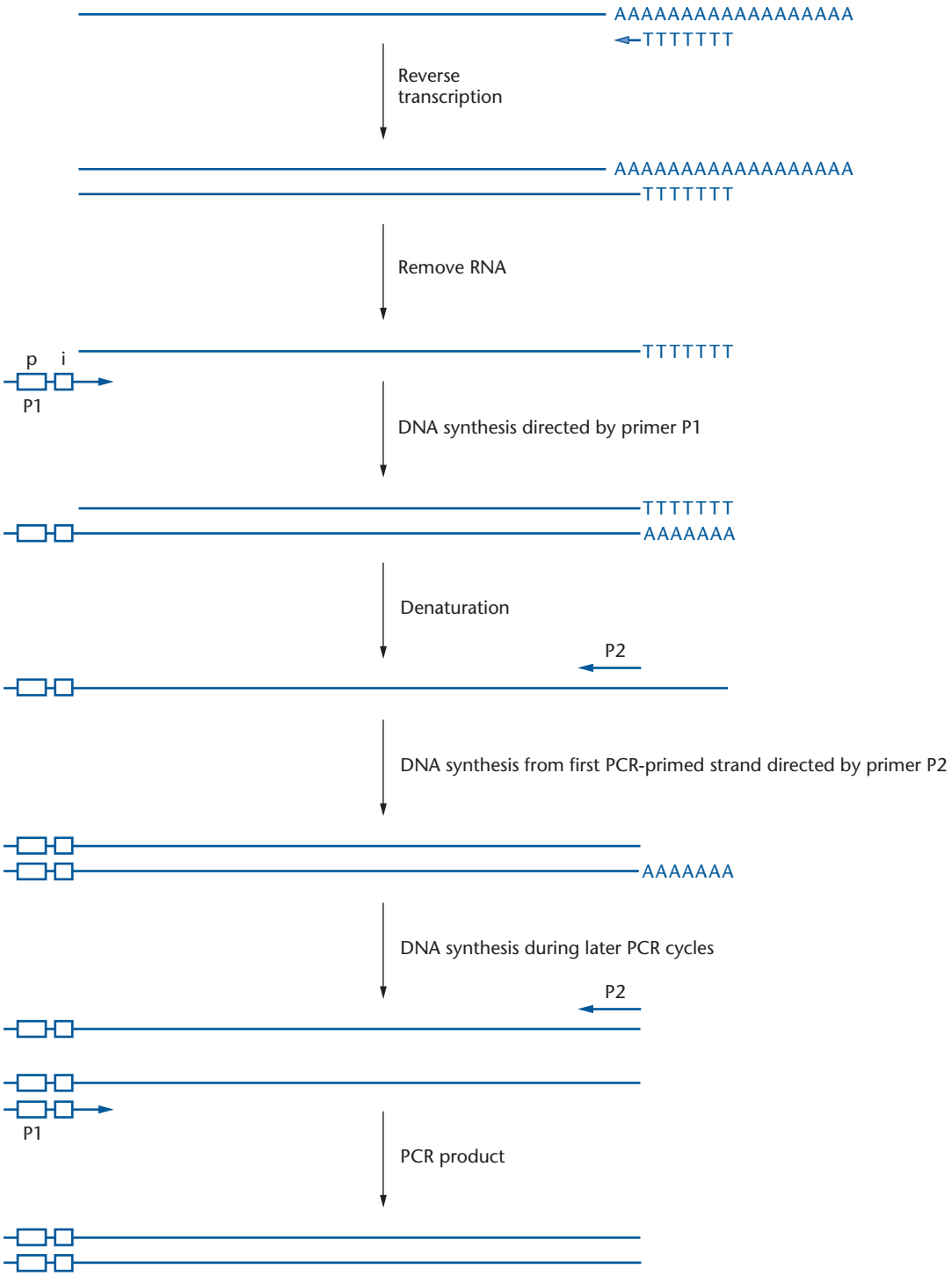


Figure 7.8 Reverse transcription-polymerase chain reaction (RT-PCR) for the protein truncation test (PTT). Reverse transcription of total RNA from the tissue expressing a disease gene is directed by an oligo(dT) primer (←TTTTTTT). Primer P1, which is complementary to sequences at the 5′ end of a specific cDNA, is used for the DNA synthesis of a second cDNA strand. The P1 primer also contains an RNA polymerase promoter (p) and a eukaryotic initiation of translation site (i). The P1-primed DNA strand acts as a template for DNA synthesis directed by primer P2. During the PCR cycles, complementary DNA sequences that are defined by primers P1 and P2 accumulate. The PCR product can be transcribed and translated in vitro.

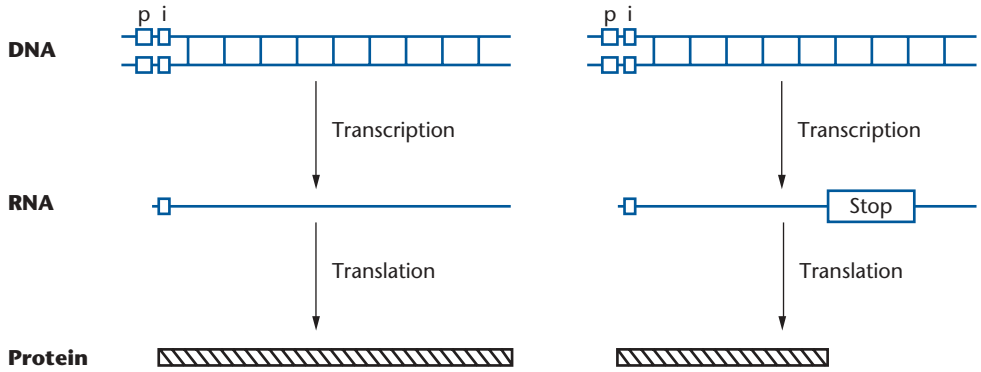


Figure 7.9 In vitro transcription and translation for the protein truncation test (PTT). The presence of a stop codon produces a shorter (truncated) protein in comparison to a full-length protein that is synthesized from a nonmutated coding region. If the PCR products originate from a person who is heterozygous for a truncation mutation, then both full-length and truncated proteins will be produced. If the source of the PCR products has no truncation mutation or is homozygous for a truncation mutation, then only full-length proteins or truncated proteins will be produced, respectively.

than 25 bp. The PTT is based on reverse transcriptase-PCR (RT-PCR). Total RNA is extracted from tissue that expresses the disease gene, and the first-strand cDNAs are synthesized from the mRNAs by reverse transcriptase with oligo(dT) as the primer. Then, primers for the first (5′) and last (3′) exons are used to produce a full-length cDNA corresponding to the putative disease gene. Or an in-frame region of a cDNA can be amplified with primers for internal exons. For the PTT, the upstream (5′) primer contains a sequence for an RNA polymerase promoter and a eukaryotic initiation of translation recognition site. These recognition sites facilitate the transcription and translation of the amplification product in vitro by a cell-free extract containing both an RNA polymerase that binds to the promoter sequence and all the components that support translation (Figure 7.8).

The newly synthesized protein is labeled with either a radioactive or a biotinylated amino acid. The proteins of the cell-free extract are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The synthesized protein is detected by either autoradiography or a streptavidin-based chemiluminescent protocol, depending on how the protein was labeled. A full-length template free of mutation will code for a full-length labeled protein. If an internal deletion greater than 25 bp, a nonsense mutation, or a splice-junction (exon-skipping) mutation is present in the gene, then a shortened (truncated) protein will be produced (Figure 7.9). Similarly, a truncating mutation within a segment of the coding region will produce a protein smaller than the one encoded by the same fragment that is free of such a mutation. DNA sequencing is required to establish the nature of the nucleotide change that causes protein truncation.

key terms

capillary electrophoresis
chemical mismatch cleavage
denaturing gradient gel electrophoresis
direct DNA sequencing
functional/candidate gene cloning
heteroduplex analysis
mutation detection assay
positional-candidate gene cloning
protein truncation test
single-strand conformation polymorphism

summary

There are two basic strategies for discovering disease-causing genes. With the functional/candidate gene cloning approach, the coding sequence of a protein is entered into the human genome database to find the corresponding gene. With the positional-candidate approach, linkage studies localize the chromosome site of the disease-causing gene and the human genome database is examined for the likely candidate genes within the region of the mapped site. Regardless of the gene discovery procedure, proof that a putative gene represents the gene for a particular disorder depends on detecting in affected individuals nucleotide changes that are not present in the same gene of those who do not have the disorder. To this end, the gene sequence information retrieved from the human genome base is used to formulate any one of a number of mutation detection assays such as single-strand conformation polymorphism, denaturing gradient gel electrophoresis, heteroduplex analysis, chemical mismatch cleavage, and direct DNA synthesis. The protein truncation test is used to detect nonsense mutations.

References

Andersen, P. S. et al. 2003. Capillary electrophoresis-based single strand DNA conformation analysis in high-throughput mutation screening. *Hum Mutat* **21**:455–465.

Andersen, P. S. et al. 2003. High-throughput single strand conformation polymorphism mutation detection by automated capillary array electrophoresis: validation of the method. *Hum Mutat* **21**:116–122.

Condie, A. et al. 1993. Detection of point mutations in the p53 gene: comparison of single-strand conformation polymorphism, constant denaturant gel electrophoresis, and hydroxylamine and osmium tetroxide techniques. *Hum Mutat* **2**:58–66.

Gite, S. et al. 2003. A high-throughput nonisotopic protein truncation test. *Nat Biotechnol* **21**:194–197.

Glavac, D. and M. Dean. 1995. Applications of heteroduplex analysis for mutation detection in disease genes. *Hum Mutat* **6**:281–287.

Grompe, M. 1993. The rapid detection of unknown mutations in nucleic acids. *Nature Genet* **5**:111–116.

Hoskins, B. E. et al. 2003. Evaluation of multiplex capillary heteroduplex analysis: a rapid and sensitive mutation screening technique. *Hum Mutat* **22**:151–157.

Jordanova, A. et al. 1997. SSCP analysis: a blind sensitivity trial. *Hum Mutat* **10**:65–70.

Li, Q. et al. 2002. Integrated platform for detection of DNA sequence variants using capillary array electrophoresis. *Electrophoresis* **23**:1499–1511.

Nature. 2001. The human genome. *Nature* **409**:814–958.

Petersen, D. C. et al. 2002. Novel mutations and SNPs identified in *CCR2* using a new comprehensive denaturing gradient gel electrophoresis assay. *Hum Mutat* **20**:253–259.

Roest, P. A. et al. 1993. Protein truncation test (PTT) for rapid detection of translation-terminating mutations. *Hum Mol Genet* **2**:1719–1721.

Science. 2001. The human genome. *Science* **291**:1177–1357.

van der Luijt, R. B. and P. M. Khan. 1996. Protein truncation test for presymptomatic diagnosis of familial adenomatous polyposis. *Methods Mol Genet* **8**:97–112.

Xiao, W. and P. J. Oefner. 2001. Denaturing high-performance liquid chromatography: A review. *Hum Mutat* **17**:439–474.

review questions

1. Explain the rationale and the key methodological features of functional/candidate gene cloning.
2. Explain the rationale and the key methodological features of positional-candidate gene cloning.
3. Why is a mutation detection assay an essential component of any gene isolation strategy?
4. Describe how SSCP is used to detect gene mutations.
5. Describe how DDGE is used to detect gene mutations.

chapter 8

Bioinformatics: Genomics, Functional Genomics, and Proteomics

Computers are useless. They can only give answers.

PABLO PICASSO (1881–1973)

Knowledge is of two kinds. We know a subject ourselves, or we know where we can find information upon it.

SAMUEL JOHNSON (1709–1784)

SCIENTIFIC INFORMATION is generally presented for expert scrutiny in peer-reviewed articles that are published in professional periodicals. However, in the mid- to late 1980s, molecular biology journals were devoting more and more pages to DNA, RNA, and protein sequences of single-cloned genes. Moreover, anyone who wanted to conduct comparative analyses of nucleotide or amino acid sequences among related genes or proteins as well as other kinds of analyses had the unenviable task of typing out all the sequences from the relevant publications. The GenBank database was established in 1988 to accommodate the flood of DNA sequences. The purpose of GenBank was the collection, management, storage, and distribution of sequence data. At first, submissions to GenBank were sporadic, but almost complete compliance was achieved when many journals made database submission a prerequisite for publication. Initially, access to the GenBank database was through servers that were linked to NSFnet (National Science Foundation Network). By current standards, the original GenBank interface was primitive and the download times were interminable. From 1990 to 1995, large-scale projects, such as genetic and physical mapping of the human genome, partial sequencing of thousands of cDNAs (ESTs), and sequencing of entire genomes required additional databases for storing and retrieving information. NSFnet was replaced in 1995 with the development of the Internet (World Wide Web). Thereafter, submissions, access, and especially retrieval (data mining) of stored molecular information became rapid and easy. The visual aspects of the online sites improved dramatically, and links among relevant databases were established. Ready access through the Internet led to the creation of specialized databases including those for gene-specific mutations, regulatory sequences, mitochondrial genes and functions, specific human

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Table 8.1 Types of molecular databases.

Bibliographic Resources
Cellular Regulation
Chromosome Aberration
Comparative Genomics
Gene Expression
Gene Identification And Structure
Gene Mutation
Gene Sequences
Genetic And Physical Maps
Genetic Disorders
Genomic Sequences
Intermolecular Interactions
Metabolic Pathways
Protein Motifs
Protein Sequences
Protein Structure
Proteome Resources
RNA Sequences
Transgenic Organisms

genetic diseases, protein-protein interactions, protein structures, gene expression data, and many other types (Table 8.1). More specifically, after 15 years, GenBank contained more than 17 billion nucleotide bases from about 14 million sequences derived from more than 100,000 different organisms. Currently, there are about 400 molecular databases that range from major DNA and protein sequence repositories (e.g., GenBank, Ensembl, UCSC Gene Browser, Genome Database, Protein Information Resource, Swiss-Prot) to bibliographic and information resources (e.g., OMIM, PubMed, RefSeq, Gene Clinics) (Table 8.2). Details about many of these databases are available at http://nar.oupjournals.org.

Similarity Search of a DNA Database

Clearly, the proliferation of molecular databases would not have been possible without either high-speed computers or the programmers who develop the means whereby the information can be used by the scientific community. Because of its distinctive nature, the extensive use of computers for storage and analysis of molecular data has become known as bioinformatics. Broadly speaking, bioinformatics is the development and application of computational tools for the submission, storage, organization, archiving, acquisition, analysis, and visualization of biological and medical data. This information is routinely accessed through Internet sites that are connected to other databases. Onsite tutorials (e.g., http://www.elsi.ac.uk/2can/home.html) provide instructions for both accessing the information and explaining how to use the software tools for sequence similarity searches, gene prediction, and many other kinds of analyses. The molecular databases are designed to meet the needs of researchers and are not meant for curious visitors.

Here, brief step-by-step demonstrations of sequence retrieval and a sequence similarity search will illustrate some of the features of DNA databases. First, the coding sequence (cDNA) of the human cystic fibrosis transmembrane

Table 8.2 Molecular databases.

Database	URL	Description
ALFRED	http://alfred.med.yale.edu	Allele frequencies and DNA polymorphisms
Alzheimer Disease Mutations	http://molgen-www.uia.ac.be/ADmutations/	All gene mutations related to Alzheimer disease
ArrayExpress	http://www.ebi.ac.uk/arrayexpress	Microarray gene expression data
Atlas of Genetics and Cytogenetics in Oncology and Hematology	http://www.infobiogen.fr/services/chromcancer/	Genes, cytogenetics, and clinical features of cancer and cancer-prone diseases
Cooperative Human Linkage Center	http://gai.nci.nih.gov/CHLC/	Integrated genetic and marker maps of human chromosomes
Database of Interacting Proteins (DIP)	http://dip.doe-mbi.ucla.edu	Protein–protein interactions
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	Single-nucleotide polymorphisms
DNA Data Bank of Japan (DDBJ)	http://www.ddbj.nig.ac.jp	All known nucleotide and protein sequences
EMBL Nucleotide Sequence Database	http://www.ebi.ac.uk/embl.html	All known nucleotide and protein sequences
Ensembl	http://www.ensembl.org/	Annotated information on eukaryotic genomes
ExPASy Molecular Biology Server	http://ca.expasy.org	Expert Protein Analysis System. Links to protein databases
GDB	http://www.gdb.org	Human genes and genomic maps
GenAtlas	http://www.citi2.fr/GENATLAS/	Human genes, markers, and phenotypes
GenBank	http://www.ncbi.nlm.nih.gov/	All known nucleotide and protein sequences
GeneCards	http://bioinfo.weizmann.ac.il/cards/	Integrated database of human genes, maps, proteins, and diseases
GeneClinics	http://www.geneclinics.org/	Medical genetics information resource
Genew	http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl	Nomenclature for human genes
HugeIndex	http://hugeindex.org	mRNA expression levels of human genes in normal tissues
HuGeMap	http://www.infobiogen.fr/services/Hugemap	Human genome genetic and physical map data
Human Gene Mutation Database	http://www.hgmd.org	Links to locus-specific mutation databases
Kyoto Encyclopedia of Genes and Genomes (KEGG)	http://www.genome.ad.jp/kegg	Metabolic and regulatory pathways
MITOMAP	http://www.gen.emory.edu/mitomap.html	Human mitochondrial genome
Online Mendelian Inheritance in Man	http://www.ncbi.nlm.nih.gov/Omim/	Catalog of human genetic and genomic disorders
PROSITE	http://www.expasy.org/prosite	Biologically significant protein patterns and profiles
Protein Information Resource (PIR)	http://pir.georgetown.edu	Comprehensive, annotated, nonredundant protein sequences
PubMed	http://www.ncbi.nlm.nih.gov/PubMed/	Abstracts of journal articles
RefSeq	http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html	Nonredundant sequences from genomes, genes, transcripts, and proteins
SNP Consortium database	http://snp.cshl.org	Single-nucleotide polymorphisms
Stanford Microarray Database	http://genome-www.stanford.edu/microarray	Raw and normalized data from microarray experiments
Swiss-Protein/TrEMBL	http://www.expasy.ch/sprot	Protein sequences
UCSC Genome Browser	http://genome.ucsc.edu/	Genome assemblies and annotation

Adapted from A. D. Baxevanis (2003). *The Molecular Biology Database Collection: 2003 Update*, *Nucl Acids Rev* **31**:1–12.

conductance regulator (*CFTR*) gene will be retrieved. Next, a segment of the CFTR cDNA will be used for a similarity search of a DNA database to find any related sequences. Finally, the results of the search will be explained.

Although cDNA sequences can be retrieved from different databases, the Genome Database (GDB) is a good place to access a known human cDNA sequence. On the GDB home page (http://www.gdb.org.gdb/), type in CFTR in the "query" box, and click on the "SUBMIT" box. Don't change the default settings. On the "Genomic Segment Query Results" page, click on GDB:120584. The "Gene CFTR" page provides a number of links pertaining to the *CFTR* gene such as cytogenetic localization and both nucleotide acid and protein sequence data. You may want to check out these links later. However, for the current exercise, under "Nucleic Acid Sequences Links:", click on "M28668 (cDNA)" to go to the "Nucleic Acid Sequence Database Preference" page. Click on "EMBL sample'. This link accesses the complete sequence of the CFTR cDNA in the GenBank format. After the CFTR sequence information is loaded, scroll down to the cDNA nucleotide sequence that begins after the SQ line with the nucleotides "aattggaagc", highlight the first 600 nucleotides including the numbers, and copy this portion of the CFTR cDNA sequence into your clipboard.

For the rest of the demonstration, go to the home page of the National Center for Biotechnology Information (http://www.ncbc.nlm.nih.gov/) and click on "BLAST" in the menu bar. Then, under the section entitled "Nucleotide", select "nucleotide-nucleotide BLAST (blastn)", which will open the BLAST input page. Paste the CFTR cDNA sequence (query sequence) from the clipboard into the "Search" box. Leave the database setting at nr (nonredundant) and click on the "BLAST!" button. The "formatting BLAST" page will provide an estimate of the time that it will take to complete the analysis. It should not take more than 60 seconds to get the results with our query sequence. After waiting a minute or so, click on the "Format!" button. Save the BLASTN results to hard drive so the results can be examined offline.

Before examining the output in detail, a brief explanation of the BLAST program is necessary. The basic local alignment sequence tool (BLAST) is a family of applications that are designed for accurate and rapid searches of sequence databases with either a nucleotide or a protein query sequence. Blastn is used for nucleotide-nucleotide alignments. A query sequence is matched with the total set of sequences in a database. A raw score is calculated based on assigned values for identities, mismatches, gaps, and the number of characters in a gap with each alignment between the query sequence and sequences of the database. This raw score, in turn, is converted with normalizing parameters into a bit score (S). Finally, based on the bit score, an E-value (expect value) for each alignment is calculated. A high bit score (S) indicates that there is a reasonable alignment between two sequences, but this value is not sufficient to conclude that an alignment is significant. The E-value takes into consideration both the S value and the length of the aligned portion of the query sequence and the total sequence length in the database. Consequently, values for S and E with a particular query sequence will change as more sequences are added to the database. If an E-value is considerably less than 1.0, then the alignment is unlikely to occur by chance and, therefore, it is probably biolog-

ically significant. In other words, the alignment is real. A BLAST tutorial is available at http:www.ncbi.nlm.nih.gov/Education/BLASTinfo/guide.html.

More specifically, as expected with our query sequence, excellent alignments were observed with CFTR sequences from humans, rhesus monkey (*Macaca mulatta*) and other primates; cattle (*Bos taurus*); sheep (*Ovis aries*), mouse (*Mus musculus*); and many other animals. The "Color Key for Alignment Scores" box of the BLAST output provides a pictorial overview of the results. The extent of an alignment with the query sequence and the location(s) of the aligned sequences with regard to the query sequence are indicated. Beneath the "Color key" and under the heading "Sequences producing significant alignments:" there is a list of aligned sequences and individual bit scores and E-values. In the third part of the output, under the heading "Alignments", each specific query-to-sequence alignment is presented. At the bottom of the "Sequences producing significant alignments:" list, the E-values that are close to 1.0 or slightly greater do not represent significant alignments despite perfect matches of segments containing about 20 nucleotides. For example, you can check whether the alignment of the sequence designated as "gi/30684800|ref/NM 118073.2|Arabidopsis thaliana disease resistance protein" or any other *Arabidopsis* sequence significantly matches any part of the query sequence by searching the *Arabidopsis* DNA database (http://www.arabidopsis.org/) with the CFTR cDNA query sequence. As you can readily confirm, a BLAST analysis reveals no significant alignments between the first 600 nucleotides of the CFTR cDNA and any *Arabidopsis* sequence.

A BLASTn report has additional features of interest that facilitate access to other relevant records. The start of each underlined blue line of the "Sequences producing significant alignments:" section is a link to a database entry for the matched sequence. The letters in the colored boxes at the end of some lines are links to sites that contain additional information about the selected sequence. The "Taxonomy reports" link yields a report of the organisms that are noted in the BLASTn output with both common names and complete taxonomic descriptions. Finally, although not part of the BLASTn output, a very helpful site that crosslinks with many databases and assembles extensive information about a gene or protein is Bioinformatics Harvester at http://harvester.embl.de. Try a Harvester search with CFTR as the query!

In sum, the adage "Necessity is the mother of invention" coined by Richard Franck (1624–1708) is more than apt for the relationship between bioinformatics and genomic research. As the amount of genomic data has expanded, accessible databases have been developed that have been augmented with additional information (annotations) and links to other sources of relevant data. Picasso was wrong. Computers are not useless. With the appropriate programs, information is accessible that otherwise would be impossible to obtain. Moreover, novel questions can now be asked that previously were considered unanswerable.

Functional Genomics

Genomics encompasses the study of all features of genomes and individual genes at the DNA level including mutations, polymorphisms, and phylogenetic relationships that are based on sequence differences. Another aspect of

genomics, which is now called functional genomics, is concerned with the patterns of transcription (gene expression), either qualitatively or quantitatively, as a function of clinical conditions, in response to natural or toxic agents, or at different times during biological processes such as the cell cycle. One of the aims of gene expression studies is to discover the genes that are up- and down-regulated under specific conditions. In the past, the transcription of only one or a few genes could be followed at a time. Currently, functional genomic methodology tracks the simultaneous transcription of thousands of genes (gene expression profiling) of either a cell or a tissue sample. The main experimental approaches for determining gene expression profiles are DNA microarrays and serial analysis of gene expression (SAGE). Because of the large amount of data that is generated from these experiments, special computational tools are required for obtaining, storing, and analyzing the results.

DNA Microarray Technology

A DNA microarray (DNA chip, gene chip) experiment consists of hybridizing a nucleic acid sample derived from the mRNAs of a cell or tissue to single-stranded probes that are bound in an ordered arrangement on a solid platform. One type of DNA microarray is formed by spotting PCR-amplified cDNA sequences from the mRNAs of a single cell or all the coding sequences of an organism onto a glass slide or nylon membrane. In this case, about 10,000 different probes can be arrayed in a 1-cm^2 area.

An alternative microarray system is made up of sets of oligonucleotide probes, usually 25 nucleotides in length, representing thousands of genes, that are synthesized directly (in situ) on a quartz wafer by photolithography. Briefly, for the formation of this type of oligonucleotide array, a common first nucleotide of every probe is tethered to a quartz wafer in a 11-mm area (probe cell). Then, the surface of the wafer is flooded with a nucleotide, say G, and, under computer control, those probe cells that do not have G as the first added nucleotide are shielded (masked) from the light that stimulates the joining reaction. In other words, nucleotide joining takes place only in unmasked probe cells. After the G-joining reaction is complete, the wafer is washed and flooded with another nucleotide, say C. The probe cells that do not have C as a first added nucleotide and those that already have an added G are masked and the joining reaction proceeds in unmasked probe cells. This process is continued until the remaining initial nucleotides, A and T, are added. Thereafter, the computer-controlled addition reactions are repeated until each probe cell contains thousands of copies of an oligonucleotide with a specific nucleotide sequence. With gene-based oligonucleotide arrays, there are 11 to 20 pairs of probes near the 3′ end of each gene. A pair of probes consists of a sense and an antisense sequence. Multiple probes are used for each gene to distinguish between specific and nonspecific hybridizations. A full oligonucleotide array may contain more than 500,000 probe cells representing as many as 30,000 genes.

Typically, for most gene expression profiling experiments with spotted cDNA microarrays, mRNAs from two different sources, such as disease and normal cells, are extracted, purified and reverse transcribed to first-strand cDNA sequences. Each batch of cDNA is labeled with a different fluorophore. Generally, a green-emitting fluorescent dye (Cy3) is used for the normal (ref-

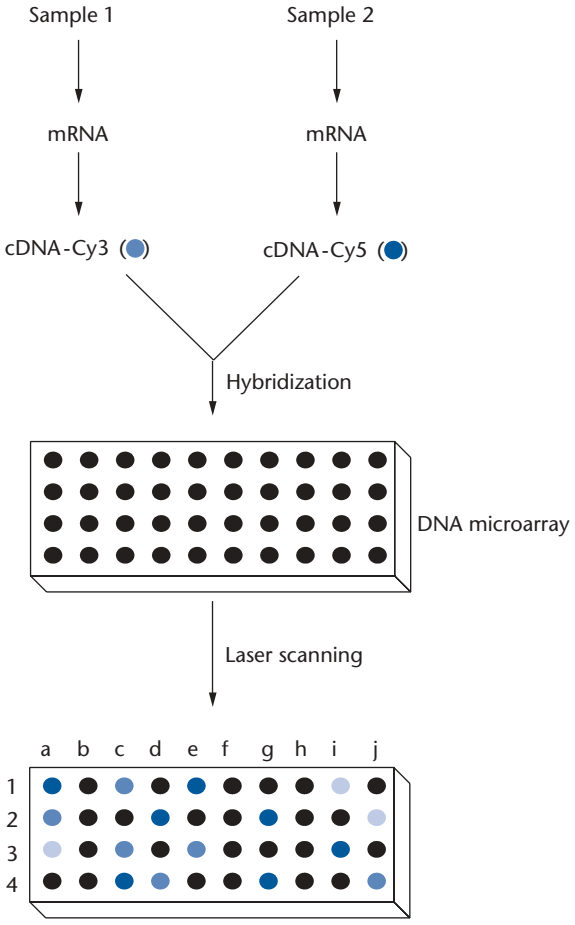

Figure 8.1 Gene expression profiling with a DNA microarray. Messenger RNA is extracted from two samples (sample 1, sample 2), and during reverse transcription the first cDNA strands are labeled with the fluorescent dyes Cy3 and Cy5, respectively. The cDNA samples are mixed and hybridized to an ordered array of either gene sequences or gene-specific oligonucleotides. After the hybridization reaction, each probe cell is scanned for both fluorescent dyes and the separate emissions are recorded. Probe cells that produce only a green or red emission represent genes that are transcribed in samples 1 and 2, respectively; yellow emissions denote genes that are active in both samples; and no emissions (black) represent genes that are not transcribed in either sample. Here, the dyes are depicted as blue and light blue and the combined spots are gray.

erence) sample and a red-emitting fluorescent dye (Cy5) for the test sample (Figure 8.1). After labeling, the cDNA samples are mixed and hybridized to the same microarray. After hybridization, a confocal scanning laser microscope is used to scan the spots. The individual emissions from the dyes at each spot are recorded and stored. Overall, with a red signal, the gene is transcribed only in the reference cells and is downregulated in the other cell population. With green fluorescence, a gene is transcribed only in the test cells, that is, upregulated. With a yellow emission, the gene is transcribed in both cell populations. The absence of any signal indicates that the gene is not transcribed in either cell population. The levels of emissions for Cy3 and Cy5 of yellow spots are used to estimate the difference in expression of genes in the two cell samples. For example, if the emission value for Cy5 from a spot is half the Cy3 emission from the same spot, then there is a twofold reduction of the expression of this gene between the test and reference samples.

Target sequences are not prepared in the same way for oligonucleotide and cDNA microarrays. For oligonucleotide microarrays, mRNA is purified with an oligo(dT) sequence that has a bacteriophage T7 RNA polymerase promoter sequence (T7 primer) extension (Figure 8.2). Double-stranded cDNA is synthesized from the mRNA. Then, with T7 RNA polymerase in the presence of a biotinylated ribonucleotide, a biotin-labeled RNA copy (cRNA, antisense

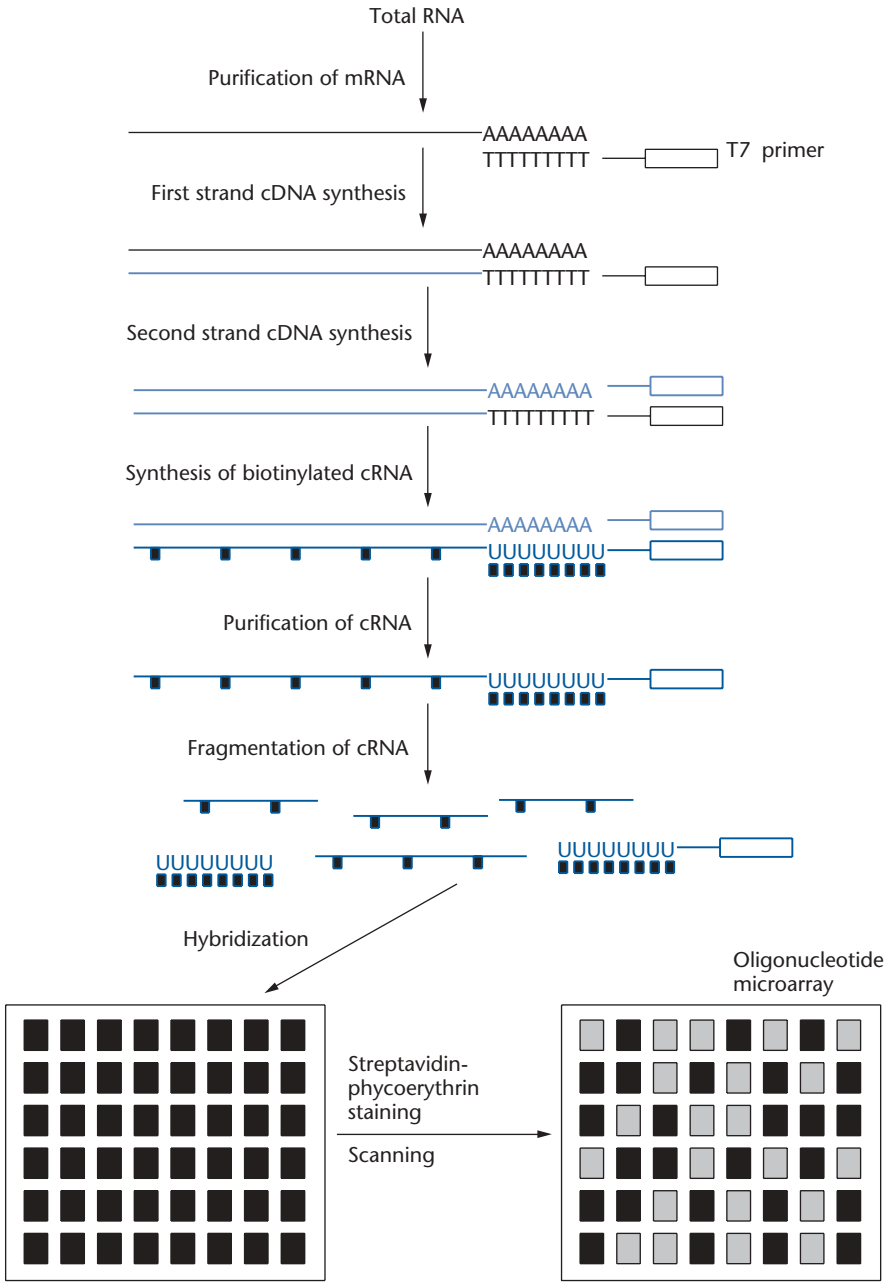

Figure 8.2 Gene expression profiling with an oligonucleotide microarray. Messenger RNA is purified with a poly(dT) sequence that has a T7 RNA polymerase primer sequence extension. After two-stranded cDNA synthesis, the second cDNA strand acts as a template for T7 RNA polymerase in the presence of biotinylated nucleotides (blue rectangles). The biotinylated cRNA is purified, fragmented into pieces from 50 to 100 nucleotides in length, and hybridized to an oligonucleotide microarray. The microarray is stained with streptavidin-phycoerythrin, and the probe cells (black) are scanned for emission from the biotin-bound streptavidin-phycoerythrin. Positive-response probe cells are shown here as gray.

RNA, aRNA) of the second cDNA strand is synthesized. The cRNA is fragmented to pieces from 50 to 100 nucleotides in length for optimal hybridization. After hybridization, the microarray is treated with streptavidin-phycoerythrin (SAPE). The streptavidin binds to biotin residues of hybridized cRNA, and the locations of the emissions from phycoerythrin that are elicited during laser scanning are recorded. For comparative gene expression profiles, separate experiments are run and the relative transcript abundance is calculated from the ratio of fluorescence values of each probe cell in the two microarrays.

Because of the vast amount of data generated by microarray experiments, specialized software has been developed to maximize the output of information. In this way, clusters of genes that are differentially expressed, that is, upregulated or downregulated, can be identified. The fundamental inference is that a group of coexpressed genes is likely coregulated and, consequently, the products of these genes participate in the same biological function. However, this supposition may not be entirely valid. Microarray experiments with *Drosophila* indicate that genes within a segment of a chromosome that do not have a common function often are transcribed at the same time. Thus, some members of a coexpressed cluster probably result from a physical change at the chromatin level that allows transcription of genes that are not involved in a particular process. If this observation holds for humans, then further research will be required to determine between functional and nonfunctional gene transcription. This notwithstanding, any consistent differences will help clinicians distinguish between diseased and normal cell states. In fact, microarray analyses have uncovered discrete transcription patterns between closely related disorders that should lead to the identification of biomarkers for defining and tracking successive stages of progressive disorders and, hopefully, identify potential targets for disease-specific therapies.

Serial Analysis of Gene Expression

Unlike DNA microarrays that rely on hybridization and signal detection, serial analysis of gene expression (SAGE) uses recombinant DNA techniques, DNA sequencing, and computer sequence searches to identify most of the genes that contribute to a population of mRNAs (Figure 8.3). Polyadenylated mRNA is captured by an oligo(dT) sequence that is labeled with biotin and attached to a streptavidin-coated magnetic bead. Double-strand cDNA is synthesized from the purified mRNA sample. A strong magnet is used to retain the magnetic beads with attached cDNAs in a single reaction tube during successive treatments and washings. The cDNAs are cut with the restriction endonuclease *Nla*III, which recognizes the sequence CATG/GTAC and cuts outside to the G:C base pairs, leaving a 3′ GTAC extension. Because *Nla*III cuts, on the average, 1 in 256 base pairs, there is a high probability that each cDNA will have at least one *Nla*III recognition site. Because the cDNAs are bound to beads, each *Nla*III cut site that is closest to the 3′ end of a cDNA is retained and all unbound fragments are washed away. In the nomenclature developed for SAGE, *Nla*III is called an anchoring enzyme. Next, the *Nla*III-digested cDNA sample is divided into two. One aliquot is ligated with adapter A and the other with adapter B. Both adapters have a CATG extension that is complementary to the extension produced by *Nla*III digestion, a 5-base pair recognition site for the restriction endonuclease *Bsm*FI, and a sequence for priming a PCR reaction. The adapters have different primer sequences to prevent base pairing (snap back) during PCR. After the adapters are ligated to *Nla*III-cut cDNA, the products are treated with *Bsm*FI. This type IIS restriction endonuclease binds to its recognition site, but unlike *Nla*III and other type II restriction endonucleases, it cuts 10 nucleotides downstream of one DNA strand and 14 nucleotides in the other strand from the recognition site regardless of the intervening nucleotide sequence. In SAGE parlance, *Bsm*FI is a tagging

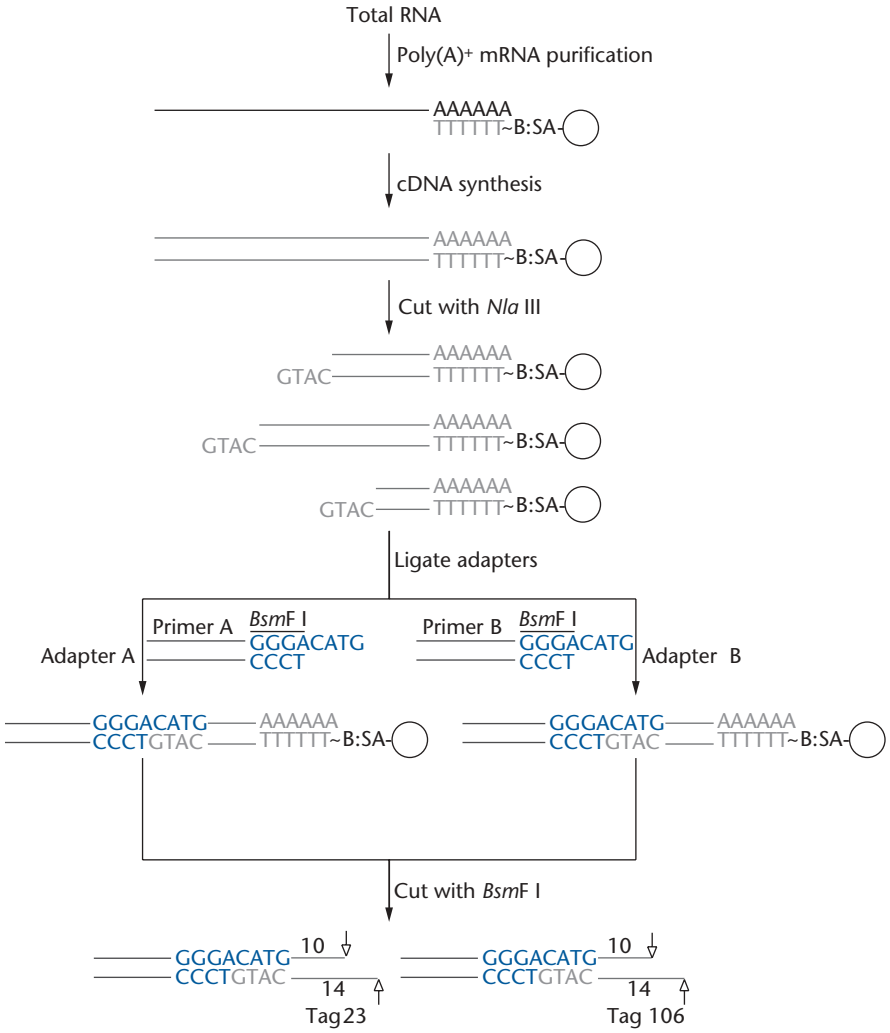

Figure 8.3 Serial analysis of gene expression (SAGE). Poly(A)$^+$-messenger RNA transcripts are isolated by poly(dT) hybridization. The poly(dT) sequence is biotinylated (B) and bound to streptavidin (SA)-coated magnetic beads (open circles). Double-stranded cDNAs are synthesized from the captured mRNAs and then cut with the restriction endonuclease *Nla*III. The fragments that are not bound to the magnetic beads are eluted. The *Nla*III-cut cDNA sample that is attached to the magnetic beads is divided into two: one sample is ligated with adapter A and the other with adapter B. Each adapter contains a 4-base extension that is complementary to the 4-base extension produced by cleavage with *Nla*III; a recognition site for the type IIS restriction endonuclease *Bsm*FI; and a different primer sequence (Primer A, Primer B). The ligated adapter-*Nla*III-cut molecules are treated with *Bsm*FI, which cleaves the DNA 10 and 14 nucleotides downstream from its recognition site (open arrows). The extensions of the *Bsm*FI fragments are filled in by DNA synthesis, and the mixture is blunt-end ligated. Some of the ligated molecules are two joined segments from different cDNAs (ditags) that are flanked by primer sequences. The ditags are amplified by PCR and then treated with *Nla*III. Ligation of *Nla*III-cut ditags forms concatemers of various ditags. Concatemers with about 20 ditags (~500 base pairs) are purified and cloned into a plasmid vector. The concatemers are sequenced, the individual tags are identified, the likely corresponding gene is determined by a similarity search, and the frequency of each tag in the sample is recorded.

enzyme. The segment of cDNA produced by the *Bsm*FI treatment is called a tag. The adapter-tag molecules are released into solution. The 4-nucleotide extension of the *Bsm*FI-cut DNA is filled in to form a blunt-end molecule. The sample is ligated. Under these conditions, among other possibilities, the blunt ends of two tags are joined to form a two-tag molecule, a ditag, that is flanked by primer sequences. Because ditag formation is completely random, tags from different cDNAs are joined during ligation. Of all the ligation products, the ditags are readily amplified during PCR. The amplified ditags are treated with *Nla*III to release the adapter sequences and produce ditags with an *Nla*III extension at each end. The *Nla*III-cut ditags are separated from the adapter sequences and ligated to form randomly joined, multiple combinations (concatemers) of ditags. Concatemers that are about 500 base pairs in length are isolated and cloned in an *E. coli* plasmid. The concatemers are sequenced, the sequence of each tag is recorded, and a specialized "tag to gene" database is searched to identify the corresponding or the most likely gene. In addition, based on the information from DNA sequencing, the number of times each tag is sequenced is determined, which represents its abundance in the initial

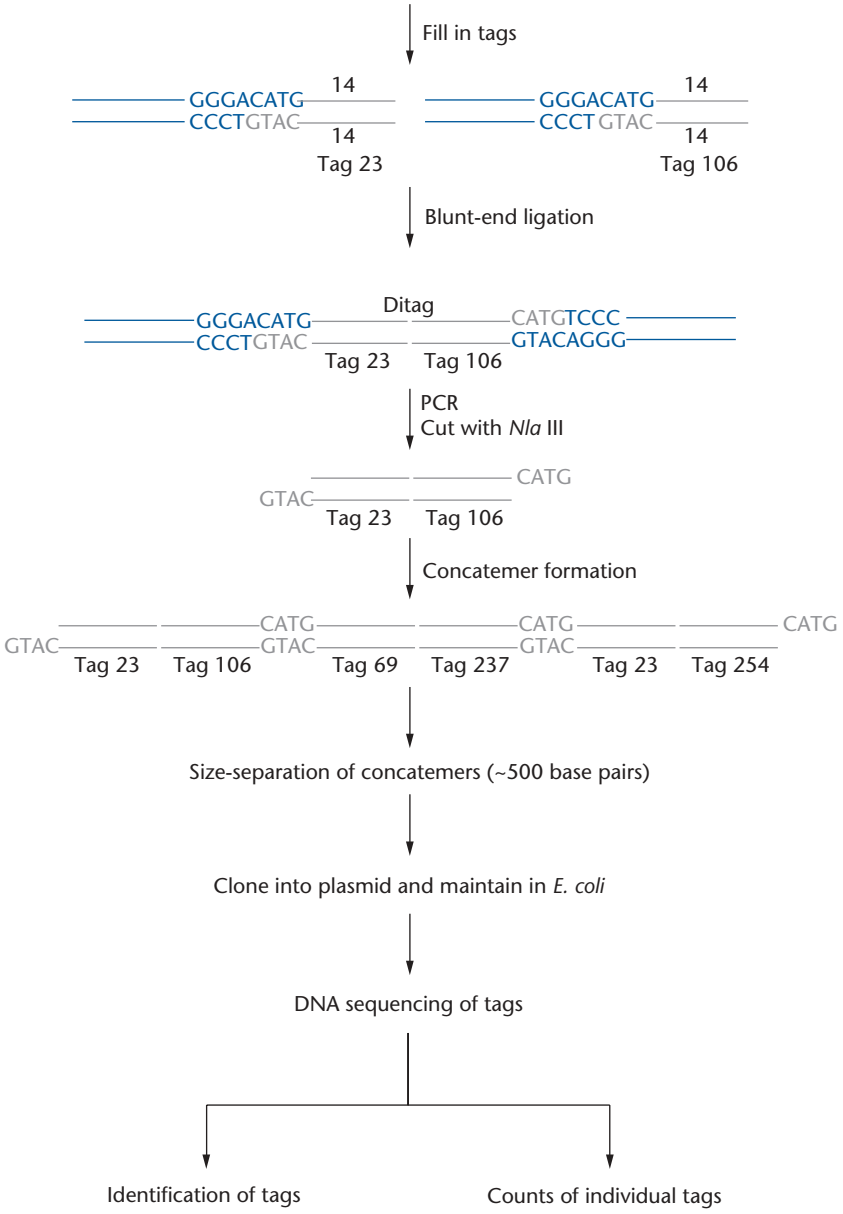

Figure 8.3 *continued*

sample. Up- and downregulated mRNAs can be identified by comparing the frequencies of tags in different samples.

Other anchoring and tag enzyme combinations have been used to identify transcribed genes that do not have a *Nla*III site and produce longer tags. Online resources (http:// www.ncbc.nlm.nih.gov/SAGE, http://www.cgap.nci.nih.gov/SAGE) are available for matching tags to likely genes, determining the frequency of a tag among various SAGE libraries, and providing other pertinent information. (See review question 10.)

Proteomics

Proteomics is the comprehensive study of all the proteins, that is, the proteome, of a cell, tissue, body fluid, or organism from a variety of perspectives includ-

ing structure, function, expression profiling, and protein-protein interactions. From a practical standpoint, this information is used to track clinical disorders and detect targets for therapeutic treatments. Generally, the suffix "omics" implies large-scale experimentation with the analysis of many samples at one time. As a consequence of this "high throughput," there is a heavy reliance on computers and computer programs for assembling, analyzing, archiving, and distributing proteomic data. Diverse computer resources have been developed to handle various kinds of proteomic information.

A number of factors complicate the study of proteins. First, there are many more proteins than genes because of alternative splicing, posttranslation modifications and, to a lesser extent, RNA editing. With about 60% to 70% of human genes undergoing alternative splicing, the human proteome may consist of 85,000 or more proteins. Second, it is impossible to account experimentally for every member of a proteome with a single technique because proteins are susceptible to degradation, have different solubilities, and range considerably in abundance. Despite these drawbacks, effective procedures have been devised for examining most of the components of many proteomes.

Separation and Identification of Proteins

Ordinarily, the study of the proteome of a multicellular organism is difficult. Thus, for convenience, the complexity is reduced by examining the protein complement of a cellular component or organelle such as the nucleolus, nuclear matrix, lysosome, endoplasmic reticulum, and so on. These protein subsets have been dubbed subproteomes. Because of its high resolving power, two-dimension polyacrylamide gel electrophoresis (2D PAGE) is frequently used to separate a population of proteins. Briefly, a protein sample is subjected to electrophoresis through an immobilized pH gradient in one dimension, that is, the first dimension, and then electrophoresed at right angles to the first dimension, that is, the second dimension, through a polyacrylamide gel. The proteins are separated in the first dimension on the basis of their net charge (isoelectric point) and in the second dimension according to molecular mass. The separated proteins form an array of spots in the gel that is visualized by staining with Coomassie Blue or silver. A 2D-polyacrylamide gel can resolve up to 2000 proteins. The pattern of stained spots is captured by densitometric scanning. Databases have been established with images of 2D PAGE gels from different cell types. Software packages are available for detecting spots, matching patterns between gels, and quantifying the protein content of the spots. Proteins with either low or high molecular weights and those that bind to membranes and are present in small amounts are not readily resolved by 2D PAGE. The next task after separation of most of the proteins of a proteome or subproteome is to identify as many of the proteins as possible. Mass spectrometry is commonly used for this purpose.

In principle, a mass spectrometer detects the masses of the ionized components of a molecule. The results are presented as a spectrum with the *x*-axis representing mass to charge (m/z) ratios and the *y*-axis representing the relative abundance of each ion with respect to the most abundant ion. In practice, mass spectrometers have different configurations according to the nature (wet or dry) of the sample (analyte), the mode of ionization of the analyte, how the

electric field(s) is established for accelerating the ions, and the method of detecting the different masses. Mass spectrometric studies of proteins and peptides have been facilitated by effective ionization methods such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). Peptide masses are usually determined by MALDI-mass spectrometry (MALDI-MS) and amino acid sequences by ESI-tandem mass spectrometry (ESI-MS/MS). Mass spectrometry is an important proteomic tool because analyses are rapid and accurate and require small amounts of starting material. Moreover, computational protocols are available for processing large amounts of mass spectrometry data.

Protein identification is straightforward because particular databases can be easily searched with either peptide mass or amino acid sequence data. To this end, a spot is cut out of a gel and then treated with trypsin, which cleaves on the C-terminal side of lysine and arginine residues. The salts, polyacrylamide, and other contaminants are removed before the peptides are concentrated. MALDI-MS is used regularly to determine the mass (m/z value) of each peptide in the sample. The values of the observed peptide masses are matched with the expected tryptic peptides for all known proteins (Figure 8.4). This type of analysis is called peptide mass fingerprinting. Online sites, such as http://US.expasy.org/tools/peptident.html, are available that rapidly find the set of peptide masses of a known protein that most likely corresponds to those of the unknown protein.

Alternatively, the amino acid sequence of a peptide can be obtained by ESI-MS/MS and used to search a protein database to identify an unknown protein (Figure 8.5). With this approach, the peptides derived from a spot in a 2D gel are first separated by mass and then one of the peptides is selected for sequencing. Fragmentation and ionization of a peptide occurs at amide bonds. When the charge from ionization is at the N-terminus it is designated a b-ion, and at the C-terminus it is a y-ion. Each ion type forms a ladder of subsequences consisting of one, two (dipeptide), three (tripeptide) and more amino acids up to the full-length peptide. An amino acid sequence is determined with the same ion type by calculating the difference of mass (Δm) between adjacent subsequences. This difference for members of a y-ion ladder represents the successive loss of an amino acid from the N-terminus. For the following y-ion subsequences VFDEFK, FDEFK, DEFK, EFK, FK, and K, the difference from one subsequence to the next is the removal of an amino acid from the N-terminus. In other words, for amino acid sequencing, the y-ions form a reverse mass ladder. The converse holds true for a b-ion spectrum. Each difference of mass is equivalent to the mass of a known amino acid except for leucine and isoleucine, which have the same mass. For example, with part of a y-ion series, the masses (m/z values) for five consecutive peaks from large to small are 1171.50, 1056.48, 942.43, 813.39, and 684.35 and the successive differences are 115.02, 114.05, 129.04, and 129.04. Thus, based on amino acid masses, the sequence is Asp-Asn-Glu-Glu. Automated programs are available that distinguish the ion types of a scan, remove as much noise as possible, and calculate the most likely amino acid sequence. Protein identification does not require complete sequencing of all of the peptides. Frequently, partial sequences of two or three peptides are sufficient for effective similarity searches of protein databases.

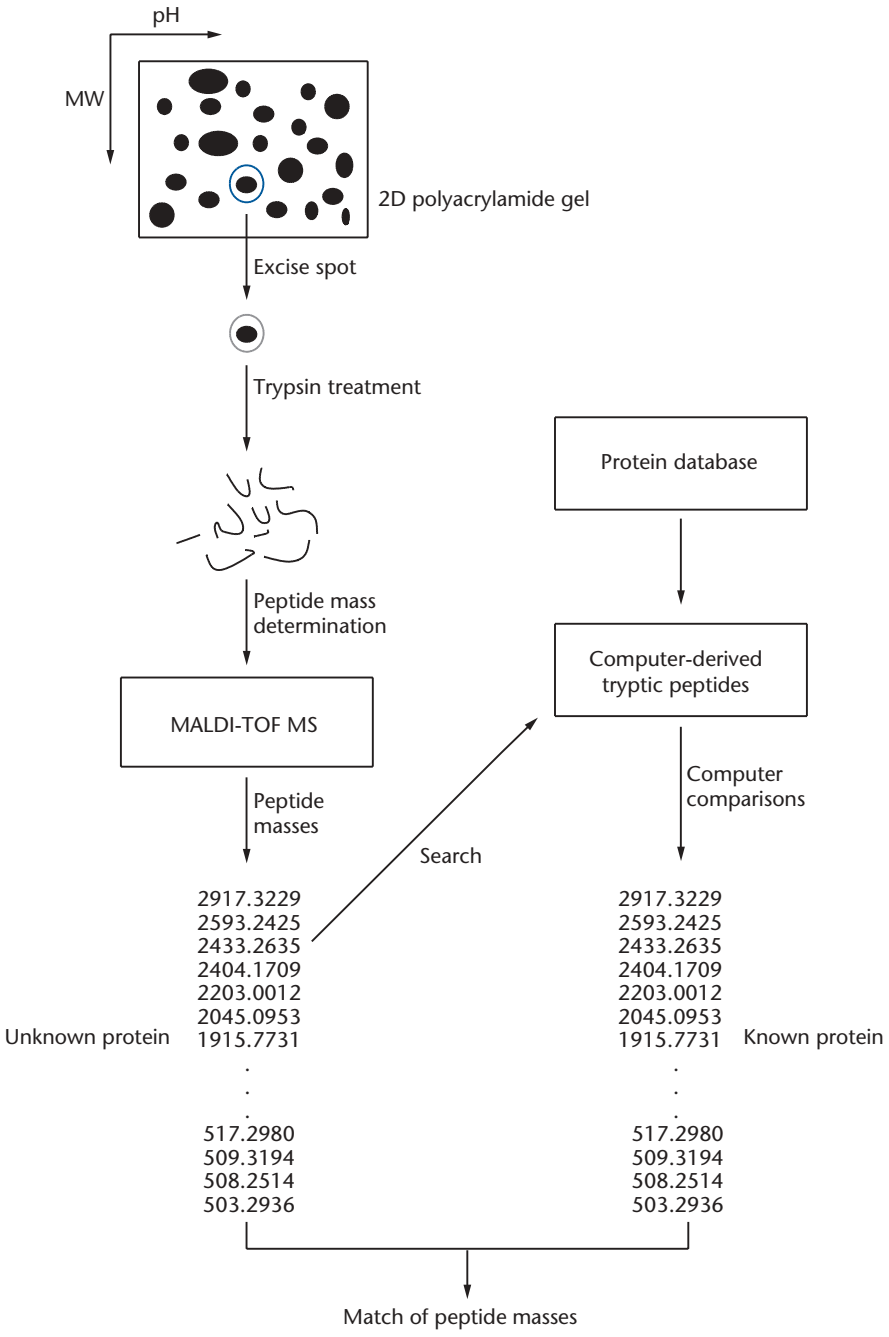

Figure 8.4 Peptide mass fingerprinting. A spot containing an unknown protein that was separated by two-dimension polyacrylamide gel electrophoresis is excised and treated with trypsin. Purified trypsin peptides are separated by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The set of peptide masses from the unknown protein are used to search a database with the masses of tryptic peptides for every known protein, and the best match is determined. Only some of the tryptic peptide masses for the unknown protein are listed in this example. (See review question 15 to identify the unknown protein.)

A nongel system called shotgun proteomics uses liquid chromatography (LC) combined with tandem mass spectrometry (LC-MS/MS) for analyzing the proteins of a proteome. In this case, a complete protein mixture is initially treated with a protease. Then, the peptides are separated by liquid chromatography and the amino acid sequence of each peptide is determined by MS/MS. Finally, the proteins are identified by database searches. Hundreds and, in some cases, thousands of proteins including those not well resolved by 2D PAGE have been recorded for proteomes and subproteomes with this approach.

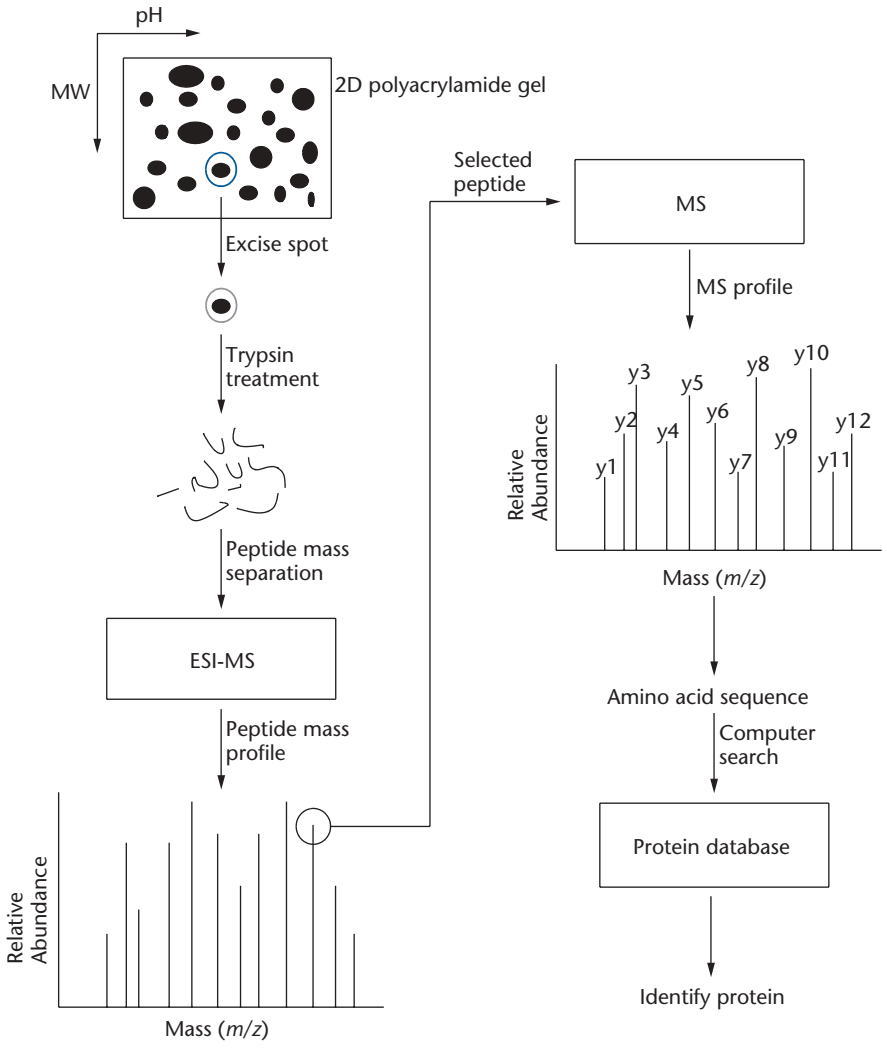

Figure 8.5 Protein identification by amino acid sequencing electrospray ionization (ESI) tandem mass spectrometry. A spot containing an unknown protein from a two-dimension polyacrylamide gel is excised and treated with trypsin to produce peptides. The tryptic peptides are separated according to their mass-to-charge ratios, and the amino acid sequence of a selected peptide is determined with a mass spectrometer (MS). Only the y-ion ladder (y1, y2, etc.) of a fragmented and ionized peptide is depicted here. The unknown protein is identified by searching a protein database with the amino acid sequences from two or more peptides.

Protein Expression Profiling

Protein expression profiling is important for pinpointing changes during pathological processes, cataloging differences between normal and cancer cells, and tracking cellular responses to toxic agents. Both gel and nongel methods have been developed for comparing the proteins of different samples.

With two-dimension differential in-gel electrophoresis (2D DIGE), proteins of one sample are labeled with the fluorescent dye Cy3 and those of a second sample with Cy5 (Figure 8.6). The labeled samples are mixed and run together in the same gel, which overcomes the variability between separate gel runs. The Cy3 and Cy5 protein patterns are visualized separately by fluorescent excitation. The images are compared, and any differences are recorded. In addition, the ratio of Cy3 to Cy5 fluorescence for each spot is determined to detect proteins that are either up- or downregulated. Unknown proteins are identified by mass spectrometry.

The isotope-coded affinity tag (ICAT) method combined with LC-MS/MS is another way of comparing proteins from different sources (Figure 8.7). An

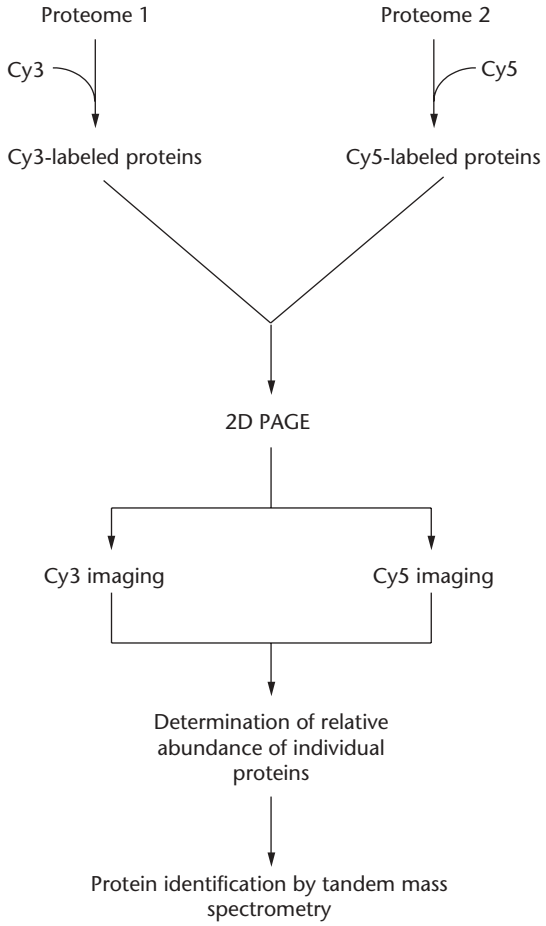

Figure 8.6 Two-dimension differential in-gel electrophoresis (2D DIGE) method for quantitative analysis of protein expression. The proteins of two proteomes are labeled with the fluorescent dyes Cy3 and Cy5, respectively. The fluorescence-labeled samples are combined and separated by two-dimension polyacrylamide gel electrophoresis (2D PAGE). The gel is scanned for each fluorescent dye, and the relative levels of the dyes in each protein spot are recorded. The gel is stained with a protein dye, and each spot with an unknown protein is excised and treated with trypsin. The peptides are separated by electrospray ionization tandem mass spectrometry, and the amino acid sequences of some selected peptides are determined. The protein is identified by searching a protein database for a likely match with amino sequences from two or more peptides.

ICAT reagent consists of an affinity tag (biotin), a carbon chain (mass-encoded linker) that is labeled with either eight hydrogen (light form, DO) or eight deuterium (heavy form, D8) atoms, and a chemical group that covalently binds to an amino acid, usually cysteine. Deuterium is a stable isotope of hydrogen that is twice its mass, that is, 2 daltons. Thus, the mass difference between a peptide that is labeled with a light and a heavy ICAT is 8 daltons. This difference is readily detected by mass spectrometry.

The proteins of one proteome are labeled with a light ICAT and those of another with a heavy ICAT. The samples are mixed, treated with trypsin, fractionated, and passed through an avidin column to capture only ICAT-labeled peptides. The purified ICAT-labeled peptides are partitioned by liquid chromatography before introduction into a mass spectrometer. The key feature of this technique is that the same ICAT-labeled peptides from the two samples will produce a pair of signals that differ by 4 or 8 daltons depending on whether the peptide is doubly or singly charged, respectively. Moreover, the ratio of light and heavy peptides with the same charge state reflects the relative amounts of the source proteins in the original samples. Finally, with ESI-MS/MS, the amino acid sequence of a peptide is determined and the protein that matches the sequence can be identified. Hundreds of proteins from different samples can be compared in this way.

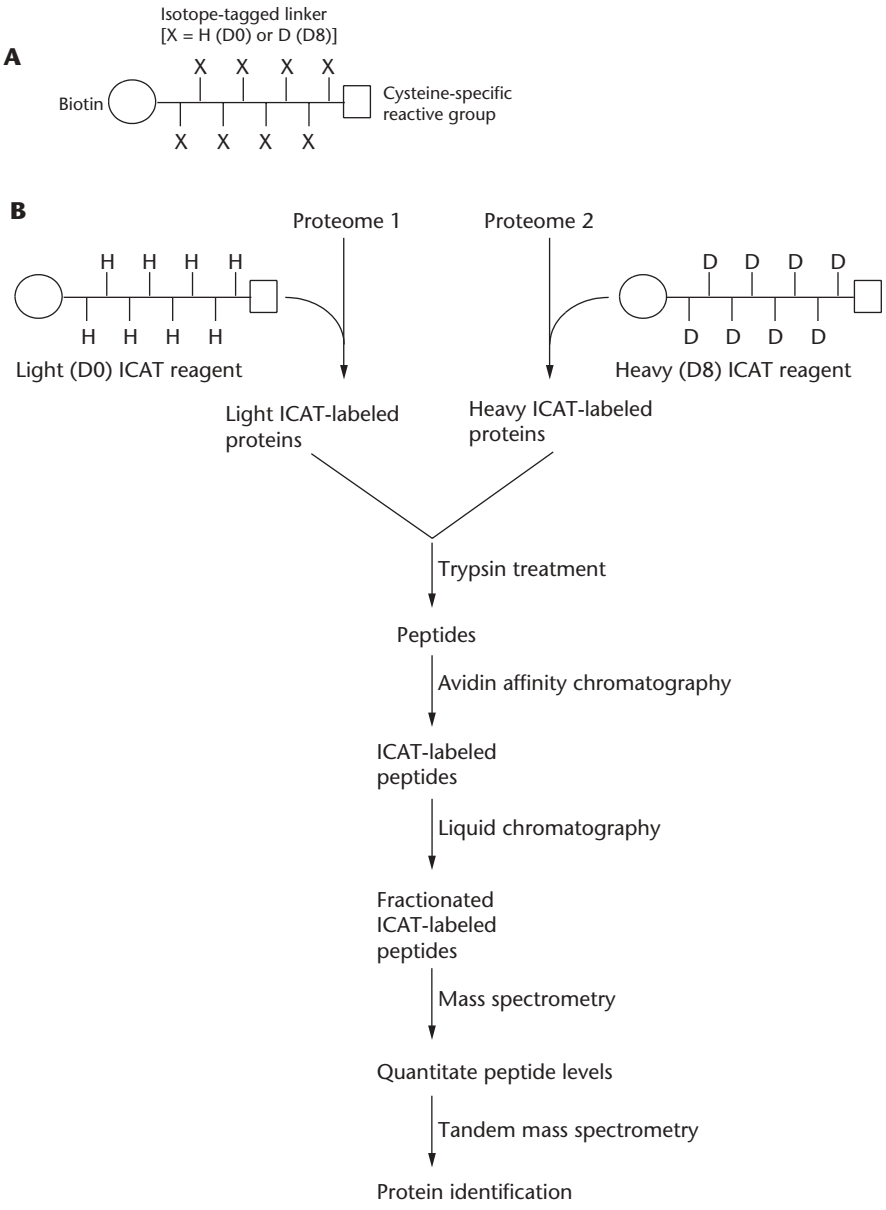

Figure 8.7 Isotope-coded affinity tag (ICAT) method for quantitative analysis of protein expression. (A) Schematic representation of an ICAT reagent. An ICAT reagent has either all hydrogen (H) or all deuterium (D) atoms at sites (X) of a linker sequence. (B) ICAT protocol. Proteins are extracted from two proteomes, and one sample is labeled with the light (D0, hydrogen only) ICAT reagent and the other with the heavy (D8, deuterium only) ICAT reagent. The samples are combined and treated with trypsin. The ICAT-labeled peptides are captured by avidin affinity chromatography and fractionated by liquid chromatography. The ratio of light to heavy (D0 : D8) versions of the same peptide is determined by mass spectrometry, which provides an estimate of the relative amounts of the protein in the two proteomes. The protein represented by a pair of heavy and light peptides is identified by amino acid sequencing with electrospray ionization tandem mass spectrometry and searching a protein database with this sequence for a likely match.

Protein-Protein Interaction Mapping

Proteins seldom act alone. On the average, one protein interacts with five others. Some protein-protein interactions are short-lived, others form stable multicomponent clusters, and, at a higher level of cellular organization, clusters interact with one another. Determining the functional interconnections among the members of a proteome is not an easy task. The strategies for examining protein-protein interactions on a large scale require a number of experimental manipulations with no guarantee that all potential interactions will be recognized. Notwithstanding the limitations of existing protocols, thousands of protein-protein interactions for proteomes of single-celled and multicellular organisms have been cataloged.

from the HUMAN GENETICS files

How Many Genes Do We Have?

The exact number of genes in the human genome has been difficult to pin down. Before the release of the draft sequences of the human genome in February 2001, it was thought that we had between 80,000 to 100,000 genes. Surprisingly, estimates based on analyses of the draft sequences ranged from 23,000 to 49,000, with a consensus value of about 30,000 genes. This gene number was considered to be a low for humans. By comparison, yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*), and roundworm (*Caenorhabditis elegans*) have about 6000, 13,6000 and 20,000 genes respectively. Consequently, based on our apparent biological complexity, it was perplexing that humans had only one-third more genes than a simple invertebrate and about as many as a tomato. To confound the gene number controversy, some researchers estimated that there were as many as 60,000, 89,000 or, quite possibly, 120,000 human genes.

Clearly, a number of questions come to mind. How are gene numbers predicted? Why do gene counts vary? What is the likely number of human genes? What is the significance of a low gene number?

One of the obstacles to an accurate human gene count is that coding genes make up only 3% to 5% of the total genome. Although a belabored cliché, gene counters are literally looking for a handful of needles hidden in a large haystack. There must be a precise identification process for any realistic tally of the total number of human genes. Unfortunately, eukaryote gene structure confounds gene enumeration. Exons are usually about 140 nucleotides in length and are separated by introns that may be thousands of nucleotides in length. As well, the sequences at the 5′ and 3′ ends (5′- and 3′-untranslated regions, 3′-UTR, 5′-UTR) of mRNAs are not translated and vary from gene to gene. However, there are some consistent landmarks that aid gene identification. The coding regions (open reading frames, ORFs) can be readily recognized. Also, at the 3′ end of most genes, there are a translation stop codon, a transcription termination site, and a sequence that signals poly(A) tailing. At the 5′ end, the translation start codon and transcription start site are consistent gene features. Finally, the splice sites that precede and follow the internal exons are regular motifs.

A number of gene prediction computer programs based on the common gene structures have been devised to scan the genome for possible genes. Generally, these programs are designated as ab initio, which signifies a "from the beginning" or, more specifically, "based on first principles" approach. The ab initio programs predict coding nucleotides with high accuracy and recognize exons with good efficiency, but are less than 50% effective at finding complete genes. In addition, the numbers of falsely identified genes (false positives) is high.

Alignments between a comprehensive set of cDNAs and the human genome is another way of identifying genes. Full-length cDNAs are an excellent resource for finding genes because they contain all the exons of a gene. However, variation in splicing of exons makes accurate gene identification somewhat arduous. More importantly, relatively few complete human cDNAs have been synthesized and sequenced, although techniques have been developed for capturing intact capped mRNAs. The major sources of cDNA sequences are expression sequence tag (EST) databases. These cDNAs are incomplete and concentrated at the 3′ end of the mRNAs. In addition, the databases contain about 10 to 15 ESTs for each gene. In some studies, a single representative sequence for each cDNA was determined to winnow down the overall number of cDNA sequences that would be needed for scanning genome sequences. The cDNA approach effectively locates exons. However, final gene counts are determined by combining the information about cDNA alignments with results from ab initio analyses. A number of genes are overlooked with this approach because not all genes are represented in the cDNA databases. As well, miscounting splice variants as a number of individual genes instead of a single gene can inflate a gene count.

Theoretically, the alignment of the complete genomic sequence of an organism such as the mouse or pufferfish to the entire human genome should locate highly conserved sequences, which would likely be exons and regulatory elements and produce poor similarity scores for introns and the DNA between genes (intergenic DNA). The principle underlying this comparative genome strategy for gene enumeration is that introns and intergenic DNA are not under the same biological constraint as exons and regulatory regions. Thus, nucleotide changes accumulate in introns and intergenic DNA, with the result that over a long duration in different species these sequences diverge from one another. By contrast, the sequence similarity of exons and regulatory elements is maintained between relatively closely related organisms because gene mutations lower reproductive fitness, which makes these sequence deviations less likely to be passed on from one generation to the next.

Generally, on the basis of different gene identification strategies, we probably have about 25,000 genes. This number will increase as genes without introns and those that do not encode proteins are discovered. Also, genes may be found when the long stretches of genomic DNA that appear devoid of genes, the so-called dark matter, are more thoroughly explored. Regardless of the exact gene number, human biological complexity is probably the consequence of intricately interconnected sets of genes combined with proteins that have multiple functions and not tens of thousands of genes acting, more or less, independently.

The two-hybrid method that was devised for studying the yeast proteome has been used extensively to determine pairwise protein-protein interactions. The underlying principle of this assay is that the physical connection between two proteins reconstitutes an active transcription factor that initiates the expression of a reporter gene. Generally, transcription factors have two domains. One region (DNA-binding domain) is required for binding to a specific DNA site, and the other region (activation domain) activates transcription (Figure 8.8A). These two domains need not be part of the same protein to function as an effective transcription factor. However, the activation domain alone will not attach to RNA polymerase. Connection with the DNA-binding domain is necessary to place the activation domain in the correct orientation and location to initiate transcription by RNA polymerase. For a two-hybrid system, the coding regions of the DNA-binding and activation domains of a specific transcription factor are isolated and individually cloned. A cDNA sequence is cloned in-frame with the DNA-binding domain sequence to form a fusion gene that produces a hybrid protein. A protein attached to the DNA binding domain is called the "bait" or target. Another cDNA sequence is cloned adjacent to the activation domain coding sequence. A protein attached to the activation domain is called the "prey." Host cells are transformed with bait and

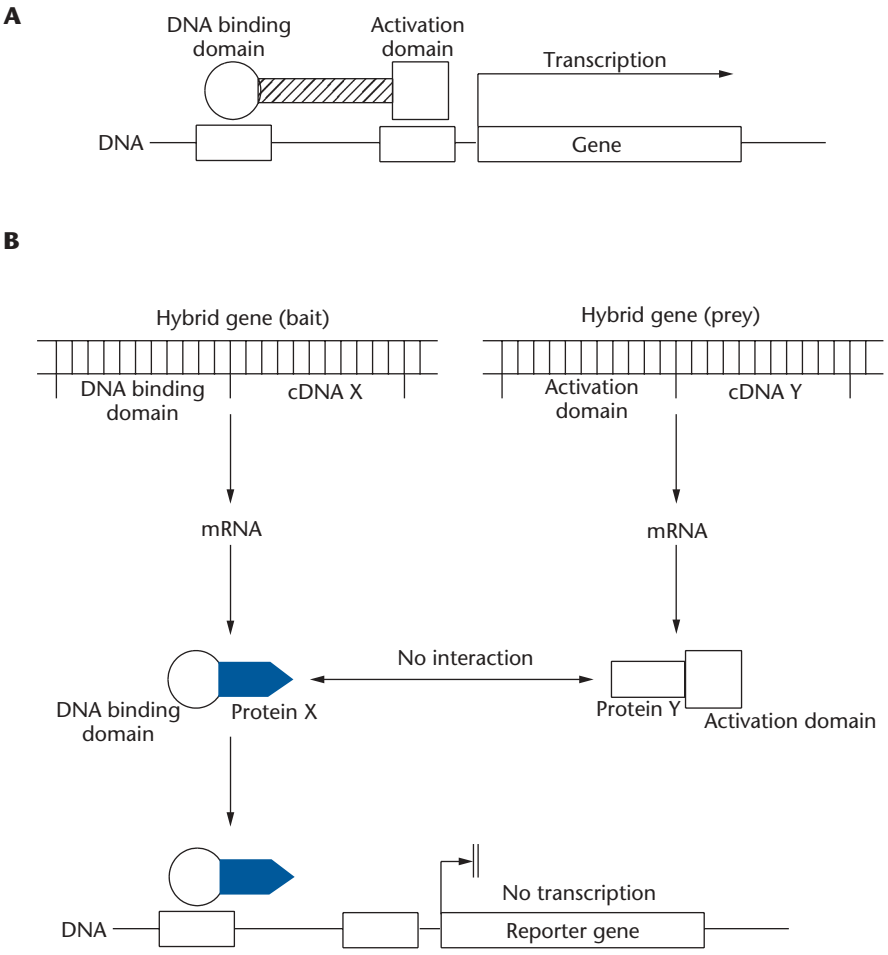

Figure 8.8 Two-hybrid assay for detecting pairwise protein interactions. (A) The DNA binding domain of a transcription factor binds to a regulatory sequence that orients and localizes the activation domain that is required for the initiation of transcription of the gene by RNA polymerase. (B) The coding sequences for the DNA binding domain and the activation domain are fused to cDNA X and cDNA Y, respectively, and both constructs (hybrid genes) are introduced into cells. After translation, the DNA binding domain-protein X fusion protein binds to the regulatory sequence of a reporter gene. However, protein Y (prey) does not interact with protein X (bait) and the reporter gene is not transcribed because the activation domain does not, on its own, associate with RNA polymerase. (C) The proteins encoded by the cDNAs of the hybrid genes interact, the activation domain is properly oriented, and the reporter gene is transcribed, demonstrating a specific protein-protein interaction.

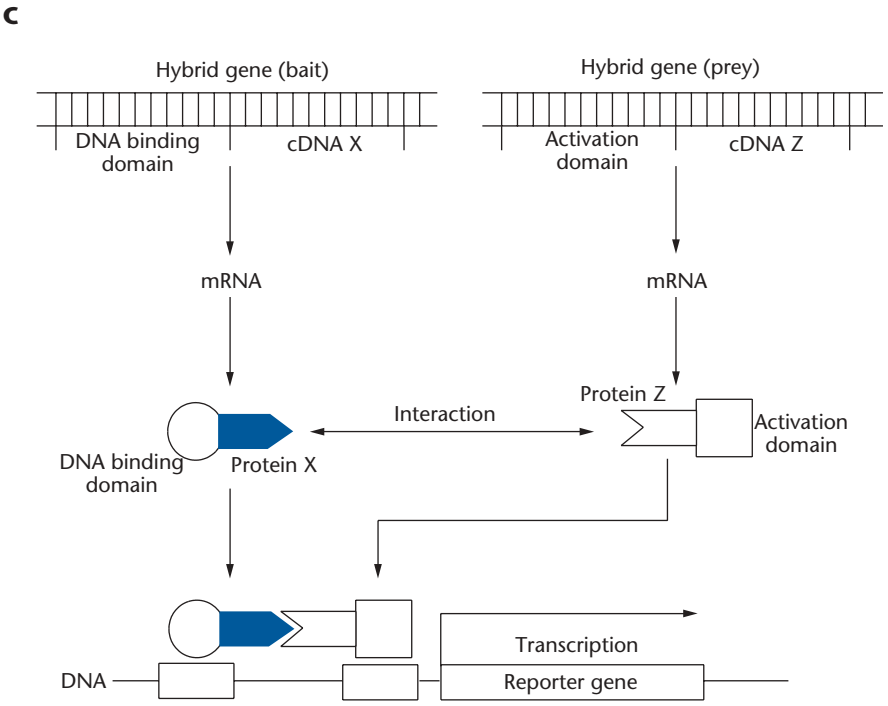

Figure 8.8 *continued*

prey DNA constructs. After expression and translation, if the bait and prey do not interact, then there is no transcription of the reporter gene (Figure 8.8B). Alternatively, if the reporter gene is transcribed, then a physical connection occurred between the bait and prey proteins that enabled the activation domain to make contact with RNA polymerase (Figure 8.8C). In other words, there is a specific interaction between the bait and prey. The product of an active reporter gene may either allow a host cell to proliferate in a specific medium or produce a colorimetric response.

A variant of the two-hybrid system has been developed for studying protein interactions in mammalian cells (Figure 8.9). With this scheme, two genetically altered subunits, α and *ω*, of an indicator enzyme (β-galactosidase) are

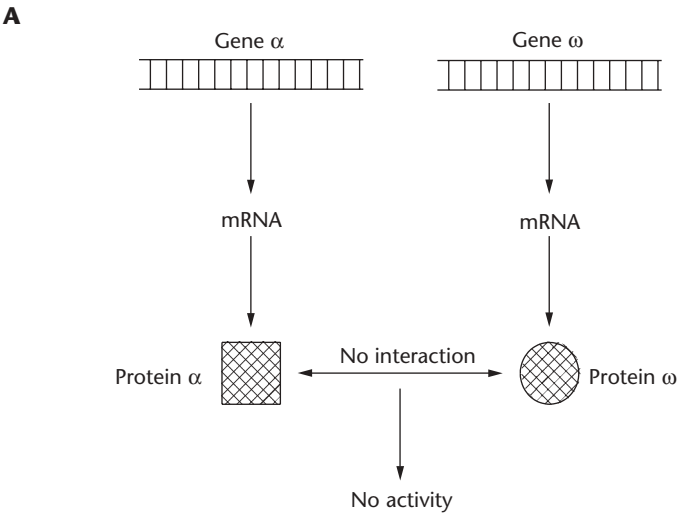

Figure 8.9 Complementation assay for detecting pairwise protein interactions. (A) Proteins α and ω must combine for activity, but, because of mutations, the two subunits are not able to interact spontaneously. (B) DNA fusion constructs of the gene encoding protein α (gene α) with a cDNA (cDNA X) and the gene encoding protein ω (gene ω) with a cDNA (cDNA Y) are introduced together into cells. Because proteins X and Y do not interact, proteins α and ω do not associate and the activity specified by the α:ω combination is not observed. (C) DNA fusion constructs of the gene encoding protein α (gene α) with a cDNA (cDNA X) and the gene encoding protein ω (gene ω) with a cDNA (cDNA Z) are introduced together into cells. Because proteins X and Z interact, proteins α and ω are brought together and the activity specified by the α:ω combination is observed.

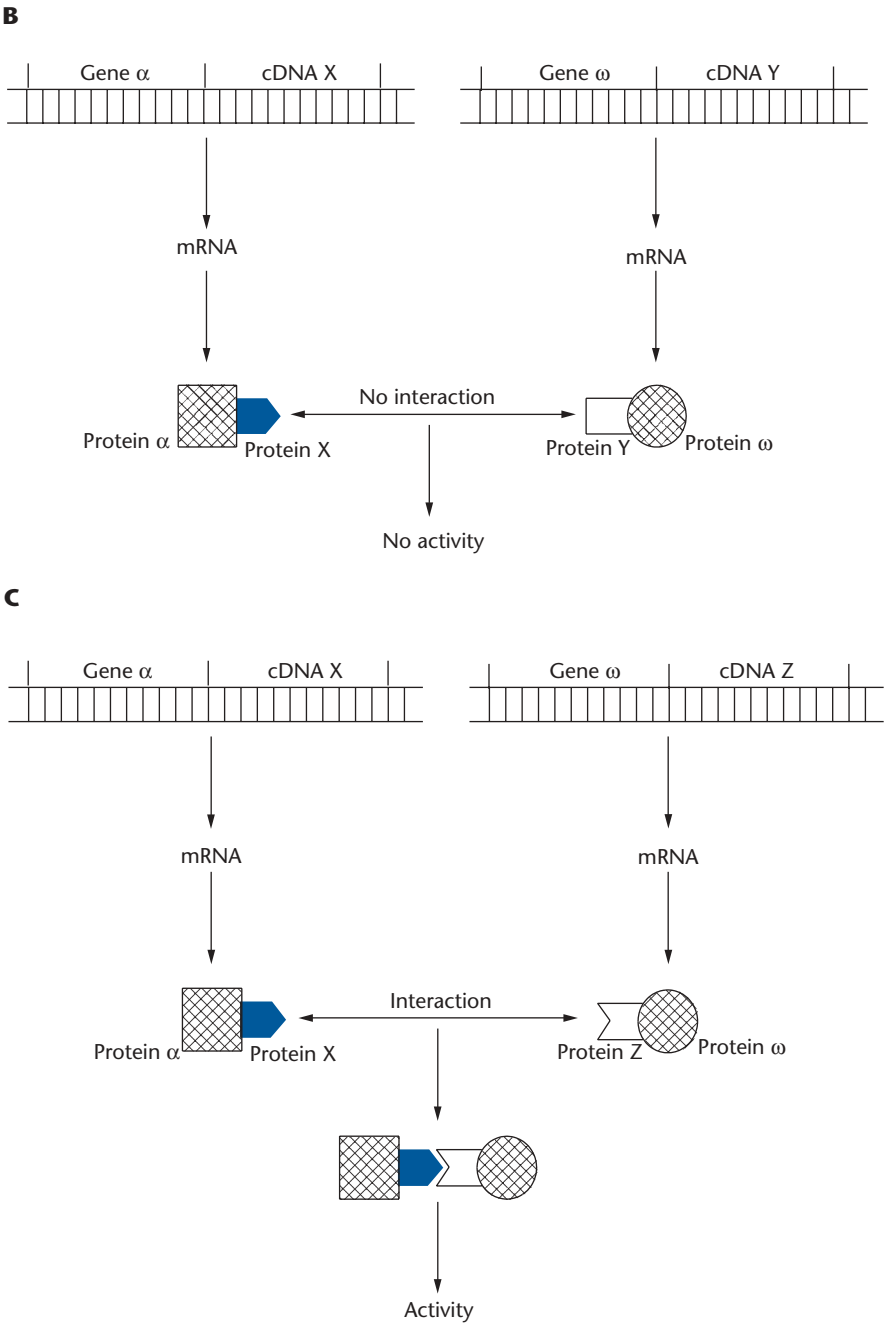

Figure 8.9 *continued*

unable to associate under normal cellular conditions and, as a result, there is no β-galactosidase activity. However, cDNAs are cloned in-frame with the genes for proteins α and *ω*, respectively, and the constructs are tested two at a time. If two cDNA-encoded proteins interact, then proteins α and *ω* are brought into close proximity and the formation of a functional β-galactosidase is detected with a colorimetric assay.

As a first step for any large-scale protein-protein interaction study, thousands of cDNAs are prepared and cloned adjacent to specified DNA sequences

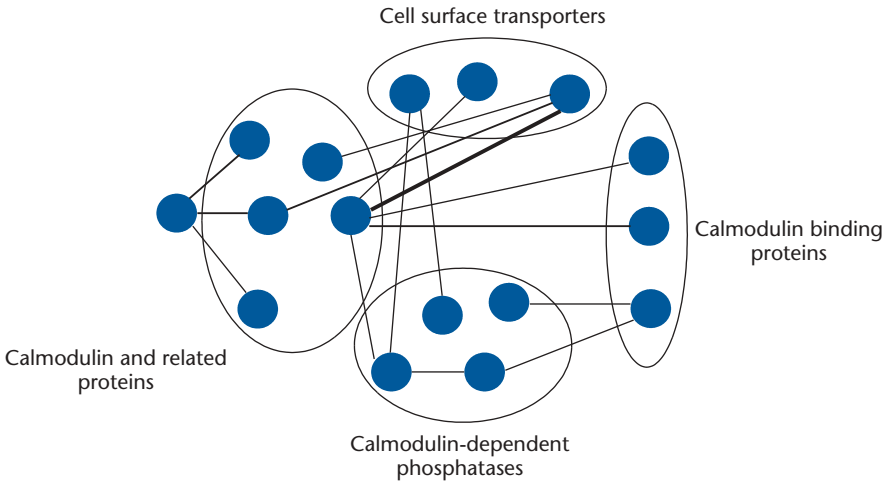

Figure 8.10 Protein interaction map of calcium signaling protein clusters of *Drosophila melanogaster*. Individual proteins (solid circles) are not named. The thickness of a connecting line denotes the extent of the interaction rating. Adapted from Fig. 5D in L. Giot et al. (2003) A protein interaction map of *Drosophila melanogaster*. *Science* **302**:1727–1736.

to form hybrid genes. Many schemes have been devised to streamline that acquisition of results. Regardless of the protocol, vast numbers of interactions are scored. Specialized computer programs are required to categorize and map all the relationships. As part of this analysis, stringent statistical criteria are used to minimize the numbers of possible false positive interactions in the final data set. With *Drosophila melanogaster*, an overall protein interaction (interactome) map of 3000 interactions with 3522 proteins was delineated. In addition, the nuclear, cytoplasmic, and extracellular locations of 2268 interactions with 2346 proteins were mapped. Finally, smaller interacting sets of proteins within cellular regions were noted (Figure 8.10). Also, a network of 5500 protein interactions was constructed for the roundworm, *Caenorhabditis elegans*. Protein interaction maps place proteins of unknown functions into contexts that provide clues about their roles in cellular processes and identify proteins with multiple functions.

Instead of studying pairwise protein interactions, the tandem affinity purification (TAP) tag procedure is designed to capture multiprotein clusters and then identify the components with mass spectrometry (Figure 8.11). In this case, a cDNA sequence that encodes the target (bait) protein is fused to a DNA sequence that encodes two short amino acid sequences (tags). An amino acid sequence tag binds with a high affinity to a specific molecule and facilitates purification of the target protein. A "two-tag" system allows two successive rounds of affinity binding to ensure that the target and its associated proteins are free of any nonspecific proteins. Alternatively, a "one-tag" system with a small protein tag that is immunoprecipitated with a specific antibody requires only a single purification step. In a number of trials, the tags did not alter the function of various test proteins.

A cDNA-two-tag construct is introduced into a host cell, where it is expressed and a hybrid protein is synthesized. Presumably, the same set of proteins combine with the target protein as normally bind to the native version. After the cells are lysed, the target protein and any interacting proteins are affinity purified. The proteins of the cluster are separated by one-dimension polyacrylamide gel electrophoresis. Individual bands are excised and treated

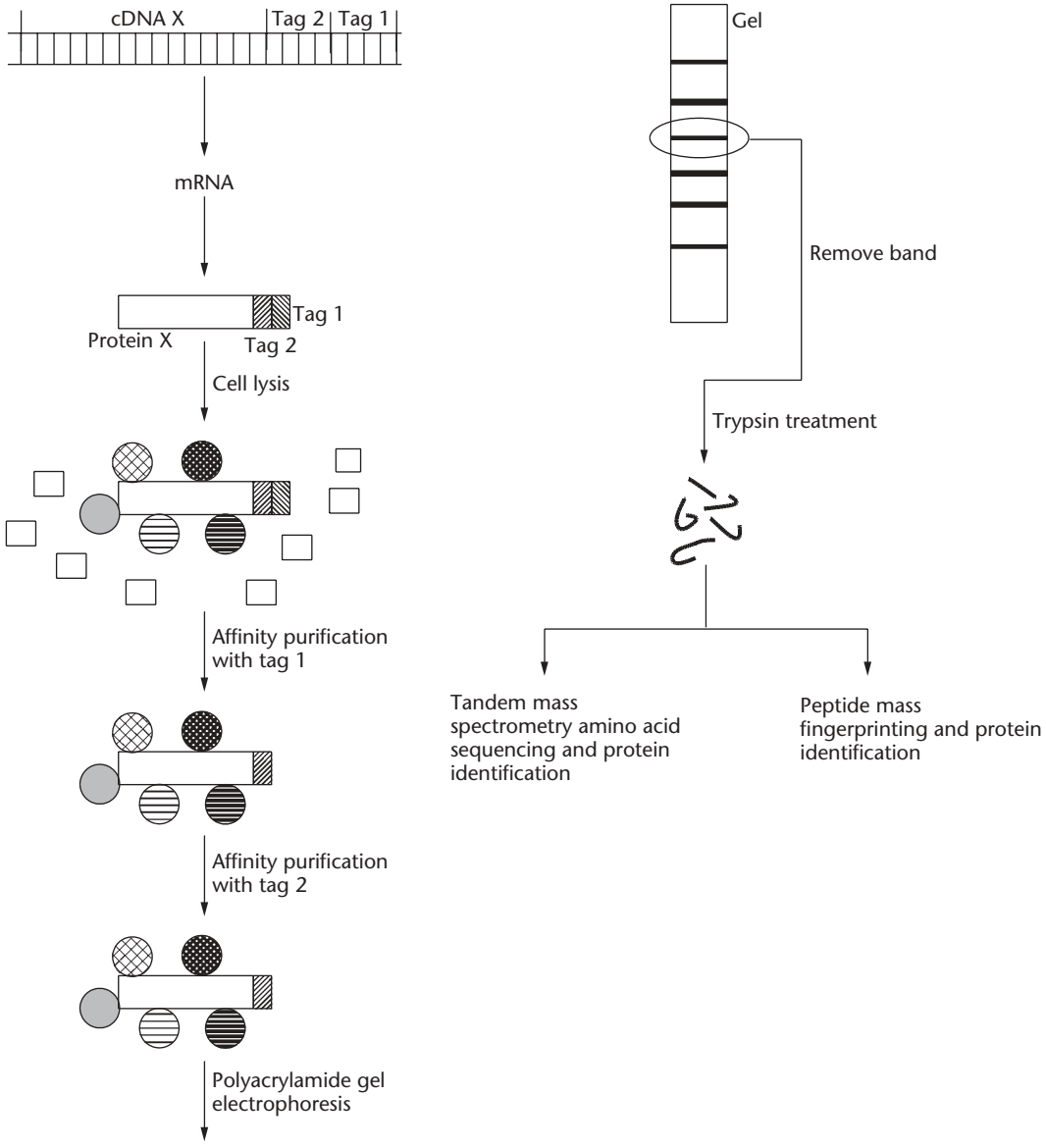

Figure 8.11 Tandem affinity purification (TAP) tag procedure for detecting protein-protein interactions. Two DNA sequences (tag 1, tag 2), each of which encoding a short amino acid sequence with a high affinity to a specific molecule, are cloned together and fused in-frame to the 3′ end of the coding region of a cDNA (cDNA X). The tagged cDNA construct is introduced into a host cell, where it is transcribed and the mRNA is translated. Proteins bind to the protein encoded by cDNA X (protein X). The cluster of protein X and its associated proteins is separated from cell components (squares) by the binding of tag 1 to its affinity partner. The cluster is eluted, tag 1 is removed, and a second purification step is carried out with tag 2 and its affinity partner. The proteins of the cluster are separated by one-dimension polyacrylamide gel electrophoresis. Single bands are excised from the gel and treated with trypsin. The protein represented by the tryptic peptides is identified by either peptide mass fingerprinting or searching a protein database with peptide amino acid sequences obtained with electrospray ionization tandem mass spectrometry.

with trypsin, and the proteins are identified with mass spectrometry. Computer programs are available for generating maps of clusters with common proteins, assigning proteins with shared interrelationships to specific cellular activities, and establishing the links between multiprotein complexes.

key terms

2D DIGE
2D PAGE
activation domain
affinity purification
alignment score
anchoring enzyme
bait
b-ion
bioinformatics
BLASTn
BLASTp
concatemer
Cy3
Cy5
database
deuterium
ditag
DNA-binding domain
EMBL
Ensembl
ESI-MS/MS
functional genomics
GenBank
Genome Database
genomics
ICAT
isotope-coded affinity tag
m/z ratio
MALDI-MS
mass spectrometry
microarray
OMIM
peptide mass fingerprinting
photolithography
phycoerythrin
prey
profiling
Protein Information Resource
protein-protein interaction
proteome
proteomics
query
RefSeq
SAGE
serial analysis of gene expression
similarity search
streptavidin
Swiss-Prot
tag enzyme
tandem affinity purification
transcriptome
two-dimension differential in-gel electrophoresis
two-hybrid assay
Type IIS restriction endonuclease
y-ion

summary

Bioinformatics grew as a field of study, for the most part, from the efforts to maintain, organize, analyze, and make accessible large amounts of gene and genomic sequence information. GenBank, a gene sequence database, was established in the late 1980s to cope with the influx of DNA sequences in the scientific literature. By the mid-1990s, a myriad of databases had been developed for genomic sequences, genetic and physical maps, expression sequence tags (ESTs), and many other types of molecular data. The expansion of the Internet and the availability of browsers led to enhanced pictorial presentation of information stored in databases and the development of computational tools for not only rapidly searching DNA or protein databases with query sequences using BLASTn or BLASTp but also other kinds of analyses. Currently, there are hundreds of public online molecular databases.

The expression of thousands of genes can be tracked simultaneously by hybridization of target sequences from a group of mRNAs to the bound probes of cDNA or oligonucleotide microarrays. Serial analysis of gene expression (SAGE) relies on sequence information from small segments of cDNAs derived from a population of mRNAs to assess which genes are up- or downregulated under various conditions.

The comprehensive study of all the proteins of a cell, tissue, body fluid, or organism, namely, the proteome, is called proteomics. High-throughput methods have been developed to identify most members of a proteome, compare the levels of individual proteins between two proteomes, and characterize thousands of protein-protein interactions within a proteome. There are two main ways of obtaining comparative protein expression profiles. With these approaches, proteomes from two different sources are analyzed together. For 2D differential in-gel electrophoresis, the proteins of two samples are labeled with the fluorescent dyes Cy3 and Cy5, respectively, and separated by 2D PAGE. Then, the specific fluorescent emissions from the individual proteins are recorded to determine the relative proportions of the proteins in each sample. Mass spectrometry analysis of peptides from different samples that are labeled with a heavy form, namely, eight deuterium atoms (D8), or a light form, namely, no deuterium atoms (D0), of an ICAT reagent reveals the relative amounts of the proteins in the original proteomes.

With the two-hybrid method, the activity of a reporter gene can only occur when two tested proteins (bait and prey) interact and, as a result, the activation domain of a transcription factor is properly oriented and located for the initiation of transcription of the reporter gene. Large networks of protein-protein interactions are formed on the basis of many thousands of pairwise assays. Alternatively, the proteins that associate with a test (bait) protein in vivo are isolated with the TAP tag procedure. In this case, the individual proteins of a purified cluster are separated by 1D polyacrylamide gel electrophoresis and identified either by peptide mass fingerprinting or mass spectrometry amino acid sequencing and a database similarity search. Protein interaction maps place proteins with unknown functions among those with known cellular roles and demarcate proteins that participate in diverse functions.

References

Aebersold, R. and M. Mann. 2003. Mass spectrometry-based proteomics. *Nature* **422**: 198–207.

Alexandersson, M., S. Cawley, and L. Pachter. 2003. SLAM: cross-species gene finding and alignment with a generalized pair hidden Markov model. *Genome Res* **13**:496–502.

Argani, P. et al. 2001. Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res* **7**:3862–3868.

Argani, P. et al. 2001. Discovery of new markers of cancer through serial analysis of gene expression: prostate stem cell antigen is overexpressed in pancreatic adenocarcinoma. *Cancer Res* **61**:4320–4324.

Bajic, V. B. and S. H. Seah. 2003. Dragon gene start finder: an advanced system for finding approximate locations of the start of gene transcriptional units. *Genome Res* **13**:1923–1929.

Baldi, P. and G. W. Hatfield. 2002. *DNA Microarrays and Gene Expression: From Experiments to Data Analysis and Modeling*. Cambridge University Press, Cambridge, UK.

Baxevanis, A. D. 2003. The Molecular Biology Database Collection: 2003 update. *Nucleic Acids Res* **31**:1–12.

Birney, E., M. Clamp, and T. Hubbard. 2002. Databases and tools for browsing genomes. *Annu Rev Genomics Hum Genet* **3**:293–310.

Boon, K. et al. 2002. An anatomy of normal and malignant gene expression. *Proc Natl Acad Sci USA* **99**:11287–11292.

Brun, C. et al. 2003. Functional classification of proteins for the prediction of cellular function from a protein-protein interaction network. *Genome Biol* **5**:R5–R13.

Clamp, M. et al. 2003. Ensembl 2002: accommodating comparative genomics. *Nucleic Acids Res* **31**:38–42.

DeRisi, J. L., V. R. Iyer, and P. O. Brown. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **278**:680–686.

Figeys, D. 2003. Novel approaches to map protein interactions. *Curr Opin Biotechnol* **14**:119–125.

Flicek, P. et al. 2003. Leveraging the mouse genome for gene prediction in human: from whole-genome shotgun reads to a global synteny map. *Genome Res* **13**:46–54.

Gavin, A. C. et al. 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415**: 141–147.

Giot, L. et al. 2003. A protein interaction map of *Drosophila melanogaster*. *Science* **302**: 1727–1736.

Graves, P. R. and T. A. Haystead. 2002. Molecular biologist's guide to proteomics. *Microbiol Mol Biol Rev* **66**:39–63.

Gu, C. C. et al. 2002. Role of gene expression microarray analysis in finding complex disease genes. *Genet Epidemiol* **23**:37–56.

Gygi, S. P. et al. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* **17**:994–999.

Han, D. K. et al. 2001. Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat Biotechnol* **19**:946–951.

Harrison, P. M. et al. 2002. A question of size: the eukaryotic proteome and the problems in defining it. *Nucleic Acids Res* **30**:1083–1090.

Haviv, I. and I. G. Campbell. 2002. DNA microarrays for assessing ovarian cancer gene expression. *Mol Cell Endocrinol* **191**: 121–126.

Heller, M. J. 2002. DNA microarray technology: devices, systems, and applications. *Annu Rev Biomed Eng* **4**:129–153.

Ho, Y. et al. 2002. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* **415**:180–183.

Kanehisa, M. and P. Bork. 2003. Bioinformatics in the post-sequence era. *Nat Genet* **33** Suppl:305–310.

Karolchik, D. et al. 2003. The UCSC Genome Browser Database. *Nucleic Acids Res* **31**:51–54.

Lash, A. E. et al. 2000. SAGEmap: a public gene expression resource. *Genome Res* **10**: 1051–1060.

Lashkari, D. A. et al. 1997. Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proc Natl Acad Sci USA* **94**:13057–13062.

Lesk, A. 2002. *Introduction to Bioinformatics*. Oxford University Press, New York.

Li, S. et al. 2004. A map of the interactome network of the Metazoan *C. elegans*. *Science* **303**:540–543.

Liang, P. 2002. SAGE Genie: a suite with panoramic view of gene expression. *Proc Natl Acad Sci USA* **99**:11547–11548.

Lipshutz, R. J. et al. 1999. High density synthetic oligonucleotide arrays. *Nat Genet* **21**:20–24.

Macoska, J. A. 2002. The progressing clinical utility of DNA microarrays. *CA Cancer J Clin* **52**:50–59.

Ota, T. et al. 2004. Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat Genet* **36**:40–45.

Parra, G. et al. 2003. Comparative gene prediction in human and mouse. *Genome Res* **13**:108–117.

Patterson, S. D. and R. H. Aebersold. 2003. Proteomics: the first decade and beyond. *Nat Genet* **33** Suppl:311–323.

Phizicky, E. et al. 2003. Protein analysis on a proteomic scale. *Nature* **422**:208–215.

Pruess, M. et al. 2003. The Proteome Analysis database: a tool for the in silico analysis of whole proteomes. *Nucleic Acids Res* **31**:414–417.

Rajeevan, H. et al. 2003. ALFRED: the ALelle FREquency Database. Update. *Nucleic Acids Res* **31**:270–271.

Rigaut, G. et al. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* **17**:1030–1032.

Rossi, F. M., B. T. Blakely, and H. M. Blau. 2000. Interaction blues: protein interactions monitored in live mammalian cells by β-galactosidase complementation. *Trends Cell Biol* **10**:119–122.

Spellman, P. T. and G. M. Rubin. 2002. Evidence for large domains of similarly expressed genes in the *Drosophila* genome. *J Biol* **1**:5.

Strausberg, R. L. et al. 2002. An international database and integrated analysis tools for the study of cancer gene expression. *Pharmacogenomics J* **2**:156–164.

Unlu, M., M. E. Morgan, and J. S. Minden. 1997. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* **18**:2071–2077.

Velculescu, V. E. et al. 1995. Serial analysis of gene expression. *Science* **270**:484–487.

Washburn, M. P., D. Wolters, and J. R. Yates, 3rd. 2001. Large-scale analysis of

the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* **19**:242–247.

Yarmush, M. L. and A. Jayaraman. 2002. Advances in proteomic technologies. *Annu Rev Biomed Eng* **4**:349–373.

Yates, J. R. 3d. 1998. Mass spectrometry and the age of the proteome. *J Mass Spectrom* **33**:1–19.

Zhang, M. Q. 2002. Computational prediction of eukaryotic protein-coding genes. *Nat Rev Genet* **3**:698–709.

Zhu, H. 2001. Global analysis of protein activities using proteome chips. *Science* **293**:2101–2105.

review questions

1. What is BLAST?
2. What is the likely source of the amino acid sequence LSPQMSGEEEDSDLAAKLGMCNREIVRRGA? Do a BLASTp search of the Swiss-Prot database at http://ncbi.nlm.nih.gov/BLAST. What additional information about the source protein and its gene can be obtained from http://harvester.embl.de/ and other sites such as http://www.ncbi.nlm.nih.gov/Omim/searchomim.html and http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch?
3. What is a DNA microarray?
4. Describe a cDNA microarray gene expression profiling system.
5. Describe how an oligonucleotide microarray is formed with photolithography.
6. How is the target sample prepared for an oligonucleotide microarray experiment?
7. What is an affinity tag?
8. What are the objectives of gene expression profiling experiments?
9. Describe the SAGE procedure.
10. With SAGE Genie (http://www.cgap.nci.nih.gov/SAGE), find all the information that you can about the following SAGE tags: ATCGCTTTCT, TTTTGTCATT, and GGCCCCAGTT.
11. Why are there so many more proteins than genes in humans?
12. What are the principal features of 2D PAGE?
13. Describe how unknown members of a proteome can be readily identified.
14. What is a peptide mass fingerprint?
15. Are you curious about the unknown protein in Figure 8.4? If so, type the peptide masses of the unknown as a column. Omit the dots. Save the column to clipboard. Go to http://us.expasy.org/tools/peptident.html. Paste the list of peptide masses into the "Enter a list . . ." box. Use the E-mail option to receive a printout of the search. Don't change the default settings. Click on "Start Peptident". The search will take about 2 min. The E-mail should arrive within 5 min. You can also save the results from your browser. What is the unknown protein? In general, account for the matches with the other proteins. (You may also want to checkout the peptide mass fingerprint program at http://www.matrixscience.com. Click on "Mascot". Click on "Peptide Mass Fingerprint". Fill in information boxes. Paste in peptide masses into "Query" box and click on the "Start Search . . ." button. Examine both the concise and full reports.)
16. The following ladder of y-ion masses (m/z ratios) were observed with mass spectrometry after fragmentation and ionization of a peptide: 1136, 980, 851, 723, 609, 512, 411, 324, 196, and 99. What is the likely amino acid sequence of the peptide? A list of amino acid masses can be obtained from http://us.expasy.org/tools/findmod/findmod_masses.html, http://matrixscience.com/help/aa_help.html, or http://www.its.caltech.edu/~ppmal/sample_prep/work3.html.
17. What is the ICAT method?
18. Describe the major features of the two-hybrid assay.
19. What is the TAP tag system?
20. What are the objectives of protein-protein interaction studies?

chapter 9

Human Population Genetics

No theory is good except on condition that one use it to go beyond.

ANDRÉ GIDE (1869–1951)

I am reluctant to intrude in a discussion concerning matters of which I have little expert knowledge, and I should have expected the very simple point which I wish to make to have been familiar to biologists. . . . Mr. Yule is reported to have suggested . . . that if brachydactyly is dominant "in the course of time one would expect, in the absence of counteracting factors, to get three brachydactylous persons to one normal." It is not difficult to prove, however, that such an expectation would be quite groundless. . . . a little mathematics of the multiplication-table type is enough to show . . . [that] . . . there is not the slightest foundation for the idea that a dominant character should show a tendency to spread over a whole population, or that a recessive should tend to die out.

G. H. HARDY (1877–1947)

The existing theories of population genetics will no doubt be simplified and systematized. Many of them will have no final importance than a good deal of nineteenth-century dynamical theory. This does not mean that they have been a useless exercise of algebraic ingenuity. One must try possibilities before one reaches even partial truth.

J. B. S. HALDANE (1892–1964)

THE GENETICS OF HUMANS can be studied at the level of the individual, the family, or a population. Mendelian principles apply to each category, although the methodological approaches are quite different. For the most part, the genetic constitution of an individual is determined by genetic testing of a large number of loci, or, less specifically, by cytogenetic analysis. With families, examination of the patterns of disorders in multigeneration pedigrees provides clues about the genetics of a particular condition and, possibly, the chromosome location of a disease-causing gene that, in turn, acts as a springboard for its isolation and characterization. Finally, the genetic constitution of an interbreeding population can be described in terms of gene and genotype frequencies. Moreover, population genetics, which is a mathematically based discipline, is concerned with the impact of population size, migration, nonrandom mating, and reduced transmission of certain alleles and genotypes from one generation to the next on the genetic composition of a population.

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Alleles, Genotypes, and Hardy–Weinberg Equilibrium

A two-allele, one locus system is the simplest way to explain the rudiments of population genetics. In this case, a single autosomal locus, designated *A*, has only two alleles (*A1*, *A2*). To determine the genetic composition of a population, a large number of individuals are screened and the frequencies of the *A1A1*, *A1A2*, and *A2A2* genotypes are recorded. By way of illustration, consider an arbitrary population with frequencies of 0.40, 0.40, and 0.20 for the genotypes *A1A1*, *A1A2*, and *A2A2*, respectively. Because these three genotypes represent all the possible diploid arrangements for locus *A*, then, obviously, the sum of the individual genotype frequencies is 1. The frequencies of the *A1* and *A2* alleles can be determined from the genotype frequencies. For example, the frequency of *A1* is equal to the frequency of the *A1A1* genotype plus half the frequency of the heterozygous genotype *A1A2*. Because each individual has two alleles, the frequency of *A1* is twice the frequency of the *A1A1* genotype plus the frequency of the heterozygous genotype (*A1A2*) divided by 2, that is, $\frac{2f(A1A1)+f(A1A2)}{2}$ or simply $f(A1A1) + \frac{1}{2}f(A1A2)$. In terms of our specific example, the frequency of *A1* = 0.40 + 1/2(0.40) = 0.60. And the frequency of *A2* is 1 minus the frequency of *A1*, that is, 1 – 0.60 = 0.40. The sum of the frequencies of the *A1* and *A2* alleles is 1 because they represent all the possible alleles at locus *A* in the population. Of course, the frequency of *A2* can also be determined directly: 0.20 + 1/2(0.40) = 0.40.

The frequencies of the *A1* and *A2* alleles can also be calculated from raw data. For example, the original sample consisted of 400, 400, and 200 individuals who were *A1A1*, *A1A2*, and *A2A2*, respectively. Thus, the frequency of $A1 = \frac{(400\times 2)+400}{1000\times 2} = 0.60$ and the frequency of $A2 = \frac{(200\times 2)+400}{1000\times 2} = 0.40$. In the numerator, the number of homozygous individuals, either *A1A1* or *A2A2*, is multiplied by 2 because homozygotes have two versions of the same allele at a locus. And, in the denominator, the times 2 (×2) function is used because the total number of alleles at locus *A* is two times the total number of sampled individuals.

The concepts of population genetics are more readily explained with two-allele systems than those with many alleles. However, the frequencies of multiple alleles at a locus can be easily calculated. For a single locus with alleles $A1, A2, \ldots An$ with allele frequencies $p_1, p_2, \ldots p_n$, there are $\frac{n+1}{2}$ possible genotypes and for any allele $p_i = f(A_iA_i) + \frac{\sum_{j\neq i} f(A_jA_i)}{2}$. For example, if the only genotypes in a population are *A1A1*, *A1A2*, *A1A3*, and *A2A2* and their frequencies are 0.5, 0.2, 0.2, and 0.1 respectively, then the allele frequencies are *A1* = 0.5 + 1/2(0.2) + 1/2(0.1) = 0.7, *A2* = 0.1 + 1/2(0.2) = 0.2, and *A3* = 1/2(0.2) = 0.1.

Now that the gene and genotype frequencies at locus *A* have been established, a major question arises: What happens to these frequencies in the next generation? However, before this question can be answered, some assumptions about various population parameters must be considered. At this point in the

discussion, for convenience, let's assume that the human population we are working with is extremely large; all matings are equally likely, with no matings between members of different generations; there is no migration in or out of the population; there is no mutation of alleles at the locus under consideration; all individuals are equally fertile (reproductively fit); and each couple produces the same number of surviving, fertile offspring. With these provisos in place, we can determine what happens in the next generation to the frequencies of alleles and genotypes of our arbitrary population where the initial frequencies of *A1* and *A2* are 0.60 and 0.40, respectively.

Because any male is equally likely to mate with any female, then there are nine possible matings with respect to genotypes. These can be represented by a 3 × 3 table (Table 9.1). The sides of the table show the genotype frequencies for males and females, and the contents show the frequency of each particular mating in the population. For example, in this generation, the *A1A1* × *A1A1* mating occurs 16% of the time.

Next, the frequencies of the genotypes among the offspring from these matings can be determined (Table 9.2). The frequencies of the genotypes of the offspring of the matings depend on both the frequency of the particular mating in the population and the Mendelian proportion expected for that particular mating. For example, all the offspring of the *A1A1* × *A1A1* matings will be *A1A1*; therefore, the frequency of offspring is 1.0 × 0.16, or 0.16. On the other hand, 0.25, 0.50, and 0.25 of the offspring of the *A1A2* × *A1A2* matings are expected to be *A1A1*, *A1A2*, and *A2A2*, respectively. Because the frequency of the *A1A2* × *A1A2* matings is 0.16, then the frequencies of the *A1A1*, *A1A2*,

Table 9.1 Frequencies of random matings in a population where the frequencies of the *A1A1*, *A1A2*, and *A2A2* genotypes are 0.40, 0.40, and 0.20, respectively.

		Females		
		0.40 *A1A1*	**0.40 *A1A2***	**0.20 *A2A2***
Males	**0.40 *A1A1***	0.16	0.16	0.08
	0.40 *A1A2*	0.16	0.16	0.08
	0.20 *A2A2*	0.08	0.08	0.04

Table 9.2 Frequencies of the genotypes of offspring after random mating of a population where the frequencies of the *A1A1*, *A1A2*, and *A2A2* genotypes in the previous generation were 0.40, 0.40, and 0.20, respectively.

Matings (Male × female)	Frequency of matings	Offspring		
		A1A1	*A1A2*	*A2A2*
A1A1 × *A1A1*	(0.40)(0.40)	0.16		
A1A1 × *A1A2*	(0.40)(0.40)	0.08	0.08	
A1A1 × *A2A2*	(0.40)(0.20)		0.08	
A1A2 × *A1A1*	(0.40)(0.40)	0.08	0.08	
A1A2 × *A1A2*	(0.40)(0.40)	0.04	0.08	0.04
A1A2 × *A2A2*	(0.40)(0.20)		0.04	0.04
A2A2 × *A1A1*	(0.20)(0.40)		0.08	
A2A2 × *A1A2*	(0.20)(0.40)		0.04	0.04
A2A2 × *A2A2*	(0.20)(0.20)			0.04
		0.36	0.48	0.16

and *A2A2* offspring are (0.25)(0.16), (0.5)(0.16), and (0.25)(0.16), respectively. Summing each genotypic class among the offspring gives genotypic frequencies for *A1A1*, *A1A2*, and *A2A2* of 0.36, 0.48, and 0.16, respectively in the next generation. Clearly, the genotype frequencies have changed from those of the parental (original) generation. However, the gene frequencies remain unchanged. In case there are any doubts, after one generation, the frequency of *A1* = 0.36 + 1/2(0.48) = 0.60 and the frequency of *A2* = 0.16 + 1/2(0.48) = 0.40.

Now, what happens to the allele and genotype frequencies in the ensuing generation? With the format from Table 9.2, the frequencies of the genotypes of the next generation can be calculated assuming a large population size, random mating, no migration, no mutation, and equal reproductive fitnesses (Table 9.3). The tallies show that the genotype frequencies remain the same as the previous generation. Moreover, if the population parameters do not change, the allele and genotype frequencies are maintained generation after generation, or, in Shakespeare's words, "to the last syllable of recorded time." The constancy of allele and genotype frequencies through successive generations represents a balanced state that was originally called genetic equilibrium.

The principle of genetic equilibrium was elucidated, independently, by G. Hardy (1877–1947), an English mathematician, and W. Weinberg (1862–1937), a German physician, in 1908. To acknowledge their contribution, the term genetic equilibrium was eventually changed to the Hardy–Weinberg equilibrium (HWE), the Hardy–Weinberg law (HWL), or the Hardy–Weinberg principle (HWP). Later, when it was realized that W. E. Castle (1867–1962), an American geneticist, had described the equilibrium principle in 1903, some scientists, to give full attribution, started to refer to genetic equilibrium as the Castle–Hardy–Weinberg law, whereas others stayed with either HWE or HWL.

Up to this point, a specific numerical example has been used to demonstrate the fundamental features of the HWE. The principle of genetic equilibrium can be readily generalized and applied to the complete range of allele frequencies. Usually, in population genetics, the frequencies of the alleles of a two-

Table 9.3 Frequencies of genotypes of offspring after random mating of a population where the frequencies of *A1A1*, *A1A2*, and *A2A2* genotypes in the previous generation were 0.36, 0.48, and 0.16, respectively.

Matings (Male × female)	Frequency of matings	Offspring		
		A1A1	*A1A2*	*A2A2*
A1A1 × *A1A1*	(0.36)(0.36)	0.13		
A1A1 × *A1A2*	(0.36)(0.48)	0.085	0.085	
A1A1 × *A2A2*	(0.36)(0.16)		0.06	
A1A2 × *A1A1*	(0.48)(0.36)	0.085	0.085	
A1A2 × *A1A2*	(0.48)(0.48)	0.06	0.12	0.06
A1A2 × *A2A2*	(0.48)(0.16)		0.04	0.04
A2A2 × *A1A1*	(0.16)(0.36)		0.06	
A2A2 × *A1A2*	(0.16)(0.48)		0.04	0.04
A2A2 × *A2A2*	(0.16)(0.16)			0.02
		0.36	0.48	0.16

Table 9.4 Derivation of the genotypes and frequencies for a two-allele, one-locus system with the p/q notation.

		Female gametes	
		***A1* (*p*)**	***A2* (*q*)**
Male gametes	***A1* (*p*)**	p^2	pq
	***A2* (*q*)**	pq	q^2

Table 9.5 Generalized form of the Hardy–Weinberg equilibrium.

Matings (Male × female)	Frequency of matings	Offspring		
		A1A1	*A1A2*	*A2A2*
A1A1 × *A1A1*	$(p^2)(p^2)$	p^4		
A1A1 × *A1A2*	$(p^2)(2pq)$	p^3q	p^3q	
A1A1 × *A2A2*	$(p^2)(q^2)$		p^2q^2	
A1A2 × *A1A1*	$(2pq)(p^2)$	p^3q	p^3q	
A1A2 × *A1A2*	$(2pq)(2pq)$	p^2q^2	$2p^2q^2$	p^2q^2
A1A2 × *A2A2*	$(2pq)(q^2)$		pq^3	pq^3
A2A2 × *A1A1*	$(q^2)(p^2)$		p^2q^2	
A2A2 × *A1A2*	$(q^2)(2pq)$		pq^3	pq^3
A2A2 × *A2A2*	$(q^2)(q^2)$			q^4

Tally of the genotypes of the offspring:

Frequency of *A1A1* = $p^4 + p^3q + p^3q + p^2q^2 = p^2(p^2 + 2pq + q^2) = p^2(1) = p^2$
Frequency of *A1A2* = $p^3q + p^2q^2 + p^3q + 2p^2q^2 + p^3q + p^2q^2 + p^3q = 2p^3q + 4p^2q^2 + 2pq^3 = 2pq(p^2 + 2pq + q^2) = 2pq(1) = 2pq$
Frequency of *A2A2* = $p^2q^2 + pq^3 + pq^3 + q^4 = q^2(p^2 + 2pq + q^2) = q^2(1) = q^2$

allele, one-locus system are designated as p and q. When dominance is present, p always signifies the dominant allele and q the recessive allele. Because p and q represent, in the simplest case, the frequencies of the only two alleles at a locus, then $p + q = 1.0$. With this terminology, the frequencies of the genotypes can be generated by a "two-by-two" table, where p^2 and q^2 denote the different homozygous genotypes and $2pq$ the heterozygous genotype (Table 9.4). Because the frequencies of p^2, $2pq$, and q^2 represent all the possible genotypes for a two-allele, one-locus system, then $p^2 + 2pq + q^2 = 1.0$. And, because $(p + q)^2 = p^2 + 2pq + q^2$, the HWE is occasionally called the square law.

With some algebraic factoring, HWE can be readily demonstrated with the p and q notation (Table 9.5). Simplification of the sum of the terms for each genotype indicates that the genotype frequencies among the offspring are the same as in the previous generation. In other words, the population is (and was) in HWE. Remember that HWE depends on five key assumptions, namely, large population size, no migration, random mating, no mutation, and equal reproductive fitness. The genotype frequencies as a function of all values of one of the alleles in a two-allele, one-locus system can be depicted graphically (Figure 9.1). For example, in a HWE population, when *A2* (q) equals 0.5, the genotypes *A1A1* (p^2), *A1A2* ($2pq$), and *A2A2* (q^2) are 0.25, 0.50, and 0.25, respectively.

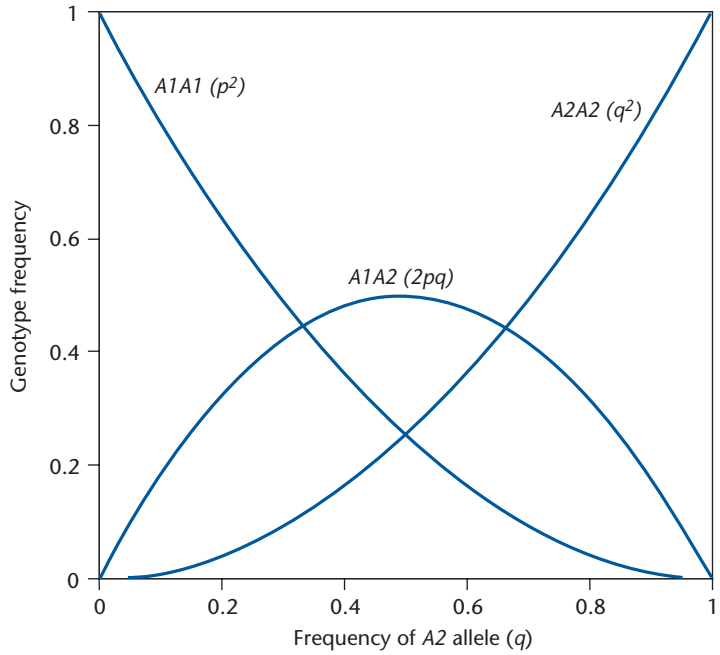


Figure 9.1 Frequency of the *A1A1* (p^2), *A1A2* (*2pq*), and *A2A2* (q^2) genotype of a two-allele, one locus system as a function of the frequency of the *A2* allele (*q*) in a Hardy–Weinberg equilibrium population.

It is of interest to determine whether individual loci in human populations comply with HWE. Of the many possible examples that could be cited, a population study of the M and N proteins, which are embedded in the cell membrane of red blood cells, convincingly addresses this issue. These proteins are encoded by the glycophorin A gene (*GYPA*), which maps to 4q28.2–q31.1. Glycophorin A is a sialoglycoprotein with the M and N variants differing from each other by two amino acids. The presence of the M, N, or both M and N proteins on red blood cells can be determined by a simple antibody test with a small blood sample. Before its discovery by mapping and gene characterization, the *GYPA* locus was designated the *L* locus and the *M* and *N* alleles were represented as L^M and L^N. The appropriate allelic designations are *GYPA*M* and *GYPA*N*. Here, for convenience, these alleles will be merely specified as *M* and *N*. With the *MN* allele system, there is a one-to-one correspondence between genotype and phenotype. In other words, the *MM*, *MN*, and *NN* genotypes are responsible for the M, MN, and N phenotypes, respectively.

In a large study, 2320 parents from 1160 families were screened for their MN blood types. The frequencies of M, MN, and N individuals in this sample were 0.311, 0.492, and 0.197, respectively. Therefore, the frequency of *M* is 0.311 + 1/2(0.492) = 0.557 and the frequency of *N* is 1 – 0.557 = 0.443. If the population is in HWE with respect to these alleles, then the expected MM, MN, and NN genotype frequencies would be $(0.557)^2 = 0.310$, $2(0.557)(0.443) = 0.494$, and $(0.443)^2 = 0.196$ for the M, MN, and N phenotypes, respectively. Clearly, the observed genotype frequencies are equivalent to those expected for HWE. In the same study, 2734 children were tested and the frequencies of the M, MN, and N blood types were 0.311, 0.493, and 0.195. Thus, the genotype frequencies in the offspring generation are the same as those of the parental generation, which is expected for a locus that is in HWE.

Because the *GYPA*M* and *GYPA*N* alleles are codominant, each genotype can be determined directly by the assay system. The status of HWE can also be ascertained for loci with completely dominant alleles, although the calculations are somewhat different because homozygous dominant individuals cannot be distinguished phenotypically from heterozygotes. In a classic study by L. H. Snyder (b. 1901), families were screened to determine those individuals who could or could not taste the chemical phenylthiocarbamide (PTC). The data were then used to verify whether the PTC taste sensitivity trait was in agreement with HWE. When this study was conducted, the ability to taste PTC was believed to be an excellent example of a simple dominant autosomal condition. However, with more discriminating criteria for PTC taste sensitivity, the genetic basis of the phenotype has been found to be more complex. Possibly, two or more loci are involved. Or perhaps, a major incompletely dominant locus along with some modifier genes are responsible for the phenomenon. Regardless of the uncertainty concerning the mode of inheritance of PTC tasting, Snyder's original study, which partitioned the samples into discrete categories consisting of tasters and nontasters, is instructive. Currently, the phenylthiocarbamide locus, which maps to 7q, is designated *PTC*. Previously, the symbol *T* was used to specify the dominant allele for PTC taste sensitivity and *t* the recessive allele for PTC taste insensitivity. For simplicity, this traditional nomenclature will be used here.

Among the offspring of taste-sensitive (taster; *TT*, *Tt*) × taste-insensitive (nontaster; *tt*) matings, 483 tasters and 278 nontasters were scored, with the nontasters comprising $\frac{278}{278+483} = 0.36$ of the total offspring. To determine whether this value agrees with HWE, the expectation of the frequency of nontaster offspring from taster and nontaster marriages must be calculated. As shown in Table 9.6, this frequency is equivalent to $\frac{q}{1+q}$. Generally, if HWE exists in a population, the square root of the frequency of the recessive genotype equals the value of q, that is, $\sqrt{q^2} = q$. In the North American population of those of European descent, the frequency of nontasters (q^2) is 0.30 and the frequency of the *t* allele (q), assuming HWE for this locus, should be 0.55. With this value for q, the expression $\frac{q}{1+q} = \frac{0.55}{1+0.55} = 0.354$, which is

Table 9.6 Proportion of taster (*tt*) offspring from taster (*TT*, *Tt*) × nontaster (*tt*) matings.

Matings (Male × female)	Frequency of matings	Offspring		
		TT	*Tt*	*tt*
$TT \times tt$	$(p^2)(q^2)$		p^2q^2	
$Tt \times tt$	$(2pq)(q^2)$		pq^3	pq^3
$tt \times TT$	$(q^2)(p^2)$		p^2q^2	
$tt \times Tt$	$(q^2)(2pq)$		pq^3	pq^3

Proportion of nontasters (tt) among the offspring = $\frac{pq^3 + pq^3}{2p^2q^2 + 4pq^3} = \frac{2pq(q^2)}{2pq(pq + 2q^2)} = \frac{q^2}{q(p+2q)} = \frac{q}{1-q+2q} = \frac{q}{1+q}$.

Table 9.7 Proportion of taster (*tt*) offspring from taster (*TT*, *Tt*) × taster (*TT*, *Tt*) matings.

Matings (Male × female)	Frequency of matings	Offspring		
		TT	*Tt*	*tt*
$TT \times TT$	$(p^2)(p^2)$	p^4		
$Tt \times TT$	$(2pq)(p^2)$	p^3q	p^3q	
$Tt \times Tt$	$(2pq)(2pq)$	p^2q^2	$2p^2q^2$	p^2q^2
$TT \times Tt$	$(p^2)(2pq)$	p^3q	p^3q	

Proportion of nontasters (*tt*) among the offspring = $\frac{p^2q^2}{4p^3q + 4p^2q^2 + p^4} = \frac{p^2q^2}{p^2(p+2q)^2} = \frac{q^2}{(1-q+2q)^2} = \frac{q^2}{(1+q)^2}$.

virtually the same as the observed value. In other words, the *TT*, *Tt*, and *tt* genotypes are in HWE in this population.

In the same vein, the observed and expected results of the frequency of homozygous recessive offspring when both parents are tasters can be determined and compared (Table 9.7). In this case, the expected frequency of taster offspring is equivalent to $\frac{q^2}{(1+q)^2} = \frac{(0.55)^2}{(1+0.55)^2} = 0.126$. The observed numbers of taster and nontaster offspring from taster parents were 929 and 130, respectively, with the proportion of tasters among the offspring being $\frac{130}{929+130} = 0.126$. Again, there is exceptional agreement between the observed and expected values, which supports the view that the PTC taster-nontaster gene system conforms to HWE.

So far, two-allele, one-locus autosomal gene systems have been used to illustrate HWE. However, equilibrium conditions exist for multiple alleles, X-linked alleles, linked loci, and two or more independently assorting loci. For a three-allele, autosomal system, the alleles are designated p, q, and r and, therefore, in a population, $p + q + r = 1$. For HWE, the expected frequencies of the genotypes are derived by binomial expansion and would be $(p + q + r)^2 = p^2 + 2pq + 2pr + q^2 + 2qr + r^2 = 1$. With four alleles at a locus, the Hardy–Weinberg genotype frequencies are $(p + q + r + s)^2 = p^2 + q^2 + r^2 + s^2 + 2pq + 2pr + 2ps + 2qr + 2qs + 2rs = 1$ and so on for additional alleles.

Hardy–Weinberg Equilibrium with X-Linked Genes

Unlike autosomal genes that reach HWE in one generation with idealized population parameters, X-linked genes require a number of generations with these conditions before a genetic equilibrium is established. The reason for this difference is the pattern of transmission of X chromosomes from one generation to another. With a two-allele X-chromosome gene system, the female population can be homozygous for either allele (*A1A1* or *A2A2*) or heterozygous (*A1A2*). On the other hand, males have only one X chromosome and, therefore, carry one or the other allele (*A1* or *A2*). Assume that the frequency of *A2* among females (q_f) in a population is 0.32 and, among males (q_m) in the same

population, it is 0.56. After one generation with Hardy–Weinberg conditions, $q_f = \frac{0.32 + 0.56}{2} = 0.44$ because the X chromosomes of females are derived equally from mothers and fathers; therefore, q_f among daughters is the average of q_f and q_m in the previous generation, that is, $\frac{q_f + q_m}{2}$. And, in this generation, $q_m = 0.32$ because all the X-linked genes of males are inherited from their mothers. Therefore, the gene frequency of an X-linked gene among sons is equal to the frequency of that gene in the female population of the previous generation. The formula for determining the overall frequency of an X-linked recessive gene in a population is $\bar{q} = \frac{2q_f + q_m}{3}$. The parameter $\bar{q}$ is pronounced "cue-bar". For our specific example, in the initial population, $\bar{q} = \frac{2(0.32) + 0.56}{3} = 0.40$ and $\bar{p} = 0.60$. In the next generation, $\bar{q} = \frac{2(0.44) + 0.32}{3} = 0.40$ and, of course, $\bar{p} = 0.60$. In other words, with Hardy–Weinberg conditions, X-linked allele frequencies do not change from one generation to the next.

After the second generation, $q_f = \frac{0.44 + 0.32}{2} = 0.38$, $q_m = 0.44$, and, as expected, $\bar{q} = \frac{2(0.38) + 0.44}{3} = 0.40$. The extent of the "zig-zag" effect of the frequency of *A2* in males and females diminishes as equilibrium is approached (Figure 9.2). In this case, after eight generations, $q_f = 0.3997$ and $q_m = 0.4006$. Eventually, at equilibrium, $q_f = q_m = \bar{q}$, which persists in the continued presence of Hardy–Weinberg conditions.

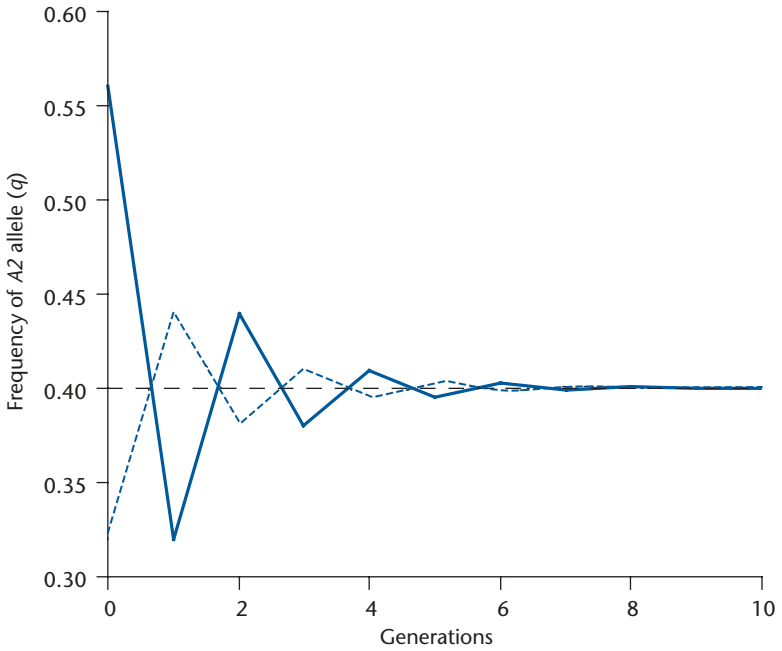


Figure 9.2 Establishing Hardy–Weinberg equilibrium for an X-linked locus. The starting frequency of the *A2* allele (q) in females is 0.32 and in males 0.56. The frequency of the *A2* allele in females (dotted line), males (solid line), and the overall population (dashed line; $q = 0.40$) are shown for successive generations after the onset of Hardy–Weinberg conditions.

Hardy–Weinberg Equilibrium with Two Loci

The establishment of genetic equilibrium for two alleles at two different autosomal loci, say alleles *A1* and *A2* at locus *A* and alleles *B1* and *B2* at locus *B*, occurs when the frequency of a gamete with a particular combination of alleles, that is, *A1B1*, *A1B2*, *A2B1*, or *A2B2*, equals the product of the frequencies of the individual alleles that comprise a gamete. If P represents the frequency of a gamete with a particular genetic composition and p and q the frequencies of the alleles, then a population is in equilibrium for the two loci when $P_{A1B1} = p_{A1}p_{B1}$, $P_{A1B2} = p_{A1}q_{B2}$, $P_{A2B1} = q_{A2}p_{B1}$, and $P_{A2B2} = q_{A2}q_{B2}$. This form of equilibrium is called gametic or linkage equilibrium. Although not noted here, the relationship between gametic and individual allele frequencies for linkage equilibrium holds for any number of autosomal loci.

When the frequency of one or more of the gametic combinations of alleles from two loci does not coincide with the combined individual frequencies of the two alleles, for example, $P_{A1B1} \neq p_{A1}p_{B1}$, then linkage disequilibrium is said to exist. In this context, the term "linkage" does not refer exclusively to loci on the same chromosome, but it also includes unlinked loci. The parameter D is used to signify linkage disequilibrium. The measure of D is the value of the product of the frequencies of gametes with the same type of alleles, that is, $P_{A1B1}P_{A2B2}$, minus the product of the frequencies of gametes with different types of alleles, that is, $P_{A1B2}P_{A2B1}$. Thus, $D = P_{A1B1}P_{A2B2} - P_{A1B2}P_{A2B1}$. When $D = +0.25$, only gametes with same type of alleles, that is, *A1B1* and *A2B2*, occur in a population; whereas when $D = 0$, the two loci are in genetic equilibrium. With linkage disequilibrium as an indicator of genetic equilibrium, the lower the frequency of recombination, the longer the time it takes for genes on the same chromosome to reach equilibrium (Figure 9.3). And fewer generations are required to establish genetic equilibrium with independently assorting loci than those that are genetically linked (Figure 9.3). After many generations, if two

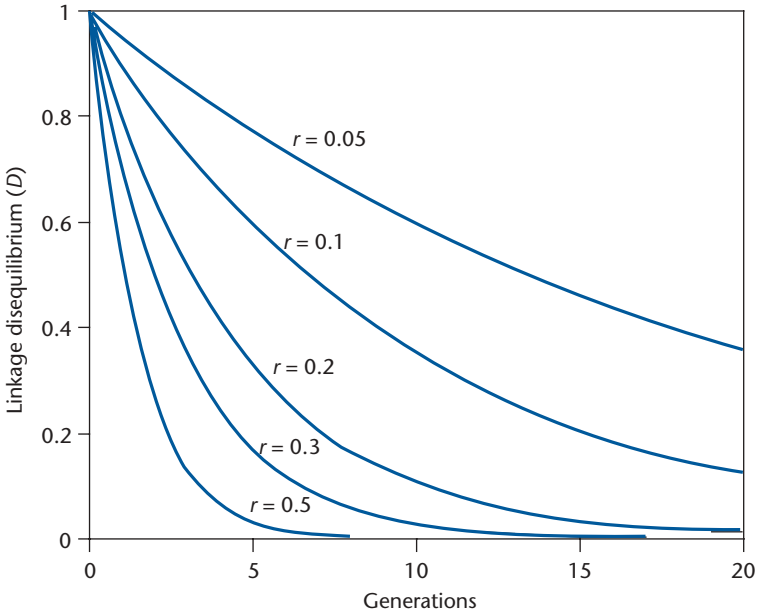


Figure 9.3 Time course for establishing linkage equilibrium ($D = 0$) for two loci with different recombination frequencies (r). Unlinked loci are represented by $r = 0.5$. Only *A1B1* and *A2B2* gametes are present in the population when $D = +0.25$.

genes do not reach genetic equilibrium, then either they are absolutely linked or viability and/or fertility is affected when they occur together, thereby invalidating one the tenets of the Hardy–Weinberg conditions.

Processes That Alter Allele and Genotype Frequencies in Populations

As has been emphasized repeatedly, HWE depends on certain assumptions about a population and its genes. Briefly, the population must be very large; all possible matings occur with no choice being made on the basis of any inherited feature; genes do not mutate; there is no flow of genes due to migration in or out of the population; and every allele has the same likelihood of being transmitted to the next generation. Specific examples of loci in HWE in human populations have been cited, and many others are known. In these cases, it is assumed that the full set of Hardy–Weinberg conditions are operating. However, these conditions need not be the only stipulations to achieve constancy of allele frequencies in a population. In some instances, phenomena such as mutation and nonrandom of transmission of alleles counterbalance each other to produce no net change in the frequencies of alleles or genotypes from one generation to the next.

Notwithstanding the significance of HWE, it doesn't require much knowledge of genetics and human biology to realize that human populations are not always large enough to approach the HWE assumption; mating is not necessarily completely random; genes mutate; intermixing between different groups is a common occurrence; and some genes diminish reproductive fitness. The terms dispersive (random, stochastic) and systematic (deterministic, vectorial) have been used to characterize the processes that alter allele and genotype frequencies in populations. With dispersive processes such as population size and mate preference, the extent but not the direction of change of the frequency of an allele is predictable, whereas with systematic processes such as migration, mutation, and differential reproductive fitness (selection) both the magnitude and direction of gene change can be predicted. Accordingly, it is of considerable interest to determine the genetic consequences when there is noncompliance with the HWE requirements.

Population Size

The consequences of a small population on gene and genotype frequencies can be illustrated in various ways. Often, a classroom demonstration of this effect entails drawing 10 beans from a bag containing 500 red and 500 white beans. In this case, the beans represent two alleles of a locus with $p = q = 0.5$. Students are asked to score the number of white and red beans in the first sample, then replace the 10 beans and draw and score another sample of 10 beans, and so on. Under these conditions, the values of p and q differ dramatically from one sample to the next. By contrast, when the bag contains 10,000 beans with equal numbers of white and red beans and 2000 beans were drawn per sample, scored, and replaced, the values of p and q in each sample are very close to the original frequencies. These results demonstrate that in a large population the

sampling of gametes has very little impact on allele frequencies in the next generation. On the other hand, in small populations, the frequencies of alleles, due to sampling, vary considerably from one generation to the next. In more formal terminology, the sampling fluctuations of gene frequencies in small populations exemplifies the phenomenon called random genetic drift. Absolute constancy of allele frequencies is statistically impossible in a small population even if all of the other Hardy–Weinberg conditions are operative.

The bean-bag model, as outlined above, has limitations. For example, it does not present a picture of what happens to the genetic composition of a relatively small population over a number of generations. Gene changes through successive generations could be tracked by setting up a new bean-bag after each sampling with the proportion of beans that were observed at the previous drawing. In addition, the results from a number of bean-bags, each with the same original allele frequencies, should be scored to obtain an overall picture of the effect of random genetic drift.

Fortunately, computer simulations, which are less arduous and considerably faster than the bean-bag strategy, can be used to demonstrate the results of random genetic drift over a number of generations. Usually, these programs are written to take into account all of the Hardy–Weinberg parameters, including nonoverlapping of generations, while setting different values for the number of mating individuals per generation. The mean number of individuals who contribute genes to the next generation is called the effective population size (N_e). When the values for N_e are small, the analyses always show fluctuating gene frequencies in successive generations. Invariably, after a number of generations, one of the alleles is lost, that is, p or $q = 0$, while the other allele, obviously, becomes the only one at the locus in the population, that is, p or $q = 1.0$ (Figure 9.4). When the frequency of an allele becomes 1.0, the gene is said to be genetically fixed. The smaller the number of breeding individuals in a population, the fewer the number of generations before genetic fixation occurs. For example, if the effective population size is 20, then genetic fixation is expected

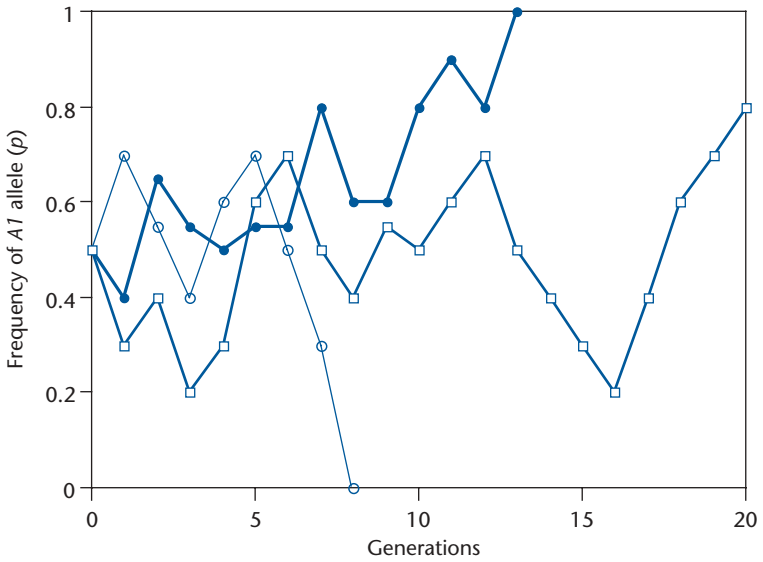


Figure 9.4 Representation of a computer simulation demonstrating the consequences of random genetic drift over time. Only three populations (●, ○, □) are shown. The initial frequency of the *A1* (p) and *A2* (q) allele in each population is the same, that is, $p = q = 0.5$. The effective population size is maintained at 10 male and 10 female parents per generation. All of the other Hardy–Weinberg conditions are maintained in successive generations.

in 80 generations. When computer models are run with a large number of independent populations of the same size and with the same initial allele frequencies, the patterns of fluctuation of allele frequency vary from population to population, and eventually one of the original alleles in each population, becomes fixed. Because genetic fixation under these conditions is strictly a random event, there is a 50:50 chance that one of the alleles will either be lost or fixed (Figure 9.5). With respect to genotypes, random genetic drift over time increases homozygosity and decreases heterozygosity.

Genetic Structure of Isolated Human Populations

Genetic models provide a conceptual framework for considering changes of allele and genotype frequencies in successive generations. However, real human populations are unlikely to conform to the parameters that are required for HWE. In addition to random genetic drift, small human groups are likely to experience nonrandom mating. And, depending on social arrangements, some individuals may transmit more of their genes, on average, to the next genera-

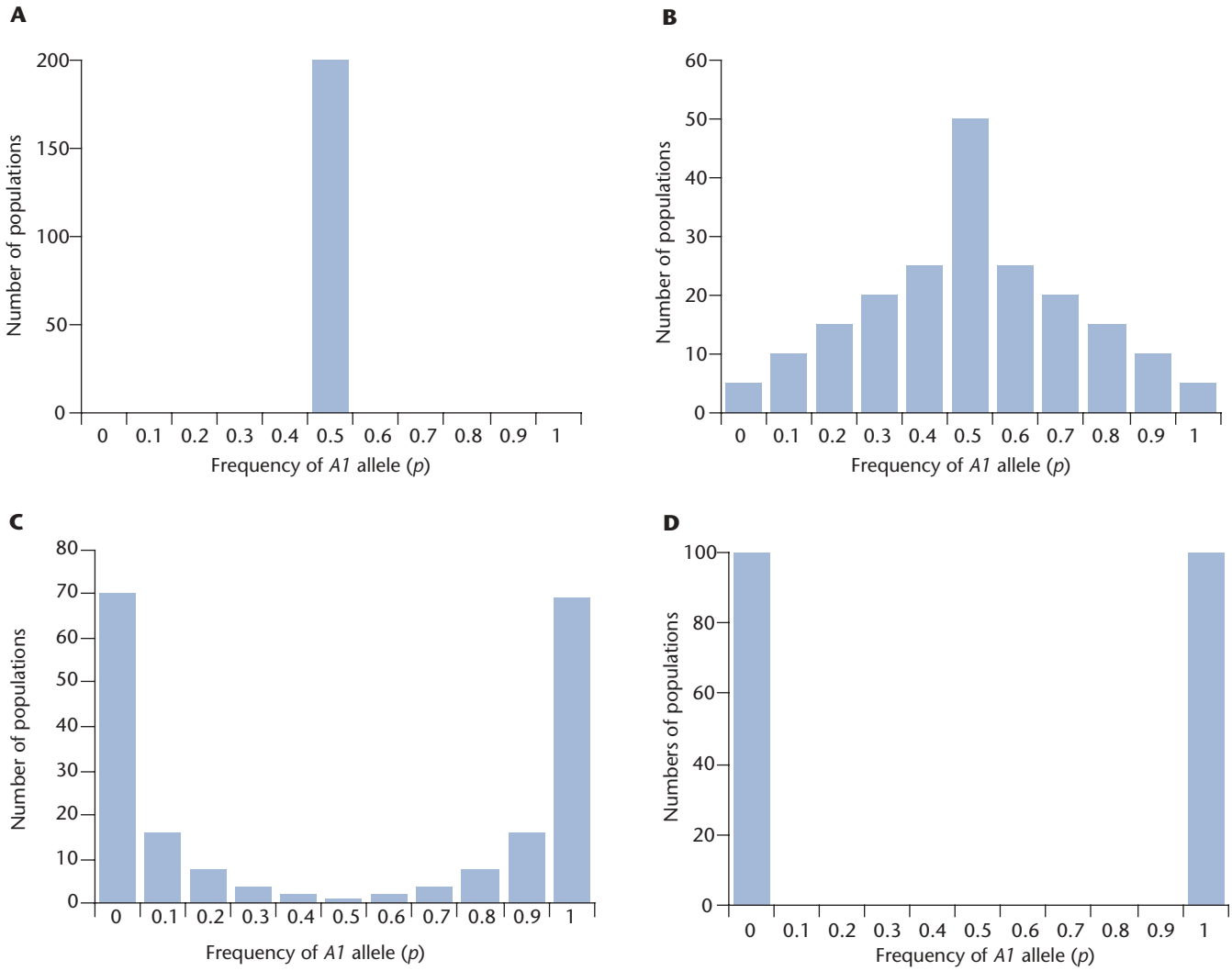


Figure 9.5 Frequency of the *A1* allele in 200 small populations over time. The effective population size is maintained at 10 male and 10 female parents per generation. All of the other Hardy–Weinberg conditions are maintained in successive generations. The initial frequency of the *A1* (p) and *A2* allele (q) in each population is the same, that is, $p = q = 0.5$ (A). (B), (C), and (D) likely frequency distribution of the *A1* allele in 200 populations after about 5, 30, and 90 generations, respectively.

tion than others. Human groups often expand but then because of pressure on the environment or, for other reasons, subdivide into smaller communities. In turn, small populations may merge with other groups. Historically, cultural mores and/or geography have isolated human populations from one another. In the 1960s, J. V. Neel (1915–2000) initiated broad-ranging studies of some extremely isolated South American tribes. Specifically, for more than twenty years, Dr. Neel and his colleagues focused on the health, diseases, nutrition, demographics, population structure, and genetics of the Yanomame tribe, which is located in the region of the Orinoco basin at the borders of southern Venezuela and northern Brazil.

The Yanomame gather food from the rain forest, hunt small animals, and maintain garden plots of plantain, manioc, and maize. They live in villages that are separated by 15 to 40 miles from each other and contain from 50 to 250 individuals. Over the period of the study, some villages fused with others and some divided into smaller enclaves. On occasion, voluntary and involuntary movement of individuals occurred between both Yanomame villages and other tribes. In other words, as expected, the Yanomame populations were subjected to the interplay of many different factors and not merely random genetic drift. With such dynamic conditions, an intriguing question was whether the Yanomame villages had similar or divergent allele frequencies.

Analysis of 16 alleles from nine gene systems in 48 Yanomame villages revealed a range of allele frequencies for many of the loci (Figure 9.6). In some instances, the frequency of an allele varied from 0.2 to 0.8. Some villages did not have an allele that was present in other villages. And genetic fixation occurred in some villages, but not in others. This pattern of disparate allele frequencies between villages of the same tribe was called genetic microdifferentiation. The principal factors other than sampling effects that contributed to genetic microdifferentiation of Yanomame villages were unequal allele frequencies between the two sexes, which was attributed to female infanticide; differential fertility because the headman in each village had more than one wife and as a result had more children on the average than the other males in

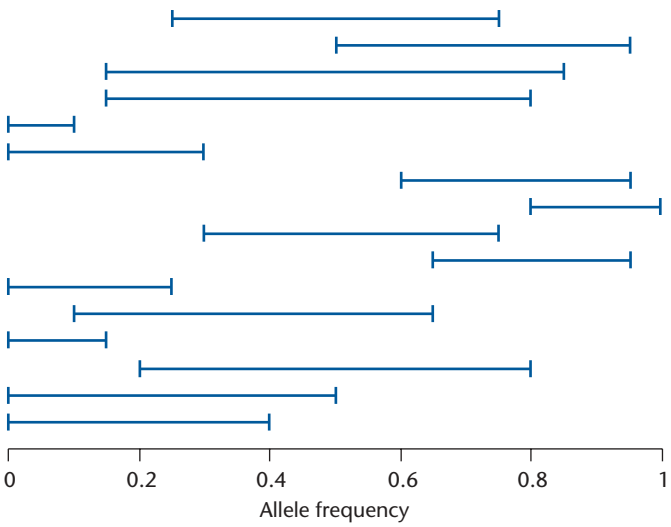


Figure 9.6 Frequency ranges of 16 alleles of nine loci in 48 Yanomame villages. Each line represents the range of frequencies of an allele from the sampled villages. Values of 0 and 1 denote genetic fixation. Adapted from J. V. Neel. 1994. *Physician to the Gene Pool*, John Wiley and Sons, NY, p.193.

the village; and marriages between individuals from different villages. It is problematic whether the genetic differences among the Yanomame villages can be extrapolated to other isolated human groups. And, as Neel has emphasized, the genetic structure of one extant, unacculturated group does not necessarily represent that of prehistoric communities in general. However, despite the distinctive nature of the genetics of the Yanomama, stochastic factors undoubtedly affect the genetics of all isolated human groups.

Founder Effect

A genetic phenomenon called the founder effect (founder principle) begins in a small population. In this case, a rare recessive allele that happens to be present in a member of an original group of settlers is transmitted through successive generations as the population expands and remains isolated geographically or culturally from neighboring groups. After 10 or so generations, under these conditions, the population can be quite large and many of the descendants carry a copy of a recessive allele that originated from one of its founders. As a result of matings between these heterozygotes, a number of children are born with a homozygous recessive condition that is rare in other populations (Figure 9.7). The initial entrapment of a particular recessive allele in a small but growing, isolated population is strictly a chance phenomenon. As well, "founder effect" heterozygotes who marry and have affected offspring are not necessarily closely related genetically. In other words, inbreeding in the contemporary population is not usually a significant feature of a founder effect. However, during the early generations, when the population was being established, differential reproduction and nonrandom mating probably occurred. It is difficult to trace the origin of a particular allele in a contemporary population to a specific founder without detailed birth records. However, when sufficient information is available, the data often indicate that one founder contributed the allele for one disorder.

There are a number of examples of founder effects in different parts of the world. In Finland, for example, genetic isolates of small numbers of founders have grown to over 5×10^6 individuals in approximately 100 generations, resulting in more than 35 genetic disorders that are collectively called the "Finnish disease heritage." In the Charlevois-Saguenay-Lac Saint Jean region of northeastern Quebec in Canada, a population that originated in 1675 with a small

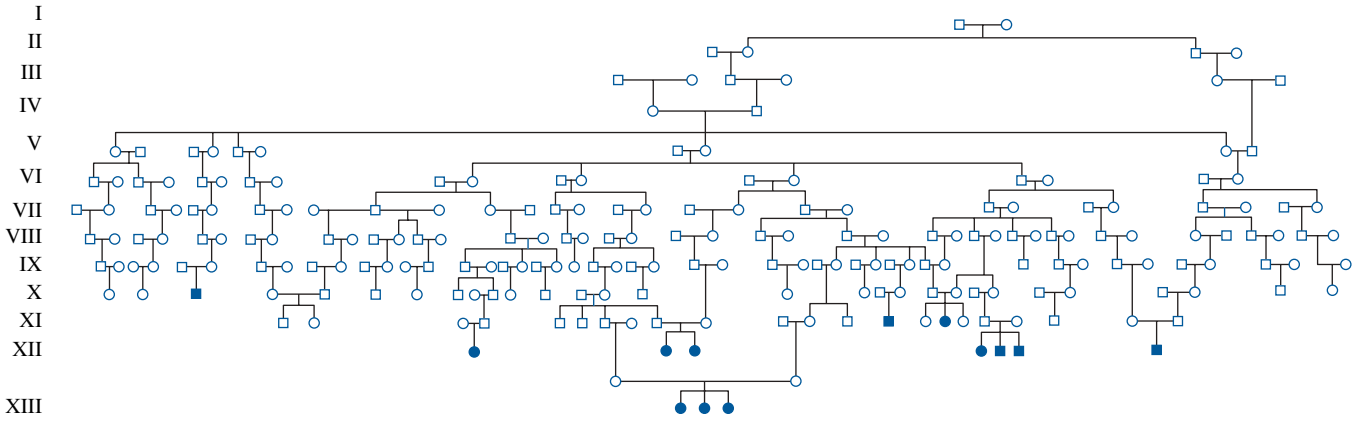


Figure 9.7 Founder effect in Yarmouth County, Nova Scotia, Canada. The original population was founded in about 1767. The individuals who are affected with a rare autosomal recessive neurodegenerative disorder called Niemann–Pick type D disease are represented by solid symbols. Adapted from Figure 1 in W. L. Greer et al. 1999. *Clin Genet* **55**:248–255.

Table 9.8 Autosomal recessive disorders that occur with increased frequencies in the Charlevoix-Saguenay-Lac Saint Jean region of Quebec, Canada.

Disorder	Clinical features	Frequency of heterozygotes
Cystinosis	Lysosomal storage disorder; excess excess excretion of amino acids and phosphate; softening of bones (osteomalacia); kidney damage	0.0256
Cytochrome *c* oxidase deficiency	Decreased blood pH and bicarbonate; muscle weakness (hypotonia); abnormal facial shape (dysmorphism); excess lactate in blood and cerebrospinal fluid (metabolic acidosis); liver malfunction	0.0323
Histidinemia	Histidine ammonia lyase (histidinase) deficiency; usually benign; occasionally mental retardation, kidney damage, and/or neurological defects	0.0313
Lipoprotein lipase deficiency	Increased triglyceride levels in the blood; abdominal pain; damage to the pancreas; enlarged liver and spleen; sudden occurrences of yellow lesions on the body	0.0233
Mucolipidosis	Lysosomal storage disorder; damage to bone, cartilage, and connective tissue	0.0256
Pseudo-vitamin D-deficient rickets	Calcium deficiency (hypocalcemia); increased secretion of the parathyroids; bone deformities and abnormal bone development (rickets)	0.0385
Pyruvate kinase deficiency	Destruction of red blood cells (hemolytic anemia); lethargy; chills; fever; back and abdominal pains	0.0156
Sarcosinemia	Sarcosine dehydrogenase deficiency; mental retardation	0.0345
Sensorimotor polyneuropathy	Sensory loss; muscle weakness and wasting; numbness; decreased tendon reflexes	0.0435
Spastic ataxia	Abnormal gait; involuntary muscle movements; lack of muscle coordination; wasting of distal muscles; loss of control of eye muscles	0.0476
Tyrosinemia type I	ρ-Hydroxyphenylpyruvate oxidase deficiency; liver and kidney damage; severe pain in arms and legs	0.0455

number of founders ($N \approx 600$) now consists of over 310,000 individuals. During the 12 generations that this population has grown and remained relatively isolated, the frequencies of heterozygotes for different disease-causing loci have increased 5 to 40 times in comparison to the general population (Table 9.8). Each of these homozygous recessive disorders occurs infrequently in other populations.

Migration

Human populations are seldom sedentary and rarely completely isolated. Individuals from one population often join other populations. These movements

(migrations) may alter the genetic structure of the population that acquires genes by immigration. The introduction of genes into a recipient population is called gene flow, and genetic admixture is the term describing the consequences of the amalgamation of two or more groups of individuals with dissimilar genetic compositions. The impact of gene flow depends on the proportion of reproducing immigrants entering a population, the size of the recipient population, and the extent of the differences of gene frequencies between immigrant (contributing) and recipient populations. Theoretically, gene flow can introduce new alleles into a population, reduce the level of homozygosity in the recipient population, and equalize genetic differences between populations.

Some of the genetic effects of migration can be demonstrated with a specific example for a two-allele, one-locus system (Figure 9.8). In this case, the extent

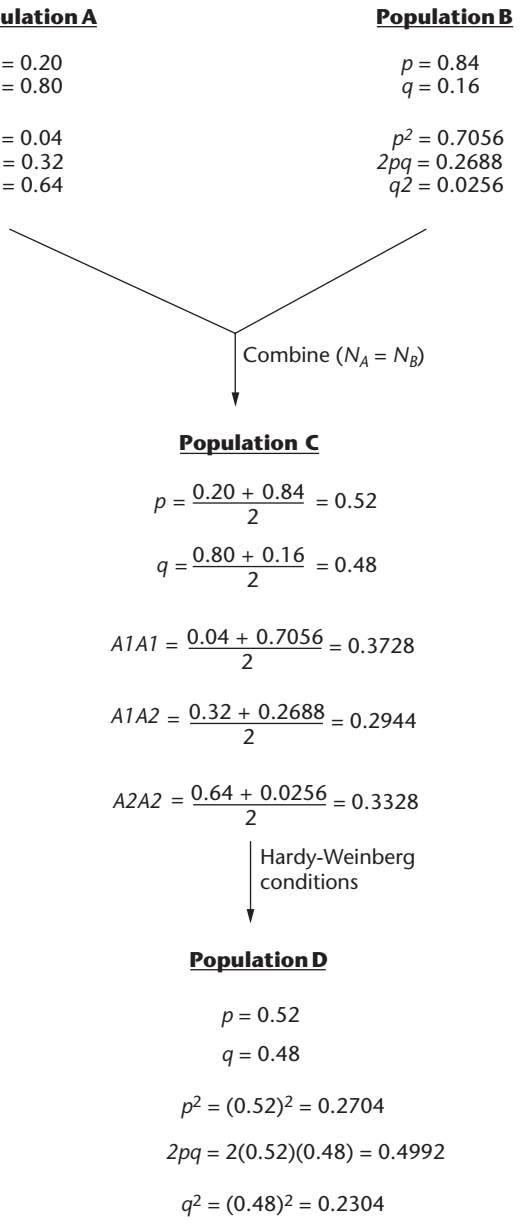


Figure 9.8 Genetic consequences of combining two populations of equal size that have different allele and genotype frequencies. The sizes of populations A and B are designated as N_A and N_B, respectively.

of migration is extreme because the combined populations have the same size. In the real world, a group of immigrants is usually much smaller than the recipient population. As shown in Figure 9.8, the two initial populations have different allele frequencies and after these populations fuse, the new population (Figure 9.8, population C) has, in comparison to either of the original populations, new allele and genotype frequencies that, if Hardy–Weinberg conditions are present, reach HWE in one generation (Figure 9.8, population D). After the original populations combine, the proportion of homozygotes, that is, homozygosity, is $\frac{p_A^2 + q_A^2 + p_B^2 + q_B^2}{2} = \frac{0.04 + 0.64 + 0.7056 + 0.0256}{2} = 0.7056$, where the subscripts A and B denote the original populations. By contrast, the frequency of homozygous genotypes of the HWE population becomes $p^2 + q^2 = 0.5008$ (Figure 9.8, population D). Clearly, the extent of overall homozygosity has diminished. This outcome reflects a general phenomenon called the Wahlund effect (Wahlund principle) that occurs when the allele frequencies of the original populations are different, regardless of the sizes of the two populations. Obviously, if the allele frequencies of the two populations are the same, then there is no genetic change and, consequently, no reduction in overall homozygosity occurs after HWE is established.

Gene flow from a large population to a smaller one can be generalized. For example, after immigrants from population 1 enter population 2, the proportion of immigrants in the combined population is m and the fraction of nonimmigrant (natives) is $1 - m$. Under these circumstances, the frequency of the *A2* allele after migration (q_3) is

$$q_3 = mq_1 + (1 - m)q_2$$

where q_1, q_2, and q_3 represent the frequencies of the *A2* allele in the contributing (population 1), premigration (population 2), and combined (population 3) populations, respectively. The equation $q_3 = mq_1 + (1 - m)q_2$ can be reorganized in terms of m:

$$q_3 - q_2 = m(q_1 - q_2),$$
$$m = \frac{q_3 - q_2}{q_1 - q_2}$$

In this form, m represents a measure of genetic admixture in the recipient population. Parenthetically, the expressions $\frac{q_3 - q_2}{q_1 - q_2}$ and $\frac{q_2 - q_3}{q_2 - q_1}$ are algebraically equivalent. For this reason, one or the other may be found in population genetics textbooks. Also, the value of m is the same regardless of which allele is used, that is, $m = \frac{q_3 - q_2}{q_1 - q_2} = \frac{p_3 - p_2}{p_1 - p_2}$.

It is difficult to determine the specific effect that migration has on the gene frequencies of human populations. Any estimate of the amount of genetic admixture depends on the allele frequencies of the contributing and premigration populations being different. The greater the difference between these allele

frequencies, the more reliable the calculation of genetic admixture. An allele that affects survival or fertility in either of these populations cannot be used for evaluating genetic admixture. Finally, if the premigration population existed centuries ago, then certain assumptions about the allele frequency in the ancestral population based on an extant descendant population must be made.

With these stipulations in mind, the extent of genetic admixture in African American populations has been calculated. The frequencies of alleles for a number of different loci were determined for various African American populations and the contributing European American population. Because, in this case, the premigration population(s) existed over 200 years ago, the gene frequencies of contemporary populations in West Africa, who occupy the regions from which African Americans were originally brought to the US as slaves, were used for the ancestral values. Specifically, for the blood group Duffy allele (*FY*A*), which is not found in West Africa, the measure of genetic admixture (m) in New York City was $\frac{0.08-0}{0.43-0}=0.186$. When a number of alleles were considered in this way, it was estimated that approximately 15% to 20% of the genes in African Americans are due to gene flow from the European American population. The range of values for m is about 7% to 25% among southern and northern cities in the US. Genes are also being contributed from the African American population to the European American population, but an estimate of the extent of this gene flow is difficult to determine because of the difference in the sizes of the two populations. However, in three US cities, the frequency of the West African version of the *FY*A* allele, that is, the *FY*O* null allele, is about 1%. If gene flow persists over a number of generations, eventually the original genes will be supplanted (Figure 9.9). And, obviously, the greater the gene flow per generation, the more rapidly the genes in the recipient population are replaced.

In the past, estimate of the amount of genetic admixture in human populations was constrained because there were too few alleles that could be used to generate reliable data. However, with the development of short tandem

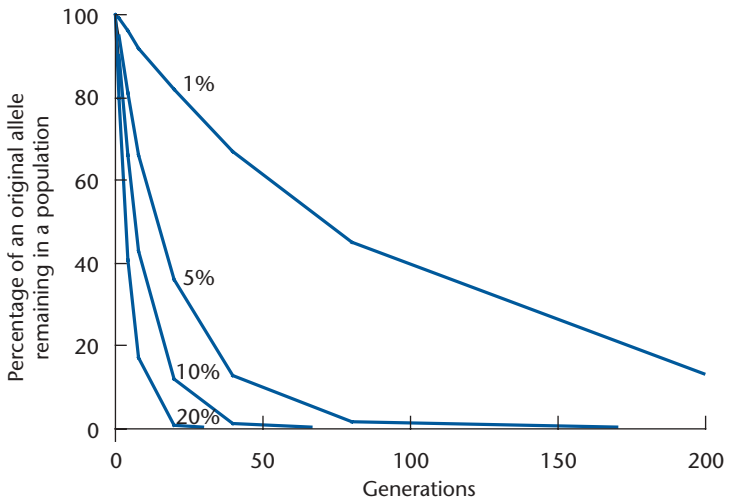


Figure 9.9 Percentage of an original allele remaining in a recipient population after a number of generations with a constant rate of gene flow per generation. The percent values are the amount of gene flow per generation. The curves are derived from the expression $(1-m)^t$, where m is the proportion of gene flow per generation and t the number of generations.

repeat and single-nucleotide polymorphism genotyping, large numbers of alleles are now available for these studies.

Mating Systems

Assortative Mating

With Hardy–Weinberg conditions, all males and females mate in each generation and who mates with whom is strictly a chance phenomenon. The converse of random mating (panmixia, nonassortative mating) is assortative mating (nonrandom mating), which occurs when mates preferentially choose one another on the basis of inherited features or when close relatives marry each other (inbreeding, consanguinity). Assortative mating can be positive (homogamous) or negative (disassortative, heterogamous). Positive assortative mating entails individuals with the same genotype or genetically determined phenotypes marrying each other. On the other hand, negative assortative mating involves choice of mates based on antithetical genotypes and phenotypes. In human populations, neither positive nor negative assortative mating is widespread. Nevertheless, it is easier to illustrate the consequences of nonrandom mating on the genetic structure of a population by assuming that it is absolute.

Consider a population with matings only between individuals with the same genotypes where the alleles *A1* and *A2* are codominant (*A1A1* × *A1A1*, *A1A2* × *A1A2*, *A2A2* × *A2A2*). Under these circumstances, in each successive generation, the frequency of heterozygotes (*A1A2*) is halved and each homozygous genotype (*A1A1*, *A2A2*) is increased by half the amount of the decrease in heterozygosity. Eventually, a novel genetic equilibrium, which is not a HWE, is established with no heterozygotes and only homozygotes in the population (Figure 9.10). In this example, the original frequencies of alleles *A1* and *A2* were 0.60 and 0.40, respectively, and after eight generations with complete positive assortative mating 60% of the genotypes are *A1A1* and 40% are *A2A2*. If the eighth-generation population consisted of 10^3 individuals, then the

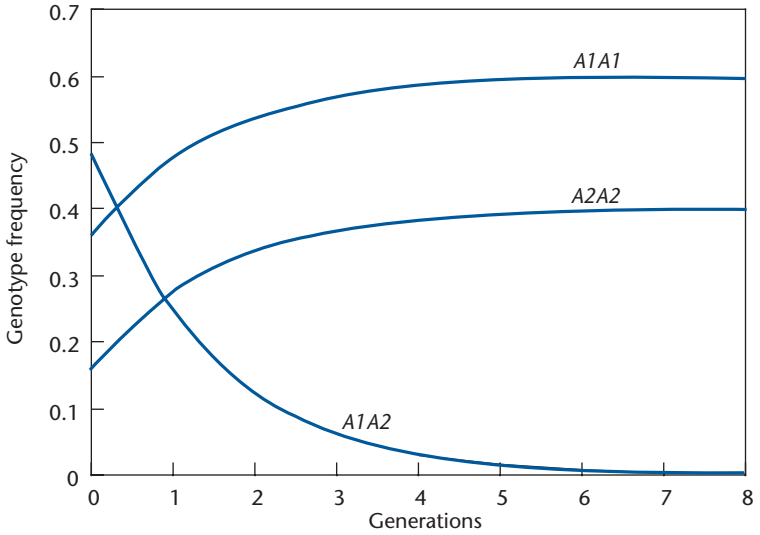


Figure 9.10 Effect of complete positive assortative mating without dominance on genotype frequencies over a number of generations. The starting frequencies of the *A1A1*, *A1A2*, and *A2A2* genotypes were 0.36, 0.48, and 0.16, respectively. At equilibrium, 60% of the population is *A1A1* and 40% *A2A2*.

frequency of $A1 = \frac{2 \times 600}{2 \times 1000} = 0.60$ and the frequency of $A2 = 0.40$. In other words, the frequency of each allele did not change from its original value despite the changes in genotype frequencies. In this case, the total homozygosity goes from 52%, that is, 0.36 + 0.16, in the initial population to 100% by the eighth generation.

Complete negative assortative mating is the reverse of complete positive assortative mating (*A1A1* × *A1A2*, *A1A1* × *A2A2*, *A1A2* × *A1A1*, *A1A2* × *A2A2*, *A2A2* × *A1A1*, *A2A2* × *A1A2*). Each of the matings with complete negative assortment produces heterozygous offspring. Over time, with codominance of the *A1* and *A2* alleles, homozygosity decreases and heterozygosity increases, but allele frequencies do not change from one generation to the next. Eventually, a genetic equilibrium that does not have Hardy–Weinberg proportions is established (Figure 9.11). On the other hand, if the *A1* allele is dominant and negative assortative mating is complete, then only those individuals with dominant and recessive phenotypes will mate with each other, that is, *A1A1* × *A2A2* and *A1A2* × *A2A2*. In this case, a genetic equilibrium is reached in one generation, with two-thirds of the genotypes being *A1A2* and one-third *A2A2*. Dominant homozygotes (*A1A1*) are no longer present in the population. And, regardless of the initial allele frequencies, the frequencies of the *A1* (p) and *A2* (q) alleles at equilibrium are always 0.25 and 0.75, respectively.

Theory notwithstanding, humans seldom consciously choose mates on the basis of their genotypes. Numerous studies of mate preference have found weak positive assortative mating for characteristics that have a genetic component such as height, weight, physical appearance, personality, and intelligence. At the genetic level, each of these traits is determined by a number of different

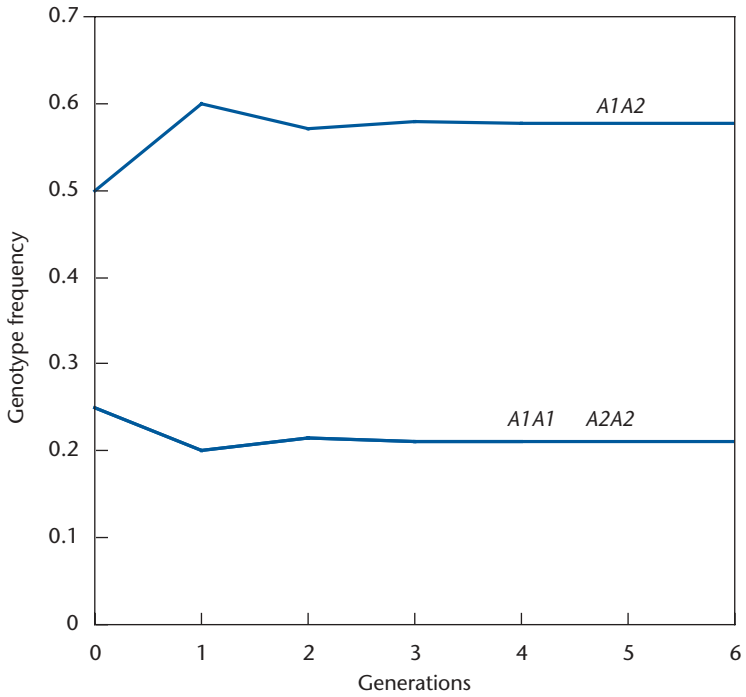


Figure 9.11 Effect of complete negative assortative mating without dominance on genotype frequencies over a number of generations. The starting frequencies of the *A1A1*, *A1A2*, and *A2A2* genotypes were 0.25, 0.50, and 0.25, respectively. The frequency of the *A1* and *A2* alleles does not change in successive generations. At equilibrium, the frequencies of the *A1A1*, *A1A2*, and *A2A2* genotypes are 0.2113, 0.5774, and 0.2113, respectively.

loci acting in concert to produce the phenotype. Moreover, these phenotypes are also affected by environmental influences. More significant correlations between mates have been noted for nongenetic features such as age, birthplace, and nationality. To date, there is no convincing example of persistent positive assortative mating in humans for a phenotype that is determined by a single gene locus.

Inbreeding

Marriages between sibs, parents and their offspring, and other close relatives are illegal in most countries and forbidden in practically all cultures. In Leviticus 18:6–18 of the Hebrew Bible, there are 17 proscribed matings. Although some of these combinations such as brother-sister and other incestuous pairings have a sound genetic basis, the original purpose of the codification of these marriage restrictions probably had nothing to do with biology and everything to do with maintaining social harmony within and between families. For example, because a father-in-law with daughter-in-law mating is banned, a married son, in theory, need not be concerned about his father's intentions toward his spouse. In some instances, there appear to be no prohibitions about who can mate with whom. Akhenaten, an Egyptian pharaoh from 1385 to 1358 BCE, and his children provide an extraordinary example of marriages between members of one family (Figure 9.12). Akhenaten married his mother, his first cousin and his daughter. Each of his sons married a half-sister, and one of these daughters married her grandfather.

Inbreeding (consanguinity) in humans is generally defined as a mating between genetically related individuals. Evidently, what constitutes a "genetically related individual" needs to be clarified. From a genetic viewpoint, the degree of inbreeding is based on the probability that an individual will inherit two alleles that are copies of genes that were present in an ancestor of both parents. In other words, as a consequence of a shared common ancestor, a homozygous genotype is formed by two alleles that are identical by descent (IBD). The relevance of inbreeding depends on the number of generations that separate a couple from their shared ancestor(s). If the interval is four or more

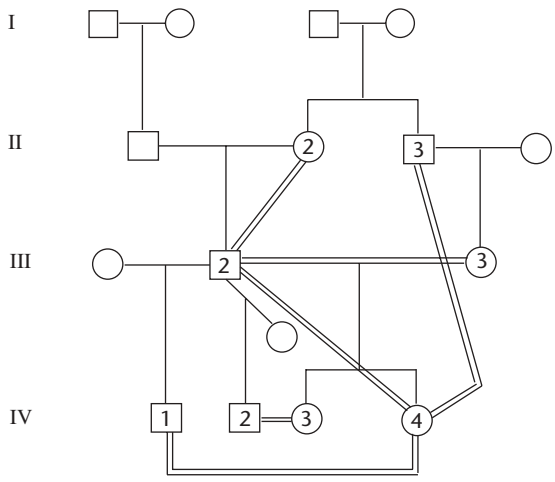


Figure 9.12 Marriages of Akhenaten, Egyptian pharaoh from 1385 to 1358 BCE, and his immediate family. A double line in a pedigree denotes a consanguineous marriage. The number designation for a specific individual is inside his/her pedigree symbol. Akhenaten (III-2) married his mother (II-2), his cousin Nefertiti (III-3), and his daughter (IV-4). Akhenaten's sons (IV-1, IV-2) each married a half-sister (IV-3, IV-4), and one of his daughters (IV-4) married her grandfather (II-3), who was Akhenaten's uncle.

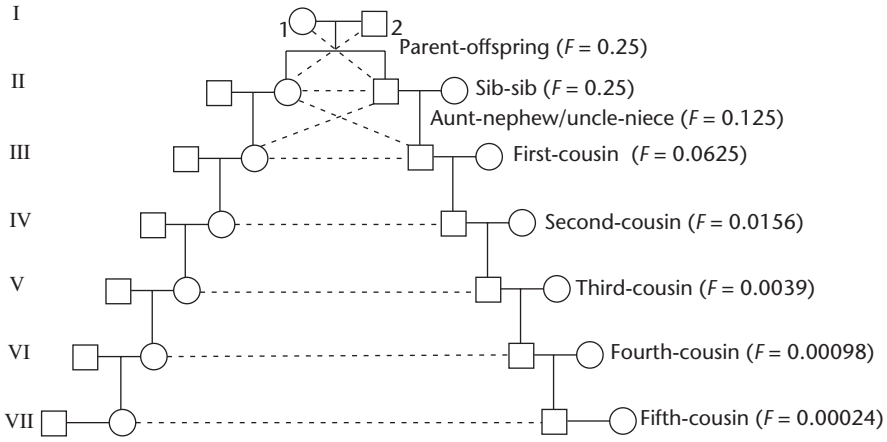


Figure 9.13 Schematic representation of a pedigree showing matings of genetically related individuals. Individuals I-1 and I-2 are the common ancestors for each mating. A dashed line indicates a mating. Both the type of mating and its inbreeding coefficient (*F*) are noted.

generations, the level of inbreeding is considered negligible because there is relatively little chance that any of the offspring will become an IBD homozygote. For example, there are four generations between a third-cousin marriage and the common ancestors who are the great-great-grandparents (Figure 9.13). In terms of consanguinity, the genetic relatedness of third cousins is of little concern. On the other hand, for a first-cousin marriage, which is common in human inbreeding, the distance between the couple and their shared ancestors is two generations (Figure 9.13). In this instance, inbreeding is a significant consideration because the increased likelihood that an offspring will be homozygous for alleles that are identical by descent.

The measure of inbreeding can be quantified by calculating the probability that an individual will have two alleles that are identical by descent. This probability is called the inbreeding coefficient (*F*). For any consanguineous marriage, the value of the inbreeding coefficient depends on (i) the number of transmissions (steps) that are required for an allele of a common ancestor to be passed to both parents and then to their offspring and (ii) the probability of transmission of a particular allele at each step. A first-cousin marriage will be used to depict how an *F* value is computed (Figure 9.14). The common ancestors of the first cousins III-1 and III-2 are their grandparents, I-1 and I-2. Theoretically, I-1 and I-2 can have four different autosomal alleles, that is, *A1, A2, A3* and *A4*, at a single locus. To accommodate four possible alleles, the genotypes of the common ancestors are *A1A2* and *A3A4*. Consequently, a putative offspring IV-1 of a first-cousin marriage could have one of four possible homozygous genotypes that are identical by descent. Simply put, IV-1 could be *A1A1, A2A2, A3A3,* or *A4A4*. The probability of transmission of each of the four possible alleles will be considered separately. Based on segregation, the probability that *A1* will be transmitted from I-1 to II-2 is 1/2, from II-2 to III-1 1/2, and from III-1 to IV-1 also 1/2. Similarly, the probability of the transmission of *A1* from I-1 to II-3 to III-2 to IV-1 is 1/2 for each of these three steps. The probability that IV-1 will be *A1A1* is the product of the probabilities for each of the six steps, that is, $(1/2)^6$. Thus, IV-1 has $(1/2)^6$ chance of being homozygous for the *A1* allele. The complete set of steps that result in a homozygous genotype that is identical by descent is often called a

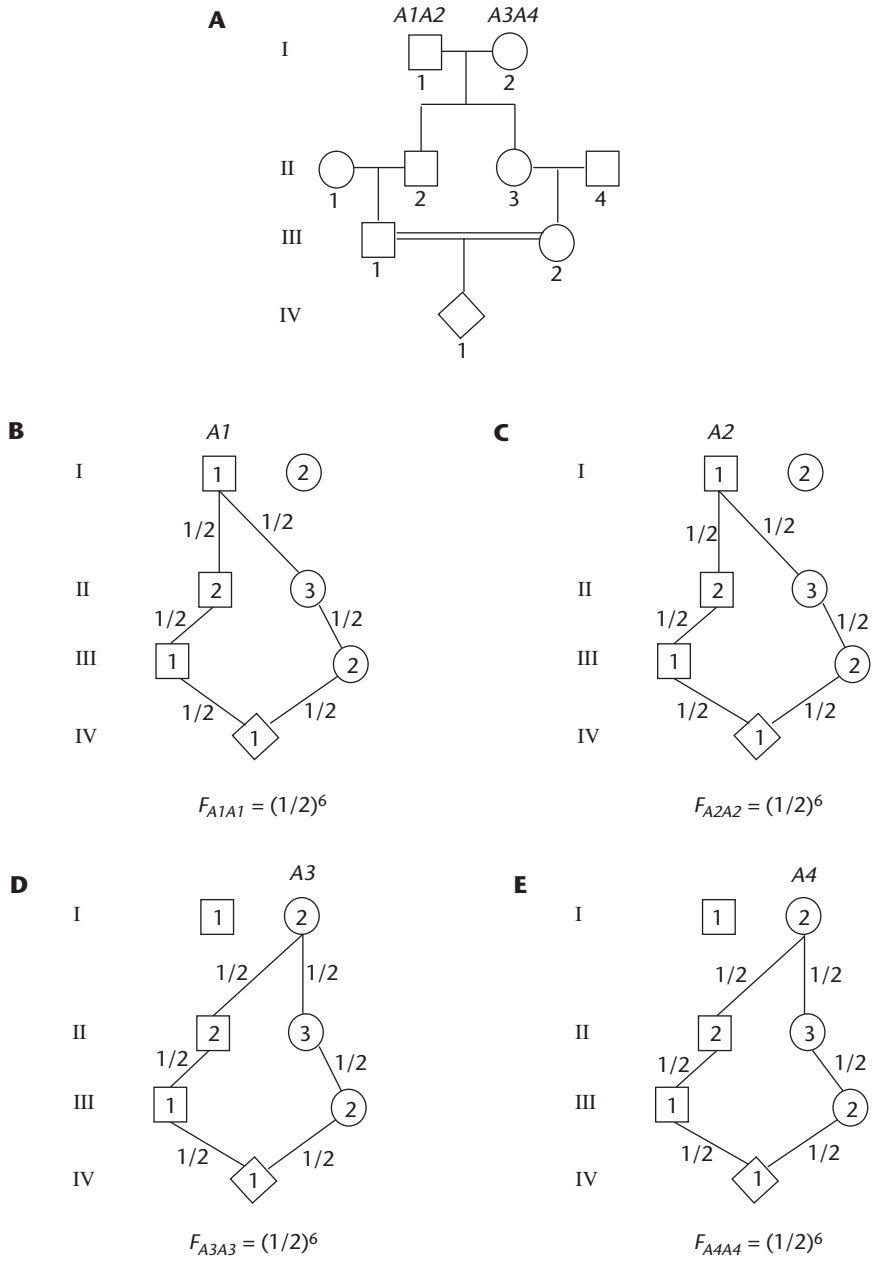


Figure 9.14 Determination of the inbreeding coefficient of an autosomal locus for a first-cousin marriage. (A) Pedigree showing a first-cousin marriage. The genotypes of the common ancestors (I-1, I-2) are *A1A2* and *A3A4*. (B–E) Marriage and descent lines have been removed from the pedigree shown in A for clarity, and the transmission paths for each allele are provided. The number designation for genetically relevant individuals is retained and placed inside his/her pedigree symbol. The probability of each step of a path that gives rise to *A1A1*, *A2A2*, *A3A3*, and *A4A4* genotypes in the offspring (IV-1) is presented in B, C, D, and E, respectively. The inbreeding coefficient (*F*) for each complete path is noted. The *F* value for a first-cousin marriage is the sum of the *F* values for each of the possible alleles at a locus of the common ancestors becoming identical by descent.

path. A complete path can be traced in different ways. One approach is to track it back from the putative offspring to a common ancestor and then down to the other spouse and back to the offspring, for example, IV-1 to III-1 to II-2 to I-1 to II-3 to III-2 to IV-1 (Figure 9.14B). Following the same reasoning as presented for *A1*, the likelihood that IV-1 will be *A2A2*, *A3A3*, or *A4A4* is $(1/2)^6$ for each genotype (Figure 9.14C, D, and E). Accordingly, the probability that IV-1 will be homozygous for any pair of alleles that are identical by descent is the sum of probabilities for each individual allele, that is, $(1/2)^6 + (1/2)^6 + (1/2)^6 + (1/2)^6 = 1/16 = 0.0625$. Thus, the inbreeding coefficient *F* for first-cousin marriages is 0.0625. Inbreeding coefficients for other consanguineous

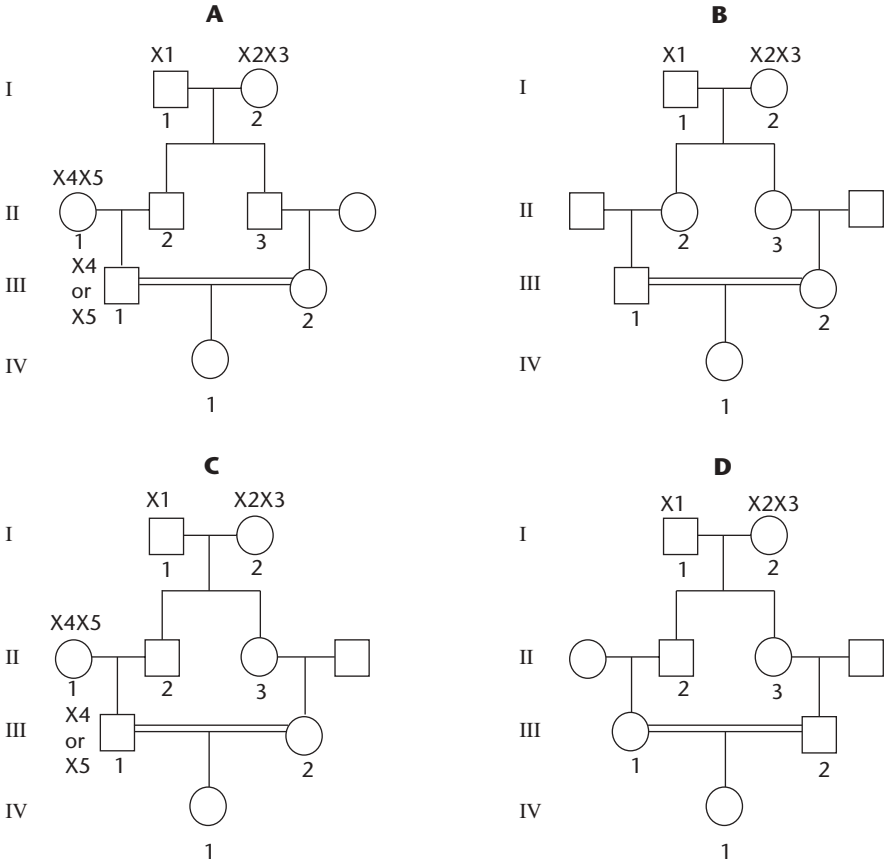


Figure 9.15 Identity by descent of X-linked alleles with first-cousin marriages. (A) None of the female offspring of the first-cousin marriage can be homozygous for X-linked alleles that are identical by descent because the X chromosome, that is, X4 or X5, carried by III-1 is from outside the direct line of descent. (B) Offspring of the first-cousin marriage can be identical by descent for any of the X-linked alleles on the X chromosomes (X1, X2, X3) of the common ancestors (I-1, I-2). (C) None of the offspring of the first-cousin marriage can be homozygous for X-linked alleles that are identical by descent because the X chromosome (X4 or X5) carried by III-1 is from outside the direct line of descent. (D) Offspring of the first-cousin marriage cannot be identical by descent for any of the alleles on chromosome X1 because it is not transmitted to II-2, but they can be identical-by-descent homozygotes for any of the alleles on chromosomes X2 or X3.

marriages can be determined in this way (Figure 9.13). For example, the F value for third-cousin matings is $4(1/2)^{10} = 0.0039$. This method of calculating F values assumes that the common ancestors were not the products of consanguineous marriages and, in theory, there was no inbreeding in the ancestors' population.

In principle, the calculation of the inbreeding coefficient for both autosomal and X-linked genes is the same. However, because males have only one X chromosome, they can never be homozygous for alleles that are identical by descent. Moreover, males do not transmit an X chromosome to their sons. With these considerations in mind, the F value for X-linked genes in first-cousin marriages depends on the sex of the two sibs whose children are marrying. There are four possible types of first-cousin family arrangements. First cousins can have fathers who are brothers (Figure 9.15A), mothers who are sisters (Figure 9.15B), the father of the male cousin who is the brother of the mother of the female cousin (Figure 9.15C), or the mother of the male cousin who is the sister of the father of the female cousin (Figure 9.15D). With the arrangement in Figure 9.15A, the male cousin (III-1) carries an X chromosome from his mother who is not in the direct line of descent from the common ancestors (I-1 and I-2). The F value for this union is zero because no identical-by-descent homozygosity can occur among his offspring. If the mothers of the first cousins are sisters, then X chromosomes from the shared ancestors can be

transmitted to the offspring of the first-cousin marriage (Figure 9.15B). Because every daughter receives an X chromosome from her father, the probability of this transmission is 1. Thus the probability of the path from the shared male ancestor (I-1) to IV-1 through the male cousin (III-1) is [(1)(1/2)(1)], and through the female cousin (III-2), it is [(1)(1/2)(1/2)]. The complete path is [(1)(1/2)(1)(1)(1/2)(1/2)]. The probabilities of the two paths for the X chromosomes from the shared female ancestor (I-2) are both [(1/2)(1/2)(1)(1/2)(1/2)(1/2)]. The sum of the probabilities of the three paths is 3/16, that is, $F = 3/16 = 0.1875$. The F value for the first-cousin arrangement in Figure 9.15C is zero because the male first cousin (III-1) does not have an X chromosome that originated in one of the common ancestors. For the arrangement in Figure 9.15D, the X chromosome (X1) of the male common ancestor (I-1) is not transmitted to his son (II-2) and, consequently, the offspring cannot be identical by descent for alleles on this chromosome. On the other hand, the alleles on either of the X chromosomes of I-2 can become identical by descent in the offspring of this first-cousin marriage. The probability of the path for each of these X chromosomes is [(1/2)(1)(1/2)(1/2)(1/2)(1)]. Therefore, $F = 1/8 = 0.125$.

Often, in an extended pedigree, a couple has a number of shared common ancestors in different generations. The formula

$$Fx = \sum_{A} (1/2)^{n+m+1}(1 + F_{\mathrm{A}})$$

was devised by Sewall Wright (1889–1988) to determine the inbreeding coefficient of any individual in a multigeneration kindred. With this equation, n and m are the number of generations from the father and mother of individual x respectively to a common ancestor A, F_{A} is the inbreeding coefficient of the common ancestor A, and F_{X} is the inbreeding coefficient of individual x. The value of F_{X} is the sum of each path, that is, $(1/2)^{n+m+1}$, from a couple to each of their common ancestors. With a first-cousin marriage, for example, where $F_{\mathrm{A}} = 0$, the number of generations from the male of the first-cousin couple to one of the nearest common ancestors is 2, that is, $n = 2$, and from the female partner to the same ancestor $m = 2$. Similarly, when the generations are counted back to the other common ancestor, $n = 2$ and $m = 2$. Thus the inbreeding coefficient for offspring of this mating is

$$F_{\mathrm{x}} = \sum (1/2)^{n+m+1} = (1/2)^{2+2+1} + (1/2)^{2+2+1} = 2(1/2)^{5} = 0.0625$$

As expected, Wright's formula is equivalent to summing the probabilities of all of the paths that may lead to the formation of a homozygote that is identical by descent.

Based on the extended pedigree in Figure 9.16, the F value for offspring of the marriage between individuals X-1 and X-2 can be determined by counting the number of generations back from this couple to each of the four common ancestors (I-3, I-4, IV-4, IV-5). In this case, $F_{\mathrm{A}} = 0$ for each ancestor, and the inbreeding coefficient is

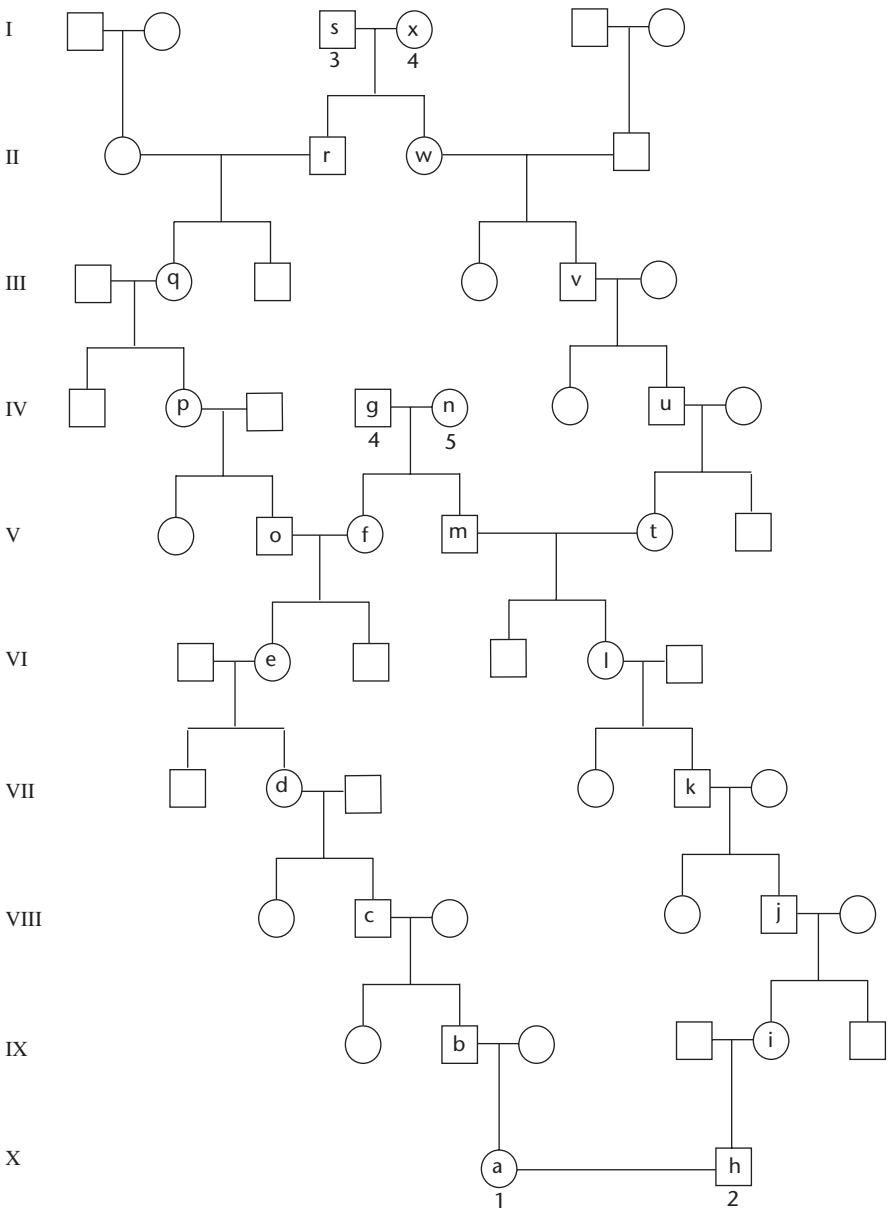


Figure 9.16 Tracking the paths of allele transmission in a multigeneration pedigree. The letters in the symbols help mark the ancestors in the lines of descent between X-1 and X-2 and their common ancestors, IV-4, IV-5, I-3, and I-4.

$$(1/2)^{19} + (1/2)^{19} + (1/2)^{13} + (1/2)^{13} = 0.000248$$

The exponent of the first term is the number of generations from X-1 to I-3 ($n = 9$) plus the number of generations from X-2 to I-3 ($m = 9$) plus 1. The exponents ($n + m + 1$) of the second, third, and fourth terms are based on the numbers of generations from both X-1 and X-2 to I-4, to IV-4, and to IV-5. Parenthetically, the F value in this example is equivalent a fifth-cousin marriage. Because using Wright's formula with "pencil and paper" can be tedious and time-consuming, computer programs have been developed for extremely large pedigrees with complex mating histories.

Table 9.9 Effect of inbreeding on a HWE population.

	Genotype frequency			Allele frequency	
	A1A1	*A1A2*	*A2A2*	*A1*	*A2*
Initial generation	0.16	0.48	0.36	0.40	0.60
Next generation ($F = 0.0625$)	0.16 + Fpq = 0.175	0.48 − $F2pq$ = 0.45	0.36 + Fpq = 0.375	0.175 + 1/2(0.45) = 0.40	0.36 + 1/2(0.45) = 0.60

Effect of Inbreeding on Populations

Inbreeding increases homozygosity and decreases heterozygosity without altering allele frequencies of a population. If a HWE population is subjected to an amount of inbreeding equivalent to F, then, after one generation, the frequency of the heterozygous genotype is reduced by $F2pq$ and the frequency of each homozygous genotype is increased by Fpq (Table 9.9). The change of heterozygosity in one generation can also be used as a measure of the extent of inbreeding if all other factors are in accord with Hardy–Weinberg conditions. In this case,

$$F = \frac{H_0 - H_1}{H_0}$$

where H_0 and H_1 represent the frequencies of the heterozygotes in the initial and following generation, respectively. With data from Table 9.9,

$$F = \frac{H_0 - H_1}{H_0} = \frac{0.48 - 0.45}{0.48} = 0.0625$$

If inbreeding persists, eventually a population approaches 100% homozygosity (Figure 9.17). The rate of accumulation of homozygotes is a function of the extent of inbreeding. Although not relevant for human populations because inbreeding is not pervasive, the inbreeding coefficient increases in successive generations with recurrent inbreeding. With self-fertilization in plants, F approaches 1.0 after 6 generations, and in animals with 16 generations of sib-sib matings, F is greater than 0.80.

The inbreeding coefficient for a population can be calculated from a representative sample of a large number of marriages with the equation

$$\sum m_i F_i$$

where m_i is the frequency of a particular mating and F_i the inbreeding coefficient for that mating. Specifically, if the frequencies of nonconsanguineous, uncle-niece, first-cousin, and second-cousin marriages in a sample are 0.909, 0.001, 0.03, and 0.06, respectively, the F value for the population is [(0.909)(0) + (0.001)(0.125) + (0.03)(0.0625) + (0.06)(0.015625)] = 0.0029. This value indicates that each person in the population has a 0.29% probability of carry-

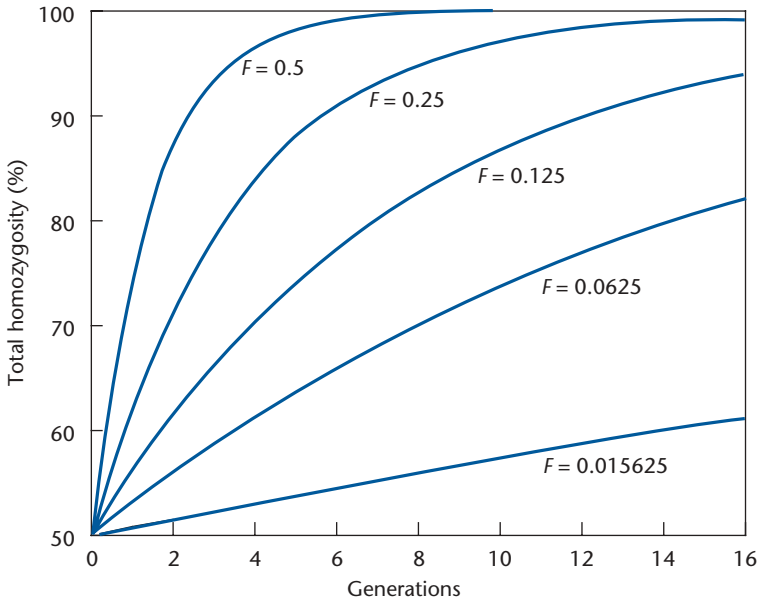


Figure 9.17 Total homozygosity in successive generations due to inbreeding. The value of the inbreeding coefficient (F), which remains constant for each generation, is shown for each curve. The curve marked $F = 0.5$ represents self-fertilization. The other curves from the highest to lowest F value are sib-sib, uncle-niece or aunt-nephew, first-cousin, and second-cousin matings, respectively.

ing two alleles that are identical by descent. For most human populations, F values that are 0.0005 or less signify a very low level of inbreeding. On the other hand, an F value of 0.008 or more implies that inbreeding occurs to some extent in the population. Population inbreeding coefficients as high as 0.03 have been observed (Table 9.10). In some of the populations with high F values, first-cousin and uncle-niece/aunt-nephew marriages are culturally promoted or, at least, not considered taboo. In other instances, consanguinity may result from small population size, ethnic separation, or geographic isolation.

Populations with $F \geq 0.02$ have more stillbirths and childhood disorders including central nervous system defects, cleft palate, cerebral palsy, congenital heart disease, and rare autosomal recessive disorders than populations with lower levels of inbreeding. In this context, the proportion of individuals with autosomal homozygous recessive conditions in a population who are the children of first-cousin marriages is of interest. If first-cousin marriages occur with a frequency of c and all non-first-cousin marriages are $1 - c$, then the total number of homozygous recessive individuals in a population is

$$q^2(1-c)+c[q^2(1-F)+q(F)]$$

where $F = 1/16 = 0.0625$ for first-cousin marriages. The total number of homozygous recessive offspring from first-cousin marriages is

$$c[q^2(1-0.0625)+q(0.0625)]$$

The fraction of homozygous recessive individuals in the population whose parents are first cousins is

$$\frac{c[q^2(1-0.0625)+q(0.0625)]}{q^2(1-c)+c[q^2(1-0.0625)+q(0.0625)]}$$

Table 9.10 Inbreeding coefficients for various populations in the world.

Location	Inbreeding coefficient (*F*)
North Jordan	0.028
Saudi Arabia	0.02–0.03
Israel	
Arabs	0.019
General population	0.004
Pakistan	
Punjab	0.023–0.03
Gujranwala	0.032
Lahore	0.027
Faisalabad	0.029
India	
Uttar Pradesh	0.017
Tamil Nadu (rural)	0.037
Tamil Nadu (urban)	0.020
Karnataka	0.033
Japan	
Hiroshima	0.003
Nagasaki	0.004
Kuwait	0.026
Nigeria	
Yoruba	0.024
Brazil	
Rio Grande de Sol	0.008
Minas Gerais	0.003–0.009
Paraná State	0.0005
Spain	
Galicia	0.002
Turkey	0.009
France	
Morbihan	0.0005
USA	
Dunkers	0.025
Hutterites	0.020

which simplifies to

$$\frac{c(1+15q)}{16q+c(1-q)}$$

The expected proportions of homozygous recessive individuals from first-cousin marriages for different values of the recessive allele (q) with $c = 0.01$, 0.03 and 0.06 are shown in Figure 9.18. When a recessive allele is frequent, the proportion of heterozygotes is high, and, consequently, most of the homozygous recessive individuals are offspring of nonconsanguineous marriages. By contrast, when a recessive allele is rare, say $q = 0.001$, then with a 1% rate of first-cousin marriages, about 40% of the homozygous recessive individuals are the offspring of these marriages, and with 6% first-cousin marriages it is 80%. Clearly, if prevalent, first-cousin marriages contribute a large number of cases of rare homozygous recessive disorders to a population.

First-cousin marriages comprise approximately 45% of all marriages in urban areas of Pakistan. In these regions, there is a 20% greater risk that

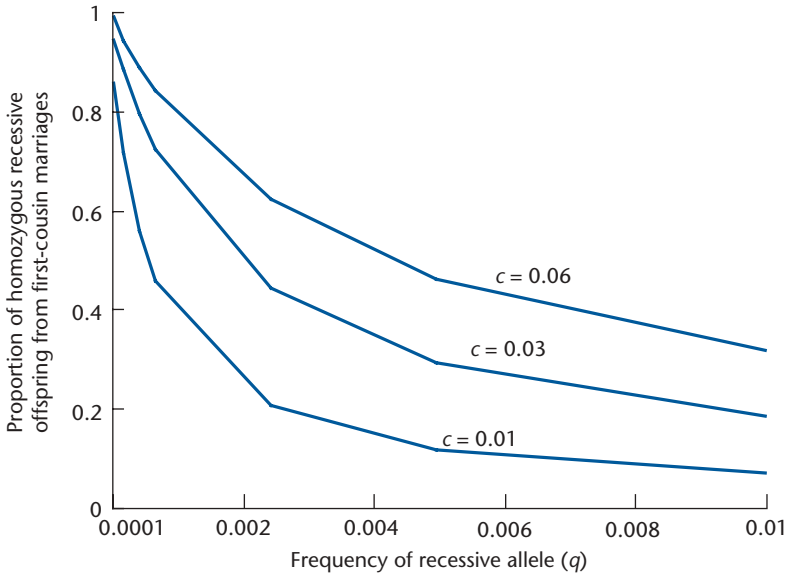


Figure 9.18 Contribution of first-cousin marriages to the proportion of homozygous recessive offspring in a population as a function of the frequency of the recessive allele (q) with different values for the frequency of first-cousin marriages (c).

offspring of first-cousin parents will die before the age of five in comparison to the children of nonconsanguineous couples. In the US, where first-cousin marriages are about 0.1% of all marriages, about 6% of the rare homozygous disorders in the population are attributed to consanguinity. The frequency of first-cousin marriages decreased from 1 to 0.1% over a 25-year period in the US. Similarly, in Japan, first-cousin marriages went from 6% to 0.4% during the same time span. Other countries are likely to experience corresponding declines in the frequency of first-cousin marriages in the future.

Both inbreeding in small isolates and founder effects have been useful for mapping rare autosomal recessive disorders and determining different loci that are responsible for the same phenotype, that is, genetic heterogeneity. An interesting example of this phenomenon was discovered by studying Bardet–Biedl syndrome (BBS) in small populations. Bardet–Biedl syndrome is a rare autosomal recessive condition that affects about 1 in 140,000 individuals worldwide. However, in Arab Bedouin populations in the Middle East its frequency is 1 in 13,500 and in the province of Newfoundland in Canada about 1 in 17,500. The phenotypic features of BBS include obesity, mental retardation, retinitis pigmentosa, polydactyly, and partial or incomplete development of the genitalia (hypogenitalism). In the Negev Desert of Israel, three isolated and genetically unrelated Bedouin tribes each showed multiple occurrences of BBS. Because marriages between relatives are common in the Bedouin culture; an increase in the frequency of homozygosity is expected in these small populations. Homozygosity mapping of a kindred from each of these tribes, surprisingly, revealed that BBS was due to a different chromosome locus in each case (Table 9.11). Another linkage study with a large, noninbred family from Newfoundland revealed a fifth *BBS* locus (*BBS5*; 2q31). The *BBS1* locus, which accounts for more than 50% of all the cases of BBS, was mapped conventionally with a large number of North American pedigrees. Additional *BBS* loci (*BBS6*, 20p12–p13; *BBS7*, 4q27; *BBS8*, 14q32.11) have been identified.

Table 9.11 Genetic heterogeneity of Bardet–Biedl syndrome (BBS).

Subtypes	Chromosome location	Source of kindreds for linkage analyses
BBS1	11q13	North American families
BBS2	16q21	Arab Bedouins
BBS3	3p12-q13	Arab Bedouins
BBS4	15q22.3-q23	Arab Bedouins
BBS5	2q31	Newfoundland

Table 9.12 Change in the number of *A1* alleles in successive generations with a forward mutation rate (μ) of 10^{-6} per gamete per generation.

Generation	Number of *A1* alleles before mutation	Number of *A2* alleles due to mutation	Number of *A1* alleles after mutation
0	2,000,000	0	2,000,000
1	2,000,000	2	1,999,998
2	1,999,998	2	1,999,996
3	1,999,996	2	1,999,994
4	1,999,994	2	1,999,992

Mutation

With the advances in DNA sequencing, changes at the nucleotide level within coding and noncoding regions are readily cataloged. These replacements, as discussed later in this chapter, are used to test for the types of forces that have acted on DNA regions. However, traditionally, population geneticists have been concerned with the theoretical consequences of recurrent mutations that have an effect on the phenotype. Gene-specific mutation rates have been determined for dominant disorders, but the accuracy of these estimates is questionable because incomplete penetrance and genetic heterogeneity confound the calculations. Generally, the rate of mutation of a dominant to a recessive allele (forward mutation, μ) ranges from approximately 10^{-5} to 10^{-6} per gamete per generation. The reverse mutation from a recessive to a dominant allele (back mutation, ν) has values of 10^{-6} to 10^{-7} per gamete per generation. A thorough study of the changes at the DNA level for almost all the cases of hemophilia B in the United Kingdom indicated that this X-linked gene (*F9*; Xq26.3–q27.1) has an overall forward mutation rate of 7.73×10^{-6} per gamete per generation. Interestingly, but without any clear explanation, the *F9* gene mutation rate is 8.6 greater in males than females.

A simplified example can be used to illustrate the consequences of mutation on the genetic structure of a population. Assume that a population has 2×10^6 *A1* alleles and no *A2* alleles, that is, $p = 1$, the mutation rate from *A1* to *A2* (μ) is 10^{-6} per gamete per generation, and there is no back mutation from the *A2* to the *A1* allele. Under these conditions, after one generation, there are two *A2* alleles and 1,999,998 *A1* alleles. During each subsequent generation with the same mutation rate, the population gains two additional *A2* alleles at the expense of two *A1* alleles (Table 9.12). Evidently, mutation changes the allele frequencies in a population, but the rate of change is exceptionally slow.

In more formal terms, the expression for the frequency of the *A2* allele in the first (t + 1) generation as a consequence of forward mutation (μ) is

$$q_{t+1} = q_t + \mu p_t = q_t + \mu(1 - q_t) = q_t + \mu - \mu q_t = \mu + (1 - \mu)q_t$$

where q_t is the original frequency of the *A2* allele, q_{t+1} the frequency of the *A2* allele in the t + 1 generation, μ the rate of forward mutation, and $+\mu p_t$ the gain in *A2* alleles from mutation of *A1* alleles in the original (t) generation. In the next (t + 2) generation, with the same conditions, then

$$q_{t+2} = \mu + (1 - \mu)q_{t+1}$$

Because $q_{t+1} = \mu + (1 - \mu)q_t$, this expression becomes

$$q_{t+2} = \mu + (1 - \mu)[\mu + (1 - \mu)q_t] = \mu + \mu(1 - \mu) + \mu(1 - \mu)^2 q_t$$

Following the pattern of this series, the value of q in the t + 3 generation is

$$q_{t+3} = \mu + \mu(1 - \mu) + \mu(1 - \mu)^2 + \mu(1 - \mu)^3 q_t$$

and, so on, for successive generations. After n generations, with the help of some algebraic machinations, the equation in terms of the *A1* allele (p) is

$$p_n = p_t(1 - \mu)^n$$

Consequently, with $\mu = 10^{-6}$ and $p_t = 1.00$, the value of p after four generations is 0.999996, and after 1024 generations it is 0.998977. It would take 690,000 generations for p to go from 1.00 to 0.5 and 4,830,000 generations to go from 0.5 to 0.0075. These data underscore what has been stressed previously: Mutation will change allele frequencies in a population, but when acting alone, the rate of change is excruciatingly slow. However, as a consequence of mutation, genetic fixation cannot occur. And, significantly, mutation introduces new alleles into the gene pool, thereby increasing genetic variability.

In actuality, gene mutation occurs in both directions with a dominant allele mutating to a recessive allele and vice versa. Generally, forward mutation (μ) is 10 to 100 times more frequent than back mutation (ν). With both forward and back mutation in a large population, the change in the frequency of the *A1* allele due to mutation is the sum of the loss of *A1* alleles by forward mutation ($-\mu p$) plus the gain of *A1* alleles by back mutation ($+\nu q$). The Greek letter delta (Δ) denotes change; therefore, the change in the frequency of the *A1* allele with two-way mutation is

$$\Delta p = -\mu p + \nu q$$

Remember that $q = 1 - p$; then

$$\Delta p = -\mu p + \nu(1 - p) = -\mu p + \nu - \nu p = \nu - p(\mu + \nu)$$

If there is no net change in the value of p from one generation to the next, then $\Delta p = 0$ and an equilibrium is established between forward and back mutation. When p is at equilibrium, it is designated as $\hat{p}$, which is pronounced "pee-hat". Thus, in terms of p, the expression $\nu - p(\mu + \nu) = 0$ becomes

$$\hat{p} = \frac{\nu}{\mu + \nu}$$

Correspondingly, the change in q (Δq) in one generation with two-way mutation is equal to the gain in *A2* alleles from *A1* alleles by forward mutation ($+\mu p$) plus the loss of *A2* alleles from back mutation ($-\nu q$). If $\Delta q = 0$, then

$$+\mu(1-q) - \nu q = 0,$$
$$\mu = q(\mu + \nu),$$

and

$$\hat{q} = \frac{\mu}{\mu + \nu}$$

When $\mu = 1.0 \times 10^{-6}$ and $\nu = 1.0 \times 10^{-7}$, at equilibrium,

$$\hat{p} = \frac{1.0 \times 10^{-7}}{1.0 \times 10^{-6} + 1.0 \times 10^{-7}} = 0.09$$

And, as expected,

$$\hat{q} = \frac{1.0 \times 10^{-6}}{1.0 \times 10^{-6} + 1.0 \times 10^{-7}} = 0.91$$

As noted previously, μ is usually 10 to 100 times greater than ν. Therefore, with ratios of 10:1 and 100:1 for $\mu:\nu$, the values for $\hat{q}$ are 0.91 and 0.99, respectively. However, recessive alleles in human populations seldom, if ever, have such high values. The observed frequencies of homozygous recessive disorders (q^2) invariably are rare and range from 0.0001 to 0.00005, with corresponding values for q being 0.01 to 0.007. Obviously, there is a significant discrepancy between a genetic equilibrium established by two-way mutation acting by itself and the observed occurrences of human recessive alleles. Quite simply, two-way mutation is not the only process at work in populations. In particular, deleterious alleles often diminish the reproductive capability of individuals, which lessens intergenerational transmission of these alleles and, as a result, prevents them from attaining exceptionally high frequencies.

Fitness and Selection

Obviously, individuals who are reproductively impaired, because of genetic or environmental factors, do not readily transfer their genes to the next generation. With Hardy–Weinberg conditions, it is assumed that everyone in a population has the same reproductive potential (reproductive success), which entails both surviving to reproductive age (viability) and, in this case, producing an equal number of offspring (fertility). In genetic parlance, the term fitness

(reproductive fitness, adaptive value), symbolized by w, is used to describe reproductive success. Selection (s), on the other hand, is the component or force that, directly or indirectly, either limits or enhances the reproductive competence of individuals with certain genotypes.

Vulnerability to predation is often used to illustrate, in broad terms, the interrelated concepts of fitness and selection. For example, equal numbers of black and gray fish of the same species were placed in gray and black tanks, and then penguins were allowed to feed. In the gray tank, 1.6 times more black than gray fish were eaten. And, in the black tank, the gray fish were consumed 2.7 times more frequently than black ones. Therefore, in this experiment, survival depended on both the environmental context and the phenotypes of the fish, with the penguins acting as the selection agent. If the color of the fish is genetically determined, then those fish that survive are more likely to reproduce and pass on the genes that are responsible for color to the next generation. Specifically, in the gray tank, the gray fish have a selective advantage and, consequently, are more reproductively fit than black fish because they will produce more offspring than the black fish. This result is reversed in the black tank, where black fish have the selective advantage over the gray fish, who are less fit in this environment and engender fewer offspring for the next generation. This predation-survival experiment demonstrates some of the aspects of fitness and selection in populations. The next step is to consider a framework for quantifying the impact of these parameters on the genetic structure of a population.

For a two-allele, one-locus system, the fitness values (fitnesses) of the *A1A1*, *A1A2*, and *A2A2* genotypes are w_{11}, w_{12}, and w_{22}, respectively. On this basis, generalization of the effect of fitness on the transmission of genes over one generation is straightforward (Table 9.13).

As shown in Table 9.13, the total of the frequencies of the genotypes in the new (t + 1) generation is $p^2w_{11} + 2pqw_{12} + q^2w_{22}$, which is often represented by the symbol $\bar{w}$, pronounced "double-yew-bar". The frequency of the *A1* allele (p_1) in the t + 1 generation is

$$\frac{p^2w_{11} + 1/2(2pqw_{12})}{\bar{w}}$$

The change in frequency of the *A1* allele from the t to t + 1 generation is

$$\Delta p = p_1 - p = \frac{p^2w_{11} + 1/2(2pqw_{12})}{\bar{w}} - p = \frac{p^2w_{11} + pqw_{12} - p\bar{w}}{\bar{w}}$$

Table 9.13 Effect of fitnesses on genotype frequencies over one generation.

	Genotype			
	A1A1	***A1A2***	***A2A2***	**Total**
Initial frequency	p^2	$2pq$	q^2	1
Fitness	ω_{11}	ω_{12}	ω_{22}	
Contribution to the next generation	$p^2\omega_{11}$	$2pq\omega_{12}$	$q^2\omega_{22}$	$p^2\omega_{11} + 2pq\omega_{12} + q^2\omega_{22}$

With some algebraic maneuvering, this expression becomes

$$\Delta p = \frac{pq[p(w_{11} - w_{12}) + q(w_{12} - w_{22})]}{p^2 w_{11} + 2pqw_{12} + q^2 w_{22}}$$

This equation is used extensively by population geneticists and evolutionary biologists. Because it will be encountered a number of times in this chapter and it has no official name, here, for convenience, it will be called the "general fitness equation."

In practice, the fitness of a genotype is a relative value based on the most fit genotype. For Hardy–Weinberg populations, the fitness values for each genotype are the same, that is, $w_{11} = w_{12} = w_{22} = 1$. As expected, the value of p_1 reduces to p when each genotype has the same fitness, that is,

$$p_1 = \frac{p^2 w_{11} + pqw_{12}}{p^2 w_{11} + 2pqw_{12} + q^2 w_{22}} = \frac{p^2 + pq}{p^2 + 2pq + q^2}$$

and because $p^2 + 2pq + q^2 + 1$ and $q = 1 - p$,

$$p_1 = p^2 + p(1 - p) = p^2 + p - p^2 = p$$

The most fit genotype is the one that produces the most offspring. Relative fitness is the ratio of the proportion of offspring from a less fit genotype to the proportion of offspring from the most fit genotype. More specifically, for a dominant lethal disorder, 108 heterozygotes had 27 offspring and 427 homozygous recessive parents had 582 offspring. The relative fitness of the heterozygotes is $\frac{27/108}{582/427} = 0.2$. Because the homozygous dominant genotype is lethal, $w_{11} = 0$. Thus, for this example, the relative genotype fitness is $w_{11} : w_{12} : w_{22} = 0 : 0.2 : 1.0$, respectively.

Fitness and selection (s) are two sides of the same coin. On the one hand, $w = 1 - s$, where s is the selection coefficient. On the other, $s = 1 - w$. Therefore, when $w_{11} : w_{12} : w_{22} = 0 : 0.2 : 1.0$, then $s_{11} : s_{12} : s_{22} = 1.0 : 0.8 : 0$. The subscripts for the selection coefficients represent the *A1A1*, *A1A2*, and *A2A2* genotypes, respectively. Selection coefficients, not fitnesses, are customarily used to measure the impact of differential reproductive capability on allele and genotype frequencies.

Analysis of the relative reproductive success of a set of genotypes depends on dominance relationships and the relative fitness of the heterozygous genotype. For example, with complete dominance, the heterozygote (*A1A2*) and one of the homozygotes (*A1A1* or *A2A2*) have the same phenotype, and, consequently, the same fitness value and these two genotypes are more fit than the third genotype. In other words, if the *A1* allele is dominant, then the fitness relationship is $w_{11} = w_{12} > w_{22}$. Alternatively, if *A2* is dominant, then $w_{22} = w_{12} > w_{11}$. With incomplete dominance, the fitness of the heterozygote lies between the fitnesses of the two homozygotes, that is, $w_{11} > w_{12} > w_{22}$ or $w_{22} > w_{12} > w_{11}$. On occasion, the heterozygous genotype is more fit than either of the two homozygous genotypes (overdominance), that is, $w_{12} > w_{11} \geq w_{22}$ or $w_{12} > w_{22}$

Table 9.14 Relative fitnesses of genotypes and heterozygous effect with different types of dominance.

Dominance	Relative fitness	Heterozygous effect
Complete dominance (*A1* dominant; *A2* recessive)	$\omega_{11} = \omega_{12} > \omega_{22}$	$h = 0$
Complete dominance (*A2* dominant; *A1* recessive)	$\omega_{22} = \omega_{12} > \omega_{11}$	$h = 1$
Incomplete dominance	$\omega_{11} > \omega_{12} > \omega_{22;}$ $\omega_{22} > \omega_{12} > \omega_{11}$	$0 < h < 1$
Overdominance	$\omega_{12} > \omega_{11} \geq \omega_{22;}$ $\omega_{12} > \omega_{22} \geq \omega_{11}$	$h < 0$
Underdominance	$\omega_{11} \geq \omega_{22} > \omega_{12;}$ $\omega_{22} \geq \omega_{11} > \omega_{12}$	$h > 1$

$\geq w_{11}$. And, rarely, the heterozygous genotype is the least fit of the three genotypes (underdominance), that is, $w_{11} \geq w_{22} > w_{12}$ or $w_{22} \geq w_{11} > w_{12}$ (Table 9.14).

To account specifically for the extent of the reproductive fitness of the heterozygote, a parameter, h, was created. It is called either the heterozygote effect or the degree of dominance with respect to the *A2* allele. When h is used, the relative fitnesses of *A1A1*, *A1A2*, and *A2A2* are always 1, $1 - hs$, and $1 - s$, respectively. With this system, the fitness of the *A1A1* genotype is invariably 1, even though in some instances it is not necessarily the most fit genotype. The value of h depends on the dominance relationship between the *A1* and *A2* alleles and the relative fitness of the heterozygous genotype (Table 9.14). With complete dominance of the *A1* allele, $h = 0$. When the *A2* allele is completely dominant, then $h = 1$. With this system, s can be positive or negative. When s is negative, *A2A2* is more fit than *A1A1*, and when it is positive, *A1A1* is more fit than *A2A2*. For incomplete dominance, h is greater than zero and less than 1 ($0 < h < 1$). For semidominance, $h = 0.5$. The h value for overdominance is less than zero ($h < 0$). Underdominance has an h value greater than 1 ($h > 1$). Because $w_{11} = 1$, $w_{12} = 1 - hs$, and $w_{22} = 1 - s$, the generalized fitness equation

$$\Delta p = \frac{pq[p(w_{11} - w_{12}) + q(w_{12} - w_{22})]}{p^2 w_{11} + 2pqw_{12} + q^2 w_{22}}$$

can be rewritten as

$$\Delta p = \frac{pqs[ph + q(1 - h)]}{1 - 2pqhs - sq^2}$$

Directional Selection

Now that a mathematical basis for fitness and selection has been considered, the consequences of reduced fitness for specific genotypes can be examined. For example, what happens to allele and genotype frequencies when the *A1* allele is completely dominant and the homozygous recessive genotype is at a selective disadvantage? This problem can be broached in two ways. First, with the general scheme shown in Table 9.15, the frequency of the *A2* allele after one generation of selection with $h = 0$ and $0 < s < 1$ is

Table 9.15 Selection with complete dominance of the *A1* allele.

	Genotype			Total
	A1A1	***A1A2***	***A2A2***	
Initial frequency	p^2	$2pq$	q^2	1
Relative fitness	1	$1 - hs$	$1 - s$	
Contribution to the next generation	p^2	$2pq$	$q^2(1 - s)$	$1 - sq^2$

$$q_1 = \frac{q^2(1-s)+1/2(2pq)}{1-sq^2} = \frac{q^2 - sq^2 + (1-q)q}{1-sq^2} = \frac{q(1-sq)}{1-sq^2}$$

And, the change in frequency of the *A2* allele (Δq) is

$$\Delta q = q_1 - q = \frac{q(1-sq)}{1-sq^2} - q = \frac{q(1-sq) - q(1-sq^2)}{1-sq^2}$$
$$= \frac{q - sq^2 - q + sq^3}{1-sq^2} = -\frac{sq^2(1-q)}{1-sq^2}$$

Alternatively, the general fitness equation with the heterozygous effect can be used to determine Δp and p_1. For example, when $h = 0$, the relationship becomes

$$\Delta p = \frac{pqs[ph + q(1-h)]}{1 - 2pqhs - sq^2} = \frac{pq^2 s}{1-sq^2}$$

And, because $\Delta p = p_1 - p$, $p_1 = p + \Delta p$.

More specifically, if $q = 0.01$ and $s = 0.05$, then after a single generation $q_1 = 0.009995$ and $\Delta q = -0.000005$. The negative sign indicates a decrease in the frequency of the *A2* allele. The frequency of the *A1* allele increases by 0.000005. Although the effect of selection is small in this case, the frequencies of the two alleles proceed in opposite directions in successive generations. Boosting the selection pressure increases the extent of change in allele frequencies per generation. If $s = 1$, then $w_{22} = 0$ and individuals with an *A2A2* genotype do not transmit any of their genes to the next generation. With $s = 1$ and $q = 0.01$, in the following generation, $q_1 = 0.009$ and $\Delta q = -0.001$. Clearly, allele and genotype frequencies change in successive generations when there is selection against the homozygous recessive genotype. The amount of change in one generation is a function of both the initial frequency of the *A2* allele and the intensity of selection (Figure 9.19).

So far, the discussion of selection with complete dominance has been restricted to changes in a single generation. It is useful to be able to estimate the approximate number of generations that are required for an allele to go from one value to another with a particular selection coefficient. The ubiquitous general fitness equation,

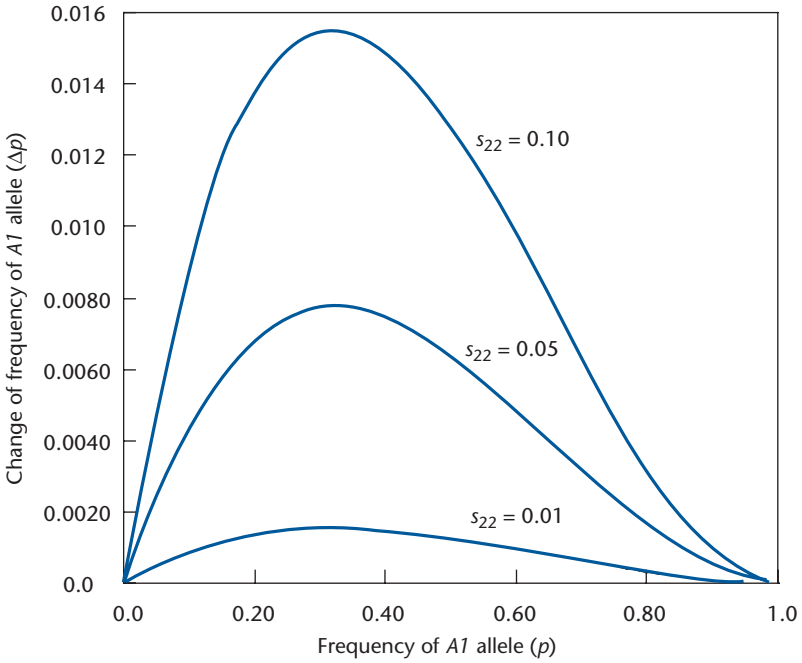


Figure 9.19 Selection with complete dominance. The relative genotype fitnesses, $w_{11} : w_{12} : w_{22}$, are 1 : 1 : 0.90 with $s_{22} = 0.10$, 1 : 1 : 0.95 with $s_{22} = 0.05$, and 1 : 1 : 0.99 with $s_{22} = 0.01$.

$$\Delta p = \frac{pqs[ph + q(1-h)]}{1 - 2pqhs - sq^2}$$

can be used as a starting point for this purpose. With complete dominance of the *A1* allele, $h = 0$ and, if the frequency of q is small (say, 0.01 or less), then the denominator ≈ 1 and the numerator is pq^2s. After integration, $\Delta p = pq^2s$ becomes

$$\ln\left(\frac{p_n}{q_n}\right) + \left(\frac{1}{q_n}\right) = \left[\ln\left(\frac{p}{q}\right) + \frac{1}{q}\right] + sn$$

By solving for n, which represents the number of generations, with assigned values for the other parameters, the time course of the increase of the *A1* allele from p to p_n or the decrease of the *A2* allele from q to q_n can be calculated (Figure 9.20). Although slow, selection with complete dominance eventually leads to the complete loss of the *A2* allele and, concomitantly, the fixation of the *A1* allele.

The effect of selection with incomplete dominance can be determined from the general fitness equation that includes the heterozygous effect. In these instances, the value of h can be derived from the relationship $w_{12} = 1 - hs$. Specifically, if $s = 0.05$ and $w_{12} = 0.98$, then $h = 0.4$, and with $p = 0.99$, the change of the frequency of the *A1* allele (Δp) is

$$\Delta p = \frac{pqs[ph + q(1-h)]}{1 - 2pqhs - sq^2} = 0.00002$$

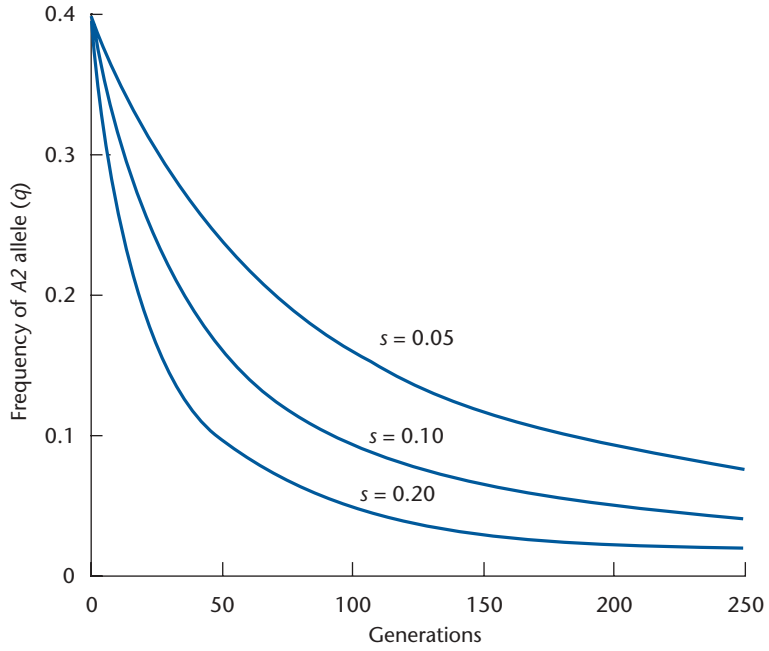


Figure 9.20 Selection with complete dominance over time. The relative genotype fitnesses, $w_{11} : w_{12} : w_{22}$, are 1 : 1 : 0.80 with $s_{22} = 0.20$, 1 : 1 : 0.90 with $s_{22} = 0.10$, and 1 : 1 : 0.95 with $s_{22} = 0.05$.

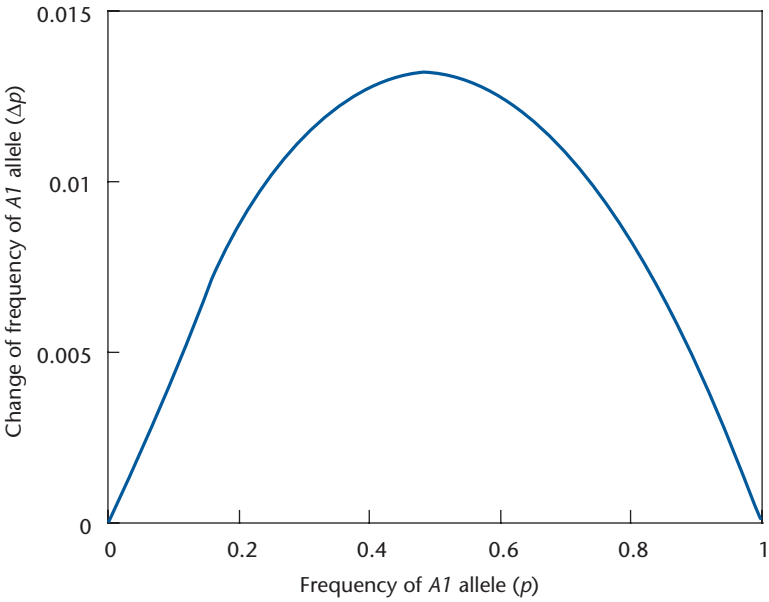


Figure 9.21 Selection with incomplete dominance. The relative genotype fitnesses $w_{11} : w_{12} : w_{22}$ are 1 : 0.95 : 0.90 with selection coefficient $s = 0.10$ and heterozygous effect $h = 0.50$.

The frequency of the *A1* allele after one generation of selection is $p_1 = p + \Delta p = 0.99002$. With incomplete dominance, the change in the frequency of the *A1* allele in one generation due to selection is more effective in the middle of the full range of frequencies than at the extremes (Figure 9.21). Moreover, because the fitnesses of two of the three genotypes are reduced when dominance is incomplete, the amount of change in allele frequency is greater per generation than when dominance is complete. Selection that leads to the disappearance of one allele and the fixation of the other one is called directional selection.

Table 9.16 Selection favoring the heterozygous genotype.

	Genotype			Total
	A1A1	*A1A2*	*A2A2*	
Initial frequency	p^2	$2pq$	q^2	1
Fitness	$1 - s_{11}$	1	$1 - s_{22}$	
Contribution to the next generation	$p^2(1 - s_{11})$	$2pq$	$q^2(1 - s_{22})$	$1 - s_{11}p^2 - s_{22}q^2$

Balancing Selection

Overdominance (heterozygote superiority, heterozygote advantage) occurs when the heterozygous genotype is more fit than either of the homozygous genotypes. Under these circumstances, the selection coefficients for the *A1A1* and *A2A2* genotypes are designated s_{11} and s_{22}, respectively. The outcome of overdominance in one generation is presented in Table 9.16. The frequency of the *A2* allele becomes

$$q_1 = \frac{(1-s_{22})q^2 + 1/2(2pq)}{1-s_{11}p^2 - s_{22}q^2} = \frac{q^2 - s_{22}q^2 + (1-q)q}{1-s_{11}p^2 - s_{22}q^2} = \frac{q - s_{22}q^2}{1-s_{11}p^2 - s_{22}q^2}$$

and the change in frequency of the *A2* allele is

$$\Delta q = q_1 - q = \frac{q - s_{22}q^2}{1-s_{11}p^2 - s_{22}q^2} - q = \frac{q - s_{22}q^2 - q(1-s_{11}p^2 - s_{22}q^2)}{1-s_{11}p^2 - s_{22}q^2}$$
$$= \frac{s_{22}q^2(q-1) + s_{11}p^2q}{1-s_{11}p^2 - s_{22}q^2} = \frac{s_{22}q^2[-(1-q)] + s_{11}p^2q}{1-s_{11}p^2 - s_{22}q^2} = \frac{s_{11}p^2q - s_{22}pq^2}{1-s_{11}p^2 - s_{22}q^2}$$

When the change in frequency of the *A2* allele (Δq) in one generation with overdominance is plotted as a function of the frequency of the *A2* allele (q), an interesting curve with positive and negative phases is formed (Figure 9.22). Unlike directional selection where one of the alleles is driven to fixation, overdominance leads to a stable equilibrium for both alleles with $q > 0$ and $p < 1$, unless, of course, p or $q = 1$. For example, when both homozygous genotypes are lethal ($s_{11} = 1$; $s_{22} = 1$), only the heterozygous genotype is maintained in successive generations. Under these conditions, a stable equilibrium, that is, balanced polymorphism, is established with $\hat{p} = \hat{q} = 0.5$ when Δp and $\Delta q = 0$. There are a number of ways that the concept of a balanced polymorphism can be generalized for any set of values for s_{11} and s_{22}. Based on the scheme in Table 9.16, but using fitnesses instead of selection coefficients, that is, $w_{11} = 1 - s_{11}$, $w_{12} = 1$, and $w_{22} = 1 - s_{22}$, the frequencies of the alleles after one generation are

$$p_1 = \frac{w_{11}p^2 + 1/2\, w_{12}\, 2pq}{w_{11}p^2 + w_{12}\, 2pq + w_{22}q^2} = \frac{p(w_{11}p + w_{12}q)}{w_{11}p^2 + w_{12}\, 2pq + w_{22}q^2}$$

and

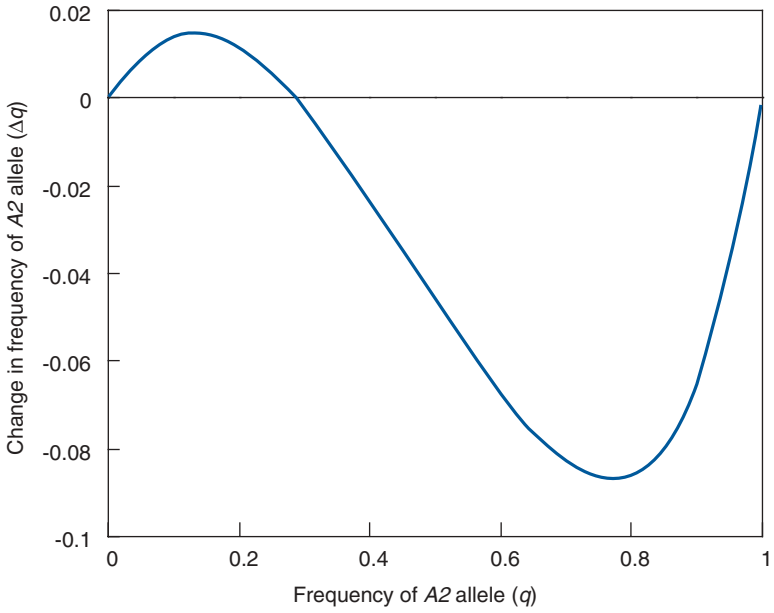


Figure 9.22 Change in frequency of the *A2* allele (q) with overdominance. The relative genotype fitnesses $w_{11} : w_{12} : w_{22}$ are $0.80 : 1 : 0.50$ with selection coefficients $s_{11} = 0.20$ and $s_{22} = 0.50$. The value of $\hat{q}$ at the stable equilibrium point, that is, $\Delta q = 0$ and $0 < q < 1$, is 0.286.

$$q_1 = \frac{w_{22}q + 1/2\, w_{12}\, 2pq}{w_{11}p^2 + w_{12}\, 2pq + w_{22}q^2} = \frac{q(w_{22}q + w_{12}p)}{w_{11}p^2 + w_{12}\, 2pq + w_{22}q^2}$$

The ratio p_1/q_1 is

$$\frac{p_1}{q_1} = \frac{p(w_{11}p + w_{12}q)}{q(w_{22}q + w_{12}p)}$$

At equilibrium, $p_1 = p$ and $q_1 = q$; therefore, $p_1/q_1 = p/q$. Consequently, after dividing both sides by p/q, this equation can be represented in terms of $\hat{p}$ and genotype fitnesses:

$$\frac{w_{11}p + w_{12}q}{w_{22}q + w_{12}p} = 1;$$
$$w_{11}p + w_{12}q = w_{22}q + w_{12}p;$$
$$q(w_{12} - w_{22}) = p(w_{12} - w_{11});$$
$$(1 - p)(w_{12} - w_{22}) = p(w_{12} - w_{11});$$
$$w_{12} - w_{22} = p[(w_{12} - w_{11}) + (w_{12} - w_{22})]$$

and

$$\hat{p} = \frac{w_{12} - w_{22}}{(w_{12} - w_{11}) + (w_{12} - w_{22})}$$

Moreover, because $w_{11} = 1 - s_{11}$, $w_{12} = 1$, and $w_{22} = 1 - s_{22}$, $\hat{p} = \dfrac{s_{22}}{s_{11} + s_{22}}$. And, if the original relationship is solved with respect to q, then $\hat{q} = \dfrac{s_{11}}{s_{11} + s_{22}}$.

Alternatively, with respect to the heterozygous effect (h), the expressions for $\hat{p}$ and $\hat{q}$ can be generated by starting with

$$p_1 = \frac{p^2 + 1/2(2pq)(1 - hs)}{1 - 2pqhs - sq^2}$$

Because $p_1 = p$ at equilibrium,

$$\begin{aligned}
&p(1 - 2pqhs - sq^2) = p[p + q(1 - hs)];\\
&1 - 2pqhs - sq^2 = p + q - qhs;\\
&1 - 2pqhs - sq^2 = p + 1 - p - qhs;\\
&-2pqhs - sq^2 = -qhs;\\
&2ph + 1 - p = h;\\
&p(2h - 1) = h - 1
\end{aligned}$$

and

$$\hat{p} = \frac{h - 1}{2h - 1}$$

Or, for $\hat{q}$, the expression $2ph + q = h$ becomes

$$\begin{aligned}
&2h(1 - q) + q = h;\\
&2h - 2hq + q = h;\\
&(2h - 1)q = h
\end{aligned}$$

and

$$\hat{q} = \frac{h}{2h - 1}$$

With overdominance, the frequencies of the *A1* and *A2* alleles are driven to a stable equilibrium. The equilibrium point corresponds to the maximum average fitness, and its location is a function of the values of the selection coefficients s_{11} and s_{22}, that is, $\hat{p} = \frac{s_{22}}{s_{11} + s_{22}}$ or $\hat{q} = \frac{s_{11}}{s_{11} + s_{22}}$. The number of generations that are required to reach equilibrium depends on the initial allele frequencies and the extent of selection against each homozygote (Figure 9.23). Generally, differential reproduction that leads to a stable equilibrium when neither p nor q is 0 is called balancing selection (balanced selection).

Sickle cell anemia in malarial regions of Africa provides one of the better examples of balancing selection in human populations. In areas with endemic malaria, heterozygotes (*HBB*A*/*HBB*S*) for the sickle cell anemia gene are more likely to survive malarial infection than homozygotes (*HBB*A*/*HBB*A*) with normal hemoglobin. The fitness of individuals who are homozygous for sickle cell anemia (*HBB*S*/*HBB*S*), in any environment, is greatly diminished

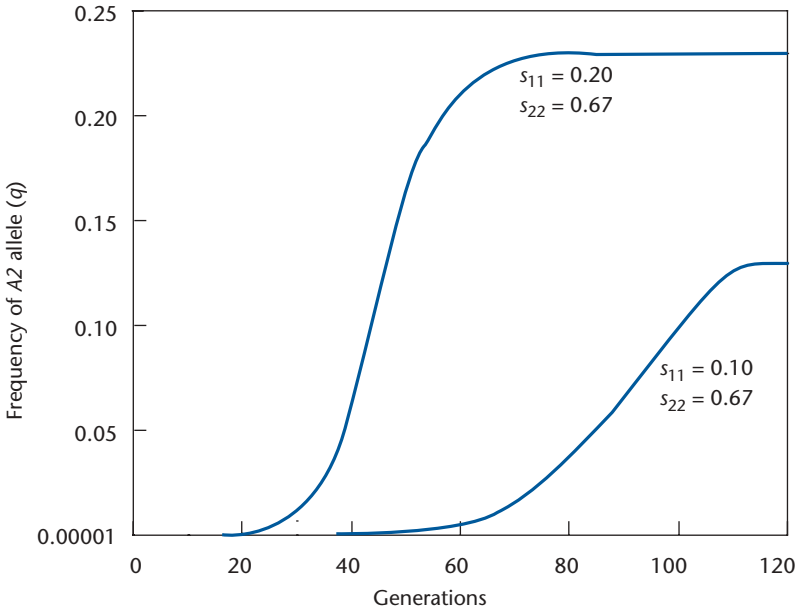


Figure 9.23 Frequency of the *A2* allele (q) over time with overdominance. The relative genotype fitnesses $w_{11}:w_{12}:w_{22}$ are 0.80:1:0.33 with $s_{11} = 0.20$ and $s_{22} = 0.67$ and 0.90:1:0.33 with $s_{11} = 0.10$ and $s_{22} = 0.67$. The selection coefficients are constant in successive generations. The equilibrium frequencies of the *A2* allele ($\hat{q}$) are 0.23 when $s_{11} = 0.20$ and $s_{22} = 0.67$ and 0.13 when $s_{11} = 0.10$ and $s_{22} = 0.67$.

by the severe anemia that accompanies the disorder. In the presence of malaria, the order of fitness of these genotypes is $w_{AS} > w_{AA} > w_{SS}$ and the relative fitness is 1:0.85:0, respectively. The expected equilibrium value for the sickle cell allele (*HBB*S*) is $\hat{q} = \frac{s_{11}}{s_{11} + s_{22}} = \frac{0.15}{0.15 + 0.10} = 0.13$, and for the normal allele (*HBB*A*) it is $\hat{p} = \frac{s_{22}}{s_{11} + s_{22}} = \frac{1.00}{0.15 + 1.00} = 0.87$. These values are very close to the observed allele frequencies in areas with high levels of malaria. This genetic interplay is an interesting example of a biological trade-off. Balancing selection maintains a deleterious gene in a population at a relatively high frequency that in a different environmental setting would be reduced to a low frequency by directional selection.

Disruptive Selection

With underdominance (heterozygote inferiority), the relative genotype fitnesses are $w_{11} \geq w_{22} > w_{12}$ or $w_{22} \geq w_{11} > w_{12}$. Under these conditions, a plot of the change of allele frequency as a function of the frequency of the allele shows a negative and a positive phase (Figure 9.24). This graph appears identical to the one with overdominance. However, the genetic consequences in these two cases are completely different. The point of minimal fitness with underdominance, unlike overdominance, corresponds to $\Delta p = \Delta q = 0$ for $0 < p$ and $q < 1$. Although exceedingly unlikely, if the original allele frequencies for a locus with underdominance happen to be equal to their equilibrium values, when both alleles are present in a population, then these allele frequencies persist as long as the selection coefficients do not change. On the other hand, when the frequency of an allele is less than its equilibrium frequency, it is driven to 0 and when greater, it goes to 1.0. In an extreme case of underdominance, if the heterozygous genotype is lethal and the homozygous genotypes are

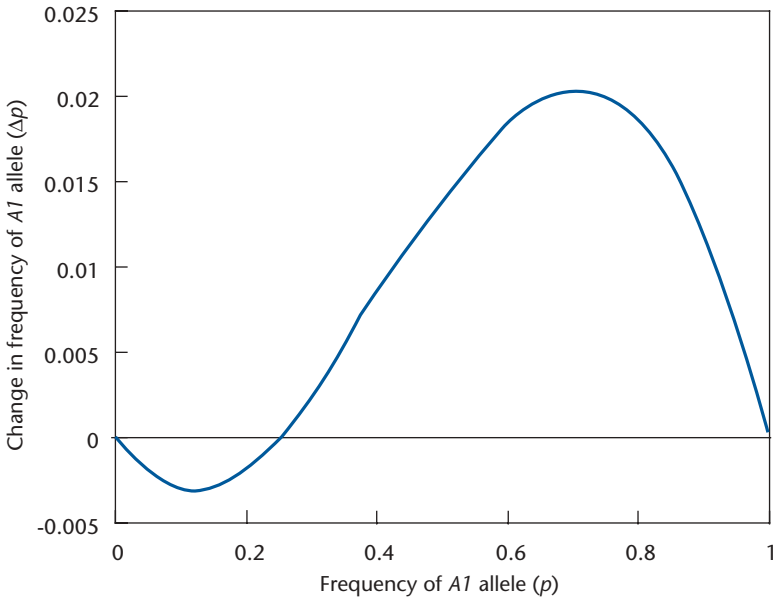


Figure 9.24 Change in frequency of the *A1* allele (Δp) in one generation with underdominance as a function of the frequency of the *A1* allele (p). The relative genotype fitnesses $w_{11} : w_{12} : w_{22}$ are $1 : 0.90 : 0.85$ with $s = 0.10$ and $h = 1.50$. The value of $\hat{p}$ at the point of unstable equilibrium when $\Delta p = 0$ with $0 < p < 1$ is 0.25.

equally fit, only homozygotes will be present in the population. The consequences of underdominance typify disruptive selection.

At this juncture, a brief review of the consequences of violating the HWE assumptions may be helpful (Table 9.17). The principles of selection have been described here with a two-allele, one-locus model with unchanging fitnesses in successive, nonoverlapping generations. Models for other genetic circumstances have been developed. However, the mathematics becomes complicated when multiple alleles (Table 9.18), unusual genetic arrangements, or various combinations of parameters are considered. Finally, in addition to the effects of selection on individual genotypes, which is designated as the viability model of selection, selection also acts on many other systems at a variety of levels (Table 9.19). For example, the fitness of a phenotype may decrease as it becomes more frequent, that is, negative frequency-dependent selection. With an organism that has a sex ratio 1:1, if one sex significantly outnumbers the other, then many individuals in the more frequent group will not find mates whereas, theoretically, all those in the less frequent group will mate. Under these circumstances, the more frequent group is less fit than the less frequent group and the sex ratio will return to a 1:1 ratio.

Mutation, Selection, and Founder Effect: A Case Study

Mutation and selection are genetic partners of sorts. On the one hand, mutations introduce new, but mostly deleterious, alleles into a population. On the other hand, selection eliminates many of these genes. The balance between mutation and selection, with different modes of dominance, can be represented mathematically. With complete dominance, the change in the frequency of the

Table 9.17 Effects of various factors on allele and genotype frequencies in populations.

Factor	Change in allele frequency	Change in genotype frequency	Consequence
Migration	Yes	Yes	Decreases genetic diversity among subpopulations
Small population size	Yes	Yes	Gene frequencies fluctuate, with one or the other allele becoming fixed eventually
Inbreeding	No	Yes	Increases homozygosity
Positive assortative mating	No	Yes	Increases homozygosity
Negative assortative mating	Yes	Yes	Increases heterozygosity
Mutation	Yes	Yes	Introduces new variants into a population
Directional selection	Yes	Yes	Eventually one allele becomes fixed and the other extinct
Balancing selection	Yes	Yes	Eventually both alleles reach a stable equilibrium with $0 < p$ and $q < 1$
Disruptive selection	Yes	Yes	Allele frequencies proceed in opposite directions from an unstable equilibrium point where $0 < p$ and $q < 1$, with one allele eventually going to fixation and the other to extinction

Table 9.18 Genotype fitnesses of a three-allele, one-locus system.

	Genotype					
	A1A1	*A2A2*	*A3A3*	*A1A2*	*A1A3*	*A2A3*
Frequency	p^2	q^2	r^2	$2pq$	$2pr$	$2qr$
Fitness	ω_{11}	ω_{22}	ω_{33}	ω_{12}	ω_{13}	ω_{23}

Note: $\bar{w} = p^2w_{11} + q^2w_{22} + r^2w_{33} + 2pqw_{12} + 2prw_{13} + 2qrw_{23}$.

Table 9.19 Some genetic and biological systems that are affected by selection.

System	Selection
Allele or genotype frequencies	Frequency-dependent selection
Allele or genotype rarity	Rare allele advantage (diversifying selection)
Altruism among genetically related individuals	Kin selection
Cellular location of a gene	Mitochondrial or chloroplast selection
Chromosome location of a gene	X-linked or Y-linked selection
Differential extinction and recolonization of subpopulations	Interdeme selection
Differential survival of gametes	Gametic selection
Fertility of mating pairs	Fecundity selection
Multiple loci	Epistatic selection
Population size	Density-dependent selection
Sex of organism	Sex-limited selection

A2 allele (Δq) in one generation due to selection is $-\frac{spq^2}{1-sq^2}$ and, for mutation, it is $\mu(1-q) - \nu q$. After combining these expressions,

$$\Delta q = -\frac{spq^2}{1-sq^2} + \mu(1-q) - \nu q$$

Because a homozygous recessive disorder is usually rare, the frequency of the recessive allele (q) is quite small. Moreover, if the disorder is severe, then the selection coefficient is about 1.0. Therefore, the denominator $1 - sq^2 \approx 1.0$ and can be ignored. As well, because the rate of back mutation (ν) is very low, the value of νq is negligible. With these approximations, the combined expression becomes $\Delta q = -spq^2 + \mu(1-q)$. At equilibrium, when $\Delta q = -spq^2 + \mu(1-q) = 0$, this equation reduces to $\hat{q}^2 \approx \mu/s$ or $\hat{q} \approx \sqrt{\mu/s}$.

With incomplete dominance, $\Delta p = pqs[ph + q(1-h)]$ for selection and $\Delta p = \nu q - \mu p$ for mutation. Remember that the numerical value for νq is so small that it can be disregarded. Thus, at equilibrium, the combined expression is $\Delta p = p^2qsh + pq^2s(1-h) - \mu p = 0$, which simplifies to $\hat{q} \approx \mu/hs$. A codominant allele that lowers the fitness of the heterozygous genotype is more readily removed from a population than a deleterious allele that is completely recessive. For example, if $s = 1$, $\mu = 1.0 \times 10^{-6}$, and, for incomplete dominance, $h = 0.20$, then $\hat{q} \approx \sqrt{\mu/s}$ is 63 times greater than $\hat{q} \approx \mu/hs$.

Certain genetic disorders occur more frequently in some populations than in others. Tay–Sachs disease (TSD), for example, is a hundred times more prevalent in the descendants of the Jews of central-eastern Europe (Ashkenazi Jews) than in the non-Ashkenazi European population. Specifically, the frequency of TSD is about 3.3×10^{-4} and 2.9×10^{-6} among Ashkenazi Jews and non-Ashkenazi populations, respectively. Tay–Sachs disease, a lysosomal storage disease, is an autosomal recessive condition of the *HEXA* gene that is located at 15q23–q24 and encodes the α-subunit of the enzyme β-hexosaminidase A. Because of the absence of β-hexosaminidase A activity, the brain-specific lipid GM2-ganglioside accumulates in children who are normal at birth. Within two to three years, brain-controlled functions are impaired, leading to paralysis, blindness, and deafness. Death invariably occurs between four and five years of age. The homozygous recessive TSD genotype is completely unfit, thus, $s = 1$. An obvious question arises: What genetic mechanism(s) accounts for the difference in occurrence of TSD in Ashkenazi and non-Ashkenazi populations?

If TSD in the non-Ashkenazi population is maintained by the interaction of selection and mutation, then agreement with the relationship $\hat{q}^2 \approx \mu/s$ is expected. In this case, $s = 1$ and, therefore, $q^2 \approx \mu \approx 2.9 \times 10^{-6}$. Because this value for the purported mutation rate of the *HEXA* gene is close to the average rate of forward mutation ($\sim 1 \times 10^{-6}$), non-Ashkenazi TSD is likely due to a balance between selection against the homozygous recessive genotype and mutation from the dominant to the recessive gene. With the same reasoning, the *HEXA* mutation rate in the Ashkenazi population would be $q^2 \approx \mu \approx 3.3 \times 10^{-4}$, which is about thirty times the average mutation rate. There is no evidence to indicate that the *HEXA* gene has a mutation rate of this magnitude

from the HUMAN GENETICS files

Dysgenics: Fact or Fiction

Human population genetics tries to account for current allele and genotype frequencies in populations based on past circumstances, and to determine what these frequencies may be under various conditions in the future. Predictions of biological phenomena, especially those relating to humans, are at best precarious. However, notwithstanding the need for caution, there are some who are convinced that the human gene pool is deteriorating. The crux of the genetic decay (dysgenics) argument is that in the past natural selection weeded out defective alleles but now, with modern medical practices, affected individuals not only survive but perpetuate defective genes that will accumulate over time and overwhelm human well-being. Superficially, dysgenics appears plausible. However, this concern is unfounded from a number of different perspectives.

First, a genetic disorder is irrelevant if a fully effective remedy exists and can be delivered to an affected individual. The cure nullifies the previous biological significance of an allele or genotype. In other words, medical treatment converts what at one time had been a deleterious genetic condition into one that is benign. Among many possible examples, shortsightedness (myopia), which probably has a genetic component, can be used to illustrate this point. In the US, about 25% of adults are myopic, and depending on the extent, if untreated, headaches, fatigue, personal injuries, and impaired learning are common symptoms. Treatment entails corrective lenses or refractive surgery. In terms of individuals, the gene pool, and the overall population, perpetuation of myopia is of minimal concern as long as the condition is recognized early and corrected. Simply put, treatment overrides genetics.

Second, although the time frame is short, there is no evidence that the frequencies of medically treated, single-locus genetic diseases are increasing as a consequence of changes in reproductive fitness. In fact, reductions in the number of affected newborns have been observed in some regions where genetic counseling and prenatal services are available.

Third, treatment of genetic disorders has had varied biological consequences. Although life expectancies from birth have been extended in some cases, full reproductive fitness has not been attained. For example, males with cystic fibrosis are usually sterile. Hemophiliacs and those with hemoglobopathies, for example, thalassemia and sickle cell anemia, are often infertile. PKU females are less fit than homozygous dominant and heterozygous individuals and require treatment to ensure successful pregnancies. Between 1984 and 1996 in the US, Canada, and Germany, PKU mothers gave birth to 331 children from 468 pregnancies. Thus, this source contributed 331 mutant *PHA* alleles to a combined gene pool of about 7.6×10^8 *PHA* alleles, thereby increasing the frequency of the recessive allele by about 0.0045%. Overall, the transmission of alleles by medically treated homozygous recessive individuals has been rather insignificant.

Fourth, the genetic consequences for future generations after reduction of selection of monogenic disorders can be represented mathematically. With a complete cure that is readily available to all affected individuals with a lethal single-locus disease, the selection coefficient goes from 1.0 to 0. If the original frequency of the recessive allele was 0.01 (i.e., $q_0 = 0.01$), it would take 70 generations or 1750 years for the frequency of the gene to double with a totally effective treatment, and by that time the frequency of the disorder would be 1 in 2500 births. If the treatment was only 50% effective with regard to fitness, then the doubling time for $q_0 =$ 0.01 would be 140 generations or 3500 years (Table A). The doubling times for dominant deleterious genes are half those of recessive alleles (Table A). Obviously, decreasing the extent of selection causes the frequencies of alleles and genotypes to increase. There is no argument on this point. However, the doubling times for most genetic disorders would require centuries before there is any quantitative impact on the gene pool. Because many of the current remedies for genetic disorders are incomplete, the expected theoretical rise in allele and genotype frequencies will be exceedingly slow. And, with the availability of diagnostic testing, preimplantation testing, and genetic counseling, there is a strong likelihood that allele frequencies for both treatable and untreatable genetic disorders will decrease over time because couples who are at risk for affected offspring will be able to make informed reproductive choices.

Fifth, unlike single-locus disorders, the long term genetic effects of correcting a multigenic (e.g., pyloric stenosis) or a genetically heterogeneous (e.g., diabetes) disorder are difficult to model mathematically. Pyloric stenosis, which is blockage of the canal between the stomach and intestine in newborns that is corrected surgically, is increasing, decreasing, fluctuating, or constant in different regions around the world. Environmental influences and not an accumulation of pyloric stenosis-causing genes are likely responsible for the increased frequencies in certain locations. Similarly, the increase of diabetes in both high- and low-incidence populations is probably due to changes in eating habits, especially obesity, and other nongenetic processes.

In sum, the claim that "the genetic quality of modern populations is deteriorating" is groundless.

Table A Doubling time in generations of recessive (R) and dominant (D) alleles as a function of the effectiveness of medical treatment for dominant and recessive conditions and initial allele frequency.

	Relative effectiveness of medical treatment									
	0.05		0.10		0.50		0.90		1.00	
Initial allele frequency	D	R	D	R	D	R	D	R	D	R
0.10	70	140	35	70	7	14	3.9	7.8	3.5	7
0.01	700	1,400	350	700	70	140	39	78	35	70
0.001	7,000	14,000	3,500	7,000	700	1,400	390	780	350	700

in the Ashkenazi population. It is also highly improbable that there would be a hundredfold difference in the mutation rates of the same gene in two neighboring populations. Consequently, inbreeding, founder effect, or overdominance could explain the frequency of Ashkenazi TSD.

Inbreeding does not appear to be responsible for the preponderance of TSD among Ashkenazi Jews. Consanguinity is not common in central-eastern European Jewish communities. For example, the frequency of first-cousin Ashkenazi marriages was two to three times less than in the non-Jewish population. However, some inbreeding probably took place when Ashkenazi communities were being established in central-eastern Europe about a thousand years ago.

In principle, selection in favor of the heterozygote is definitely one way of maintaining a lethal allele at a high frequency in a population. Assuming that equilibrium has been reached for the *HEXA* locus in the Ashkenazi population, $\hat{q} = \frac{s_{11}}{s_{11} + s_{22}}$. Because $s_{22} = 1$ and $\hat{q} = 0.017$, $s_{11} = 0.02$. Under these conditions, the homozygous dominant genotype would only be 2% less fit than the heterozygous genotype. The evidence supporting overdominance, however, is both conjectural and controversial. It was thought that TSD heterozygotes had a greater chance of surviving tuberculosis than those with the homozygous dominant genotype, even though there are no data to support this hypothesis. In fact, grandparents who have a high probability of being heterozygotes because they had grandchildren with TSD died from the same causes as grandparents who did not have TSD grandchildren. On the other hand, families who had experienced the death of a TSD child had more surviving offspring than non-TSD families. In this study, the relative fitness of the homozygous dominant to the heterozygous genotype (w_{11}/w_{12}) was 0.93 and, therefore, $s_{11} = 0.07$. This phenomenon, which is called reproductive compensation, is not based on any genetic system, but is more likely due to the desire of parents to have viable offspring. If widespread and persistent, reproductive compensation could keep a lethal gene in a population by increasing the frequency of heterozygotes. Specifically, if $s_{11} = 0.07$, then it would have taken about 37 generations or 925 years for the frequency of a TSD allele to go from 0.0017 to 0.017. Coincidentally, Jewish communities in central-eastern Europe were undergoing expansion in the eleventh and twelfth centuries. Alternatively, if $s_{11} = 0.02$, then the tenfold increase of the frequency of a TSD allele would have required about 215 generations or 5375 years, which predates by thousands of years the establishment of Jewish communities in Europe. To date, there is no evidence indicating that the major TSD allele was present in Jewish populations before to the rise of the Ashkenazim. For example, contemporary Roman Jews who trace their lineage back 2000 years and, presumably, contributed individuals to the Jewish population in central Europe during the Middle Ages, do not have the major TSD allele. In all likelihood, the major TSD allele was acquired at the time the central-eastern European Ashkenazi communities were forming. On the whole, the evidence for selective forces acting on the major Ashkenazi *HEXA* mutation is sparse.

Two independent founder events are most likely responsible for the increased frequency of TSD among Ashkenazi Jews. Coalescence analysis, which assigns a probable time of origin for a mutation (from the Human Genetics Files),

from the HUMAN GENETICS files

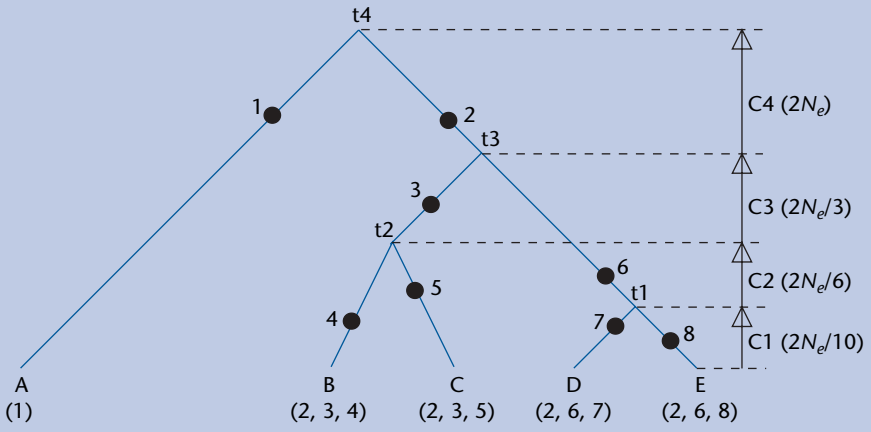
Coalescence

The coalescent process, which is mathematically complex, has become a popular way to estimate the number of generations from a set of contemporary haplotypes (alleles) to the most recent common ancestor (MRCA). In broad terms, the coalescent represents the state when all the lineages (branches) of a gene tree have been traced back with a geometric probability distribution to the point where only one branch remains. Briefly, a tree consists of branches, branch points (nodes), and tips (Figure A). The tips are extant representatives that are, in this case, DNA sequences with different nucleotide substitutions. A node is the point at which one sequence diverged from another to form two branches (lineages). Generally, with molecular data, a mutational event creates a divergence. A branch represents a lineage between divergences and from the last divergence to the present.

A gene tree is constructed by deducing the most likely pattern of sequential nucleotide substitutions that accounts for the differences among the DNA sequences. In Figure A, haplotypes D and E differ by one substitution and have a common ancestor at t_1. Thus, in tracing the branches backward, the coalescence time occurs at t_1 and the number of branches in the tree is reduced by 1. The next coalescence time occurs at t_2, the next at t_3, and the final one, that is, the coalescent, at t_4. In a diploid population, the probability that two alleles are derived from the same source is $1/2N_e$ and the probability that they have different origins is $1 - 1/2N_e$, where N_e is the effective population size. Calculation of the coalescent in a gene tree depends on the number of contemporary alleles. With five prevalent tips, the first coalescence time is expected to have occurred $2N_e/10$ generations ago, the next one $2N_e/6$ generations from the first one, the third coalescence time $2N_e/3$ generations from the second one, and the last one $2N_e$ generations from the previous one (Figure A). Formally, the expected time in generations for a coalescence is $\frac{2N_e}{k/2}$, where k is the number of branches preceding the coalescence time multiplied by those at the coalescence time. The value for the first coalescence time for a 5-allele tree is $\frac{2N_e}{5\times 4/2} = \frac{2N_e}{10}$, and so on for the subsequent coalescence times. Overall, the time to the coalescent in a gene tree is $4N_e(1 - 1/n)$, where n is the number of contemporary alleles.

In practice, the determination of the coalescent requires an estimate of the population-mutation parameter (θ), where $\theta = 4N_e\mu$ and μ is the mutation rate. (See the Neutral Theory section of this chapter for an explanation of the population-mutation parameter.) In this context, the effective population size (N_e) is equivalent to $\theta/4\mu$ for autosomal genetic diversity, $\theta/3\mu$ for X chromosome sites, and $\theta/2\mu$ for Y chromosome and mitochondrial DNA haplotypes. Both nucleotide diversity (π) and a segregating sites computation (K/a_n) approximate the population-mutation parameter. Neither the pattern (topology) nor the occurrences of the coalescences in a real-world genealogy, unlike Figure A, are straightforward. Consequently, the data are analyzed by computer simulations with numerous models that take into consideration factors such as gene flow, recombination, differential mutation rates in different DNA regions, as well as population effects including bottlenecks, expansions, and varying growth rates. The best fit between the data set and a particular model provides an estimate of the most likely duration in N_e generations from the present to the MRCA. Finally, an N_e-generation value is readily converted into years.

Figure A The coalescent for a tree with five haplotypes. The haplotypes (A–E) with each mutation signature (bracketed numbers) are shown at the tips of the tree. The numbers by the dots represent mutation events. Successive coalescence times (t_1–t_4), which correspond to the nodes of the tree, are noted. The coalescent is at t_4, which corresponds to the most recent common ancestor (MRCA). The estimated number of generations for each interval between coalescence times (C1–C4) is shown in the brackets on the right, where N_e is the effective population size.



indicates that the most common *HEXA* mutation (1278insTATC) arose about 1100 years ago at the time the Ashkenazi population was being established in central Europe and the second most common TSD mutation (*HEXA* IVS12 + 1) occurred about 500 years ago in Poland and Lithuania when the Ashkenazi population was depleted. In this context, although not necessarily applicable to TSD, potential Ashkenazi founder events that have been dated to about 900 years ago may account for a number of other genetic disorders such as idiopathic dystonia (*DYT1*; 9q34), familial dysautonomia (*DYS*; 9q31–33), Bloom syndrome (*BLM*; 15q26.1), Gaucher disease (*GBA*; 1q21), factor XI deficiency type III (*F11*; 4q35), and breast or ovarian cancer (*BRCA1*; 17q21) that occur with an increased frequency in the Ashkenazi population. Because selection is doubtful at so many loci within a particular population, founder effect in an expansion after a population constriction (bottleneck) is the more likely explanation for the observed allele frequencies including TSD.

Neutral Theory

The discovery of a large number of polymorphic loci in various organisms raised the question of whether balancing selection (overdominance) could account for the allelic frequencies in every instance. Mathematical analyses indicated that overdominance involving a large number of loci at one time was not feasible. The biological cost due to selection against the various homozygous genotypes was too great. Consequently, Motoo Kimura (1924–1994) postulated that nearly all polymorphisms are selectively neutral and that the changes of allele frequencies at these loci are due to random genetic drift (drift) and rarely to selection. These tenets form the basis of the neutral theory (neutral allele theory).

By definition, neutral mutations have no effect on the reproductive fitness of an organism. Some neutral mutations change a nucleotide of a codon without altering the encoded amino acid. Others result in a different amino acid in a part of the protein that has no affect on its activity. Of course, many neutral mutations occur in the noncoding regions of the genome. Because of their high occurrence, neutral mutations contribute significantly to the total genetic variation found within a species.

The theoretical behavior of neutral mutations in small (finite) populations has been examined in detail. As part of this mathematical approach, every new neutral mutation is considered unique. In other words, it has never occurred previously. This assumption, which is called the infinite-alleles model, is not farfetched. For example, a protein with 250 amino acids is encoded by 750 nucleotides and with four possible nucleotides at each site, the number of potential alleles is 4^{750} or 3.5×10^{451}.

In a finite population of diploid organisms with N individuals and neutral mutation rate μ, the rate of introduction of a new neutral mutation into the population is $2N/\mu$. The loss of a neutral mutation because of drift is $1/2N$. At equilibrium, the introduction of a neutral mutation is offset by loss from drift. Under these conditions, the homozygosity of a population is $\hat{F} = \frac{1}{1+4N\mu}$. Because each neutral mutation is initially one of a kind, then two alleles

of a homozygote must be identical by descent. Thus, the inbreeding coefficient (F) is appropriate for defining the balance between neutral mutation and drift. Heterozygosity (h) in a population at mutation-drift equilibrium is $1-\hat{F}=\frac{4N\mu}{4N\mu+1}$.

In an ideal finite population, the population size is constant from one generation to the next, mating is random, the same number of each sex contribute equally to the next generation, and each generation is discrete. In real-world populations, not all individuals have the same reproductive capacity. Those individuals that have offspring are designated as the effective population size, N_e. For a randomly mating population with males and females, $N_e=\frac{4N_fN_m}{N_f+N_m}$, where N_f and N_m represent the number of females and males, respectively. The expected heterozygosity at equilibrium is $\frac{4N_e\mu}{1+4N_e\mu}$, where $4N_e\mu$ represents the mean number of nucleotide substitutions at neutral sites. The population-mutation parameter, $4N_e\mu$, which is often designated as θ, plays an important role in determining population structure, the extent of migration experienced by a population, and other features of finite populations. Estimates of θ also provide methods of testing whether selection is acting on an allele or if its frequency in a population is due solely to chance.

Testing for Selection

A number of statistical measures have been developed to test whether nucleotide substitutions in a population are behaving as neutral alleles, whether they are being subjected to selective pressure, or whether other conditions are responsible for the observed allele frequencies. Significant deviation from neutrality indicates that either some form of selection or a demographic change such as rapid expansion is in effect. DNA sequences from many individuals provide the raw data for neutral theory analyses.

Tajima's D statistic is a popular way of determining whether a gene is under selection pressure. This test is based on two different estimators of the population-mutation parameter. Under the neutral theory, these two values should be the same. If the estimates are significantly different, then factors other than neutrality must be considered. One estimator of $4N_e\mu$ is calculated from the number of segregating sites (K/a_n) (Figure 9.25). A segregating site (K) has one or more different nucleotides at a particular location among the sequences. The number of segregating sites per nucleotide adjusted for the sample size (n) is K/a_n, where $a_n=1+\frac{1}{2}+\frac{1}{3}+\ldots\frac{1}{n-1}$. For neutral alleles, K/a_n is proportional to $4N_e\mu$. In this case, it is assumed that each new mutation occurs at a site that has not previously mutated, that is, the infinite-sites model.

The second estimator of $4N_e\mu$ is nucleotide diversity (π), which is a measure of the mean pairwise difference per nucleotide of a sample of DNA sequences that are usually the same length (Figure 9.26). Briefly, for one pair of sequences, say sequence 1 and sequence 2, the number of nucleotide differences (d_{12}) are counted and divided by the sequence length (L). Next, this value is multiplied

A

Site	1	**2**	3	4	**5**	6	7	**8**	9	**10**	11	12	**13**	14	**15**
Seq1	A	T	T	C	G	T	A	T	G	A	A	C	T	T	T
Seq2	A	T	T	C	C	T	A	G	G	A	A	C	C	T	G
Seq3	A	T	T	C	G	T	A	G	G	A	A	C	T	T	G
Seq4	A	A	T	C	G	T	A	G	G	A	A	C	T	T	G
Seq5	A	T	T	C	G	T	A	T	G	C	A	C	T	T	G

B

Total number of segregating sites (S) = 6

Nucleotide length (n) = 15

Segregating sites per nucleotide length (K) = 6/15 = 0.4

$a_n = 1 + 1/2 + 1/3 + \ldots 1/14$

$K/a_n = 0.4/3.25 = 0.123$

Figure 9.25 Calculating K/a_n. (A) Aligned sequences. The segregating sites are marked with bold numbers. (B) Calculation of K/a_n.

A

Seq1	A	T	T	C	G	T	A	T	G	A	A	C	T	T	T
Seq2	A	T	T	C	C	T	A	G	G	A	A	C	C	T	G
Seq3	A	T	T	C	G	T	A	G	G	A	A	C	T	T	G
Seq4	A	A	T	C	G	T	A	G	G	A	A	C	T	T	G
Seq5	A	T	T	C	G	T	A	T	G	C	A	C	T	T	G

B

Pairwise comparisons		Nucleotide difference (d_{ij})	d_{ij}/L	Sequence frequencies		d_{ij}/L x f(i) x f(j)
i	j			f(i)	f(j)	
Sequence 1	Sequence 2	4	4/15	1/5	1/5	0.011
Sequence 1	Sequence 3	2	2/15	1/5	1/5	0.008
Sequence 1	Sequence 4	3	3/15	1/5	1/5	0.008
Sequence 1	Sequence 5	2	2/15	1/5	1/5	0.0052
Sequence 2	Sequence 3	2	2/15	1/5	1/5	0.0052
Sequence 2	Sequence 4	3	3/15	1/5	1/5	0.008
Sequence 2	Sequence 5	4	4/15	1/5	1/5	0.011
Sequence 3	Sequence 4	1	1/15	1/5	1/5	0.0028
Sequence 3	Sequence 5	2	2/15	1/5	1/5	0.0052
Sequence 4	Sequence 5	3	3/15	1/5	1/5	0.008
						0.0724

Uncorrected nucleotide diversity: $\pi = 0.0724$.

Nucleotide diversity with small sample size correction: $\pi = 5/4 \times 0.0724 = 0.0905$.

Figure 9.26 Calculating nucleotide diversity (π). (A) Aligned sequences. (B) Calculation of mean pairwise difference per nucleotide with and without correction for small sample size.

by the frequency of each sequence of the pair in the sample, that is, $f(\text{seq1}) \times f(\text{seq2}) \times d_{12}/L$. This calculation is repeated for each pair of sequences. Nucleotide diversity (π) is the sum of these values. With n sequences, there are $n(n-1)/2$ pairwise comparisons. Small sample sizes are corrected by multiplying the nucleotide diversity by $n(n-1)$, where n is the number of sequences.

More formally, nucleotide diversity is $\pi = \sum_{ij} x_i x_j \pi_{ij}$, where x_i and x_j are the frequencies of the ith and jth types of sequences and π_{ij} is the difference of nucleotides between the ith and jth sequences divided by the sequence length.

With neutrality, nucleotide diversity (π) should approximate the population-mutation parameter. Thus, at mutation-drift equilibrium, $\pi - K/a_n = 0$. For Tajima's D test, the difference between the two estimators of $4N_e\mu$ is normalized by the standard deviation of this difference, that is,

$$D = \frac{\pi - K/a_n}{\sqrt{\mathrm{Var}(\pi - K/a_n)}},$$

where Var is the variance.

A Tajima's D value equal to or close to zero indicates that the two estimators are equal and the data conform to the neutral model. A significant positive D value ($\pi > K/a_n$) denotes an excess of intermediate frequency mutations that might be due, in part, to balancing selection. The presence of excess rare mutations produces a significant negative D value ($\pi < K/a_n$) that could represent negative (purifying) selection. As a test for selection, Tajima's D depends on a constant population size over time. Otherwise, recent extensive migration or reduction in effective population size will produce a positive D value. A negative D value may indicate a rapid expansion from a population that had been drastically reduced (bottleneck). Carrying out other tests for neutrality as well as understanding the demographic history of the population under study helps clarify the significance of Tajima's D values.

The ratio of nonsynonymous (K_a, d_N) to synonymous (K_s, d_S) changes in the codons of genes provides a test for selection that is not as dependent on population dynamics as Tajima's D statistic. Briefly, with a nonsynonymous mutation, one amino acid is substituted for another. For example, a mutation of TTT to TAT results in phenylalanine being replaced by tyrosine. By contrast, there is no change in the encoded amino acid with a synonymous (silent) mutation. For example, with a TTA to TTG mutation, both triplets code for leucine. If a nonsynonymous mutation is selectively neutral, then it should be fixed at the same rate as a synonymous mutation and $K_a/K_s = 1$. If an amino acid change is detrimental, then purifying selection will reduce its fixation rate with respect to a synonymous mutation and K_a/K_s should be less than 1 ($K_a/K_s < 1$). Finally, if an amino acid change has a selective advantage (positive selection, directional selection), then it will be fixed at a higher rate than a synonymous mutation and $K_a/K_s > 1$.

A single nucleotide difference between a pair of codons can be readily scored as either a synonymous or nonsynonymous change. However, often the observed difference at a site may be due to two or more sequential substitutions without the intermediate mutations being present in the sample. In these instances, the nature of the changes that make up the intervening steps must be deduced. Methods have been developed for estimating the numbers of nonsynonymous and synonymous substitutions that not only take into consideration the possible pathways from one codon to another but also differences in the rates of transitions (T ↔ C, A ↔ G) and transversions (A ↔ T, G ↔ C,

A ↔ C, G ↔ T) as well as codon usage. Various statistical approaches have been devised for testing whether the values for K_a and K_s are significantly different.

The average K_a/K_s for complete human genes and miscellaneous exons is 0.175. Less than 1% of these sequences have K_a/K_s values significantly greater than 1.0. In these cases, positive selection can be distinguished from a change in effective population size by analyzing a large number of genes at a time because many genes will be affected by a population shift whereas only a small number of genes are subjected to positive selection.

The McDonald–Kreitman (M-K) test is based on the ratio of nonsynonymous (replacement) and synonymous sites within a species, compared to the ratio of nonsynonymous and synonymous sites between species. According to neutral theory, these ratios should be equal. For the M-K test, coding regions of a gene from a number of individuals in two related species are sequenced. A nucleotide site is considered polymorphic if there is any difference in one or both species. A site is fixed if there are no variants within a species and it is different between species. Finally, the nonsynonymous and synonymous changes for the polymorphic and fixed sites are determined (Figure 9.27) and the results are organized into a 2 × 2 table, for example,

	Fixed	**Polymorphic**
Nonsynonymous	a	b
Synonymous	c	d

A lack of significance by Fisher's exact test establishes agreement with neutral theory, whereas a significant outcome demonstrates that the gene is behaving in a nonneutral manner. If cell a (upper left) has a higher value than the other cells, then the gene is likely subjected to balancing selection. If the number in cell b (upper right) is greater than the other cells, then the gene is probably undergoing purifying selection. And, if the nonsynonymous-to-synonymous ratio between species (cell a/cell c) is much larger than the nonsynonymous-to-synonymous ratio within the species (cell b/cell d), then positive selection is likely.

Neutrality testing has established that a number of human genes have undergone positive selection (adaptive evolution) (Table 9.20). In this context, one interesting example is the abnormal spindlelike microcephaly-associated gene (*ASPM*). Homozygotes with loss-of-function *ASPM* mutations have at least a 70% reduction of the cerebral cortex (microcephaly) without any other consistent physical or neurological abnormalities. Mental impairment ranges from mild to severe. Individuals with extreme ASPM microcephaly cannot read or write, and their communication skills are rudimentary. This phenotype is consistent with the cerebral cortex being responsible for higher brain functions such as language and abstract thinking. The progressive enlargement of the human brain, especially the cerebral cortex, is one of the major features of the evolution of humans. Thus, it was of interest to examine the evolutionary behavior of the *ASPM* gene. Neutrality assays including the M-K test and K_a/K_s analysis indicate that the *ASPM* gene has been subjected to positive selection.

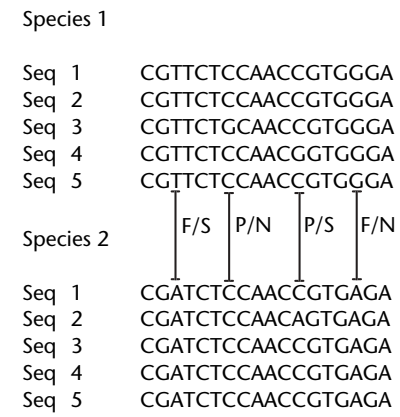


Figure 9.27 McDonald–Kreitman test. DNA sequences from the coding region of different individuals of two species are aligned and compared. Fixed, synonymous (F/S), polymorphic, nonsynonymous (P/N), polymorphic, synonymous (P/S) and fixed, nonsynonymous (F/N) differences are noted. Sequence 1 of species 1 is in frame and encodes RSPTVG.

Table 9.20 Positively selected human genes.

Gene(s)	Function
ASPM (Abnormal spindlelike microcephaly-associated)	Possibly proliferation of neuronal progenitor cells
BRCA1 (Breast cancer 1, early onset)	Negative regulation of cell growth, DNA repair, and other functions
CCT subunits (Chaperonin containing tailless complex genes)	Protein folding
DRD4 (Dopamine receptor D4)	G protein-coupled dopamine signaling
FOXP2 (Foxhead box P2)	Transcription factor involved in the development of speech and language
FY (Duffy blood group)	Receptor for chemokines and the malarial parasite *Plasmodium vivax*
GYPA (Glycophorin A)	Erythrocyte surface protein with MN blood group and possibly pathogen binding receptors
IL4 (Interleukin 4)	Immune system signaling
Morpheus gene cluster	Unknown
MSMB (Prostatic secretory protein 94, β-microseminoprotein)	Unknown
PRM1 (Protamine 1)	Sperm DNA condensation
PRM2 (Protamine 2)	Unknown, associated with sperm DNA
SPINK2 (Acrosin-trypsin inhibitor)	Serine protease inhibitor
TAS2R gene family	Bitter taste receptors
VN1R gene family (Type 1 vomeronasal receptor genes)	Pheromone receptors

Although the function of the human *ASPM* gene is not known, the orthologous gene in *Drosophila* encodes a protein that controls the organization of spindle structures during mitosis. It is likely that the human ASPM protein facilitates the proliferation of neuronal progenitor cells. Possibly, *ASPM* gene mutations altered the orientation of the progenitor cerebral cortex cells that facilitated additional cell divisions, which, in turn, led to an increased cerebral cortex cell content.

Wright's F_{ST} Statistic

For most organisms, a population usually consists of a collection of small units (demes, subpopulations). If subpopulations are isolated and small, they become subject to drift, founder effects, and inbreeding, which increases homozygosity and reduces heterozygosity. Moreover, over time, if isolation is maintained, the genetic compositions of the subpopulations diverge from one another as different alleles become fixed. Sewall Wright devised measures (*F* statistics, fixation indices) for assessing the extent of reduction of heterozygosity in a subdivided population.

An *F* statistic in population genetics, which should not be confused with *F* statistics for evaluating variances in statistics, is a comparison of the frequencies of heterozygotes between various categories (individuals, subpopulations, regions, total population) of a population. For example, one *F* statistic (F_{IT}) is equivalent to the inbreeding coefficient among individuals (I) in the total population (T). Another fixation index (F_{IS}) delineates the decrease in heterozygosity among individuals (I) relative to their subpopulations (S). Finally, the

A

Subpopulation	Allele (p) frequency	Expected heterozygosity ($2pq$)
1	0.4	0.48
2	0.2	0.32
3	0.7	0.42

$$H_S = \frac{\Sigma(2pq)}{n} = \frac{0.48 + 0.32 + 0.42}{3} = 0.407.$$

B

$$\text{Average frequency } p\ (\bar{p}) = \frac{0.4 + 0.2 + 0.7}{3} = 0.433.$$

$$\text{Average HWE heterozygosity} = 2\bar{p}\bar{q} = H_T = 0.491.$$

C

$$F_{ST} = \frac{H_T - H_S}{H_T} = 0.17.$$

Figure 9.28 Wright's F_{ST} statistic. (A) The average heterozygosity for subpopulations (H_S) is the sum of the frequencies of expected heterozygotes ($2pq$) for each subpopulation divided by the number of subpopulations. (B) The average heterozygosity for the total population (H_T) is equivalent to the frequency of heterozygotes ($2\bar{p}\bar{q}$) derived from the mean values of the observed subpopulation allele frequencies. (C) Calculating F_{ST}.

most important fixation index (F_{ST}) examines the relationship of heterozygosity of subpopulations (S) with respect to the total population (T).

The frequencies of heterozygotes within subpopulations (H_S) and the total population (H_T) under the assumption of HWE are required for computing F_{ST}. The calculation of H_S entails obtaining the frequencies of alleles for each subpopulation, computing the expected frequency of heterozygotes ($2pq$) in each subpopulation, and then averaging the heterozygote frequencies (Figure 9.28). On the other hand, H_T is the overall expected heterozygosity ($2\bar{p}\bar{q}$) derived from the mean allele frequencies of the subpopulations. The F_{ST} fixation index is equivalent to $\frac{H_T - H_S}{H_T}$.

According to Wright, although the cutoff points are somewhat arbitrary, F_{ST} values that range from 0 to 0.05 indicate little genetic differentiation (genetic diversity) among subpopulations; 0.05 to 0.15, moderate; 0.15 to 0.25, great; and >0.25 very great. In other words, a low F_{ST} value indicates that most, if not all, subpopulations are close to the mean allele frequency (centroid) and to each other, whereas the opposite applies for high F_{ST} values. Generally, $F_{ST} > 0.25$ denotes the presence of subspecies within the total population.

Estimates of F_{ST} values can include loci with multiple alleles and many different loci. Moreover, there are a number of ways of calculating F_{ST} values. In addition, approaches have been developed that take into account subpopulation size differences, the nature of the genotype data, and other factors. For human populations, F_{ST} determinations with diverse autosomal markers, such as blood groups, protein polymorphisms, RFLPs, STRPs, and SNPs, invariably range from about 0.10 to 0.15. Thus, human populations show moderate genetic differentiation, with about 15% of the genetic variation due to differences among subpopulations and the remainder falling among individuals within subpopulations. On a global scale, about 85% of human genetic variation is found among individuals in populations, about 5% among populations,

from the HUMAN GENETICS files

Using DNA Polymorphisms to Infer Human History

Distinctive sets of polymorphic sites accumulate in populations that have been, more or less, isolated for a period of time. These population-specific combinations of polymorphisms are used to analyze admixture in contemporary populations and to track human migrations of the past. However, recombination disrupts the arrangement of polymorphic loci of autosomes and X-linked chromosomes, blurring genetic signatures and haplotypes of heterozygotes are somewhat difficult to establish. By contrast, mitochondrial DNA (mtDNA) and the nonpseudoautosomal portion of the human Y chromosome (nonrecombining region of the Y chromosome, NRY) are single entities that do not recombine. As a result, not only do arrays of polymorphic loci of mtDNA and the NRY remain intact, but they are readily scored. Moreover, the genetic contributions of females and males can be examined separately because mtDNA and the NRY represent maternal and paternal inheritance, respectively. Consequently, mtDNA and NRY polymorphic loci are often preferred for studies of human populations.

Combinations of mtDNA restriction fragment length polymorphisms (RFLPs) (haplotypes) from individuals around the world can be organized into haplogroups. For example, among Native American populations there are five mtDNA haplogroups (Table A) and about 18 different haplogroups worldwide. In some instances, haplogroups with shared sites form a macrohaplogroup (superhaplogroup). And, within haplogroups, there may be distinctive subhaplogroups. Although there is some confusion about the designations and membership of certain haplogroups, Africa, which has the highest mtDNA haplotype diversity in the world, has three haplogroups (L1, L2, L3), Europe nine (H, I, J, K, T, U, V, W, X), and Asia six (A, B, C, D, F, G). Of the Asian haplogroups, A, B, C and D are found, more or less, exclusively in the Western Hemisphere. Haplogroup X, which is present in Europe, occurs with a low frequency (~3%) among Native Americans.

The DNA polymorphisms that are located on the NRY include a large number of biallelic single-nucleotide polymorphisms (SNPs) and some multiallelic short tandem repeat polymorphisms (STRPs). About 15 major Y chromosome haplogroups (A–E, G–J, L–O, Q–R) where the haplotypes of each haplogroup are all derived from a common ancestor, and 3 paragroups (F, K, P) where only some of the contemporary haplotypes can be traced to a common ancestor have been identified globally. Of these, A, B, and E are found mostly in Africa, D, H, F, N, L, O, and P in Asia, C, K, and M in the Pacific islands (Oceania), R and I and to a lesser extent J and G in Europe/Middle East, and Q in the Americas.

Mitochondrial DNA and NRY polymorphisms have been used to clarify aspects of genetic heritage in a number of different countries such as Mexico, Colombia, Brazil, Iceland, Finland, Spain, Hungary, Ireland, Estonia, and India. For example, the Magyars were a nomadic Asian group who successfully invaded central Europe in ca. 895 CE. After being defeated in 955 CE, they settled in large numbers in the region of Europe that now constitutes modern Hungary. A lasting legacy of the Magyars is the Hungarian language, which is a member of the Uralic language family from Asia and is quite distinct from the Indo-European languages that are spoken throughout most of Europe. On this basis, it seemed likely that the DNA of contemporary Hungarians would reflect Magyar/Asian ancestry. Among a sample of 98 Hungarians, only two had mitochondrial DNA haplotypes that are likely Asian in origin and none of the 46 males carried a common Asian-specific Y-chromosome polymorphism. Thus, notwithstanding the perpetuation of the Magyar language, the genetic contribution of this group to the current Hungarian gene pool seems negligible.

Traditional sources attribute the colonization of Iceland from 870 to 930 CE mainly to Norse Vikings from Scandinavia and Viking settlements in the British Isles. Undoubtedly, natives from the British Isles were members of the founding Icelandic population. However, the extent of this non-Norse contribution has been controversial. To resolve this issue, maternal and paternal origins were assessed by comparing Icelandic and European haplogroups. About 50% of the founding Icelandic females were probably British/Gaelic in origin, and 80% of the original males had Scandinavian ancestry. Thus, significant numbers of individuals from both Scandinavia and the British Isles helped populate Iceland.

In Jewish heritage, all of the direct male descendants of Moses' brother Aaron are considered priests (Cohanim, pl.) who have specific religious privileges, responsibilities, and restrictions. Generally, in

Table A Sites that define the mitochondrial DNA haplogroups of Native American populations.

Haplogroups	Haplogroup-associated markers
A	663 + *Hae*III
B	8271–8281 9-bp deletion; 16517 + *Hae*III
C	13259 – *Hin*cII/13262 + *Alu*I
D	5176 – *Alu*I
X	1715 – *Dde*I; 10394 – *Dde*I; 14465 + *Acc*I

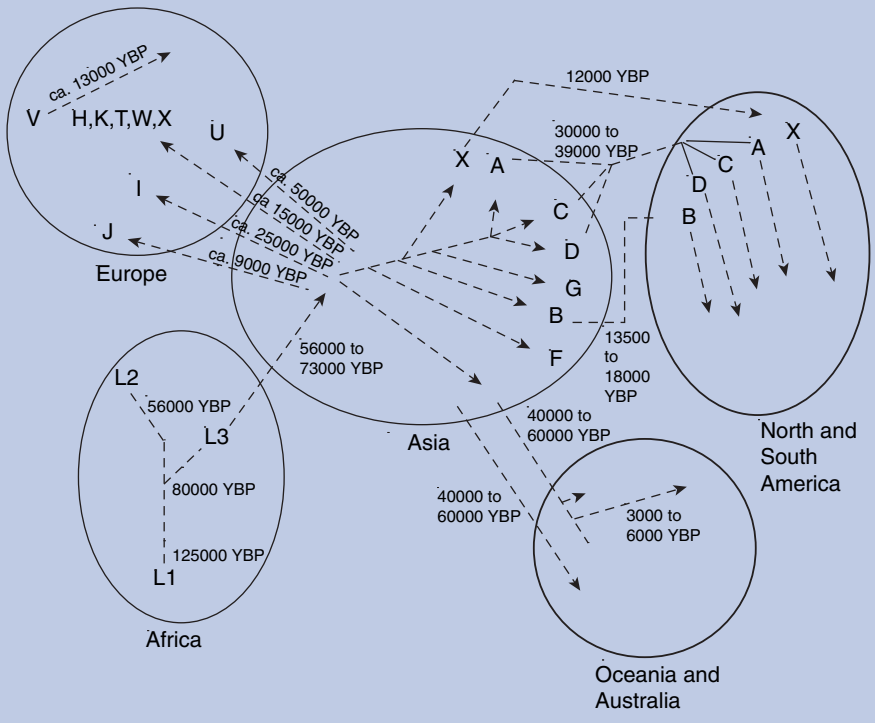
Note: A number preceding a plus (+) or minus (–) sign denotes the first nucleotide position of the designated restriction endonuclease recognition site. A plus sign represents the presence of a restriction endonuclease site, and a minus sign its absence. The 9-base pair deletion that is a characteristic feature of haplogroup B removes nucleotides 8272 to 8280.

modern times, knowledge of this type of priestly distinction is based on word of mouth from father to son. If the original dictum has been followed for the last two to three thousand years, it is highly possible that as a result of patrilinear inheritance of the Y chromosome contemporary presumptive Cohanim, regardless of geographic origin, share a common Y chromosome haplotype. To test this hypothesis, DNA samples were collected from 306 Jewish males that included 97 self-identified Cohanim. Six STR and six biallelic single nucleotide Y chromosome polymorphisms were haplotyped. Among the Cohanim of European origin (Ashkenazim) and Mediterranean/Middle Eastern descent (Sephardim), 96% and 88%, respectively, had the same Y chromosome haplotype, which was designated the Cohen modal haplotype (CMH). The CMH is present, but to a lesser extent, in other individuals of the sample and it is either absent or occurs with a very low frequency in non-Jewish Mediterranean and Asian populations. Analysis of the coalescent places the origin of the CMH to about 2100 to 3250 years ago, which corresponds, more or less, to the time when Jews worshipped at their First Temple (Solomon's Temple) and the participation of the Cohanim in religious ceremonies was mandatory.

The CMH has been used to unravel an intriguing anthropological mystery in southern Africa. The Lemba are a Bantu-speaking group of Christian and Muslim clans who are located in the Republic of South Africa and Zimbabwe. Their oral history declares that they are directly descended from Jews. Although some of their current customs are suggestive of a Jewish tradition, they could have been derived from African and Muslim practices. To test the genetic ancestry of the Lemba, Y chromosome haplotypes from Lemba clans were compared with samples from Bantu (African), Yemeni (Arab), and Jewish populations. The Jewish data set excluded both Cohanim and Leviim, who belong to another Old Testament priestly class. The CMH and a Bantu-specific haplotype occur frequently among the Lemba. About 9% of the sampled Lemba and 10% of the Sephardic component of the Jewish sample in this study have the CMH. The CMH did occur in one individual from Yemen, but it was not found in the Bantu population. The CMH was likely acquired from a Jewish, and not an Arab, source. However, the origin of the Lemba ancestors remains problematic. Jewish artisans may have left the city of Sa'na (Sena) in Yemen about 2000 to 2500 years ago, settled in Africa, and took local wives, and today their descendants are represented among the Lemba.

Reconstruction of human history for the last 150,000 years or so, although far from complete, depends on information from archeology, cultural anthropology, linguistics, and human genetics. Studies of mtDNA and NRY polymorphisms have been instrumental in broadly defining the pattern and timing of demographic expansions throughout the world. With a coalescence time of approximately 113,000 to 249,000 YBP (years before present), the L1 African mtDNA haplogroup is the oldest in the world. The two other African-specific mtDNA haplogroups L3 and L2 arose about 80,000 and 56,000 years ago, respectively. One or more L3 subhaplogroups left Africa about 60,000 years ago by way of southern Arabia and moved into India. Other groups with these subhaplogroups passed from Ethiopia into the Middle East and from there expanded throughout Asia. Eventually, Asia became the distribution center for populating, at various times, Oceania, Europe, and North America (Figure A).

Figure A Schematic representation of the timing (YBP, years before the present) and migration of many of the major human mitochondrial haplogroups (upper case letters) throughout the world. The arrows indicate, in broad terms, the directions of haplogroup dispersions. All dates are approximate.



and 10% among major geographic regions (Africa, Asia, Europe). These percentages are inconsistent with the notion that humans can be readily grouped into discrete biological entities (races).

The low F_{ST} value for human populations can be explained in part by migration between populations. With the infinite-alleles model and the assumption that migration between all populations is equally likely, the relationship between F_{ST} and migration is $F_{ST} = \frac{1}{1+4N_e m}$, where m is the proportion of alleles replaced by alleles contributed by migrant individuals. Because an individual introduces two alleles per locus and the total number of alleles in a population is $2N$, $N_e m$ represents the number of migrants that enter a population per generation. Specifically, with $F_{ST} = 0.15$, about 1.4 migrants are expected per generation or 140 migrants for every 2500 years. In other words, a very small amount of gene flow among subpopulations is sufficient to maintain moderate genetic differentiation and prevent fixation. In addition, there is no need for individuals to travel long distances to affect the genetic makeup of populations because alleles that are introduced by migrants into one subpopulation can be spread by other population members to a neighboring subpopulation and, so on, through a series of subpopulations.

key terms

adaptive evolution
admixture
assortative mating
back mutation
balancing selection
bottleneck
consanguineous marriage
directional selection
disruptive selection
dysgenics
effective population size
emigration
***F* statistics**
fertility
fixation
fixation index
forward mutation
founder effect
gene flow
gene pool
genetic fixation
genetic microdifferentiation
Hardy–Weinberg equilibrium
heterozygosity
heterozygous effect
homozygosity
identity by descent
immigration
inbreeding
inbreeding coefficient (*F*)
infinite-alleles model
infinite-sites model
linkage disequilibrium
linkage equilibrium
migration
M-K (McDonald–Kreitman) test
negative assortative mating
neutral mutation
neutral theory
nonassortative mating
nonrandom mating
nonsynonymous substitution
nucleotide diversity
overdominance
population
population-mutation parameter
positive assortative mating
purifying selection
random genetic drift
random mating
reproductive fitness
segregating site
selection
synonymous substitution
systemic process
Tajima's *D* statistic
underdominance
viability

summary

Human population genetics is concerned with the frequencies of alleles and genotypes in populations and how these frequencies are affected by various parameters. With a two-allele, one-locus model, the alleles *A1* (p) and *A2* (q) and the genotypes *A1A1* (p^2), *A1A2* ($2pq$), and *A2A2* (q^2) are maintained with the same frequencies in successive generations if the population is very large, mating is random, and other major population features such as migration, mutation, and selection do not occur. This constancy of allele and genotype frequencies in successive generations is called Hardy–Weinberg equilibrium (HWE). In the presence of HWE conditions, a genetic equilibrium is established in one generation for a two-allele autosomal locus, whereas a number of generations are required before HWE occurs for either X-linked genes or two or more separate loci. Linkage equilibrium occurs when two or more genes are randomly associated and linkage disequilibrium when they are not. A number of loci for blood cell proteins and many other traits are in HWE in contemporary human populations.

Allele and genotype frequencies change in unexpected ways in successive generations with dispersive processes such as reduced population size and migration. Conversely, the direction of allele and genotype changes over time can be predicted with systematic processes that include nonrandom mating, mutation, and selection. Random genetic drift is a feature of populations with a small number of mating individuals. Under this condition, allele frequencies fluctuate from one generation to the next because of the randomness of the transmission process until, by chance, one of the alleles is lost and the other is fixed. A founder effect occurs when a small number of individuals establish a colony that thrives and expands through a number of generations and remains, more or less, isolated socially or geographically. After 10 or so generations, this type of population has a large number of individuals who carry a copy of a deleterious recessive gene that was present in one of the original settlers. As a consequence of marriages between these heterozygotes, who for the most part are not close relatives, the population experiences an increased number of individuals with a homozygous recessive condition that is rare in most other populations.

Migration of a large number of individuals with genotype frequencies that differ from those in a recipient population decreases homozygosity of the recipient population. With a continuous rate of gene flow per generation over a large number of generations, an allele in the recipient population will be eventually supplanted by the one from the contributing population.

Random mating tends to be the rule for most human populations. Preferential mating between individuals with identical genotypes (positive assortative mating), if prevalent, would increase homozygosity in the population. On the other hand, mating between individuals with different genotypes (negative assortative mating), if frequent, would increase heterozygosity in a population. In genetic terms, inbreeding represents an increased likelihood that an individual will acquire two alleles that are identical by descent from an ancestor of both parents. If the number of generations that separate the parents and a common ancestor is three or fewer, then the extent of inbreeding has genetic relevance. The inbreeding coefficient for a particular mating is the sum of the probabilities that any allele at a locus in every common ancestor of both parents will be identical by descent in their offspring. If widespread, inbreeding increases homozygosity. In some human populations, a high frequency of inbreeding, mostly from first-cousin marriages, increases the number of individuals who are born with deleterious homozygous recessive disorders.

Mutation causes miniscule changes in allele frequencies. Nevertheless, it is an important source of genetic variation. Generally, forward mutation from a dominant to a recessive allele is ten to one hundred times more frequent than back mutation, which occurs in the reverse direction.

Reproductive fitness encompasses both viability and fertility. In some instances, a genotype produces a phenotype that is more (less) fit than other genotypes at the same locus and, as a result, the alleles of this genotype are more (less) likely to be transmitted from one generation to another. A more fit genotype is at a selective advantage, and one that is less fit is at a selective disadvantage. With either complete or incomplete dominance and selection against the *A2* allele, its frequency is reduced to zero over many generations, while the *A1* allele goes to fixation. This pattern of allele change is called directional selection. With overdominance, selection against both homozygous genotypes eventually drives the alleles to a stable equilibrium point, where the frequencies of the *A1* (p) and *A2* (q) alleles are greater than 0 and less than 1. This phenomenon is designated balancing selection. Underdominance occurs when the heterozygous

continued

genotype is less fit than either homozygous genotype. Over time, underdominance leads to one allele being lost and the other being fixed, unless the initial allele frequencies happen to agree precisely with those expected for the unstable equilibrium point. With this exceptional circumstance, the allele frequencies remain the same through successive generations. The consequences of underdominance are called disruptive selection. Genetic equilibrium between mutation and selection can be established. Overall, population genetics provides a mathematical basis that accounts for either conventional or unusual allele and genotype frequencies in populations.

In the 1960s, researchers discovered that organisms have a large number of polymorphic loci. Theoretical analysis showed that so many polymorphic loci, if each were maintained by balancing selection, would be too great a biological burden for an organism to manage. As a consequence, the neutral theory was developed. It was postulated that most mutations have no impact on reproductive fitness, that is, they are effectively neutral. Moreover, the high frequencies of these alleles were due to random genetic drift. The heterozygosity of a population at neutral mutation-drift equilibrium is $\frac{4N_e\mu}{1+4N_e\mu}$, where μ is the neutral mutation rate, N_e the effective population size, and $4N_e\mu$ the mean number of nucleotide substitutions at neutral sites. Neutral theory analyses are, for the most part, carried out with DNA sequence data. With Tajima's D statistic, the population-mutation parameter ($4N_e\mu$) is determined two different ways and the estimates are compared to test whether DNA sites deviate from neutrality. Additional tests for selection within coding regions include the ratio of nonsynonymous to synonymous substitutions (K_a/K_s) and the ratio of nonsynonymous and synonymous changes within and between species (M-K test). Most genes conform to the neutral theory, some undergo purifying selection, and a few are subjected to positive selection. In addition to selection, nonneutrality may represent a recent population change such as constriction or rapid expansion.

Wright's F_{ST} statistic is an estimation of the genetic differentiation among subpopulations. The moderate F_{ST} values that have been recorded for human populations can be attributed to modest amounts of gene flow.

References

Baum, J., R. H. Ward, and D. J. Conway. 2002. Natural selection on the erythrocyte surface. *Mol Biol Evol* **19**:223–229.

Bittles, A. H. and J. V. Neel. 1994. The costs of human inbreeding and their implications for variations at the DNA level. *Nat Genet* **8**:117–121.

Boyce, A. J. 1983. Computation of inbreeding and kinship coefficients on extended pedigrees. *J Hered* **74**:400–404.

Brown, M. D. et al. 1998. mtDNA haplogroup X: An ancient link between Europe/Western Asia and North America? *Am J Hum Genet* **63**:1852–1861.

Cann, R. L. 2001. Genetic clues to dispersal in human populations: retracing the past from the present. *Science* **291**:1742–1748.

Carmi, R. et al. 1995. Use of a DNA pooling strategy to identify a human obesity syndrome locus on chromosome 15. *Hum Mol Genet* **4**:9–13.

Cavalli-Sforza, L. L. and W. F. Bodmer. 1971. *The Genetics of Human Populations*. W. H. Freeman and Company, San Francisco.

Colombo, R. 2000. Age estimate of the N370S mutation causing Gaucher disease in Ashkenazi Jews and European populations: A reappraisal of haplotype data. *Am J Hum Genet* **66**:692–697.

Ding, Y. C. et al. 2002. Evidence of positive selection acting at the human dopamine receptor D4 gene locus. *Proc Natl Acad Sci USA* **99**:309–314.

Donnelly, P. and S. Tavaré. 1995. Coalescents and genealogical structure under neutrality. *Annu Rev Genet* **29**:401–421.

Enard, W. et al. 2002. Molecular evolution of *FOXP2*, a gene involved in speech and language. *Nature* **418**:869–872.

Evans, P. D. et al. 2004. Adaptive evolution of *ASPM*, a major determinant of cerebral cortical size in humans. *Hum Mol Genet* **13**:489–494.

Falconer, D. S. and T. F. C. Mackay. 1996. *Introduction to Quantitative Genetics*. 4th ed. Addison Wesley Longman, Ltd., England.

Fares, M. A. and K. H. Wolfe. 2003. Positive selection and subfunctionalization of duplicated CCT chaperonin subunits. *Mol Biol Evol* **20**:1588–1597.

Fay, J. C. and C. I. Wu. 2001. The neutral theory in the genomic era. *Curr Opin Genet Dev* **11**:642–646.

Fay, J. C. and C. I. Wu. 2003. Sequence divergence, functional constraint, and selection in protein evolution. *Annu Rev Genomics Hum Genet* **4**:213–235.

Fay, J. C., G. J. Wyckoff, and C. I. Wu. 2001. Positive and negative selection on the human genome. *Genetics* **158**:1227–1234.

Frisch, A. et al. 2004. Origin and spread of the 1278insTATC mutation causing Tay-Sachs disease in Ashkenazi Jews: genetic drift as a robust and parsimonious hypothesis. *Hum Genet* **114**:366–376.

Giannelli, F., T. Anagnostopoulos, and P. M. Green. 1999. Mutation rates in humans. II. Sporadic mutation-specific rates and rate of detrimental human mutations inferred from hemophilia B. *Am J Hum Genet* **65**:1580–1587.

Gillespie, J. H. 1998. *Population Genetics: A Concise Guide*. Johns Hopkins University Press, Baltimore, MD.

Green, P. M. et al. 1999. Mutation rates in humans. I. Overall and sex-specific rates obtained from a population study of hemophilia B. *Am J Hum Genet* **65**:1572–1579.

Greer, W. L. et al. 1999. Linkage disequilibrium mapping of the Nova Scotia variant of Niemann-Pick disease. *Clin Genet* **55**: 248–255.

Haldane, J. B. S. 1964. A defense of beanbag genetics. *Perspect Biol Med* **7**:343–359.

Hamblin, M. T., E. E. Thompson, and A. Di Rienzo. 2002. Complex signatures of natural selection at the Duffy blood group locus. *Am J Hum Genet* **70**:369–383.

Hammer, M. F. and S. L. Zegura. 2002. The human Y chromosome haplogroup tree: Nomenclature and phylogeography of its major divisions. *Annu Rev Anthropol* **31**:303–321.

Hardy, G. H. 1908. Mendelian proportions in a mixed population. *Science* **28**:49–50.

Hartl, D. L. and A. G. Clark. 1997. *Principles of Population Genetics*. 3rd ed. Sinauer Associates, Sunderland, MA.

Helgason, A. et al. 2000. Estimating Scandinavian and Gaelic ancestry in the male settlers of Iceland. *Am J Hum Genet* **67**:697–717.

Heyer, E. 1999. One founder/one gene hypothesis in a new expanding population: Saguenay (Quebec, Canada). *Hum Biol* **71**:99–109.

Huttley, G. A. et al. 2000. Adaptive evolution of the tumour suppressor *BRCA1* in humans and chimpanzees. Australian Breast Cancer Family Study. *Nat Genet* **25**:410–413.

Jobling, M. A. and C. Tyler-Smith. 2000. New uses for new haplotypes the human Y chromosome, disease and selection. *Trends Genet* **16**:356–362.

Johnson, M. E. et al. 2001. Positive selection of a gene family during the emergence of humans and African apes. *Nature* **413**: 514–519.

Jorde, L. 1992. Genetic diseases in the Ashkenazi population: Evolutionary considerations. In: Bonné-Tamir, B. and A. Adam. (eds.) *Genetic Diversity Among Jews: Diseases and Markers at the DNA Level*. Oxford University Press, New York, pp. 305–318.

Kreitman, M. 2000. Methods to detect selection in populations with applications to the human. *Annu Rev Genomics Hum Genet* **1**:539–559.

Kruglyak, L. 1999. Genetic isolates: separate but equal? *Proc Natl Acad Sci USA* **96**:1170–1172.

Kwitek-Black, A. E. et al. 1993. Linkage of Bardet-Biedl syndrome to chromosome 16q and evidence for non-allelic genetic heterogeneity. *Nat Genet* **5**:392–396.

Labuda, D., E. Zietkiewicz, and M. Labuda. 1997. The genetic clock and the age of the founder effect in growing populations: a lesson from French Canadians and Ashkenazim. *Am J Hum Genet* **61**: 768–771.

Labuda, M. et al. 1996. Linkage disequilibrium analysis in young populations: pseudo-vitamin D-deficiency rickets and the founder effect in French Canadians. *Am J Hum Genet* **59**:633–643.

Leppert, M. et al. 1994. Bardet-Biedl syndrome is linked to DNA markers on chromosome 11q and is genetically heterogeneous. *Nat Genet* **7**:108–112.

Li, C. C. 1967. Castle's early work on selection and equilibrium. *Am J Hum Genet* **19**:70–74.

Li, W.-H. 1997. *Molecular Evolution*. Sinauer Associates, Sunderland, MA.

Long, J. C. and R. A. Kittles. 2003. Human genetic diversity and the nonexistence of biological races. *Hum Biol* **75**: 449–471.

Luis, J. R. et al. 2004. The Levant versus the Horn of Africa: Evidence for bidirectional corridors of human migrations. *Am J Hum Genet* **74**:532–544.

Macaulay, V. et al. 1999. The emerging tree of West Eurasian mtDNAs: a synthesis of control-region sequences and RFLPs. *Am J Hum Genet* **64**:232–249.

Motulsky, A. G. 1995. Jewish diseases and origins. *Nat Genet* **9**:99–101.

Mundy, N. I. and S. Cook. 2003. Positive selection during the diversification of class I vomeronasal receptor-like (V1RL) genes, putative pheromone receptor genes, in human and primate evolution. *Mol Biol Evol* **20**:1805–1810.

Neel, J. V. 1978. The population structure of an Amerindian tribe, the Yanomama. *Annu Rev Genet* **12**:365–413.

Neel, J. V. 1994. *Physician to the Gene Pool: Genetic Lessons and Other Stories*. John Wiley and Sons, New York.

Niell, B. L. et al. 2003. Genetic anthropology of the colorectal cancer-susceptibility allele APC I1307K: evidence of genetic drift within the Ashkenazim. *Am J Hum Genet* **73**:1250–1260.

Ober, C., T. Hyslop, and W. W. Hauck. 1999. Inbreeding effects on fertility in humans: evidence for reproductive compensation. *Am J Hum Genet* **64**:225–231.

Oddoux, C. et al. 1999. Mendelian diseases among Roman Jews: implications for the origins of disease alleles. *J Clin Endocrinol Metab* **84**:4405–4409.

Ohta, T. and J. H. Gillespie. 1996. Development of neutral and nearly neutral theories. *Theor Popul Biol* **49**:128–142.

Parra, E. J. et al. 1998. Estimating African American admixture proportions by use of population-specific alleles. *Am J Hum Genet* **63**:1839–1851.

Peltonen, L., A. Jalanko, and T. Varilo. 1999. Molecular genetics of the Finnish disease heritage. *Hum Mol Genet* **8**:1913–1923.

Ptak, S. E. and M. Przeworski. 2002. Evidence for population growth in humans is confounded by fine-scale population structure. *Trends Genet* **18**:559–563.

Richards, M. et al. 2000. Tracing European founder lineages in the Near Eastern mtDNA pool. *Am J Hum Genet* **67**:1251–1276.

Richards, M. et al. 2002. In search of geographical patterns in European mitochondrial DNA. *Am J Hum Genet* **71**: 1168–1174.

Risch, N. et al. 1995. Genetic analysis of idiopathic torsion dystonia in Ashkenazi Jews and their recent descent from a small founder population. *Nat Genet* **9**:152–159.

Risch, N. 2001. Molecular epidemiology of Tay-Sachs disease. In: Desnick, R. J. and M. M. Kaback. (eds) *Tay-Sachs Disease*. Academic Press, San Diego, CA, pp. 233–252.

Risch, N. and H. Tang. 2003. Selection in the Ashkenazi Jewish population unlikely—Reply to Zlotogora and Bach. *Am J Hum Genet* **73**:440–441.

Risch, N. et al. 2003. Geographic distribution of disease mutations in the Ashkenazi Jewish population supports genetic drift over selection. *Am J Hum Genet* **72**: 812–822.

Rockman, M. V. et al. 2003. Positive selection on a human-specific transcription factor binding site regulating *IL4* expression. *Curr Biol* **13**:2118–2123.

Rosenberg, N. A. and M. Nordborg. 2002. Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. *Nat Genet* **3**:380–390.

Scriver, C. R. 2001. Human genetics: lessons from Quebec populations. *Annu Rev Genomics Hum Genet* **2**:69–101.

Semino, O. et al. 2000. mtDNA and Y chromosome polymorphisms in Hungary: inferences from the palaeolithic, neolithic and Uralic influences on the modern Hungarian gene pool. *Eur J Hum Genet* **8**:339–346.

Sheffield, V. C. et al. 1994. Identification of a Bardet-Biedl syndrome locus on chromosome 3 and evaluation of an efficient approach to homozygosity mapping. *Hum Mol Genet* **3**:1331–1335.

Shi, P. et al. 2003. Adaptive diversification of bitter taste receptor genes in mammalian evolution. *Mol Biol Evol* **20**:805–814.

Snyder, L. H. 1932. Studies in human inheritance. IX. The inheritance of taste deficiency in man. *Ohio J Sci* **32**:436–440.

Thomas, M. G. et al. 1998. Origins of Old Testament priests. *Nature* **394**:138–140.

Thomas, M. G. et al. 2000. Y chromosomes traveling south: the cohen modal haplotype and the origins of the Lemba—the "Black Jews of Southern Africa". *Am J Hum Genet* **66**:674–686.

Torroni, A. et al. 1998. mtDNA analysis reveals a major late Paleolithic population expansion from southwestern to northeastern Europe. *Am J Hum Genet* **62**: 1137–1152.

Weinberg, W. 1908. On the demonstration of heredity in man. In: Boyer, S. H. (ed) *Papers on Human Genetics*. Prentice-Hall, Englewood Cliffs, NJ, pp. 4–15.

Wolfe, K. H. and W. H. Li. 2003. Molecular evolution meets the genomics revolution. *Nat Genet* **33** Suppl:255–265.

Wyckoff, G. J., W. Wang, and C. I. Wu. 2000. Rapid evolution of male reproductive genes in the descent of man. *Nature* **403**:304–309.

Yang, Z. and J. P. Bielawski. 2000. Statistical methods for detecting molecular adaptation. *Trends Ecol Evol* **15**:496–503.

Yang, Z. and R. Nielsen. 2000. Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. *Mol Biol Evol* **17**:32–43.

Young, T. L. et al. 1999. A fifth locus for Bardet-Biedl syndrome maps to chromosome 2q31. *Am J Hum Genet* **64**:900–904.

Young, T. L. et al. 1999. A founder effect in the Newfoundland population reduces the Bardet-Biedl Syndrome I (*BBS1*) interval to 1 cM. *Am J Hum Genet* **65**: 1680–1687.

Zhang, J. 2003. Evolution of the human *ASPM* gene, a major determinant of brain size. *Genetics* **165**:2063–2070.

Zlotogora, J. and G. Bach. 2003. The possibility of a selection process in the Ashkenazi Jewish population. *Am J Hum Genet* **73**:438–440.

review questions

1. What are the basic conditions that are required for Hardy–Weinberg equilibrium (HWE)?

2. Based on the frequency of the *A1A1* genotype in the following HWE populations, what are the frequencies of the *A1* and *A2* alleles?

Population	Frequency of *A1A1*
1	0.36
2	0.16
3	0.04
4	0.09
5	0.49

3. Based on the genotypes of sampled individuals from various populations, what are the allele frequencies in each population?

Population	*A1A1*	*A1A2*	*A2A2*
1	100	500	400
2	200	500	300
3	300	400	300
4	200	600	200
5	600	300	100

4. Based on the frequency of the *A1* allele in each of the following HWE populations, what are the frequencies of the genotypes?

Population	Frequency of *A1*
1	0.40
2	0.60
3	0.25
4	0.01
5	0.99

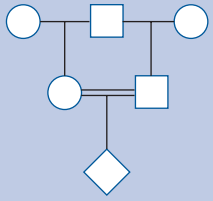
5. Which autosomal loci in the following populations are in HWE? Which are not? Of the loci that are in HWE, what are the allele and genotype frequencies? Of the loci that are not in HWE, what are the allele and genotype frequencies after HWE is established?

Population	Frequency of *A2*	Frequency of *A2A2*
1	0.35	0.1225
2	0.45	0.3225
3	0.65	0.4225
4	0.75	0.2225
5	0.85	0.7225

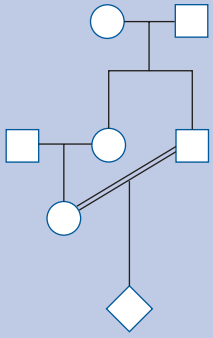
6. What is the frequency of an X-linked allele in females, males and the overall population in each of the first five generations after the establishment of HWE conditions when the original frequency of the allele in females and males was 0.65 and 0.40, respectively?

7. What is the inbreeding coefficient (F) of an autosomal locus for each of the following consanguineous marriages?

A



B



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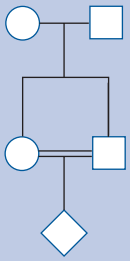
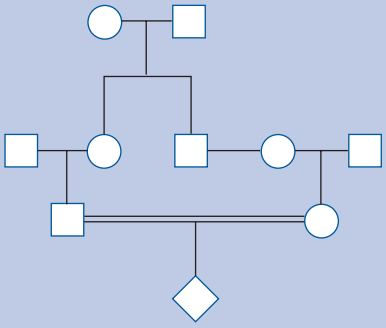


D



8. What is the inbreeding coefficient (F) of an X-linked locus for each of the consanguineous marriages in Question 7?

9. **A.** What are the expected frequencies of gametes with linkage equilibrium when the frequencies of the *A1*, *A2*, *B1*, and *B2* alleles are 0.8, 0.2, 0.4, and 0.6, respectively? Show that in this case $D = 0$. **B.** If the frequencies of gametes *A1B1*, *A1B2*, *A2B1*, and *A2B2* are 0.4, 0.1, 0.1, and 0.4, respectively, what is the value of D?

10. What is the genetic admixture (m) if the frequency of an allele (q) is 0.1 in the original population, 0.3 in the combined population, and 0.6 in the contributing population? Show that m is the same if p is used instead of q.

11. With complete dominance and $q = 0.1$, what is the proportion of homozygous recessive offspring (*A2A2*) from matings between individuals who have only dominant phenotypes?

12. If the forward mutation rate is 10^{-6}; back mutation is negligible, and the initial frequency of the dominant gene is 1.0, what is the frequency of the dominant allele (*A1*) after 10, 50, 100, and 1000 generations?

13. If the ratio of forward to back mutation is 8 : 1, what are the frequencies of the *A1* and *A2* alleles at equilibrium?

14. What is the frequency of the completely dominant allele (*A1*) after one generation when the initial frequency of the *A2* allele and the reproductive fitness of the homozygous recessive genotype (*A2A2*) are 0.50 and 0.90, respectively?

15. What is the change in frequency of the *A1* allele in one generation with semidominance?

16. With complete dominance of the *A1* allele and a lethal homozygous recessive condition, what are the changes in the frequencies of the *A1* and *A2* alleles after one generation if the initial frequency of the *A1* allele was 0.80?

17. When the heterozygous effect for a two-allele, one-locus system is –0.50, what is the frequency of the *A1* allele at equilibrium?

18. If both homozygotes (*A1A1*, *A2A2*) are 99% as reproductively fit as the heterozygous genotype, what is the frequency of the *A1* allele at equilibrium?

19. In a Hardy–Weinberg population, if a homozygous recessive condition causes the childhood death of 1 in 20 offspring and the rate of forward mutation is 10^{-6}, what is the frequency of the recessive allele (*A2*)?

20. For an incomplete dominant condition, if the reproductive fitnesses of the *A1A1*, *A1A2*, and *A2A2* genotypes are 1, 0.8, and 0.7, respectively, and the rate of forward mutation is 10^{-6}, what is the frequency of the *A2* allele (q) at equilibrium?

21. What is the population-mutation parameter?

22. What is nucleotide diversity? What is the nucleotide diversity for the following sequences?

Seq1	A	T	G	C	T	A	A	G	C	A
Seq2	A	T	C	C	T	A	A	G	G	T
Seq3	A	T	G	G	T	A	A	G	C	A
Seq4	A	T	G	G	T	A	A	G	C	A
Seq5	A	A	G	C	T	A	A	G	C	A

continued

23. Outline the main features of Tajima's *D* test.

24. What is K_a/K_s? What is the significance of $K_a/K_s > 1$? $K_a/K_s < 1$? $K_a/K_s = 1$?

25. The following information—23 fixed, nonsynonymous, 21 fixed, synonymous, 5 polymorphic, nonsynonymous, and 29 polymorphic, synonymous sites—was observed with sequences of the coding region of a gene from two related species. Test whether these data conform to the neutral theory. Discuss the meaning of the result. (There are interactive online sites for some statistical tests, e.g., http://www.graphpad.com/quickcalcs/contingency1.cfm and http://faculty.vassar.edu/lowry/fisher.html.)

chapter 10

Molecular Genetics of Complex Disorders

There is no limit to how complicated things can get, on account of one thing always leading to another.

E. B. WHITE (1899–1985)

Most often complexity seems to have been thought of as associated with the presence of large numbers of components with different types of behavior, and typically also with the presence of extensive interconnections and interdependencies.

STEPHEN WOLFRAM (b. 1959)

This is an extremely intricate subject.

CHARLES DARWIN (1809–1882)

ORGANISMS, ESPECIALLY HUMANS, are complex entities with sets of genes that contribute to a myriad of biochemical networks and structural arrays. How all these functions are coordinated in time and space to produce a distinctive, viable individual is a marvel. In some instances, as exemplified by Mendelian inheritance, a single locus is responsible for a discrete phenotype. To date, about 1200 genes that produce major phenotypic effects have been mapped and cloned. Interestingly, the "one locus-one phenotype" relationship is not the rule for most monogenic conditions. In fact, a number of these traits are not as simple as initially imagined. For example, affected family members with the same mutation may have very different phenotypes. Moreover, some traits arise from the combined action of a few genes (2 to ~15; oligogenic inheritance) and numerous others are the consequence of many genes (>15; multigenic inheritance, polygenic inheritance) acting together, with each gene having a small effect. A polygenic trait often results from both genetic and environmental (nongenetic) inputs. Not only do various combinations of alleles produce a range of phenotypes, but interactions at a locus (dominance) and between loci (epistasis), environmental influences, and gene-environmental interactions also contribute to the final phenotype. A conceptual snapshot of this type of phenotype determination, which is called multifactorial inheritance, is illustrated in Figure 10.1.

About 75% of inherited human disorders at birth and the vast majority of those that arise later in life are multifactorial. Some examples of multifactorial

An Introduction to Human Molecular Genetics, Second Edition, by Jack J. Pasternak
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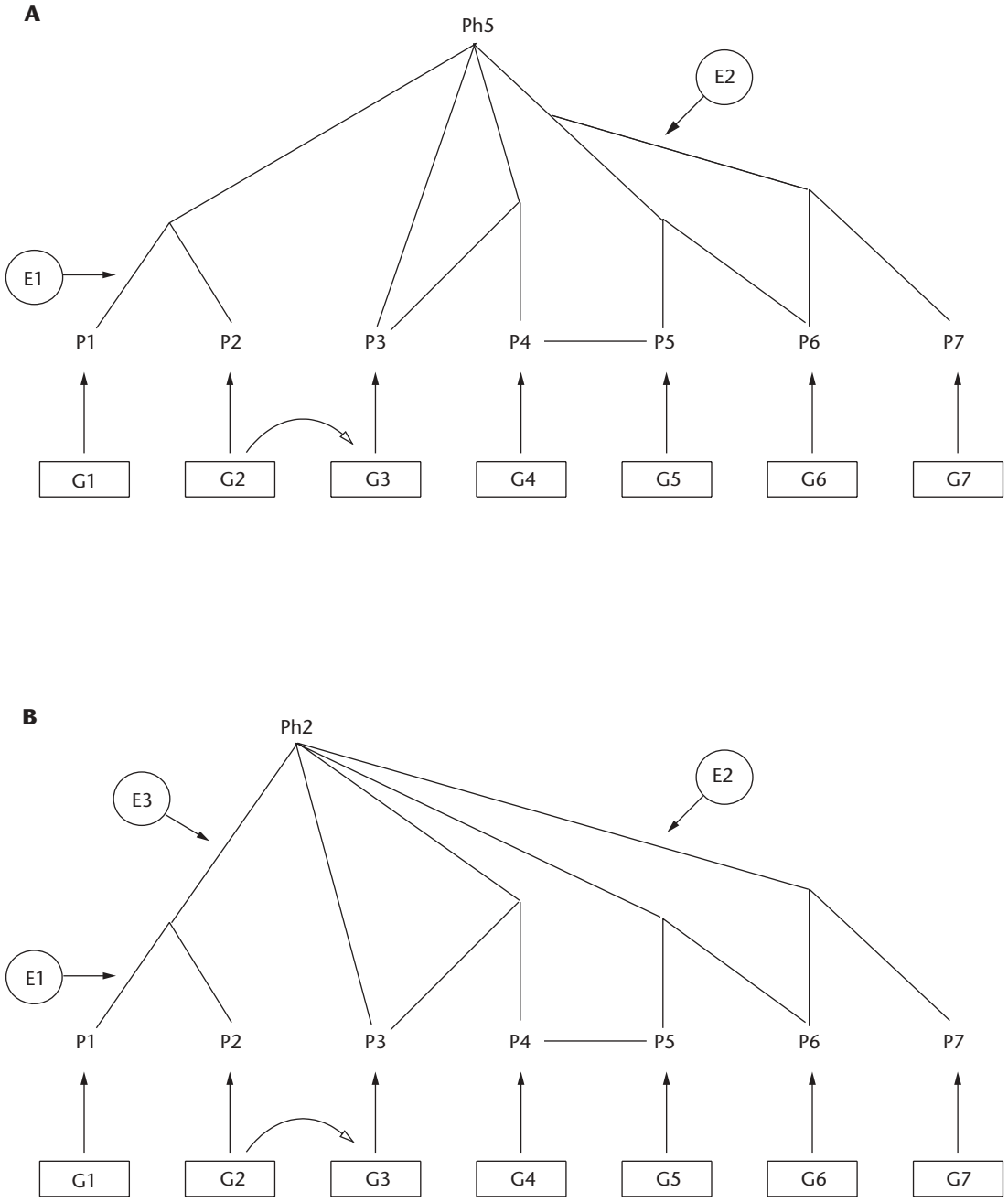


Figure 10.1 Schematic representation showing how phenotypes of a multifactorial trait are determined. (A) The primary products (P1–P7) of seven normal genes (G1–G7) form various protein-protein assemblies and with environmental factors (E1, E2) produce the Ph5 phenotype. The open arrow indicates interaction (epistasis) between genes 2 and 3. (B) The phenotypic outcome (Ph2) is due to the additional involvement of environmental factor E3. (C) A mutation in gene 5 (G5′) alters properties of the primary protein (P5′), which, in turn, leads to the Ph7 phenotype. (D) A mutation in gene 4 (G4*) significantly alters the primary protein (P5*), prevents the formation of a functional complex with P5, and results in the Ph0 phenotype.

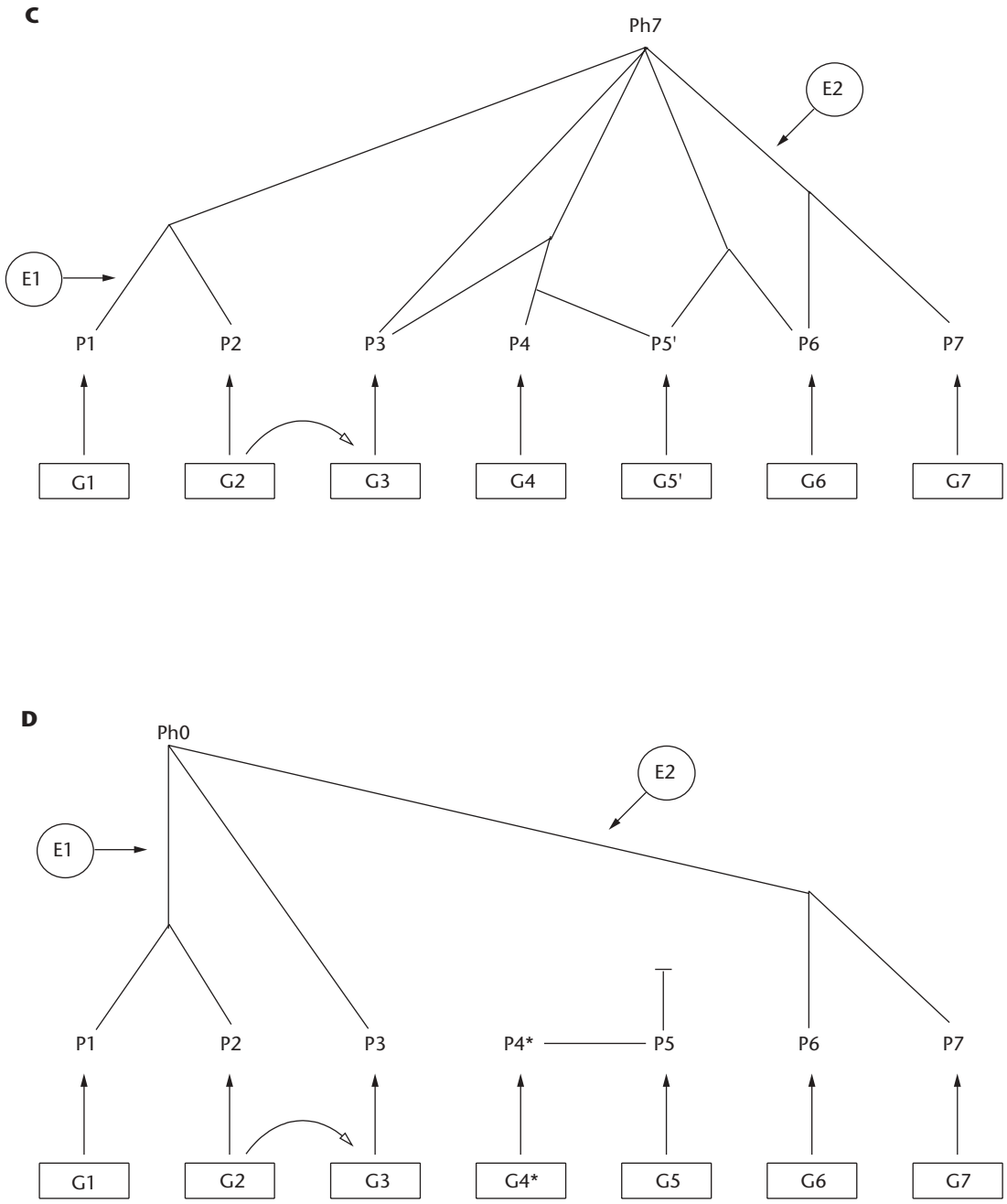


Figure 10.1 *continued*

(complex) human disorders include autism, asthma, diabetes, obesity, alcoholism, hypertension, and schizophrenia. Generally, there are three categories of complex disorders. Some have two states, namely, affected and nonaffected (dichotomous trait, binary trait), others consist of a large number of separate classes with integral (metric) values (meristic trait), and many are continuous based on a measurable feature of the phenotype. A symmetrical, bell-shaped curve that often approximates a normal distribution is observed when large numbers of individuals with either a meristic or a continuous trait are scored

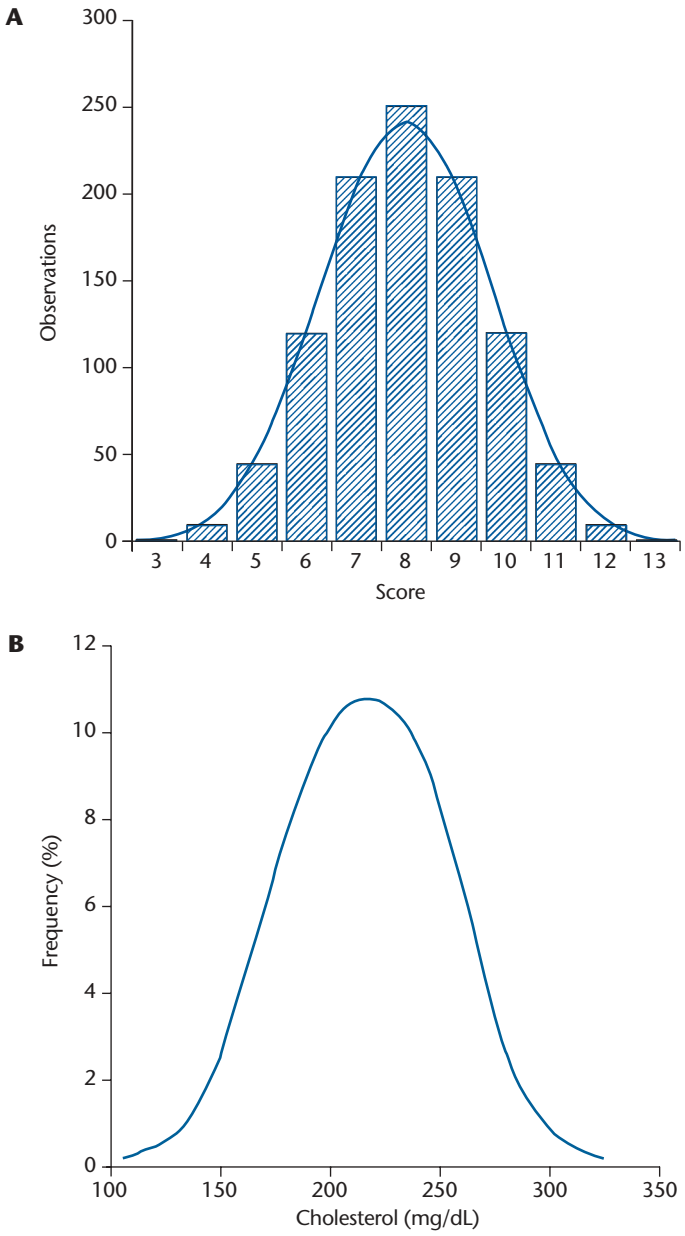


Figure 10.2 Polygenic traits. (A) Meristic trait. The histogram (hatched bars) represents the frequencies of the discrete scores. The solid line indicates the best-fit curve. (B) Continuous trait. Frequency distribution of serum cholesterol concentrations from a sample of >350,000 males. (Modified from Figure 1 in Martin et al. 1986. *Lancet* **2**:933–936.)

(Figures 10.2A and B). It is assumed that individuals with multifactorial dichotomous traits are also normally distributed. The conventional explanation for a multifactorial two-state phenotype is that for some traits affected individuals fail to reach a biochemical or physiological level (threshold) that guarantees a normal phenotype (Figure 10.3A) and with other traits exceeding a threshold results in an abnormal phenotype (Figure 10.3B). Many complex disorders are defined by a unit of measurement, hence they have been designated quantitative traits. A gene that contributes to such a phenotype is called a quantitative trait locus (QTL). QTLs (quantitative trait loci) are the ensemble of genes that determine a polygenic trait.

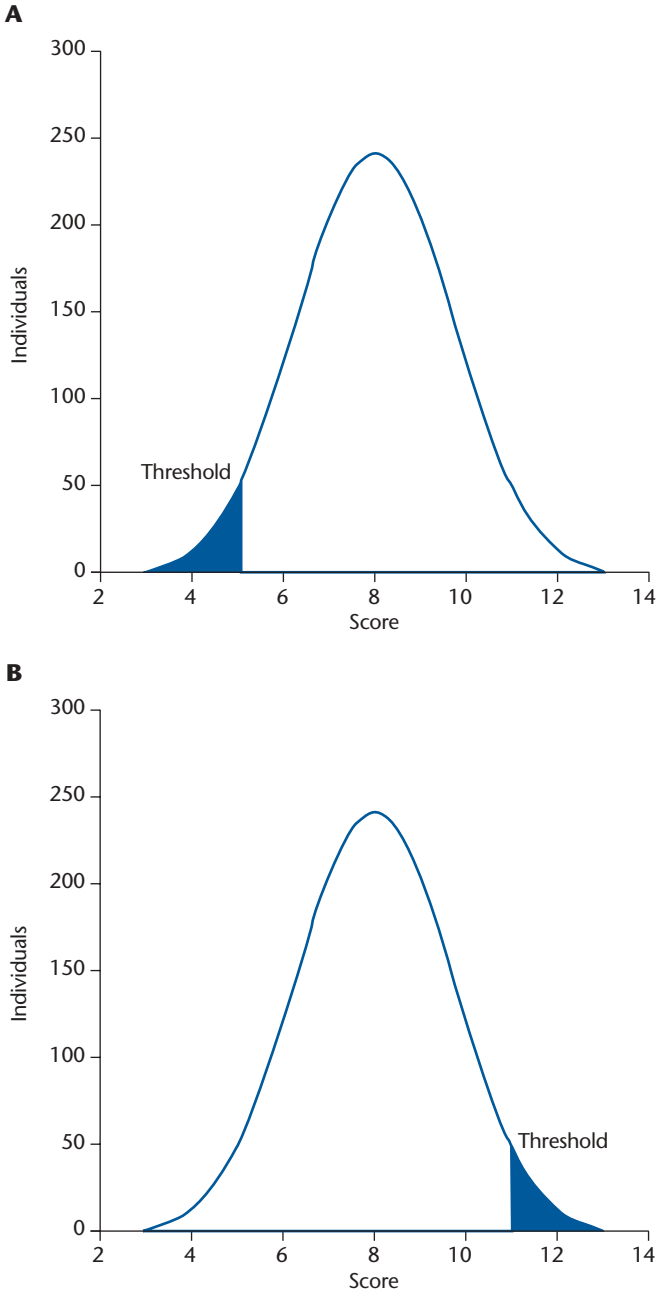


Figure 10.3 Model for dichotomous polygenic traits. (A) Affected individuals (blue) do not exceed a required biochemical or physiological threshold level. (B) Affected individuals (blue) exceed a biochemical or physiological threshold level.

Multifactorial traits are difficult to study genetically because there is no definite pattern of inheritance within multigeneration families. However, understanding how multifactorial phenotypes are determined has become feasible with the availability of high-density polymorphic DNA marker systems and the ability to genotype large numbers of individuals relatively easily and inexpensively. Various strategies and methods have been devised for analyzing the genetic basis of complex disorders, and searches are under way for the QTLs that contribute to these traits.

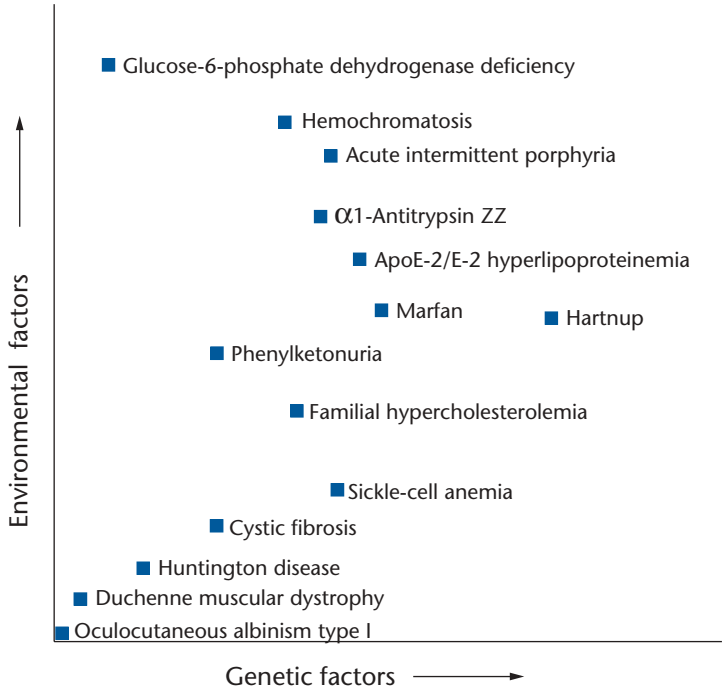


Figure 10.4 Relative contribution of environmental and genetic factors to the phenotype of some monogenic disorders.

Phenotypic Variation of Monogenic Disorders

For decades, geneticists have known that individuals with the same genotype at a particular locus can have diverse phenotypes. Terms such as incomplete penetrance and variable expressivity were created to describe these circumstances. These phenomena were thought to be the consequences of environmental agents and (or) modifier genes, although the precise impact of these effects on a particular phenotype was obscure. However, with the ease of DNA sequencing of specific genes and the increased number of molecular studies, there is a better understanding of genotype-phenotype relationships and a greater appreciation of the roles that both environmental and genetic factors play in determining phenotypic outcomes of single-gene disorders (Figure 10.4). By way of illustration, three examples from Figure 10.4 are considered here.

Oculocutaneous Albinism Type 1

Oculocutaneous albinism type 1 (OCA1) is one of the few disorders that results almost exclusively from mutations of a single gene. Albinism is a genetically heterogeneous condition, with at least five different genes independently leading to reduced pigmentation (hypopigmentation) of the hair, skin, and eyes. OCA1 is an autosomal recessive condition that occurs with a frequency of 1 in 40,000 in most populations. In addition to decreased levels of melanin in the hair, skin, and eyes, there is a loss of visual acuity, avoidance of light (photophobia), jerking of the eyes (nystagmus), misrouting of the optic nerves that produces crossed eyes (strabismus), and impaired stereoscopic vision. There are two subtypes of OCA1: OCA1A and OCA1B. The difference between types A and B lies predominantly in the extent of pigmentation during

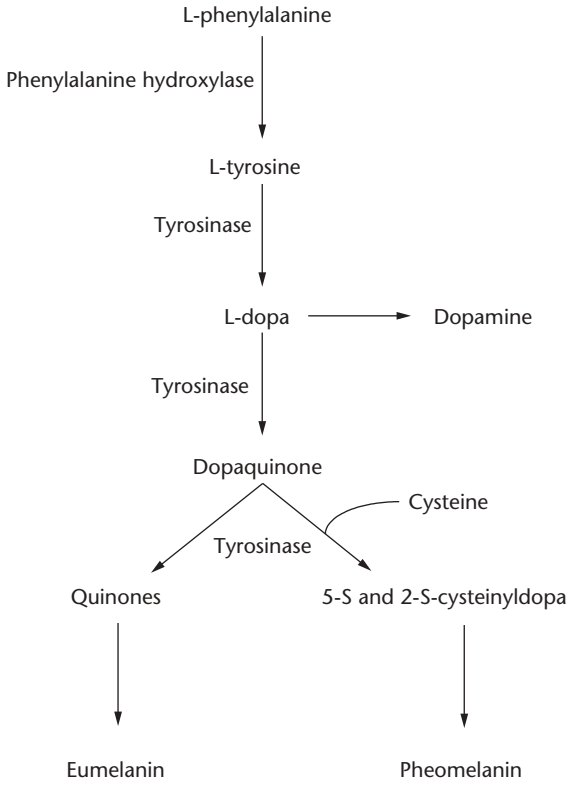


Figure 10.5 Biosynthesis of melanins.

the lifetime of an affected individual. With OCA1A, albinism is lifelong, whereas with OCA1B, the hair, skin, and eyes darken with age.

Mutations of the tyrosinase (*TYR*) gene at 11q14–11q21 are responsible for OCA1. Most of the identified *TYR* mutations are missense and produce an inactive product, that is, *TYR*-null mutations, in OCA1A individuals. *TYR* mutations with reduced activity, that is, *TYR* hypomorphic mutations, are associated with the OCA1B phenotype. Tyrosinase is an unusual enzyme because it catalyzes three separate successive steps in the pathway that precedes the synthesis of eumelanin (black pigment) and pheomelanin (red pigment) (Figure 10.5). Consequently, when a *TYR* mutation is homozygous, there is a triple impact on the pigment production pathway and the precursors for either eumelanin or pheomelanin do not accumulate to any significant extent. The strict genotype-phenotype relationship with OCA1 is the exception among monogenic disorders. In contrast to OCA1, the phenotype associated with glucose-6-phosphate dehydrogenase (G6PD) occurs almost exclusively in response to environmental factors.

Glucose-6-Phosphate Dehydrogenase Deficiency

G6PD deficiency (favism) is an X-linked recessive trait that affects about 400 million people worldwide. The enzyme G6PD catalyzes the first step in the pentose phosphate pathway. Pentose sugars are integral components of many molecules such as ATP, CoA, NAD, FAD, RNA, and DNA. As well, the G6PD reaction generates reduced nicotinamide adenine dinucleotide phosphate (NADPH) that maintains the reduced state of both glutathione (GSH)

and some protein amino acids. Reduced glutathione is an important agent that protects cells from oxidative (oxidant) stress. Unlike other cells, the sole source of NADPH in red blood cells is G6PD. Thus, when stressed, the red blood cells of G6PD-deficient individuals are not protected and, subsequently, they disintegrate. The destruction of red blood cells (hemolysis) leads to anemia.

The two major clinical features of G6PD deficiency are prolonged neonatal jaundice and hemolytic anemia. Neonatal jaundice is treated with special lights (bile lights). Nasal oxygen and bed rest alleviate hemolytic anemia. If red blood cell loss is severe, blood transfusions are given. Most postnatal G6PD-deficient individuals are free of hemolytic anemia unless they experience an infection, ingest certain oxidative compounds, or eat broad (fava) beans. Infection is the most common trigger of hemolysis, with infectious hepatitis, pneumonia, and typhoid fever eliciting the severest responses. The susceptibility to drugs was initially discovered when individuals were given primaquine to treat malaria and those who were G6PD-deficient, as ensuing studies established, had severe hemolytic episodes. Later, other drugs were discovered that induced hemolytic anemia in G6PD-deficient individuals (Table 10.1). The broad bean constituents divicine, convicine, and isouramil act as initiators of hemolysis. Once G6PD deficiency is diagnosed, affected individuals avoid broad beans and the medications that induce hemolytic anemia. To date, no significant benefits have been observed in G6PD-deficient individuals who were given dietary antioxidants.

Geographically, G6PD deficiency occurs in tropical and subtropical regions. Its prevalence correlates with areas that formerly and currently have high rates of malaria. Mutant alleles of the *G6PD* gene have increased in frequency over time in malaria-infested regions because the oxidative stress response after infection of red blood cells with the malaria parasite leads to hemolysis, thereby preventing the development of full-blown malaria. In other words, there has been an evolutionary trade-off. Individuals with G6PD deficiency are more likely to survive malaria infection than those with a wild-type *G6PD* gene. As a result, an increased number of mutant *G6PD* genes, despite the biological burden they impose, are passed on to the next generation. Because of this selec-

Table 10.1 Medications that trigger acute hemolysis in individuals with G6PD deficiency.

Analgesics/Antipyretics
acetanilid, acetophenetidin (phenacetin), probenicid, pyramidone
Antimalarials
hydroxychloroquine, mepacrine (quinacrine), pamaquine, pentaquine, primaquine, quinocide
Cytotoxic/Antibacterial
chloramphenicol, co-trimoxazole, furazolidone, furmethonol, nalidixic acid, neoarsphenamine, nitrofurantoin, nitrofurazone, *ρ*-aminosalicylic acid
Sulfonamides/Sulfones
dapsone, sulfacetamide, sulfamethoxypyrimidine, sulfanilamide, sulfapyridine, sulfasalazine, sulfisoxazole
Miscellaneous
α-methyl-DOPA, dimercaprol (BAL), hydralazine, mestranol, methylene blue, nalidixic acid, naphthalene, niridazole, phenylhydrazine, toluidine blue, trinitrotoluene, urate oxidase, pyridium

tive advantage, mutant *G6PD* alleles accumulate in populations living in areas with endemic malaria. Most of the *G6PD* gene variants are single missense mutations that occur in different exons. Mutations for a severe form of *G6PD* deficiency called chronic nonspherocytic hemolytic anemia (CNSHA) tend to be clustered in exon 10, which is involved in G6PD dimer formation. In sum, G6PD deficiency is an interesting example of a monogenic trait that depends principally on environmental factors.

Cystic Fibrosis

Cystic fibrosis (CF) is the most common life-limiting autosomal recessive disorder among individuals of Northern European ancestry. It occurs with a frequency of 1 in 3200 live births in these populations. The CF phenotype is diverse, with different organ systems being affected to varying degrees. The vast majority of those with CF suffer pancreatic insufficiency (PI) due to plugged ducts of the secretary glands (exocrine glands) of the pancreas. This blockage prevents secretion of digestive enzymes into the duodenum. In a few cases, pancreatic ductless secretory glands (endocrine glands) are also impaired. In addition to the pancreatic ducts, passageways of the lungs, liver, male reproductive tract, and intestine are often blocked. Of these, lung damage as a result of mucus clogging and bacterial infection of the airways is the main cause of ongoing distress and eventually death by about 30 years of age. In about 15% to 20% of CF individuals, blockage of the intestine (meconium ileus) is a serious problem, both at birth and later in life. The primary chemical feature of CF for diagnostic purposes is an increased chloride concentration in sweat.

The genetic basis of CF was established when a cloned gene, later designated as the cystic fibrosis transmembrane conductance regulator (CFTR) gene, was found with the same mutation in a number of CF individuals. This mutation is a 3-base pair deletion of the phenylalanine codon (F508del; ΔF508) that codes for the amino acid at position 508 of the CFTR protein. The ΔF508 mutation is the most common of the more than 1000 different *CFTR* gene mutations that have been identified. Depending on the population, the frequency of the ΔF508 mutation ranges from 30% to 80%.

The CFTR protein is a multidomain, multifunctional chloride ion channel of epithelial cells (Figure 10.6) that not only regulates intracellular chloride ion concentration by conducting chloride ions out of the cell, but regulates other channel and transporter proteins. As well, CFTR is subjected to regulation by a number of proteins.

CFTR gene mutations that affect production, different domains, and various functions of CFTR have been organized into six classes. Briefly, class I (production) mutations cause protein truncation, and no active CFTR is produced. Class II (processing) mutations are nontruncating and produce less than normal CFTR activity. Class III (channel gating) mutations affect the nucleotide binding domains (NBD1, NBD2) and decrease chloride transport. The NBD and R domains play essential roles in opening and closing the CFTR channel. Class IV (conductance) mutations alter amino acids of the transmembrane domains that make up the pore of the channel. Class C (down-regulation) mutations decrease the amount of functional CFTR that is

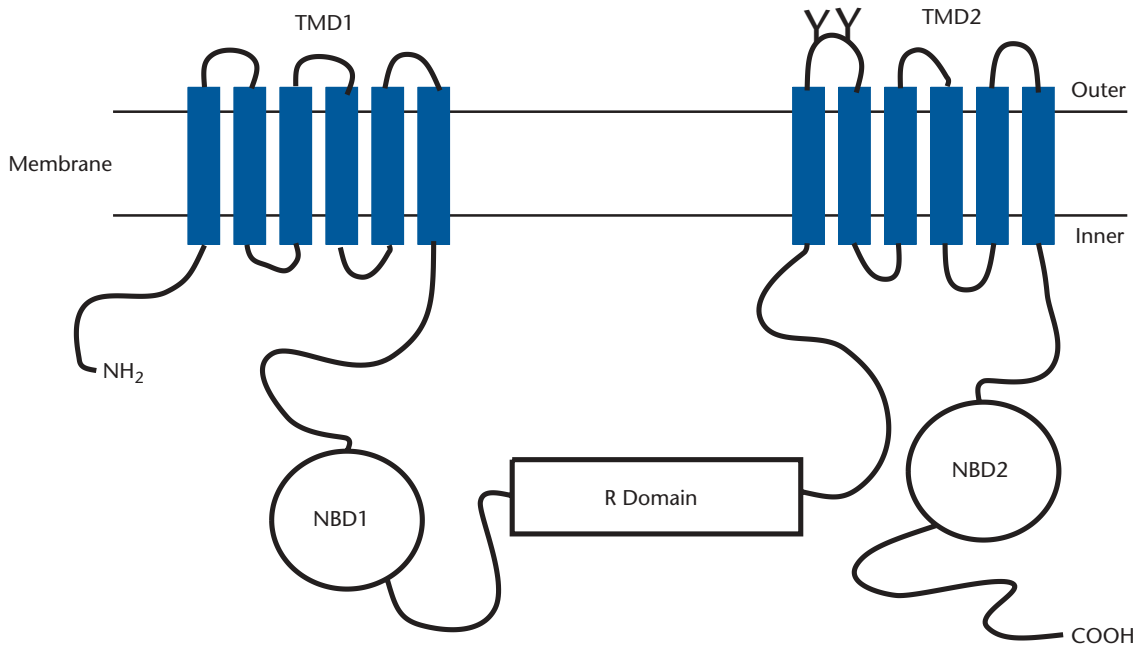


Figure 10.6 Schematic representation of CFTR chloride channel. R domain, regulatory domain; NBD1 and NBD2, nucleotide domains 1 and 2; TMD1 and TMD2, transmembrane domains 1 and 2. The Y-shaped structures denote glycosylation sites. The inner and outer membrane surfaces are noted.

produced. Class VI (regulatory) mutations affect the regulatory functions of CFTR. Generally, classes I, II, and III account for >85% of the cases of CF. The homozygous ΔF508 genotype usually produces a more severe phenotype than any of the other *CFTR* gene mutations. However, among ΔF508 homozygotes, the extent of lung dysfunction is variable, ranging from mild to severe. The lack of a significant correlation between *CFTR* genotypes and CF phenotypes suggests that other genes (modifier genes) and (or) environmental factors determine the phenotypic outcome.

The search for modifier genes often entails conducting candidate gene-association studies and (or) allele-sharing linkage analyses with either affected sib pairs (ASPs) or sets of close relatives. (These methods are described in detail later in this chapter.) Usually, a candidate gene for a CF association study has at least one highly polymorphic site that affects the amount of gene product that is synthesized. Alleles that prevent the synthesis of any functional protein, that is, null mutations, are preferred for these studies. Briefly, samples of both CF individuals and carefully matched non-CF control subjects are genotyped for the polymorphic alleles of the candidate gene. The CF individuals are also genotyped for *CFTR* mutations. In most instances, the majority of the CF sample is homozygous for the ΔF508 mutation. If the frequencies of the candidate gene alleles are the same in the CF and control groups, then there is no association. Alternatively, if the CF group has a significant preponderance of a particular candidate gene allele, then an association is established. In a similar manner, an association of a candidate gene allele with a severe or mild form of CF can be tested, except, in this case, only CF individuals with different phenotypes are screened. A number of alleles of various genes have been associated with severity of CF pulmonary disease (Table 10.2). Further studies are required to determine how a specific allele affects the CF phenotype because

Table 10.2 Cystic fibrosis modifier genes.

Protein	Function	CF-associated allele	Amount of modifier gene product	Pulmonary disorder
Mannose-binding lectin	Phagocytosis of bacteria	*MBL2-0* (null allele)	None	Severe
Glutathione-*S*-transferase	Antioxidant; detoxification of oxidative stress	*GSTM1-0* (null allele)	None	Severe
Transforming growth factor-β	Modulates fibroblast proliferation and collagen production in airway epithelium	1. Low-producing *TGFB1* alleles 2. High-producing *TGFB1* alleles	1. Low 2. High	1. Decreased severity 2. Increased severity
Tumor necrosis factor-α	Proinflammatory cytokine	Increased production *TNF2* allele	High	Increased severity
β-2 adrenergic receptor	Stimulation of adenylyl cyclase activity	Downregulating *ADRB2* allele	Low	Decreased severity
Nitric oxide synthase	Nitric oxide homeostasis	Lowered nitric oxide production *NOS1* allele	Low	Increased severity due to enhanced risk of early colonization by *Pseudomonas aeruginosa*
Major histocompatibility complex, class II antigens	Processing and presentation of antigens to T cells	*HLA-DRB1*DR4; HLA-DRB1*DR7*	Normal	Increased severity due to enhanced risk of early colonization by *Pseudomonas aeruginosa*

an association study does not reveal the mode of action of the modifying allele. In addition, the cystic fibrosis modifier 1 gene (*CFM1*) at 19q13.2–q13.4 that is responsible for meconium ileus was identified by a linkage study. A CF modifier gene that affects gastrointestinal function and another one that leads to persistent nasal congestion with sinus cavity obstruction (rhinosinusitis) have also been identified.

Association studies have been used to detect whether certain environmental factors affect the final CF phenotype. In this context, low socioeconomic status is positively associated with increased severity of lung dysfunction. And, in the absence of exposure to *Pseudomonas aeruginosa*, respiratory function is enhanced in CF individuals.

Oligogenic Disorders

The primary phenotype of monogenic traits such as CF is determined by a single major locus despite the additional complexity of modifier genes and environmental factors. On the other hand, there are some conditions that require the combined activity of alleles at a few loci to produce a phenotype. To date, the best examples of oligogenic traits are various two-locus (digenic) systems (Table 10.3). It is important to distinguish between digenic inheritance and a modifier gene effect. With the former, mutations at two different loci are required to produce a clinical phenotype. By contrast, modifier genes may diminish or enhance the phenotypic impact of alleles at another locus.

Table 10.3 Digenic inheritance.

Condition	Gene 1	Gene 2
Bardet–Beidel syndrome	*BBS2*	*BBS1*, *BBS4*, or *BBS6*
	BBS1	*BBS4*, *BBS6*, or *BBS7*
Hirschsprung disorder	*RET*	*EDNRB*
Nephrotic syndrome	*NPHS1*	*NPHS2*
Nonsyndromic recessive deafness	*GJB2*	*GJB6*
Retinitis pigmentosum	*ROM1*	*RDS*
Severe insulin resistance	*PPARG*	*PPP1R3A*
Waardenburg syndrome type 2 and ocular albinism	*MIFT*	*TYR*

Table 10.4 Monogenic and digenic inheritance of nonsyndromic deafness.

Genotype	Phenotype	Inheritance
$GJB2^{+}/GJB2^{+}$; $GJB6^{+}/GJB6^{+}$	Normal	
$GJB2^{-}/GJB2^{-}$; $GJB6^{+}/GJB6^{+}$	Deafness	Monogenic
$GJB2^{+}/GJB2^{+}$; $GJB6^{-}/GJB6^{-}$	Deafness	Monogenic
$GJB2^{+}/GJB2^{-}$; $GJB6^{+}/GJB6^{-}$	Deafness	Digenic

Autosomal Recessive Nonsyndromic Deafness

The hereditary component of hearing is extremely heterogeneous, with over 60 different genes contributing directly or indirectly to deafness. Hearing loss can be one of a number of symptoms (syndromic deafness) or the only clinical feature (nonsyndromic deafness). About 70% of all cases of inherited deafness are nonsyndromic and, of these, 75% are autosomal recessive (AR). The *GJB2* gene (also designated *DFNB1*) that encodes gap junction protein connexin 26 is mutated in about half the cases of AR nonsyndromic deafness. Connexin 26 molecules form hexameric cylindrical cell surface structures that bind with other connexin 26 hexamers in adjacent cells to form channels that facilitate potassium ion exchange between cells.

DNA sequencing studies of individuals with nonsyndromic deafness indicated that one-third of them had a single *GJB2* mutation, that is, they were heterozygotes. In these cases, a second mutation at another locus was a likely possibility. In some of these *GJB2* heterozygotes, the *GJB6* gene that encodes connexin 30 was mutated. Overall, the analyses revealed that the loss of any two of the four alleles at the *GJB2* and *GJB6* loci leads to nonsyndromic deafness (Table 10.4).

Bardet–Biedl Syndrome

Bardet–Biedl syndrome (BBS) is a rare, heterogeneous recessive disorder with at least eight identified loci (*BBS1* to *BBS8*). The BBS clinical features include retinal dystrophy, obesity, polydactyly, kidney defects, underdeveloped testes, and cognitive impairment. Studies with *BBS6*-based BBS families indicated that some affected individuals were heterozygotes at the *BBS6* locus. This result contradicts the expected mode of autosomal recessive inheritance for this condition. In one case, an affected *BBS6* heterozygote was homozygous for a mutation at the *BBS2* locus. Subsequent DNA screening of BBS families with *BBS6*

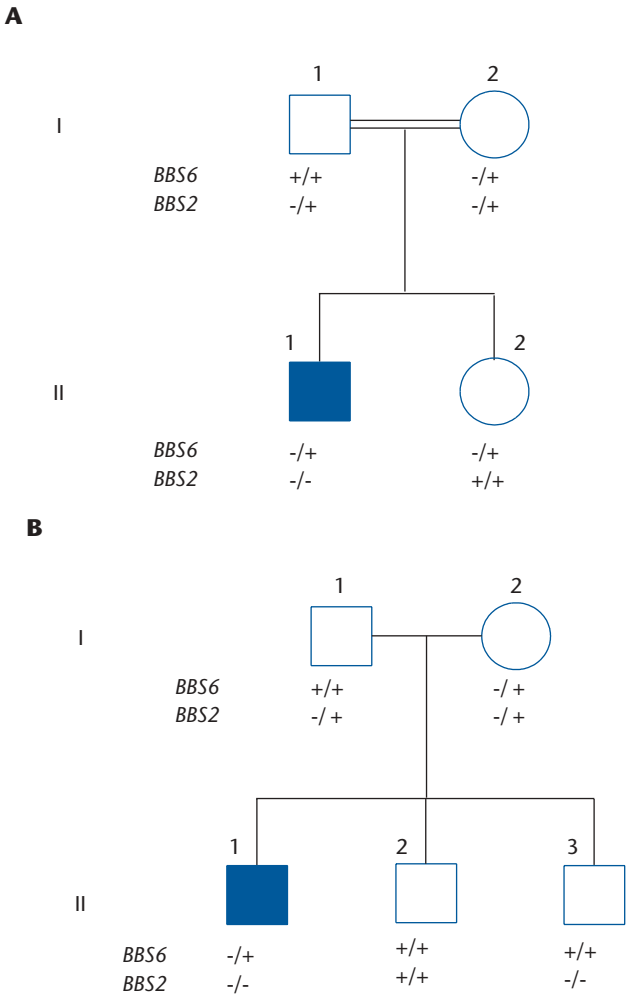


Figure 10.7 Triallelic inheritance associated with Bardet–Beidel syndrome (BBS). Genotypes of the *BBS6* and *BBS2* genes in two pedigrees (A and B) are shown. Affected individuals are depicted with solid symbols. Individual II-3 in pedigree B is noteworthy because he is homozygous recessive for the *BBS2* gene and is unaffected.

mutations for mutations of the *BBS2* gene revealed that, in some instances, a total of three mutant alleles at the *BBS2* and *BBS6* loci were required to give rise to the BBS phenotype (Figure 10.7). A normal phenotype was observed when there were two mutant alleles at the *BBS2* locus (–/–), and the *BBS6* locus was homozygous wild type (+/+). Three mutant alleles at two loci producing a distinctive phenotype has been designated triallelic digenic inheritance. This mode of inheritance also occurs between other *BBS* loci, for example, *BBS2* (–/–) and *BBS1* (–/+), *BBS1* (–/–) and *BBS4* (–/+), and with other combinations of *BBS* loci (Table 10.3). The mode(s) of action of the proteins of diallelic or triallelic digenic systems is not clear. Among various possibilities, the proteins of the two loci probably interact in some manner and when three mutations are present too few functional complexes are formed, which then leads to the abnormal phenotype.

Polygenic Inheritance

In the latter part of the nineteenth century, Francis Galton (1822–1911), a British scientist, devised weather maps, developed important statistical proce-

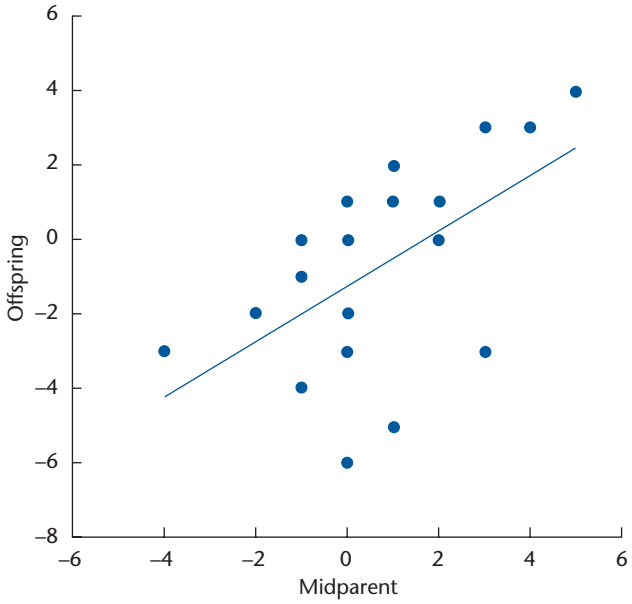


Figure 10.8 Offspring-midparent relationship for a quantitative trait. The solid line represents best-fit curve.

dures, recommended the use of fingerprints for identification, and initiated studies designed to determine whether continuous (quantitative) human traits had a genetic component. He reasoned that if heredity contributed to a particular condition then the closer the biological relationship, the greater the resemblance. This contention was tested by comparing height, among other traits, between parents and their offspring. As other researchers have shown since Galton's initial analyses, there are a number of ways that parent-offspring comparisons can be presented graphically. In Figure 10.8, the *x*-axis represents the mean score of both parents (midparent value) minus the mean value of the population and the *y*-axis is the value of the offspring minus the mean value of the population. In other words, the values for the midparents and offspring are deviations from the population mean. Negative and positive values are below and above the mean, respectively. The zero denotes the same value as the mean. The relationship in Figure 10.8, based on the best-fit curve, indicates that tall parents tend to have tall offspring and short parents short offspring. Thus, at this level of analysis, the trait appears to have a genetic basis. On the other hand, a best-fit horizontal curve would indicate that there is little relationship between parents and offspring and that the trait was randomly determined (Figure 10.9). Although somewhat informative, these data do not directly indicate either the nature or the extent of the genetic input to a trait. Also, the effect of environmental influences, which can be inferred from the scatter of the points of a plot, cannot be assessed.

In 1918, R. A. Fisher (1890–1962) established that the correlation between the genotypes of parents and offspring for a continuous trait could be explained by the combined action of a large number of genes, each with a small additive effect. With statistical analyses, the variation of a phenotype could be partitioned into genetic and environmental contributions. Moreover, both of these components can be further subdivided into additional categories. This theoretical framework has been used successfully to allocate the genetic and envi-

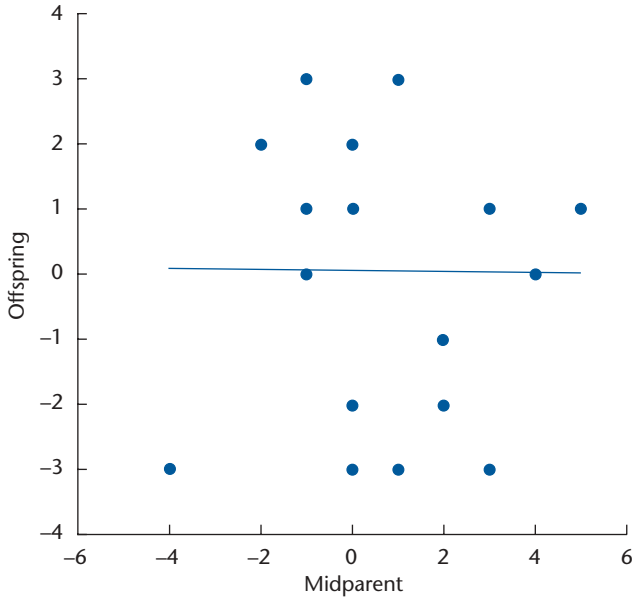


Figure 10.9 Offspring-midparent relationship for a quantitative trait. The solid line represents best-fit curve.

ronmental effects on traits of organisms that can be selectively crossed and backcrossed. However, effective statistical analysis of human traits is complicated and burdened with limiting assumptions, and it lacks the precision that is achieved with studies on agricultural crops and domestic animals. Notwithstanding, approaches using affected human relatives and human twins have been devised to determine the genetic and environmental contributions to multifactorial traits.

Familial Risk Ratio

For autosomal recessive disorders, the offspring of homozygous recessive parents are all expected to be homozygous recessive. Thus, after the first affected child is born, the risk of recurrence (recurrent risk) of a second affected offspring is 1. If two parents are heterozygous for an autosomal recessive disorder, then after the birth of an affected offspring, the recurrent risk is 0.25. Recurrent risks are readily calculated for monogenic conditions if the genotypes of the parents are known. With more complicated family situations, Bayesian statistics are routinely used by genetic counselors to determine recurrence risks. (This methodology and its application are described in Chapter 17 on genetic counseling.)

For polygenic disorders, recurrence risks are calculated from population and family data. For example, affected and unaffected siblings are enumerated from a large number of families with the same disorder and the number of affected siblings divided by the total number of siblings in the sample represents the recurrent risk for siblings. In a similar way, recurrent risks can be computed for other first-degree relatives (parent-offspring, dizygotic twins), second-degree relatives (half siblings, grandparents-grandchildren, aunt or uncle-niece or nephew), and third-degree (cousin) relationships. Genetic relationships correspond to the proportion of alleles that are held in common among relatives. For example, monozygotic (MZ) twins are genetically identical and have the

same genomes; that is, they are 100% genetically related. First-, second-, and third-degree relatives share 50%, 25%, and 12.5% of their genes, respectively. The calculated value of a recurrent risk for multifactorial disorders may vary from one geographic location to another because of diverse environmental effects. Generally, the recurrence risk is high if a disorder is severe, if it occurs in the less common sex when there is a difference in the frequency between males and females, and if it has an early onset.

A more effective parameter for determining genetic aspects of complex disorders is the familial (recurrence) risk ratio. The familial risk ratio (FRR; λ) is the frequency of the disorder in a class of relatives of an affected individual with the same relationship divided by the frequency of the disorder in the population, that is,

$$\lambda_R = \frac{\text{Frequency of the disorder in relatives of an affected individual}}{\text{Frequency of the disorder in the population}}$$

where R denotes the familial relationship. Each specific familial risk ratio (λ) is designated with a subscript where S, P, O, MZ, DZ, 1, 2, and 3 usually represent siblings, parents, offspring, monozygotic twins, dizygotic twins, and first-, second-, and third-degree relatives, respectively. Generally, if $\lambda_R = 1$, then a relative of an affected individual is no more likely to develop the disorder than anyone else in the population. Large λ_R values, that is, >1, indicate that a disorder is clustered within families, which suggests that it has a genetic basis. However, shared environmental factors may also be responsible for the aggregation of affected individuals within families. This issue can be resolved by compiling familial risk ratios for the complete range of genetic relationships. If a trait has a significant genetic component, then the familial risk ratios should correspond, more or less, to the degree of genetic relatedness, with the highest value for monozygotic twins, next for first-degree relatives, and so on (Table 10.5).

Familial risk ratios are useful for deducing whether each gene of an ensemble contributes more or less equally (additively) to a phenotype, or whether there is extensive gene interaction (epistasis; multiplicative gene effects). Dif-

Table 10.5 Familial risk ratios of monozygotic twins (λ_{MZ}) and first-degree relatives (λ_1) for some disorders.

Trait	Population prevalence (%)	λ_{MZ}	λ_1
Anorexia nervosa	0.1	710	41
Attention-deficit/hyperactivity disorder	~8	~14	~6.5
Autism	~0.035	~2500	~150
Cleft lip/palate	0.1	~500	40
Crohn disease	~0.01	840	25
Diabetes mellitus (type 1)	~0.6	80	12
Manic-depressive disorder	0.8	60	7
Multiple sclerosis	0.1	800	24
Schizophrenia	1	48	11

Table 10.6 Recurrence risks and familial risk ratios for ankylosing spondylitis among different familial relationships.

Relationship	Recurrence risk (%)	Familial risk ratio
Monozygotic twins	63	630
Offspring	7.9	79
Siblings	8.2	82
First degree	8.2	82
Second degree	1.0	10
Third degree	0.7	7

Adapted from Table 2 in Brown et al. 2000. *Ann Rheum Dis* **59**:883–886.

Table 10.7 Modeling the genetic basis of ankylosing spondylitis with familial risk ratios.

		Model				
Relationship	Familial risk ratio	Single locus	Polygenic multiplicative	Two locus multiplicative	HLA, residual polygenes	Five locus multiplicative
MZ twins	630	163	6694	282	3202	780
First degree	82	82	82	82	82	82
Second degree	10	41	9	27	11	15
Third degree	7	21	3	11	4	5

Note: The observed familial risk ratio for first-degree relatives was used for each model. HLA, human leukocyte antigen B27, which is significantly associated with ankylosing spondylitis; MZ, monozygotic.
Adapted from Table 1 in Brown et al. 2000. *Ann Rheum Dis* **59**:883–886.

ferent values for sets of familial risk ratios are expected for the additive and multiplicative models. For the additive model, $\lambda_R - 1$ decreases by half for each degree of decreasing relationship. By contrast, for the multiplicative model, the stepwise decrements are the square root of $\lambda_R - 1$.

Familial risk ratios are also used for modeling the genetic underpinnings of complex disorders. This strategy has been applied to ankylosing spondylitis (AS). Ankylosing spondylitis is a chronic arthritic disorder that predominantly affects the spine. It causes extreme suffering (ankylosing) and severe inflammation (spondylitis) of the spinal joints. The hips and other sites are also affected in 40% of the cases. The prevalence of AS in European and North American populations is about 1 in 1000. The recurrence risks and familial risk ratios for AS have been determined (Table 10.6). The pattern of familial risk ratios indicates that AS has a strong genetic component. Of the various models that were tested, a five-locus, multiplicative model gave the best fit for the observed set of familial risk ratios (Table 10.7). This model excludes the *HLA*B27* gene, which is significantly associated with AS in some, but not all, affected individuals. The *HLA*B27* gene is a risk factor for AS. In sum, on the basis of analysis of familial risk ratios, AS appears to be an oligogenic trait with interacting genes.

Twin Studies

Francis Galton, among his other accomplishments, conducted the first studies of twins to determine the contributions of heredity and upbringing. Although

the initial results were ambiguous at best, the importance of twin studies for genetic research has not diminished over the last 125 years. Currently, twin studies rely on sophisticated statistical analyses and multifaceted model fitting to reveal genetic and environmental influences on a variety of disorders, behaviors, cognitive abilities, and physical characteristics.

The essence of twin studies lies in the fact that there are two types of twins. Monozygotic twins (MZ, one egg, identical) occur after an embryo splits within the first 14 days from the initial cell division of a zygote to produce two embryos that are genetically identical. Dizygotic twins (DZ, two egg, fraternal) are produced when two ova are fertilized by different sperm and both zygotes develop simultaneously in the uterus. Thus, DZ twins have half of their genes in common and, except for sharing the same intrauterine environment during development, they are no different than any two siblings. Because DZ twins can be opposite sexes, only same-sex DZ twins, that is, female-female (FF) and male-male (MM), are used in twin studies.

The frequency of MZ twinning is fairly constant worldwide at about 3.5 per 1000 live births, which suggests that it is a random event and not under genetic control. On the other hand, DZ twinning varies among populations. In East Asia, sub-Saharan Africa, and both the Middle East and Europe, the rate of DZ twin births is about 1, 12.5, and 4.5 per 1000 live births, respectively. It is assumed that DZ twinning has a genetic component, although a role of population-specific environmental factors, such as a diet, that induce double ovulation cannot be discounted. The frequency of twinning within the first weeks after fertilization is not known, but it may occur in as many as 10% to 15% of all conceptions. Of twin pregnancies revealed by ultrasound examination 6 to 8 weeks after gestation, only half resulted in a twin birth. In the remainder of the cases, a single child was born. This phenomenon has been called the "vanishing twin syndrome." Losses of co-twins are also likely to occur earlier in development.

Twin studies are based on two fundamental postulates. First, because of completely shared genes and very similar environmental influences, any phenotypic difference between MZ twins ought to be due to environmental factors. Second, because members of twin pairs share very similar environmental influences, the greater the frequency of the same trait in both members of MZ pairs in comparison to DZ pairs, the greater the likelihood that the trait has a genetic component.

The cooperation of a large number of subjects, accurate determination of MZ and DZ twins (zygosity), and the correct assignment of the phenotype under study are essential for an effective twin study. Twins apparently readily volunteer for research studies. Large national and local registers have been assembled, with some studies including tens of thousands of twin pairs. A comprehensive questionnaire is used in most studies to establish zygosity, which has been shown to be about 95% accurate. If necessary, genotyping provides definitive proof of monozygosity. A reliable designation of a trait is obtained from clinical examination, health records, detailed questionnaires, and personal interviews.

The frequency of both twins (co-twins) having the same trait can be computed. These measures provide a means of indirectly assessing the environ-

mental and genetic contributions to a complex trait. For dichotomous (binary) traits, the term concordance (concordant) is used when both members of a twin set have the same trait and discordance (discordant) when one twin has a trait and the other does not. Concordance values can be calculated in various ways. The simplest computation is pairwise concordance for either MZ or DZ twins, which is the ratio of concordant pairs (C) for a particular trait to the sum of concordant (C) and discordant (D) pairs, that is,

$$C_{\text{Pairwise}} = \frac{C}{C + D}$$

Probandwise (casewise) concordance is often used when an experiment entails recruiting large numbers of affected twins. Under these conditions, the members of concordant twin pairs (C_2) are both examined and the phenotype in each case is verified by the researchers. However, there are also some cases where only one affected member of a twin pair is available. In these instances, the examined twin (proband) informs the researcher that his/her co-twin has the trait, that is, secondary ascertainment. Although the source of the information may be reliable, the data are not obtained independently, which undermines the experimental design. To compensate for this possible source of error, a specific formula was developed to take into consideration these twins (C_1). Probandwise concordance is

$$C_{\text{Probandwise}} = \frac{2C_2 + C_1}{2C_2 + C_1 + D}$$

where D represents the discordant twins.

In general terms, when a MZ concordance value is high and much greater than the DZ concordance, the trait is thought to have a genetic basis. However, concordances are not definitive estimates of the genetic and environmental inputs into a trait; rather, they provide researchers an important clue about how their studies of a particular trait should proceed. MZ and DZ concordances have been calculated for almost every imaginable disorder, behavior, cognitive ability, and physical feature (Table 10.8). Interestingly, very few MZ concordances are greater than 75%, which means that for many traits the role of environmental factors is important.

Concordances cannot be calculated for traits that do not readily fall into two discrete categories. Consequently, another method of numerical comparison between MZ and DZ twins is required for meristic and continuous traits. A correlation coefficient is often used as part of standard statistical analysis to assess the relationship between two sets of observations (variables). The more closely related two samples are, the higher the correlation coefficient. If there is no difference between samples, then the correlation coefficient is 1.0. With twin studies, there is no absolute rule for assigning the value of a trait for one twin to one data column (X variable) and that of the other twin to the other column (Y variable). In other words, the assignments are arbitrary. The solution to this problem is to calculate an intraclass correlation coefficient. One of the formulae for intraclass correlation coefficient (r) is

Table 10.8 MZ and DZ twin concordances.

Trait	MZ concordance (%)	DZ concordance (%)
Age-related maculopathy	37	19
Alcoholism	~35	~24
Alzheimer disease	31	9
Ankylosing spondylitis (without HLA-B27)	63	23
Anorexia nervosa	~54	~8
Asthma	45	12
Autism	~64	~9
Autoimmune hyperthyroidism	55	0
Bulimia nervosa	~46	~26
Cerebral palsy	~40	0
Celiac disease	~80	~16
Crohn disease	~47	~3
Diabetes mellitus type 1	~43	~8
Diabetes mellitus type 2	34	16
Epilepsy	62	18
Extraversion	~51	~26
Huntington disease	100	50
Idiopathic chronic fatigue	55	19
Idiopathic epilepsy	~85	~32
Migraine (with aura)	34	12
Migraine (without aura)	43	31
Multiple sclerosis	~27	~2
Neuroticism	~47	~24
Parkinson disease	16	11
Peptic ulcer	24	15
Schizophrenia	~35	~8
Simple goiter	42	13
Specific language impairment	~99	~60
Spoken language disorders	84	50
Strabismus (esotropia; eyes turn in)	51	15
Strabismus (exotropia; eyes turn out)	33	45
Suicide	11	2
Written language disorders	75	43

$$r = \frac{2\sum (x - \bar{x})(x' - \bar{x})}{\sum (x - \bar{x})^2 + \sum (x' - \bar{x})^2}$$

where x and x' represent the observations for twin 1 and twin 2 of each twin pair, respectively, and $\bar{x}$ is the mean of all observations. Intraclass correlation coefficients for MZ (r_{MZ}) and DZ (r_{DZ}) twins have been determined for a myriad of conditions (Table 10.9). Generally, a trait with both a high value for r_{MZ} and a large difference between the observed r_{MZ} and r_{DZ} values is likely to have a genetic component. A more specific measure called heritability (h^2) is used to estimate the proportions of the total variation from the mean of a normally distributed trait that could be attributed to genetic and environmental factors.

For twin studies, a commonly used estimate of broad-sense heritability is $h^2_B = 2(r_{MZ} - r_{DZ})$, where r_{MZ} and r_{DZ} are the intraclass correlation coefficients for MZ and DZ twins, respectively. Thus, loosely speaking, if the heritability estimate for a trait is 0.35, then 35% of the total (phenotypic) variation can be

Table 10.9 MZ and DZ twin intraclass correlation coefficients.

Trait	r_{MZ}	r_{DZ}
Aldosterone excretion	0.69	0.29
Attention-deficit/hyperactivity disorder	0.83	0.42
Blood pressure	0.81	0.44
Bone formation (postmenopausal)	0.67	0.48
Bone mineral density	0.90	0.58
Bone turnover	0.78	0.31
Breast tissue density	0.64	0.26
Cerebral hemisphere volume	0.87	0.56
Extraversion	0.51	0.26
General intelligence	0.86	0.60
Heart rate (beats/minute)	0.45	0.35
Height	0.89	0.57
Memory	0.45	0.34
Neuroticism	0.47	0.24
Peptic ulcer	0.65	0.35
Plasma cholesterol concentration	0.80	0.68
Premenstrual syndrome	0.55	0.28
Spatial ability	0.62	0.34
Spatial reasoning	0.65	0.45
Verbal ability	0.76	0.43
Verbal reasoning	0.77	0.52

assigned to genetic factors and 65% to environmental influences. In some twin studies, heritability estimates are greater than 1.0. Such values bring into question the assumption that the shared (common) environmental effect is the same for both MZ and DZ twins. In these cases, MZ twins experience environmental influences to a greater extent than DZ twins. On the other hand, negative heritability estimates suggest that a genetic-environmental interaction has made a significant contribution to the trait. For example, prenatal (in utero) competition between MZ twins may create a phenotypic difference between them, thereby lowering the value of the intraclass correlation coefficient.

The parameter heritability was originally formulated to assess the selective breeding potential of quantitative traits in animals. One of the main statistical measures for determining a heritability estimate is the variance. Briefly, the variance (S^2) represents the extent of dispersion from the mean of a normally distributed sample and is the sum of the square of each observation minus the mean divided by the sample size minus 1, that is,

$$S^2 = \frac{\sum (x - \bar{x})^2}{N - 1}$$

The capital letter V is often used as a descriptive symbol for the variance. The total variance for a quantitative trait is called the phenotypic variance (V_P), which is the sum of the genotypic (genetic) variance (V_G) plus the environmental variance (V_E), that is, $V_P = V_G + V_B$. And, broad-sense heritability (h_B^2) is defined as $\frac{V_G}{V_P} = \frac{V_G}{V_G + V_E}$. Theoretically, the genotypic variance can be partitioned into specific genetic effects. For example, there are additive, dom-

inant, and interaction (epistatic) gene effects that, in terms of variance, are designated V_A, V_D, and V_I, respectively. Additive genetic variance encompasses the effects of a large number of alleles acting independently to contribute to the final phenotype. Dominance and gene interaction variances represent gene interaction at single loci and at different loci, respectively. Because the effects of dominance and epistasis are difficult to disentangle experimentally, the two variances are often combined under the designation "nonadditive genetic variance." A second heritability estimate that is called narrow-sense heritability (h^2_N) is the additive genetic variance divided by the phenotypic variance, that is, $\frac{V_A}{V_P}$. The environmental variance can be subdivided into common (shared) environmental variance (V_C) and individual-specific (nonshared, within) environmental variance (V_E). With twins, the common environmental influence affects both members of a twin pair, whereas only one twin is affected by an individual-specific environmental effect. The contribution of additive genetic, common environmental, individual-specific environmental, and nonadditive genetic variances can be inferred from the numerical relationships of MZ and DZ intraclass correlation coefficients (Table 10.10).

Clearly, estimates of the magnitudes of the various genetic and environmental influences on a trait would be desirable for understanding the inputs that contribute to a final phenotype. To this end, various models are formulated, expected values for r_{MZ} and r_{DZ} are computed for each model, and the model that best fits the observed values is identified. For example, one model assumes that the phenotypic outcome is explained by a combination of additive genetic (A), common environmental (C), and individual-specific environmental (E) influences, namely, the ACE model. Other models are AE, CE, and ADE, where D denotes a nonadditive genetic component. The DE model is often ignored because nonadditive genetic effects rarely occur without the contribution of additive genetic factors. The ACED model cannot be tested with data only from studies of twins reared together because estimates of common environmental and nonadditive genetic variances are derived from the same information. However, other sources of correlations such as parent-offspring and/or sibling-sibling data can be used with twin data to analyze the goodness of fit of the ACED model.

Table 10.10 MZ and DZ twin intraclass correlation coefficient relationships and sources of variation.

	Variance			
Relationship	V_A	V_C	V_E	V_D
$r_{MZ} = r_{DZ} = 0$			+	
$r_{MZ} = r_{DZ} > 0$		+	+	
$r_{MZ} = r_{DZ} = 1$		+		
$r_{MZ} = 2r_{DZ}$ with $r_{MZ} < 1.0$	+		+	
$r_{DZ} > 1/2\ r_{MZ}$	+	+	+	
$r_{DZ} < 1/2\ r_{MZ}$	+		+	+

Note: V_A, additive genetic effect; V_C, common environmental effect; V_E, individual-specific environmental effect; V_D, nonadditive genetic effect.

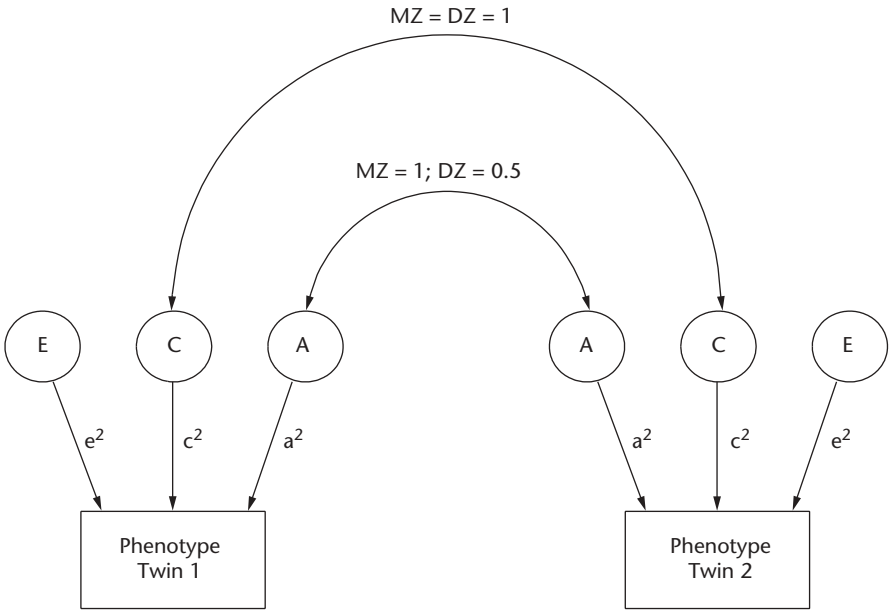


Figure 10.10 Path diagram of ACE model for MZ and DZ twins reared together. A, additive genetic influences; C, common environmental influences; E, individual-specific environmental influences; a^2, proportion of phenotypic variance due to additive genetic effects; c^2, proportion of phenotypic variance due to common environmental effects; e^2, proportion of phenotypic variance due to individual-specific environmental effects. The correlations for additive genetic and common environmental influences for MZ and DZ twins are noted. Although not shown here, the correlations for nonadditive genetic influences (D) are 1 for MZ twins and 0.25 for DZ twins.

Structural equation modeling (SEM) is routinely used for model-fitting analyses of twin data. This procedure can be represented graphically as a path diagram. In this format, the ACE model (Figure 10.10) consists of the latent (unobserved) variables A, C, and E (circles). The correlations (two-headed arrows) are marked with the specific values for MZ and DZ twins. The rectangles denote the observed phenotypes of the twin pairs. The one-headed arrows indicate the direction from the source of the effect to the variable that is affected. The square of each path coefficient (one-headed arrow) is the variance of a latent variable and is represented as the square of the lower case letter equivalent to the latent variable, namely, a^2, c^2, and e^2. Parenthetically, the value of a^2 from the best-fit model is the narrow-sense heritability.

In addition to models based on A, C and E, more complex pathways with additional latent and observed variables and combinations of interactions between variables for twins and other genetic relations can be constructed and tested. The Mx computer program that is commonly used for model-fitting analyses is based on the liability threshold model (LTM), which assumes the trait under consideration is normally distributed, the genetic and environmental effects are numerous and small, an affected status occurs when a threshold is either exceeded or not reached, and the latent variables make essential contributions to the phenotype.

The apportionment of variances for various conditions, behaviors, and attitudes based on model fitting is presented in Table 10.11. For some traits, heritability values change as a function of maturation. As shown in Table 10.11, heritability for middle ear disease is lower in two year olds ($h^2 = 0.49$), when the immune system is not fully developed, than two years later ($h^2 = 0.71$) when antibody production is apparently more efficient and infections that represent a common environmental factor have less of an impact. Similarly, heritability estimates for intelligence quotient (IQ) scores increase with age and the

Table 10.11 Estimated variances for best-fitting models for various traits.

Trait	a^2	c^2	e^2	d^2
Aggression	0.44	0.06	0.50	
Binge eating with bulimia nervosa	0.46		0.54	
Cerebral volume	0.94	0.06		
Chronic fatigue (duration ≥6 months)	0.19	0.69	0.12	
Criminality	0.33		0.25	0.42
Doing crossword puzzles	0.02		0.55	0.43
Healthy eating (≥50 y; frequency)	0.33	0.09	0.58	
Heart rate (beats/minute)	0.23	0.23	0.54	
Middle ear disease (otitis media)				
2 years old	0.49	0.41	0.10	
3 years old	0.66	0.22	0.12	
4 years old	0.71	0.13	0.14	
Moles (nevi; number)	0.62	0.33	0.05	
Obliging behavior		0.23	0.77	
Roller coaster rides	0.52		0.48	
Schizophrenia (APA classification)	0.88		0.12	
Schizophrenia (First-rank symptoms only)	0.72		0.29	
Schizophrenia (WHO classification)	0.83		0.17	
Unhealthy eating (≥50 y; frequency)	0.33	0.08	0.59	
Vomiting with bulimia nervosa	0.70		0.30	

Note: a^2, additive genetic variance; c^2, common environmental variance; e^2, individual-specific environmental variance; d^2, nonadditive genetic variance; APA, American Psychiatric Association; WHO, World Health Organization.

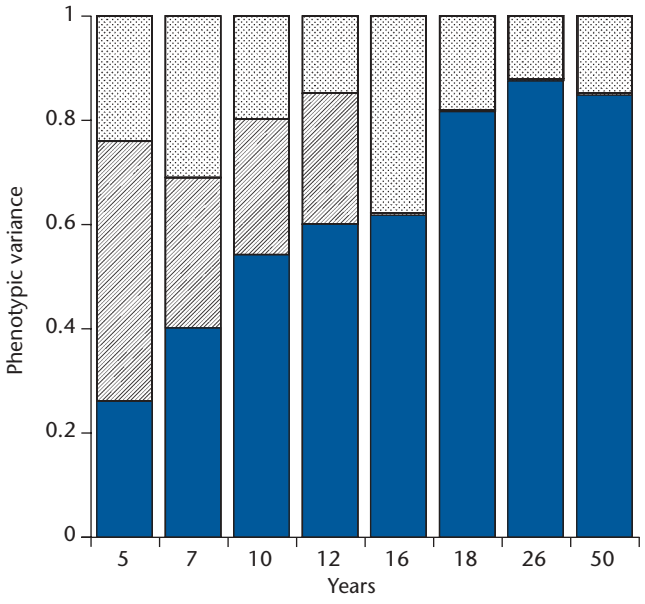


Figure 10.11 Apportionment of phenotypic variance for IQ scores from twin studies as a function of age. Additive genetic variance, solid; common environmental variance, hatched; individual-specific environmental variance, stippled. Adapted from Figure 1 at http://www.tweelingenregister.org/nederlands/twinfos/IQ%20gewijzigd.htm (accessed July 2003).

common environmental variance disappears by adolescence (Figure 10.11). The changes in heritability estimates in this case suggest that an interplay between genetic and environmental factors is important for cognitive functions during certain periods of human development. In sum, a heritability estimate reflects the genetic variance under a specific set of conditions and should not be considered immutable.

Locating Quantitative Trait Loci

Despite the insights provided by twin studies, the genes that actually contribute to complex traits are not disclosed. Linkage analyses of multigenerational families with monogenic disorders has been an effective method for localizing genes. However, the conventional LOD score method is not feasible with polygenic traits because recombinants cannot be detected and the mode of inheritance of a particular QTL is not easily modeled. Consequently, other methods are used to locate and identify QTLs.

Case-Control Association Studies

Case-control association studies detect nonrandom co-occurrences between alleles and traits. These studies are technically straightforward, although there is a high likelihood of erroneous results if certain design features are ignored. A common approach is to select a candidate gene that has one or more polymorphic sites in exons, promoter sequences, and (or) regulatory regions. Single-nucleotide polymorphisms (SNPs) are a good source of gene markers for these studies. Individuals with a trait (cases) and those without it (controls) are genotyped for the marker alleles. Sample sizes should be large (>500) because the effect of each gene with most complex traits is small. The results are compiled and arranged in a contingency table. A 2 × 2 table is used for analyzing two alleles (Figure 10.12), a 2 × 3 table for genotypes, and larger tables (2 × *n*) for multiple alleles. For example, the results of a two-allele system in Figure 10.12

A

	Marker locus		
	A1	*A2*	
Affected	80 (a)	45 (b)	125
Nonaffected	40 (c)	65 (d)	105
	120	110	230

B

$\frac{120 \times 125}{230} = 65.22$ (a)	$\frac{110 \times 125}{230} = 59.78$ (b)
$\frac{120 \times 105}{230} = 54.78$ (c)	$\frac{110 \times 105}{230} = 50.22$ (d)

C

$$\chi^2 = \sum \frac{(\text{obs} - \text{exp})^2}{\text{exp}} = \frac{(80 - 65.22)^2}{65.22} + \frac{(45 - 59.78)^2}{59.78} + \frac{(40 - 54.78)^2}{54.78} + \frac{(65 - 50.22)^2}{50.22} = 15.35$$

$df = (r - 1)(c - 1) = 1;\ p < 0.001$

Figure 10.12 A test for the association of different alleles of a locus with affected (case) and nonaffected (control) individuals. (A) The numbers of individuals with a genetic disease (affected) and those that do not have the disease (nonaffected) with either allele *A1* or *A2* of a two-allele locus are presented in the cells (a, b, c, d) of a 2 × 2 table. The sums of the rows and columns and the total number of the sample (230) are shown. (B) How the expected numbers of individuals for each cell of the 2 × 2 table in A are calculated under the assumption that each joint occurrence is equally likely is shown. (C) Results of the chi-square (χ^2) test are shown using the observed and expected values from the tables in A and B. The value for the degrees of freedom (df) is the number of rows (r) in the table minus 1 times the number of columns (c) in the table minus 1. The value for *p* of <0.001 supports the hypothesis that the alleles of the marker locus of affected and nonaffected individuals are not randomly associated.

from the HUMAN GENETICS files

Genetics of Human Intelligence

Human intelligence is almost impossible to characterize with any certainty. The conventional dictionary definition is "the ability to create and understand complex ideas." Psychologists have devised various ways of measuring intelligence. In light of the consistent correlations of individuals' performances with different types of psychometric intelligence tests, many researchers have concluded that basic cognitive functions such as verbal comprehension, perceptual organization, working memory, and processing speed are rooted in the same biological process, which has been designated the general ability factor (*g* factor; *g*). The role of *g* can be modeled in various ways because nothing concrete is known about its nature. Of these schemes, a hierarchic model is currently favored. Specifically, *g* affects major cognitive functions and, in turn, each of these functions controls a more finely tuned subset of activities. At the genetic level, a large number of different genes probably determine the major cognitive components, with gene products participating in the pathways that lead to different capabilities. Other researchers argue that the *g* factor is not a real biological entity but, rather, reflects culture-based knowledge and/or linguistic skills. For some, intelligence is not viewed as a hierarchy under the aegis of the *g* factor but as a composite of eight or more intelligences (modules) consisting of linguistic, logic-mathematical, spatial, musical, intrapersonal, bodily, kinesthetic, and naturalist capabilities.

Notwithstanding how intelligence is understood, there is undoubtedly a genetic component to this phenomenon. Heritability estimates for *g* vary from about 25% to 80%, with a mean value of about 50%. Searches have been undertaken for QTLs that influence intelligence. Candidate gene studies have not produced any likely sites. A genome-wide screen with IQ test scores as a proxy for *g* has been conducted with 1842 simple-sequence repeat DNA markers that are located about every 2 cM. Linkage analyses included a case-control association study and transmission/disequilibrium tests. In one part of the study, the IQ test scores of the case and control individuals were 136 and 100, respectively. In another phase, the members of one sample had IQ scores greater than 160 and the scores of the comparison group were 100. No significant linkage was detected with either approach, although several markers were categorized as suggestive. In sum, intelligence is likely the consequence of the action of many genes, each with a small effect. Discovery of QTLs for *g* will require larger samples and increased numbers of very closely spaced polymorphic DNA markers.

show that the alleles of the marker locus are significantly associated with the trait under study. A significant association may indicate that the candidate gene is a QTL or that it is linked to the QTL. Further studies are required to distinguish between these two possibilities.

Association studies produce false positive results if the control sample contains genetically diverse subgroups. This phenomenon is called population stratification. There are, however, various remedies that avoid this confounding factor such as using nonaffected family members as controls. Also, population stratification can be detected by screening the case and control samples with additional polymorphic DNA markers from a number of chromosomes and determining whether there are differences in allele frequencies.

Often, to increase the sensitivity of the analysis, individuals with a very specific and consistent feature of the overall phenotype, that is, an endophenotype, are selected as cases. This strategy enhances the probability of detecting a significant association by reducing the effect size. An endophenotype is likely determined by a subset of the total complement of genes that contribute to the full phenotype. For example, when elite-performance athletes were subdivided into categories based on the duration of their events, significant associations were observed. One angiotensin-converting enzyme allele was associated with short-duration activity and another one with moderate-duration activity. No significant associations were observed when elite athletic ability was used as the phenotype for the cases.

Many case-control association studies do not produce significant results because the choice of a candidate gene is often not much more than an educated guess. Moreover, because the biological basis of many complex disorders is poorly understood, the probability of identifying a successful candidate gene is very low. Fortunately, other protocols such as genomewide (genome, genomic) scans can be used to search for QTLs, sparing the effort and resources that go into case-control association studies.

Genome Scans

Unlike the direct approach of case-control association studies with candidate genes, genome scanning (screening) is an indirect strategy that does not rely on conjecture. Basically, either affected individuals, usually siblings, from a number of families or families with two or more affected individuals are genotyped with polymorphic DNA markers that cover the entire chromosome complement. A set of about 400 short tandem repeat polymorphic markers that are spaced at about every 10 cM is used for most genome scans. This level of resolution has been enhanced with the assembly of about 3000 simple sequence repeat polymorphic markers that are about 1.5 cM apart. Single-nucleotide polymorphic sites (SNPs) are preferred for genome scans because they are uniformly distributed about every 300 bases throughout the genome and easily identified with automated equipment. Eventually, sets of SNPs will supercede short tandem repeat polymorphic sequence marker systems.

Genome scans are designed to detect linkage disequilibrium (LD) between a marker site and a QTL. A variety of analytical procedures have been devised for this purpose. Some of the LD tests use estimates of various parameters such as the mode of inheritance, penetrance, and other genetic features, that is, parametric tests. Other tests are model free (nonparametric tests) and detect significant associations between a DNA marker(s) and a chromosome site in affected individuals. The results of genome scans are often presented as LOD scores and plotted as a function of the location in centimorgans (cM) along each chromosome (Figure 10.13). The criterion for significance is problematic, but commonly a LOD score ≥3.6 is considered significant, >2.2 suggestive, and >1.5 interesting. Regions with suggestive and interesting linkages are examined with additional polymorphic markers in follow-up studies.

Potential QTLs for a number of complex traits have been localized to chromosome sites with genome scans (Table 10.12). However, in a number of cases, a significant linkage and (or) association with one site in one study was not observed in another study. This lack of consistency is a major concern and is not readily explained. Under the assumption that these discrepancies are due to technical difficulties, larger samples are being studied, greater care is being taken to ensure that subjects are drawn from the same population, and more powerful analytical methods are being developed.

The human genome appears to be structured into regions (blocks) of high linkage disequilibrium that range from 10 to >100 kb and are separated by segments with little or no linkage disequilibrium. Each high-LD block has a small number of haplotypes. An advantage of this pattern for genome scans is that a few SNPs (tag SNPs) will be sufficient to detect linkage with any gene within a block. Consequently, with knowledge of the haplotype structure, the number

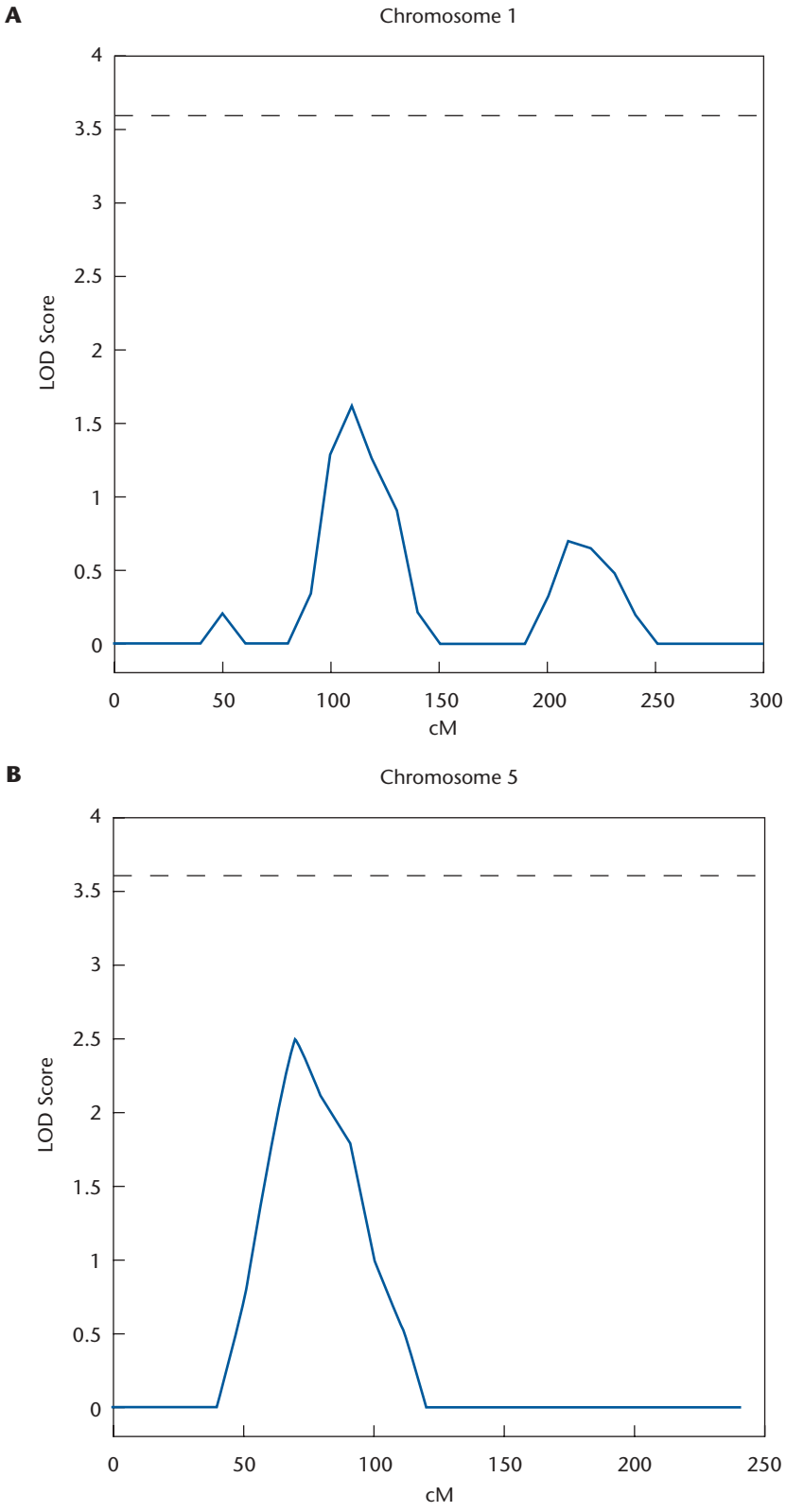


Figure 10.13 Genome scans. The results from only three chromosomes (A, B, C) are shown. The *x*-axis represents the genetic distance (centimorgan, cM) from the terminus of the short arm of each chromosome. The dashed line marks the level of a significant LOD score. Negative LOD scores were set to zero.

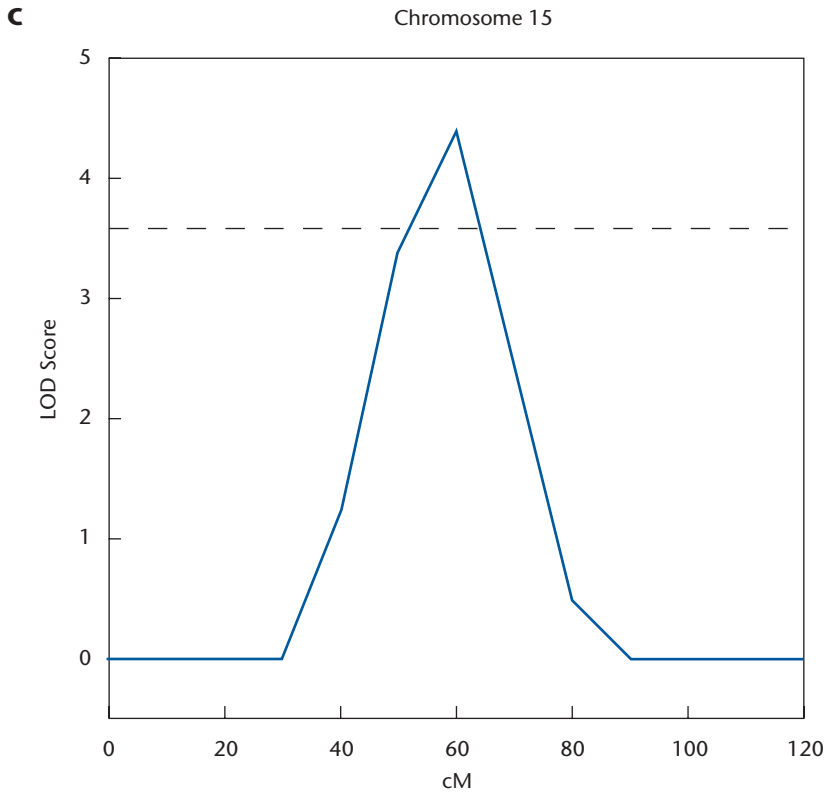


Figure 10.13 *continued*

Table 10.12 Putative quantitative trait loci (QTLs) detected by genome scans.

Trait	Chromosome	Location (cM)
Cleft palate	16	51
Abdominal subcutaneous fat	4	160
	11	15
Age-related maculopathy	12	107
Alcoholism[a]	4	121
Ankylosing spondylitis	16	109
Body mass index	7	13
	13	42
Bulimia nervosa	10	44
Creatine clearance[b]	3	66
Diabetes mellitus type 2	14	7
Dyslexia	18	36[c]
Height	6	159
	7	150
	12	56
	13	80
High-density lipoprotein cholesterol	5	40
Obesity	2	128
	20	78
Panic disorder	9	106
Relative hand skill	2	38
Vitiligo[d]	1	82

Notes:
[a] Endophenotype, maximum number of alcoholic drinks within a 24-h period.
[b] Indicator of kidney function.
[c] Distance from the most telomeric 18p marker in the set of DNA markers.
[d] Spontaneous irregular loss of pigmentation of the skin.

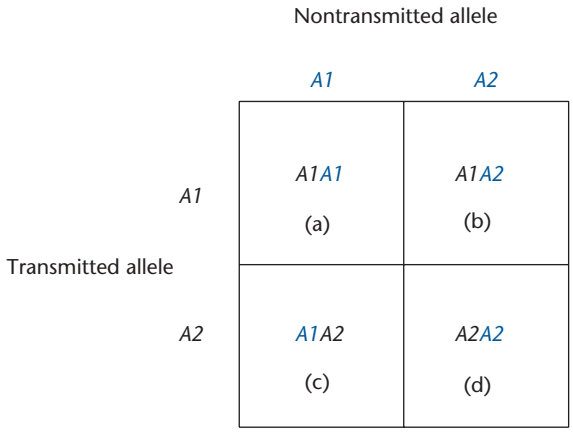


Figure 10.14 Transmitted (black) and nontransmitted (blue) alleles for *A1A1*, *A1A2*, and *A2A2* genotypes.

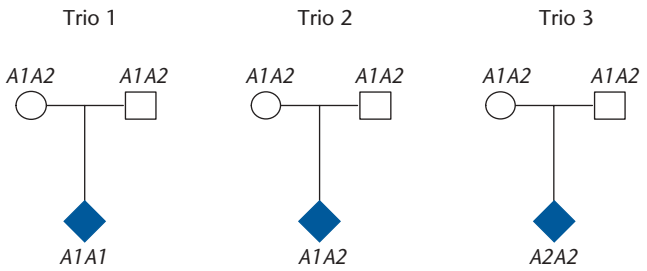
of sites that must be genotyped will be reduced without sacrificing high resolution. Construction of a human haplotype map (HapMap) by a consortium of laboratories is under way.

Transmission/Disequilibrium Test

The transmission/disequilibrium test (TDT) was originally developed to overcome the confounding effect of population stratification encountered with case-control association studies. The method effectively detects linkage with association. The key features of the TDT are tracking the transmission of alleles from heterozygous parents to affected offspring and determining whether the segregation is random or whether one allele is transmitted to an affected offspring more frequently than the other one. A significant difference between the number of transmitted alleles indicates linkage in the presence of association. More specifically, in this case, the recombination frequency (θ) is less than 0.5 and linkage disequilibrium (δ) is not zero. The possible combinations of transmission and nontransmission of alleles for *A1A1*, *A1A2*, and *A2A2* genotypes can be depicted with a four-cell table (Figure 10.14). Cells a and d represent the results for homozygotes. Cells b and c denote the two possibilities with a heterozygous genotype. It is not possible to distinguish which allele is transmitted to an offspring from homozygotes. On the other hand, the transmission of a particular allele can be readily tracked from a heterozygote. Therefore, only the numbers of transmissions of alleles recorded in cells b and c are used for the TDT. Significance is determined by a chi-square test (McNemar test), where $\chi^2 = \frac{(b-c)^2}{b+c}$, with one degree of freedom. The TDT requires genotyping of both parents, one of whom must be heterozygous, and an affected child. Such a grouping is designated a trio or triad.

In practice, a large number of parents and their affected children are recruited. Parents and affected offspring are genotyped with a set of polymorphic DNA markers that cover the entire genome or for sites within candidate genes. Trios are identified. The numbers of transmitted alleles from heterozygous parents are determined and the significance test is run (Figure 10.15). The original TDT protocol has been extended to siblings (sib-TDT), multiple

A



B

		Transmitted alleles	
Trio	Number of trios	*A1*	*A2*
1	20	40	
2	10	10	10
3	5		10
		50 (b)	20 (c)

C

$$\chi^2 = \frac{(b - c)^2}{b + c} = \frac{(50 - 20)^2}{70} = 12.86;\ 1df;\ p<0.001.$$ The difference is significant.

Figure 10.15 Transmission/disequilibrium test. (A) In this fabricated example, only trios with heterozygous parents were scored for autosome markers. Affected offspring can be either male or female (solid diamonds). Although not shown here, the transmission of alleles can be scored for X-linked markers and analyzed in the same manner as autosome markers. (B) Determination of the numbers of transmitted alleles based on the numbers of the different trios that were observed. (C) Chi-square test (McNemar's test) of the TDT data in B.

alleles, and other genetic conditions. Because the TDT relies on parents and affected offspring, the discovery of genes for late-onset disorders, when parents are not readily available, depends on other linkage detection techniques.

Affected Sib Pair Linkage Analysis

The primary objective of affected sib pair (ASP) linkage analysis is to determine whether large numbers of sib pairs with the same trait share one or more polymorphic loci. To discover significant sharing, the polymorphic alleles in the sib pairs must be identical by descent (identity-by-descent; IBD). IBD denotes an allele that is transmitted to both sibs from one of the parents. By contrast, when the same allele is present in two sibs regardless of its origin, the alleles are said to be identical by state (identity-by state; IBS). The distinction between IBD and IBS is illustrated in Figure 10.16. In Figure 10.16A, the siblings have the same genotype. The *A1* allele in both sibs is derived from the mother and the *A2* allele from the father. Thus, the sib pair has two alleles that are IBD, that is IBD2, and two that are IBS, that is, IBS2. By contrast, in Figure 10.16B, each sib acquired an *A1* and *A2* allele from a different parent. Consequently, neither allele is IBD, that is, IBD0, whereas the two alleles are identical by state, that is, IBS2.

In a large population, the proportion of sib pairs with 2, 1, and 0 IBD alleles is 1/4:1/2:1/4 for an autosomal locus. This ratio can be demonstrated by considering two parents with genotypes *A1A2* and *A3A4*. Theoretically, this mating will produce four kinds of offspring (*A1A3*, *A1A4*, *A2A3*, *A2A4*), each with the same frequency. The probabilities of pairs of sibs with 2, 1, and 0 IBD alleles can be computed from a 4 × 4 table (Figure 10.17). For example, in the top leftmost cell in Figure 10.7, both sibs received the *A1* allele from the same

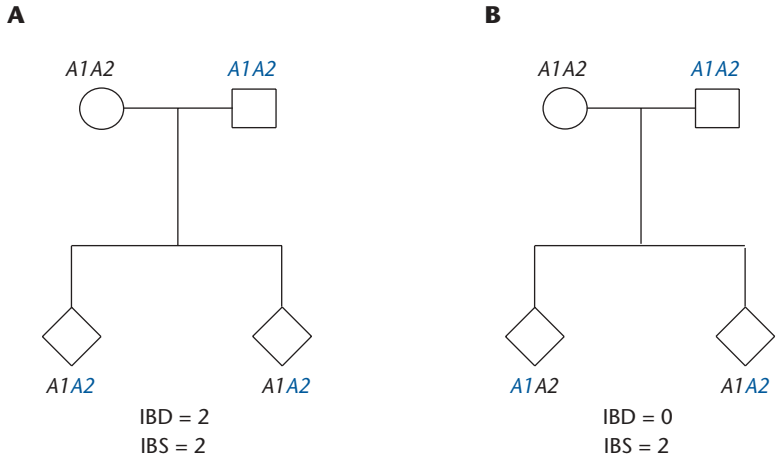
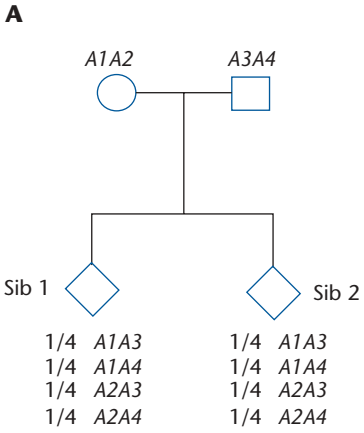


Figure 10.16 Identity by descent (A) and identity by state (B). The alleles are color coded (black, blue) to track the parental source in the sibs.



B

		Sib 2			
		1/4 *A1A3*	1/4 *A1A4*	1/4 *A2A3*	1/4 *A2A4*
Sib 1	1/4 *A1A3*	2	1	1	0
	1/4 *A1A4*	1	2	0	1
	1/4 *A2A3*	1	0	2	1
	1/4 *A2A4*	0	1	1	2

Tally: 2 IBD = 4/16 = 25%; 1 IBD = 8/16 = 50%; 0 IBD = 4/16 = 25%

Figure 10.17 Probabilities of sib pairs with 2, 1, and 0 IBD alleles. (A) Heterozygous parents with different genotypes can have offspring with four equally likely genotypes. (B) The frequencies of shared alleles that are identical by descent between all possible genotypes for sibs 1 and 2 noted in A.

parent and the *A3* allele from the other parent; thus, the two alleles are identical by descent, that is, IBD2, and so on. The tally is 1/4 IBD2, 1/2 IBD1, and 1/4 IBD0. ASP linkage analysis detects markers that deviate significantly from the expected ratio.

The optimal conditions for an ASP study are heterozygous parents who do not have the same genotype, that is, *A1A2* × *A3A4*. However, alleles that are IBD can be deduced from other parental combinations and, if necessary, from unaffected relatives. Affected sib pairs and their parents are genotyped with either polymorphic DNA markers that cover the entire genome or those

that occur within candidate genes. Specialized software is used to determine the numbers of ASPs with 2, 1, or 0 IBD alleles for each marker and whether there is a significant difference from the expected ratio. ASP linkage analysis is also used to detect X-linked QTLs. In this case, the theory is the same as with autosomal loci except that the expected ratio of IBD alleles among sib pairs is different. Significantly shared loci may represent QTLs directly or sites linked to them.

The ASP method has been extended to large pedigrees, multiple alleles, and other genetic conditions. A variant strategy compares only sib pairs where, for example, one sib is affected and the other is not for the trait under study. In this case, the sibs are genotyped and the numbers of nonmatching alleles in affected and unaffected sibs are tested for statistical significance. ASP linkage analysis and related procedures have uncovered possible chromosome locations for neuroticism, type 2 diabetes, attention-deficit/hyperactivity disorder, psoriasis, asthma, spinal ossification, and many other traits.

key terms

additive gene effect
affected sib pair linkage analysis
ASP
binary trait
broad-sense heritability
candidate gene
case-control association study
common (shared) environment
continuous
dichotomous trait
digenic
dizygotic
dominant gene effect
epistasis
familial risk ratio
favism
***g* factor**
genome scan
heritability
identical by descent
identical by state
individual-specific environment
intraclass correlation coefficient
liability threshold model
meristic
modifier gene
monogenic
monozygotic
multifactorial
multigenic
narrow-sense heritability
normal distribution
oligogenic
path diagram
polygenic
population stratification
QTL
quantitative inheritance
quantitative trait locus
sibling risk ratio
structural equation modeling
TDT
transmission/disequilibrium test
triallelic digenic inheritance
trio
variance

summary

Genetic systems are inherently intricate. To date, much attention has been devoted to isolating and characterizing genes that have a major effect on a phenotype. Detailed analyses have shown that these monogenic traits are not simple genetically; rather, both modifying genes and environmental (nongenetic) factors contribute to the final phenotype in many instances. As well, tools are now available to study traits that are determined by either a few or many genes.

There are not many examples of oligogenic traits. Of these, two-gene (digenic) inheritance has been documented for some disorders.

Many traits result from the actions of a large number of genes, each with a small effect. Generally, these polygenic traits fall into three categories: meristic, continuous, and dichotomous. The phenotypes with meristic and continuous traits are usually normally distributed. With dichotomous polygenic traits, the underlying biochemistry of the phenotype probably forms a bell-shaped curve with a large random sample of individuals and the two states arise because either too little or too much of a compound is produced. In other words, if a critical threshold is not reached then those individuals that fall below this amount are affected. Similarly, if the threshold is

continued

exceeded those individuals that are above the threshold are affected.

The familial risk ratio (λ_R) is a useful parameter for determining the genetic basis of complex traits. Also, concordances and intraclass correlation coefficients derived from studies of MZ and DZ twins are important indicators of the genetic and environmental underpinnings of complex traits. In this context, heritability estimates are helpful because they are used to assign the proportion of the total phenotypic variance that is due to genetic effects (broad-sense heritability) or additive gene effects (narrow-sense heritability). Structural equation modeling with twin data sets supplies further insights into the proportion of the total phenotypic variance of a complex trait that can be attributed to gene effects (additive, nonadditive) and environmental influences (common, individual-specific).

Various methods such as case-control association studies, genome scanning, the transmission/disequilibrium test, and affected sib pair linkage analysis have been developed for detecting quantitative trait loci (QTLs). Generally, linkage/association analyses require the genotyping of large numbers of affected individuals with very closely spaced polymorphic DNA markers that cover the entire genome. In some instances, candidate genes may be targeted with the appropriate polymorphic DNA markers. A number of putative QTLs for various complex disorders have been identified with these approaches. Reliable identification of QTLs will be more likely when there is a better understanding of the haplotype structure of the human genome and a comprehensive set of polymorphic SNPs is available.

References

Allison, D. B. and M. C. Neale. 2001. Joint tests of linkage and association for quantitative traits. *Theor Popul Biol* **60**:239–251.

Altmüller, J. et al. 2001. Genomewide scans of complex human diseases: true linkage is hard to find. *Am J Hum Genet* **69**:936–950.

Aron, Y. et al. 1999. HLA class II polymorphism in cystic fibrosis. A possible modifier of pulmonary phenotype. *Am J Respir Crit Care Med* **159**:1464–1468.

Badano, J. L. and N. Katsanis. 2002. Beyond Mendel: an evolving view of human genetic disease transmission. *Nat Rev Genet* **3**:779–789.

Baird, P. A. et al. 1988. Genetic disorders in children and young adults: a population study. *Am J Hum Genet* **42**:677–693.

Bakker, S. C. et al. 2003. A whole-genome scan in 164 Dutch sib pairs with attention-deficit/hyperactivity disorder: suggestive evidence for linkage on chromosomes 7p and 15q. *Am J Hum Genet* **72**:1251–1260.

Beales, P. L. et al. 2003. Genetic interaction of *BBS1* mutations with alleles at other *BBS* loci can result in non-Mendelian Bardet-Biedl syndrome. *Am J Hum Genet* **72**:1187–1199.

Beutler, E. 2001. Discrepancies between genotype and phenotype in hematology: an important frontier. *Blood* **98**:2597–2602.

Boehnke, M. and C. D. Langefeld. 1998. Genetic association mapping based on discordant sib pairs: the discordant-alleles test. *Am J Hum Genet* **62**:950–961.

Botstein, D. and N. Risch. 2003. Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nat Genet* **33** Suppl:228–237.

Brown, M. A., A. M. Crane, and B. P. Wordsworth. 2002. Genetic aspects of susceptibility, severity, and clinical expression in ankylosing spondylitis. *Curr Opin Rheumatol* **14**:354–360.

Brown, M. A. et al. 2000. Recurrence risk modelling of the genetic susceptibility to ankylosing spondylitis. *Ann Rheum Dis* **59**: 883–886.

Buckland, P. R. 2001. Genetic association studies of alcoholism—problems with the candidate gene approach. *Alcohol Alcohol* **36**:99–103.

Bulik, C. M. et al. 2000. Twin studies of eating disorders: a review. *Int J Eat Disord* **27**:1–20.

Cardno, A. G. and I. I. Gottesman. 2000. Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. *Am J Med Genet* **97**:12–17.

Cardon, L. R. and J. I. Bell. 2001. Association study designs for complex diseases. *Nat Rev Genet* **2**:91–99.

Daly, M. J. et al. 2001. High-resolution haplotype structure in the human genome. *Nat Genet* **29**:229–232.

del Castillo, I. et al. 2002. A deletion involving the connexin 30 gene in nonsyndromic hearing impairment. *N Engl J Med* **346**:243–249.

Dipple, K. M. and E. R. McCabe. 2000. Modifier genes convert "simple" Mendelian disorders to complex traits. *Mol Genet Metab* **71**:43–50.

Dipple, K. M. and E. R. McCabe. 2000. Phenotypes of patients with "simple" Mendelian disorders are complex traits: thresholds, modifiers, and systems dynamics. *Am J Hum Genet* **66**:1729–1735.

Ewens, W. J. and R. S. Spielman. 2001. Locating genes by linkage and association. *Theor Popul Biol* **60**:135–139.

Ewens, W. J. and R. S. Spielman. 2001. The transmission/disequilibrium test. In: Balding, D. J., M. Bishop, and C. Cannings (eds.) *Handbook of Statistical Genetics*. John Wiley & Sons, New York, pp. 507–518.

Fain, P. R. et al. 2003. A genomewide screen for generalized vitiligo: confirmation of *AIS1* on chromosome 1p31 and evidence for additional susceptibility loci. *Am J Hum Genet* **72**:1560–1564.

Feingold, E. 2001. Methods for linkage analysis of quantitative trait loci in humans. *Theor Popul Biol* **60**:167–180.

Feingold, E. 2002. Regression-based quantitative-trait-locus mapping in the 21st century. *Am J Hum Genet* **71**:217–222.

Fullerton, J. et al. 2003. Linkage analysis of extremely discordant and concordant

sibling pairs identifies quantitative-trait loci that influence variation in the human personality trait neuroticism. *Am J Hum Genet* **72**:879–890.

Gauderman, W. J., J. S. Witte, and D. C. Thomas. 1999. Family-based association studies. *J Natl Cancer Inst Monogr* **26**:31–37.

Glazier, A. M., J. H. Nadeau, and T. J. Aitman. 2002. Finding genes that underlie complex traits. *Science* **298**:2345–2349.

Goldstein, D. B. 2001. Islands of linkage disequilibrium. *Nat Genet* **29**:109–111.

Grasemann, H. et al. 2000. Airway nitric oxide levels in cystic fibrosis patients are related to a polymorphism in the neuronal nitric oxide synthase gene. *Am J Respir Crit Care Med* **162**:2172–2176.

Grasemann, H. et al. 2003. Endothelial nitric oxide synthase variants in cystic fibrosis lung disease. *Am J Respir Crit Care Med* **167**:390–394.

Guo, S. W. 1998. Inflation of sibling recurrence-risk ratio, due to ascertainment bias and/or overreporting. *Am J Hum Genet* **63**:252–258.

Hoggart, C. J. et al. 2003. Control of confounding of genetic associations in stratified populations. *Am J Hum Genet* **72**: 1492–1504.

Holmans, P. 2001. Nonparametric Linkage. In: Balding, D. J., M. Bishop, and C. Cannings (eds.) *Handbook of Statistical Genetics*. Johns Wiley & Sons, New York, pp. 487–505.

Huang, J. and Y. Jiang. 2003. Genetic linkage analysis of a dichotomous trait incorporating a tightly linked quantitative trait in affected sib pairs. *Am J Hum Genet* **72**:949–960.

International Psoriasis Genetics Study. 2003. The International Psoriasis Genetics Study: Assessing linkage to 14 candidate susceptibility loci in a cohort of 942 affected sib pairs. *Am J Hum Genet* **73**: 430–437.

Johnson, G. C. et al. 2001. Haplotype tagging for the identification of common disease genes. *Nat Genet* **29**:233–237.

Katsanis, N. et al. 2001. Triallelic inheritance in Bardet-Biedl syndrome, a Mendelian recessive disorder. *Science* **293**: 2256–2259.

King, R. A. Oculocutaneous Albinism Type 1. In: *GeneReviews* at GeneTests: Medical Genetics Information Resource (database online.) Copyright, University of Washington, Seattle. 1997–2003. Available at http://www.genetests.org. Accessed June 2003.

Lerer, I. et al. 2001. A deletion mutation in *GJB6* cooperating with a *GJB2* mutation in trans in non-syndromic deafness: A novel founder mutation in Ashkenazi Jews. *Hum Mutat* **18**:460.

Lesch, K. P. et al. 1996. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. *Science* **274**:1527–1531.

Mackintosh, N. J. 1998. *IQ and Human Intelligence.* Oxford University Press, Oxford, U.K.

Martin, M. J. et al. 1986. Serum cholesterol, blood pressure, and mortality: implications from a cohort of 361,662 men. *Lancet* **2**:933–936.

Matise, T. C. et al. 2003. A 3.9-centimorgan-resolution human single-nucleotide polymorphism linkage map and screening set. *Am J Hum Genet* **73**:271–284.

McKone, E. F. et al. 2003. Effect of genotype on phenotype and mortality in cystic fibrosis: a retrospective cohort study. *Lancet* **361**:1671–1676.

Mehta, A., P. J. Mason, and T. J. Vulliamy. 2000. Glucose-6-phosphate dehydrogenase deficiency. *Baillieres Best Pract Res Clin Haematol* **13**:21–38.

Mekus, F. et al. 2003. Genes in the vicinity of *CFTR* modulate the cystic fibrosis phenotype in highly concordant or discordant F508del homozygous sib pairs. *Hum Genet* **112**:1–11.

Merlo, C. A. and M. P. Boyle. 2003. Modifier genes in cystic fibrosis lung disease. *J Lab Clin Med* **141**:237–241.

Ming, J. E. and M. Muenke. 2002. Multiple hits during early embryonic development: digenic diseases and holoprosencephaly. *Am J Hum Genet* **71**: 1017–1032.

Nadeau, J. H. 2001. Modifier genes in mice and humans. *Nat Rev Genet* **2**:165–174.

National Institute of Mental Health Genetics Workgroup. 1997. *Genetics and Mental Disorders.* National Institute of Mental Health, Washington, DC. Available at http://www.nimh.nih.gov/researcg/genetics.htm. Accessed July 2003.

Nazarov, I. B. et al. 2001. The angiotensin converting enzyme I/D polymorphism in Russian athletes. *Eur J Hum Genet* **9**: 797–801.

Neisser, U. et al. 1996. Intelligence: Knowns and unknowns. *Am Psychologist* **51**:77–101.

Ogdie, M. N. et al. 2003. A genomewide scan for attention-deficit/hyperactivity disorder in an extended sample: suggestive linkage on 17p11. *Am J Hum Genet* **72**: 1268–1279.

Olson, J. M. et al. 2001. The heritability of attitudes: a study of twins. *J Pers Soc Psychol* **80**:845–860.

Plomin, R. et al. 2003. *Behavioral Genetics in the Postgenomic Era*. American Psychological Association, Washington, DC.

Plomin, R. et al. 2001. A genome-wide scan of 1842 DNA markers for allelic associations with general cognitive ability: a five-stage design using DNA pooling and extreme selected groups. *Behav Genet* **31**: 497–509.

Plomin, R. and F. M. Spinath. 2002. Genetics and general cognitive ability (*g*). *Trends Cogn Sci* **6**:169–176.

Rao, D. C. and M. A. Province (eds.). 2001. *Genetic Dissection of Complex Traits.* Advances in Genetics, Vol 42. Academic Press, San Diego, CA.

Reich, D. E. and D. B. Goldstein. 2001. Detecting association in a case-control study while correcting for population stratification. *Genet Epidemiol* **20**:4–16.

Rhee, S. H. and I. D. Waldman. 2002. Genetic and environmental influences on antisocial behavior: a meta-analysis of twin and adoption studies. *Psychol Bull* **128**: 490–529.

Riazuddin, S. et al. 2000. Dominant modifier DFNM1 suppresses recessive deafness *DFNB26*. *Nat Genet* **26**:431–434.

Rietveld, M. J. et al. 2003. Heritability of attention problems in children: I. cross-sectional results from a study of twins, age 3–12 years. *Am J Med Genet* **117B**:102–113.

Risch, N. 1990. Linkage strategies for genetically complex traits. I. Multilocus models. *Am J Hum Genet* **46**:222–228.

Risch, N. 2001. The genetic epidemiology of cancer: Interpreting family and twin studies and their implications for molecular genetic approaches. *Cancer Epidemiol Biomarkers Prev* **10**:733–741.

Risch, N. J. 2000. Searching for genetic determinants in the new millennium. *Nature* **405**:847–856.

Rivolta, C. et al. 2002. Retinitis pigmentosa and allied diseases: numerous diseases,

genes, and inheritance patterns. *Hum Mol Genet* **11**:1219–1227.

Rovers, M. et al. 2002. Heritability of symptom domains in otitis media: a longitudinal study of 1,373 twin pairs. *Am J Epidemiol* **155**:958–964.

Schaid, D. J. 1998. Transmission disequilibrium, family controls, and great expectations. *Am J Hum Genet* **63**:935–941.

Schechter, M. S. et al. 2001. The association of socioeconomic status with outcomes in cystic fibrosis patients in the United States. *Am J Respir Crit Care Med* **163**: 1331–1337.

Scriver, C. R. and P. J. Waters. 1999. Monogenic traits are not simple: lessons from phenylketonuria. *Trends Genet* **15**: 267–272.

Snedecor, G. W. and W. G. Cochran. 1980. *Statistical Methods.* 7th ed. Iowa State University Press, Ames, IA.

Spector, T. D., H. Snieder, and A. J. MacGregor (eds.). 2000. *Advances in Twin and Sib-Pair Analysis*. Greenwich Medical Media, London.

Tabor, H. K., N. J. Risch, and R. M. Myers. 2002. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet* **3**: 391–397.

Tait, J. F. et al. Cystic fibrosis In: *GeneReviews* at GeneTests: Medical Genetics Information Resource (database online.) Copyright, University of Washington, Seattle. 1997–2003. Available at http://www.genetests.org. Accessed June 2003.

Thomas, D. C. and J. S. Witte. 2002. Population stratification: a problem for case-control studies of candidate-gene associations? *Cancer Epidemiol Biomarkers Prev* **11**:505–512.

Vankeerberghen, A., H. Cuppens, and J.-J. Cassiman. 2002. The cystic fibrosis transmembrane conductance regulator: an intriguing protein with pleiotropic functions. *J Cystic Fibrosis* **1**:13–29.

Weatherall, D. J. 2001. Phenotype-genotype relationships in monogenic disease: lessons from the thalassemias. *Nat Rev Genet* **2**:245–255.

Weiss, K. M. and A. G. Clark. 2002. Linkage disequilibrium and the mapping of complex human traits. *Trends Genet* **18**:19–24.

Wright, A. F., A. D. Carothers, and M. Pirastu. 1999. Population choice in mapping genes for complex diseases. *Nat Genet* **23**:397–404.

Zielenski, J. et al. 1999. Detection of a cystic fibrosis modifier locus for meconium ileus on human chromosome 19q13. *Nat Genet* **22**:128–129.

review questions

1. What is multifactorial inheritance?
2. Account for polygenic dichotomous traits.
3. What is a meristic trait?
4. Why are monogenic disorders not as simple as the term "monogenic" implies?
5. What is favism? What is its relevance to genetics?
6. What is a null mutation? A hypomorph?
7. How were digenic systems discovered?
8. Discuss how digenic systems might function.
9. What is a sibling risk ratio? Describe how familial risk ratios are used to determine whether a complex trait has a genetic component.
10. What are the differences between MZ and DZ twins? What is the relevance of these differences for genetic studies?
11. Based on the following data sets, what are the concordances for the MZ and DZ twins?

MZ twins		DZ twins	
Twin 1	Twin 2	Twin 1	Twin 2
+	+	−	+
+	+	+	+
+	−	−	−
+	+	−	−
−	+	+	−
−	−	−	+
+	+	+	+
−	+	−	+
+	+	+	−
−	−	−	+

Note: +, affected; −, unaffected.

12. What is probandwise concordance? When and why is it used in genetic studies?

13. Based on the information in Table 10.8, discuss the biological basis of Huntington disease, epilepsy, and alcoholism.

14. Based on the following data, what are the intraclass correlation coefficients for the MZ and DZ twins? Discuss what you conclude from these results? Assume that these data are representative of much larger samples.

MZ twins		DZ twins	
Twin 1	Twin 2	Twin 1	Twin 2
105	110	101	121
96	89	96	78
94	101	98	126
78	82	104	84
110	96	112	94
121	129	91	110
101	111	78	94
119	126	86	71
101	89	134	112
84	96	106	109

15. What is heritability?

16. What are the heritability estimates for height, neuroticism, and attention-deficit/hyperactivity disorder? See Table 10.9.

17. Based on the data in the following table, describe the likely sources that contribute to the total phenotypic variance for each trait.

	r_{MZ}	r_{DZ}
Trait 1	0.41	0.45
Trait 2	0.31	0.65
Trait 3	0.62	0.10

17. What is population stratification? What is its relevance for case-control association studies?

18. Discuss the essential features of the TDT.

19. Distinguish between IBD and IBS. Discuss the essential features of ASP linkage analysis.

20. What is HapMap and its significance for the identification of QTLs? (See www.genome.gov and www.hapmap.org.)

chapter 11

Genomic Imprinting: An Epigenetic Modification

No rule is so general, which admits not some exception.

ROBERT BURTON (1577–1640)

In judging of a matter that is not self-evident the wise should be governed by the principle of greatest probability.

CICERO (106–43 BCE)

Parent-of-Origin Effect

One of the tenets of Mendelian genetics is that reciprocal crosses with autosomal loci produce the same ratios and phenotypes among the offspring. For example, parents who are each homozygous for different autosomal alleles have only heterozygous offspring with the same phenotype regardless of the type of dominance (Figure 11.1). An indication of a non-Mendelian process is the consistent lack of phenotypic equivalence from reciprocal matings. Such a situation may arise when an allele from one parent always determines the phenotype and the other allele, although present and not mutated, is not expressed (Figure 11.2A and B). In these instances, either the paternal (Figure 11.2A) or the maternal (Figure 11.2B) allele at a particular locus is functional and the other allele derived from the mother or father, respectively, is repressed (silenced, inactivated), or, at least, downregulated. Theoretically, some X-linked loci may also show a parent-of-origin pattern of inheritance (Figure 11.2C and D). In Figure 11.2C, the allele at locus *C* from the maternal parent is inactivated and the one derived from the paternal parent is functional. Because of hemizygosity, an inactivated essential gene inherited by a male may cause an abnormality or embryonic lethality. By contrast, in Figure 11.2D, an allele at locus *D* is maternally expressed and paternally inactivated. In each of these four examples of non-Mendelian inheritance, the activity of a particular allele is predicated on the sex of the parent.

The process that leads to the expression of an allele that is inherited from one parent and the inactivation of its counterpart in the other parent is an epigenetic phenomenon called genomic imprinting. Epigenetics is the study of heritable traits from parent to offspring that are not the result of DNA mutation. The two salient features of genomic imprinting are heritability and

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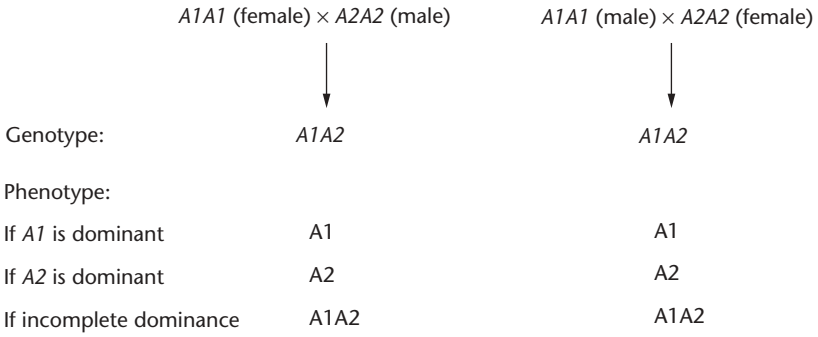

Figure 11.1 Phenotypic equivalence of a reciprocal cross with an autosomal locus. The same phenotype occurs among the offspring regardless of the parent of origin of an allele and the type of dominance.

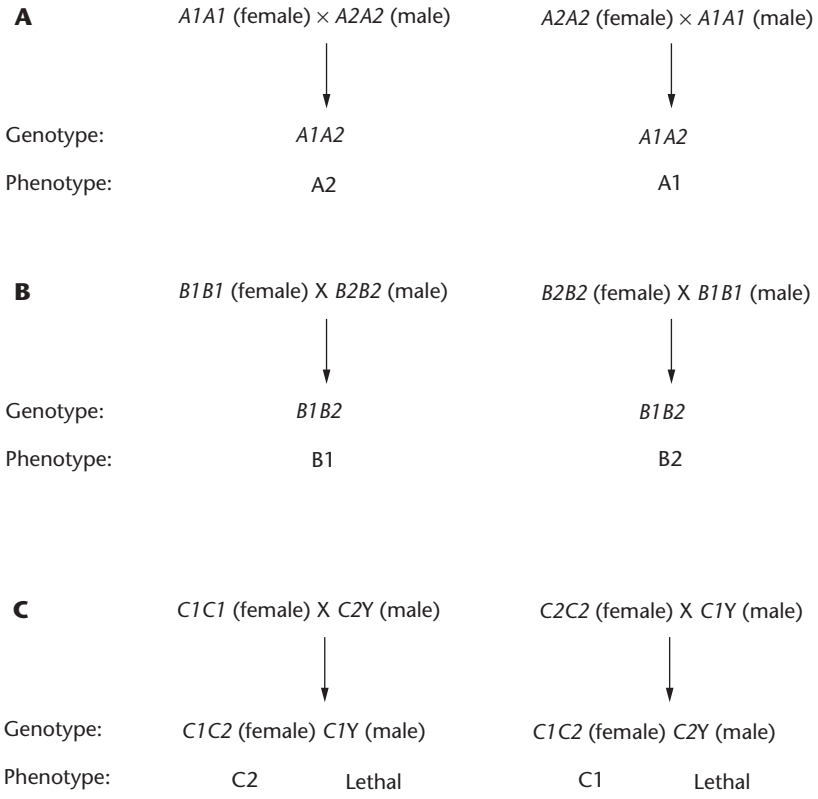

Figure 11.2 Parent-of-origin effect for autosomal and X-linked alleles. The phenotypes among the offspring are determined by an autosomal allele inherited from a male (A) or female (B) parent and by an X-linked allele from a male (C) or female (D) parent. A lethal condition is likely to occur when an inactivated essential X-linked gene is inherited by a male (C).

reversibility. In this context, heritability signifies the biological transmission of either an active or an inactive allele from a parent to an offspring. Reversibility denotes the ability of the transcriptional status of an allele to be wiped clean during each generation before the establishment of a new imprint that is determined by the sex of the parent.

There are about 50 known loci, and possibly as many as 200, where the parent-of-origin specifies whether an allele is transcribed or inactivated in human somatic cells. Many of the loci that are subject to genomic imprinting occur in clusters. Often, such a cluster has some genes that are paternally expressed and maternally silenced and others that are maternally expressed and paternally silenced. In some cases, an activated allele is expressed in all somatic cells. For other loci, transcription of the active allele occurs in a specific tissue or cell type. And, in some instances, only one allele is transcribed (monoallelic expression) in a particular tissue, whereas both are active (biallelic expression) in other cell types.

A comment about terminology is necessary at this point. Generally, the terms "imprinted gene," "genomic imprint," or "imprint" refer only to the allele that has been silenced. The active allele is assumed to occur by default. A broader approach is adopted here. An imprinted gene is one that is either active or inactive depending on its parent of origin. The combined, and somewhat cumbersome, descriptions maternally expressed, paternally silenced and paternally expressed, maternally silenced will be used occasionally to make both the parent of origin and the transcriptional status of a pair of alleles explicit. However, if one allele is said to be paternally expressed, then it is understood that the other allele is maternally silenced, and vice versa.

Gene Silencing

Genomic imprinting is a cyclical process that normally endures for only one generation (Figure 11.3). Both imprints from the previous generation are erased in primordial germ cells and replaced with a paternal and a maternal imprint during spermatogenesis, and oogenesis, respectively. The imprint of each allele at a locus is usually maintained through all somatic cell divisions following fertilization. If an active imprinted allele is not transcribed in all cell types, it is turned on at the appropriate time and cellular site. The specificity of the parent-of-origin effect remains the same from generation to generation. In other words, a paternally silenced allele is always inactivated during spermatogenesis and a maternally silenced allele during oogenesis.

Very little is known about the mechanisms of erasing, establishing, maintaining, and reading imprints. However, studies of the molecular differences between pairs of oppositely imprinted alleles have identified some gamete-specific processes that contribute to the regulation of individual alleles. In particular, attention has focused on DNA methylation as the principal means of parent-of-origin allele silencing and, less often, activation. Specifically, DNA methylation entails the addition of a methyl (CH_3) group to the 5-position of a cytosine residue in DNA that is immediately 5′ to a guanine residue, that is, the cytosine of a CpG dinucleotide is methylated (Figure 11.4). DNA methylation is established and maintained by DNA methyltransferases. Generally,

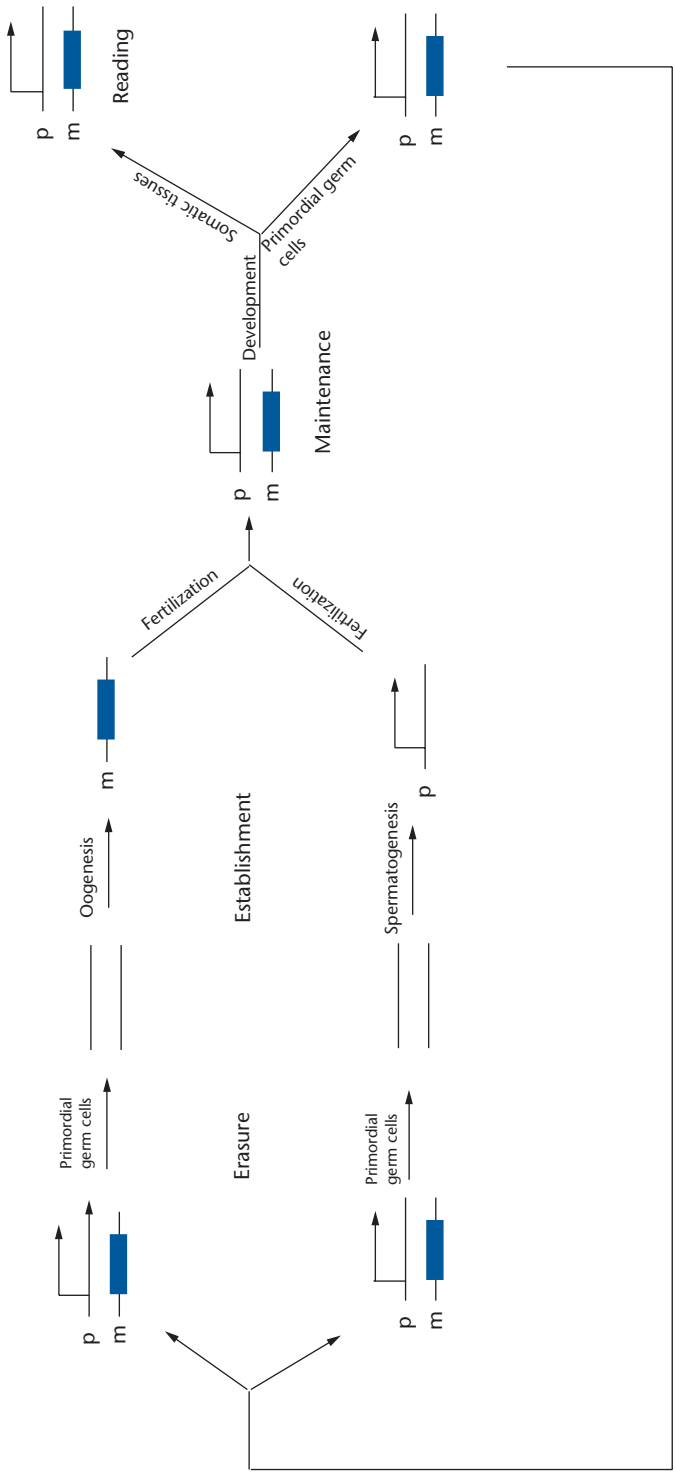

Figure 11.3 Genomic imprinting of a locus with paternally expressed, maternally silenced alleles during human sex cell formation and somatic cell development. A right-angled arrow represents a transcriptionally active allele, a solid rectangle represents a transcriptionally inactive allele, and horizontal lines are chromosomes without an established imprint. The letters p and m denote paternally and maternally derived chromosomes.

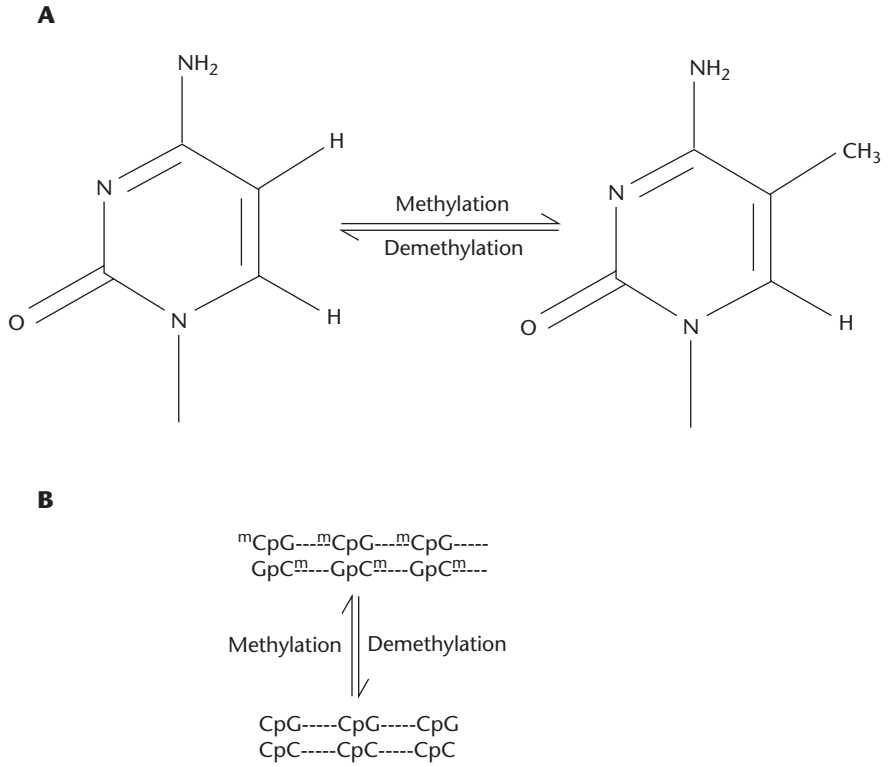

Figure 11.4 Methylation and demethylation of cytosine (A) and CpG dinucleotides (B) in DNA. CH_3, methyl group; ^mC, 5-methylcytosine.

the extent of DNA methylation is inversely correlated with gene activity. The regions of CpG repeats (CpG islands) that precede commonly expressed genes (housekeeping genes) are not methylated. On the other hand, genes that are transcriptionally inactive are frequently heavily methylated (hypermethylated).

During early mammalian development, the level of methylation of genomic DNA undergoes dramatic changes. After the zygote is formed, almost all of the chromosomal DNA is demethylated. As development proceeds, both general and gene-specific DNA methylation is restored. By the time somatic cell differentiation is complete, the overall patterns of DNA methylation are established and, subsequently, maintained through ensuing cell division cycles. It is not known how the extent of methylation of different genes is determined. And, despite the well-documented relationship between DNA methylation and repression, some genes require methylation to be active.

DNA methylation can affect gene transcription either directly or indirectly. Briefly, a methylated promoter region may block transcription by preventing the binding of transcription factors. Or the DNA methyl groups may bind a protein that, in turn, binds other proteins that change the conformation of the chromosome and make the promoter region inaccessible to transcription. On the other hand, DNA methylation could enable gene expression if the methyl groups are required for the binding of specific transcription factors or if methylation prevents repressor molecules from binding to a promoter region.

Among methylated, nonimprintable genes, the same pattern of methylation is maintained in both alleles in somatic cells. On the other hand, it is common for a parent-of-origin silenced allele to be more heavily methylated than the

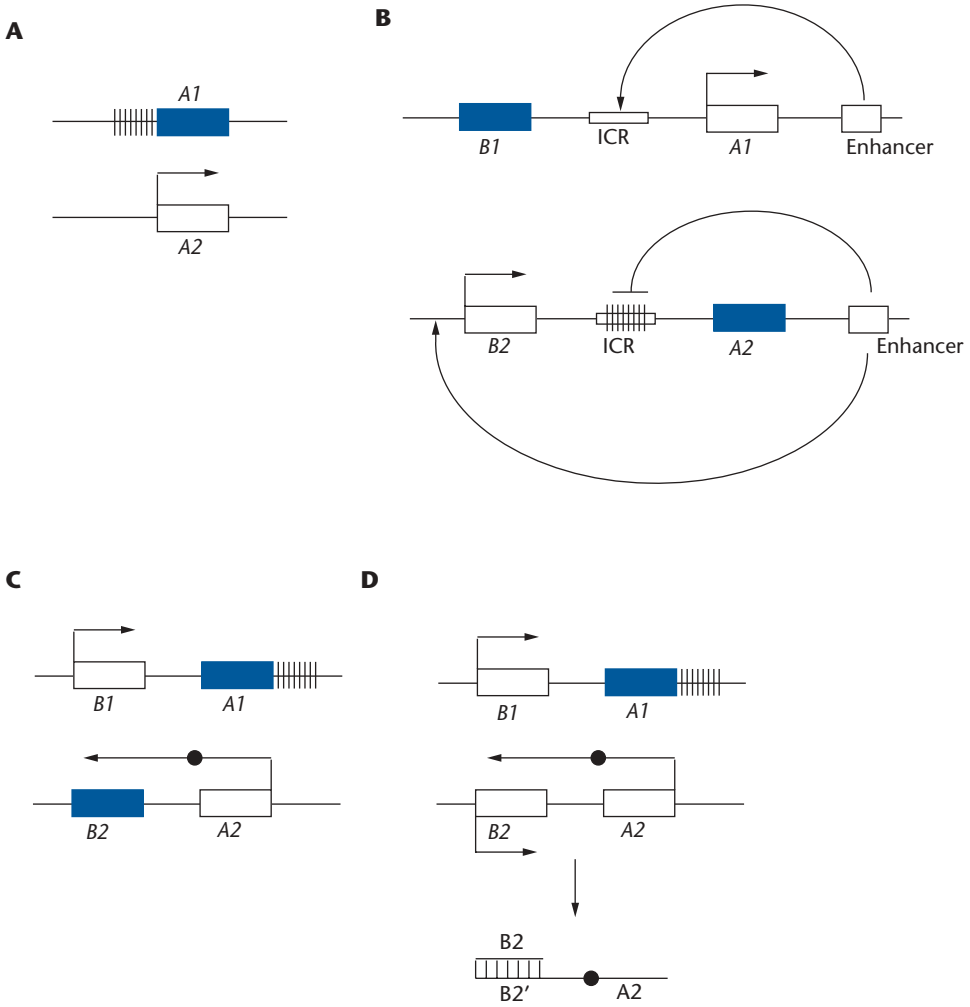

Figure 11.5 Models for silencing imprinted genes. Horizontal lines represent chromosomal DNA, closely spaced vertical lines methylated DNA, solid rectangle transcriptionally inactive gene, and open rectangle with right-angled arrow transcriptionally active gene. (A) On one chromosome, allele-specific methylation silences the *A1* allele. On the homologous chromosome, the nonmethylated *A2* allele is transcribed. (B) On one chromosome, the binding of an enhancer DNA element to an imprinting control region (ICR) facilitates the transcription of the *A1* allele, and without an available DNA enhancer element(s) the *B1* allele is silenced. On the other chromosome, the *A2* allele is silenced because the ICR is methylated and the enhancer DNA element is free to activate allele *B2* because it cannot bind to the ICR. (C) On one chromosome, DNA methylation prevents the transcription of the *A1* allele and the nonmethylated *B1* allele is transcribed. On the homologous chromosome, the nonmethylated *A2* allele is transcribed. This transcription proceeds through the *B2* allele and prevents its transcription. An internal stop codon (●) enables production of the A2 protein. (D) On one chromosome, DNA methylation prevents the transcription of the *A1* allele and the nonmethylated *B1* allele is transcribed. On the homologous chromosome, the nonmethylated *A2* allele is transcribed. This transcription proceeds through the coding strand of the *B2* allele but does not interfere with transcription from the noncoding strand of this allele. The antisense RNA sequence (B2′) from transcriptional read-through of the *B2* allele hybridizes with its complementary sequence (B2) and prevents transcription. An internal stop codon (●) enables production of the A2 protein.

allele that is expressed. As already noted, the basis of gametogenesis-specific DNA methylation is a mystery. Clearly, these methylated imprints either evade the global demethylation that follows fertilization or they are erased and re-established afterwards. The simplest mechanism for a silencing an imprintable allele would be methylation during either spermatogenesis or oogenesis of a promoter region that directly blocks access of RNA polymerase and other members of the transcription complex (Figure 11.5A). A more complicated

scheme for imprinted gene silencing requires enhancer DNA elements and DNA methylation of a discrete small regulatory sequence called the imprinting control region (ICR). Enhancer DNA sequences are located a considerable distance either upstream or downstream from their target DNA sequences, and when these sequences combine, transcription of a gene is facilitated. The interaction of an enhancer DNA element(s) and an ICR causes the transcription of a nearby gene while a more distant gene remains silent because the enhancer elements are not available to expedite its transcription. If the ICR is methylated, then the enhancer DNA element(s) cannot bind to it, transcription of the nearby gene is blocked, and the enhancer DNA element is free to initiate transcription of the second gene (Figure 11.5B).

Monoallelic silencing may occur independently of DNA methylation. When transcription of the noncoding strand of one gene proceeds through the coding strand of a second gene, either the initiation or the elongation of transcription from the noncoding strand of the second gene is curtailed (Figure 11.5C). Or if RNA molecules are synthesized from both the noncoding (sense) and coding (antisense) strands of the same gene, these complementary sequences can base pair and prevent translation (Figure 11.5D). An internal stop codon ensures that a functional protein is produced from an extended mRNA.

Genomic Imprinting and Human Disease

The biological significance of genomic imprinting is not apparent. Most imprintable genes affect fetal growth, nutrient transfer through the placenta, cell proliferation, or brain development. Generally, the genes that are paternally expressed promote growth and those that are maternally expressed constrain cell proliferation. Thus, genomic imprinting may equalize these conflicting tendencies. Moreover, mutations of imprinted genes would be expected to have an impact on these processes.

In practice, genomic imprinting creates a hemizygous condition. Consequently, when a chromosome with either a mutated or a deleted imprintable gene is inherited from the parent that ensures expression of this allele, no functional gene product is produced because the active allele is mutated and the one from the other parent is silenced (Figure 11.6A and B), whereas a mutated allele that is silenced has no effect (Figure 11.6A1 and B1). A disease phenotype is likely to occur when no functional gene product is synthesized.

The pattern of inheritance of a mutated imprinted gene in a multigeneration pedigree is determined by the parent that generates the active gene. With a paternally expressed, maternally silenced locus, the offspring are only affected when the mutated or deleted imprinted gene is transmitted through the male germ line (Figure 11.7A). Alternatively, for a maternally expressed, paternally silenced locus, the disorder is passed on exclusively by mothers (Figure 11.7B).

Chromosomes are not always distributed properly during meiosis. Nondisjunction of a whole chromosome can occur during either meiosis I or II. In either case, a gamete may carry an extra chromosome and, after fertilization, a trisomic zygote is formed (Figure 11.8). In humans, autosomal trisomy is lethal. Occasionally, one of the chromosomes of a trisomy is lost during the initial embryonic cell divisions and, as a consequence, development proceeds normally. This form of restoring disomy is called trisomy rescue. One-third of the

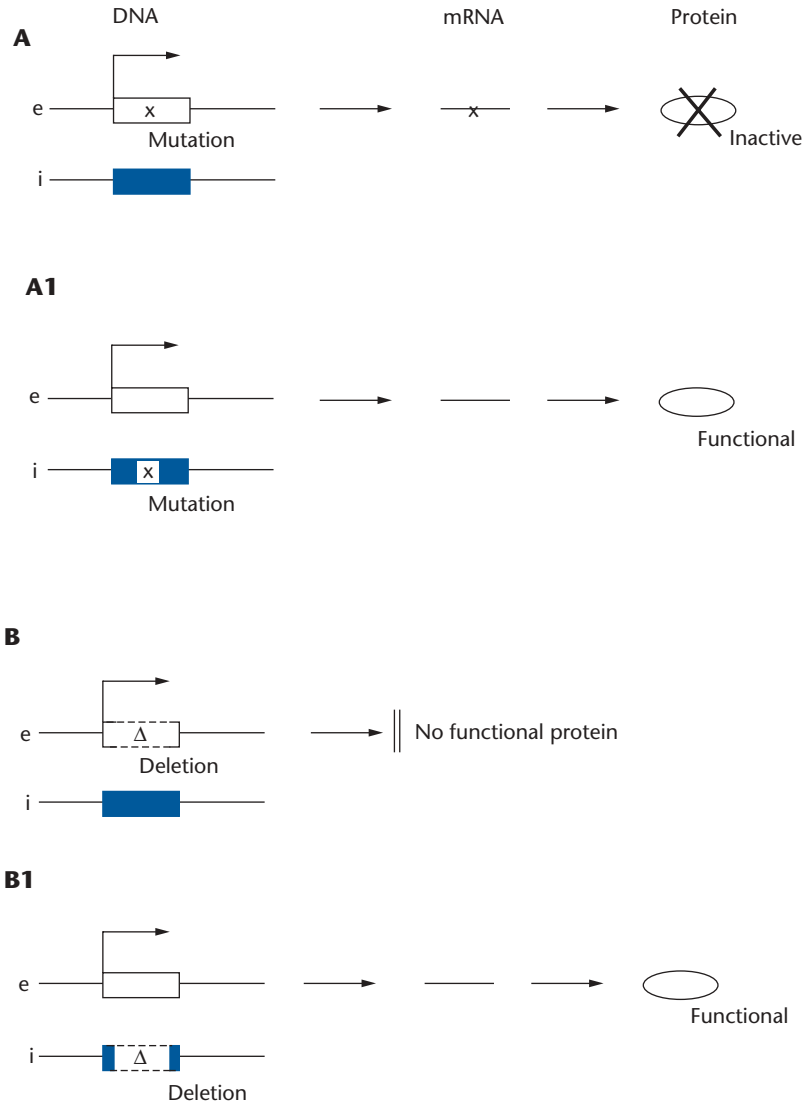

Figure 11.6 Consequences of a mutation (A) and a deletion (B) of an imprinted gene. Chromosomes with an expressed (e) or inactive (i) allele are shown. Open rectangle with a right-angled arrow and solid rectangle represent transcriptionally active and inactive alleles, respectively. The symbols x and Δ denote a mutation and a deletion, respectively. In B, the reading frame of the active allele is disrupted by a deletion (Δ) and translatable mRNA is not produced.

time the two "rescued" chromosomes are from the same parent, which is called uniparental disomy (UPD). There are two types of uniparental disomies. Heterodisomy (Figure 11.8A) and isodisomy (Figure 11.8B) represent two nonidentical and two identical chromosomes from the same parent, respectively. The parental origin and nature of a uniparental disomy can be determined by polymorphic marker haplotyping. If UPD chromosomes carry silenced imprinted genes, then no functional gene product is formed and some developmental disorder is likely to occur. Alternatively, if UPD chromosomes have two sets of active imprinted genes, then overproduction of these products may upset developmental processes.

Prader–Willi Syndrome

The Prader–Willi syndrome (PWS) is a rare neurodevelopmental disorder with a prevalence of about 1 in 12,500 that affects both sexes. Newborns and infants

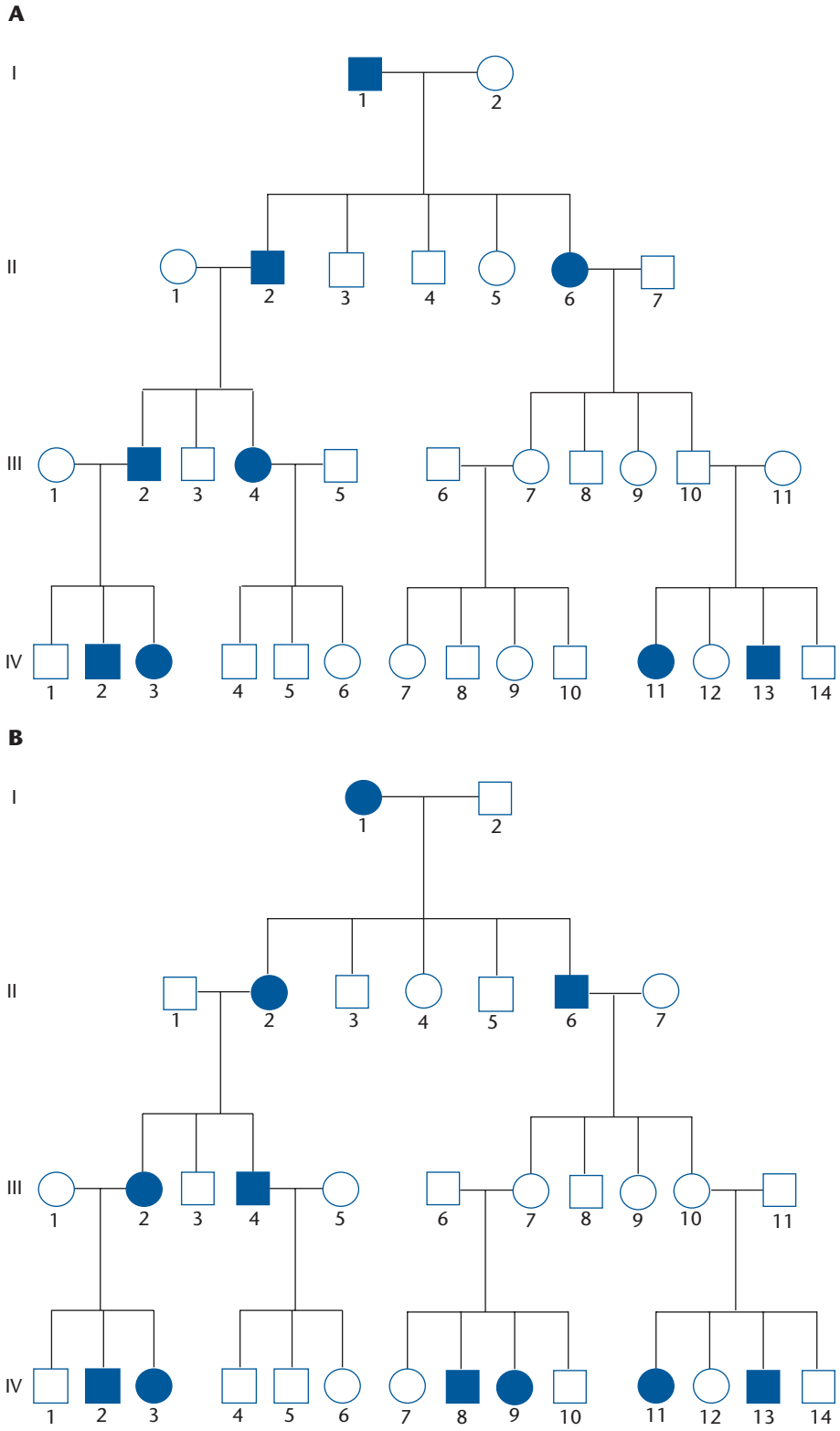

Figure 11.7 Pedigrees with a disease-causing mutation of a paternally expressed, maternally silenced (A) and a maternally expressed, paternally silenced (B) locus, respectively. Solid symbols represent affected individuals.

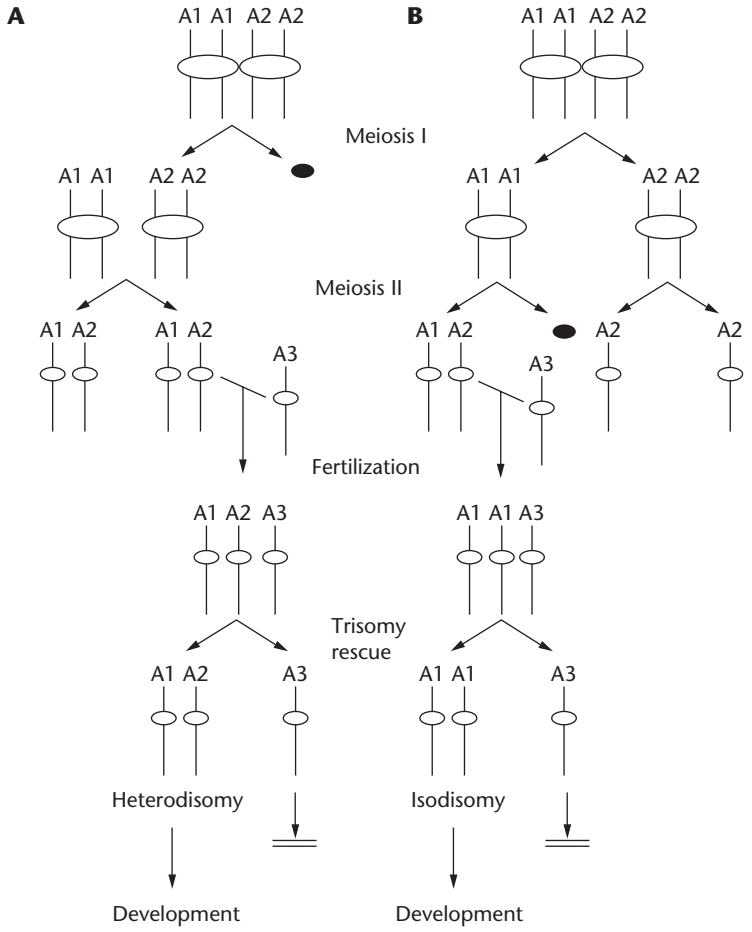

Figure 11.8 Uniparental disomy. Nondisjunction at either meiosis I (A) or II (B) and the formation of heterodisomy and isodisomy after trisomy rescue are depicted schematically. Chromosomes A1 and A2 are from one parent and A3 from the other parent. The solid ovals represent meiotic cells that do not receive the chromosomes that are being considered. A downward arrow that points to two parallel horizontal lines denotes embryonic lethality due to monosomy. Trisomy rescue is a random process, and the probability that two chromosomes from the same parent will segregate together is 0.33.

have reduced muscle tone (hypotonia) that often prevents proper sucking, and, as a result, there is a failure to thrive unless forced feeding is implemented. From 1 to 6 years of age, weight gain is exceedingly rapid, leading to extreme obesity. Developmental motor skills are delayed. During adolescence and adulthood, dietary intake must be stringently controlled; otherwise, it can be excessive. Stealing and hoarding of food are common behaviors. Infertility results from underdeveloped sex organs. Learning disabilities are mild to moderate. Early death by 30 years of age often occurs from obesity and related complications.

About 70% of the cases of PWS have a 3- to 5-Mb deletion of 15q11–q13. In these cases, the deleted chromosome is invariably inherited from the father. Another 25% of PWS patients have maternal uniparental disomy for chromosome 15 [upd(15)mat]. Both of these genetic features indicate that there is one or more imprinted gene within the 15q11–q13 region and PWS occurs when no functional product(s) from a paternally expressed gene(s) is formed (Figure 11.9A and B).

There are at least nine paternally expressed, maternally silenced genes and one with the opposite parent-of-origin effect in the 15q11–q13 region. In addition to large deletions and maternal uniparental disomy, a number of PWS

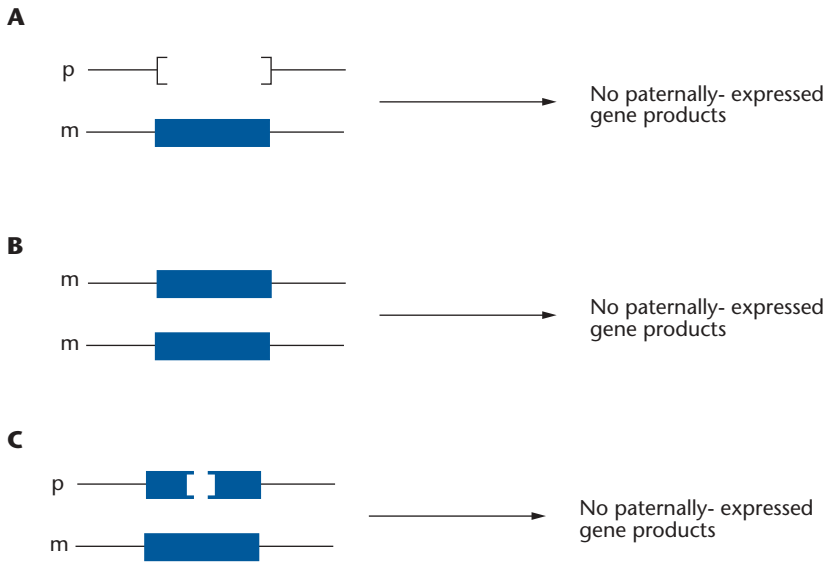

Figure 11.9 Chromosome constitutions that are frequently associated with Prader–Willi syndrome. (A) The paternal (p) chromosome has a large deletion (bracketed gap) that removes the 15q11–q13 region and a maternally derived chromosome (m) with silenced genes (solid rectangle) from the 15q11–q13 region. (B) Maternal uniparental disomy of chromosome 15. Both maternal chromosomes (m) carry silenced genes from the 15q11–q13 region. (C) Microdeletion (bracketed gap) within the 15q11–q13 region of a paternal chromosome causes silencing of genes during spermatogenesis that otherwise would be expressed and a maternally derived chromosome (m) with silenced genes (solid rectangle) from the 15q11–q13 region. In each of these cases, no functional products are synthesized.

patients have small deletions (microdeletions) within a restricted section of the 15q11–q13 cluster that affects both transcription and methylation of distant imprintable loci on the same chromosome. These microdeletions demarcate an imprinting control region (ICR; imprinting center, IC) that is required for establishing the paternal imprint. Thus, the PWS-ICR mutations silence genes that normally would have been activated during spermatogenesis (Figure 11.9C). It is not known which paternally expressed gene(s) is responsible for PWS.

Angelman Syndrome

Interestingly, the loss of all activity of the only maternally expressed, paternally silenced gene (*UBE3A*) of the 15q11–q13 cluster causes Angelman syndrome (AS), which is phenotypically distinct from PWS. The clinical features of AS include severe developmental delay, mental retardation, awkward gait, tremors, seizures, protruding tongue, absence of speech, frequent uncontrolled outbursts of laughter, and a happy disposition. The disorder becomes clinically evident from 12 to 24 months after birth. The life span is normal if many medications are administered.

The *UBE3A* gene encodes ubiquitin protein ligase 3A that is monoallelically expressed only in the brain. This enzyme adds the protein ubiquitin to intracellular proteins, which marks them for degradation. The prevalence of AS is about 1 in 16,000. About 70% of the cases of AS have a maternal chromosome with a deleted *UBE3A* gene, 7% paternal uniparental disomy of chromosome 15 [upd(15)pat], 4% mutations of the imprinting center that silence the *UBE3A* gene during oogenesis, and 5% point mutations in the *UBE3A* gene. Functional ubiquitin ligase 3A is not produced under any of these conditions. It is not understood why the absence of this enzyme causes AS.

from the HUMAN GENETICS files

X-Chromosome Inactivation

The inactivation of one of the X chromosomes in human females is a mechanism for equalizing X-linked gene expression between XX and XY chromosome constitutions. The choice of which X-chromosome is inactivated is usually random. The silencing of a set of X-linked genes requires expression of the *XIST* (X inactive specific transcript) gene at Xq13.2 that is initiated in 5- to 10-cell embryos. Shortly thereafter, transcription of the *XIST* gene is confined to the inactive X chromosome (Xi) and not the active homolog (Xa). The product of the *XIST* gene is a 17-kb noncoding, spliced and polyadenylated RNA. The chromosome that expresses a *XIST* gene becomes coated with XIST molecules. On the other hand, these transcripts do not readily bind to either an X chromosome with an inactive *XIST* gene or autosomes. Subsequently, a XIST-painted chromosome undergoes further modifications that distinguish it from an Xa chromosome. The CpG islands of an Xi chromosome become highly methylated, and the DNA histone proteins H3 and H4 have a low level of added acetyl groups. In general, hypermethylation and hypoacetylation are hallmarks of gene silencing. Also, the DNA of an inactive X chromosome is condensed, replicates late during the S phase, and is associated with a large amount of the unique histone macroH2A1.2.

Proof of the role of the *XIST* gene in X chromosome inactivation is based, in part, on the consequences of chromosome aberrations. First, the genes of an X chromosome with a deleted *XIST* gene are never inactivated. Second, when a portion of an X chromosome with an active *XIST* gene is translocated to an autosome, many of the autosomal genes are silenced. Although XIST molecules adhere to chromosome DNA in general, the extent of the spread of gene silencing is greater on an X chromosome than on an autosome. This difference suggests that an X chromosome has a large number of XIST-specific recognition sites. In this context, it has been postulated that the distribution of LINE-1 (long interspersed nuclear element, type 1; L1) elements in a chromosome may determine the effectiveness of XIST-induced inactivation. There are about 500,000 L1 elements scattered throughout the human genome, with the relative frequency of L1 elements being twice as great in the X chromosome than in any autosome. Within the X chromosome, L1 elements are clustered near the *XIST* gene and well-represented in the rest of the Xq arm, but sparsely distributed in parts of the Xp arm.

Once an Xi is established, gene inactivation is maintained through successive somatic cell divisions despite loss of some XIST molecules. During oogenesis, the chromatin of an inactivated X chromosome is restored to a normal state. And, because the process of inactivation is generally random, an Xi chromosome in one generation can be active in the next one.

However, there are exceptions to the expected 50:50 distribution of inactivated X chromosomes within a cell population. Sometimes the ratio can be as high as 90:10. Skewing of X chromosome inactivation occurs with various disorders such as recurrent spontaneous abortion, sideroblastic anemia, Mohr–Tranebaerg syndrome (XL deafness), and familial incontinenta pigmenti (XL pigmentation defect). The basis of the Xi distortion has not been fully delineated. In some cases, the X chromosome inactivation process may be defective and one of the X chromosomes is preferentially inactivated. In other cases, the origin of the imbalance of Xi chromosomes is secondary and, probably, among other possibilities, selection for or against an allele on the Xa chromosome leads to an excess or deficiency of a particular Xi chromosome in a specific tissue.

Certain genes on Xi chromosomes are never silenced. In a study of 224 X-linked transcripts, which represents about 10% of all X-linked genes, 80% were inactivated and of those that were not, many are localized to the short arm of the X chromosome (Xp). The genes that escape silencing (escapees) are interspersed among regions of inactivated genes. Some of these escapees have expressed loci on the Y chromosome and, therefore, there is no need for dosage compensation. In other instances, monoallelic expression is compatible with normal development. Finally, the portion of Xp that contains many escapees is of recent evolutionary origin and, coincidentally, has few L1 elements.

A number of questions remain about the process of X chromosome inactivation and how certain genes escape silencing. What is the signaling mechanism that turns off the *XIST* gene in an active X chromosome and turns it on in an inactive X chromosome? What restricts the binding of XIST molecules to the X chromosome that expresses the *XIST* gene? What is the counting strategy that enables embryonic cells to inactivate all but one of their X chromosomes? In this regard, it has been known for some time that the greater the number of inactivated X chromosomes, the more severe the abnormalities. For example, XXY individuals, who have one Xi chromosome, usually function normally, whereas those with an XXXXY constitution and three Xi chromosomes are often profoundly mentally handicapped. Perhaps, overproduction from the genes that escape silencing causes developmental defects.

Beckwith–Wiedemann Syndrome

The major characteristic of the Beckwith–Wiedemann syndrome (BWS) is excessive fetal and postnatal growth. About 80% of BWS infants and children are greater than the 90th percentile for height and weight. After 6 years of age, overgrowth is not pronounced. Generally, the tongue is oversized. The abdominal body wall often fails to close at the base of the umbilical cord (omphalocele) because of enlarged internal organs (visceromegaly). Consequently, the intestines protrude from the body cavity at birth. About 60% of BWS patients have an abnormally low level of blood sugar (hypoglycemia) because an enlarged pancreas produces excess insulin. Hypoglycemia causes general weakness, increased appetite, and, occasionally, convulsions, coma, and irreversible brain damage. Wilms tumor, adrenocortical carcinoma, hepatoblastoma, rhabdomyosarcoma, or other cancers occur in 5% to 10% of BWS children. The incidence of severe BWS is about 1 in 14,000. About 15% of the cases of BWS are familial, which enabled mapping of the *BWS* locus to 11p15.5.

Paternal uniparental disomy for chromosome 11 occurs in about 25% of BWS patients. In addition, aberrant chromosomes with breakpoints in the 11p15.5 region, are usually maternally derived. These observations indicate a parent-of-origin effect and implicate imprinting defect(s) as the cause of BWS. Genes that are absolutely essential for fetal development must be present in the 11p15.5 region, because, unlike PWS and AS, no significant deletions are associated with BWS.

The BWS chromosome region spans 1 Mb and contains at least eight imprinted genes. Some of these loci are paternally expressed, maternally silenced and others have the opposite pattern of imprinting. Neither the mechanism(s) for imprinting genes in the 11p15.5 region nor which gene(s) is responsible for BWS are known. Research has focused on two genes. First, the insulin-like growth factor 2 gene (*IGF2*) encodes a fetal growth factor and is paternally expressed. Second, the maternally expressed $p57^{KIP2}$ (*CDKN1C*) gene produces an inhibitor of G_1 cyclin-CDK complexes. Thus, with paternal uniparental disomy, there is biallelic expression of the *IGF2* genes and no transcription from the silenced $p57^{KIP2}$ genes. The overproduction of insulin-like growth factor 2 likely stimulates cell growth, and the absence of the $p57^{KIP2}$ gene product relaxes control of the cell division cycle. Both of these effects could induce overgrowth of the fetus and enable malignant cells to proliferate. Overall, biallelic expression of *IGF2* genes occurs in about 60% of the cases of BWS. In non-UPD BWS, mutations presumably prevent maternal silencing of the *IGF2* gene. The regulation of imprinting of the 11p15.5 gene cluster is complex. The *IGF2* gene and its neighboring loci are likely under the control of one imprinting center, and the $p57^{KIP2}$ gene and its nearby loci are controlled by another center. Defects of the *IGF2* gene imprinting system seem primarily to increase the probability of malignancy, whereas derangement of the $p57^{KIP2}$ gene system leads to the characteristic features associated with BWS.

None of the other imprinted gene clusters within the human genome has been examined in as much detail as the 15q11–q13 and 11p15.5 regions. For example, there are at least two imprinted genes at 7q32 that are expressed monoallelically in fetal tissues and biallelically in many adult cells. Parent-of-

origin effects have also been attributed to various disorders including Crohn disease, epilepsy, and spina bifida. In addition, changes of imprinting patterns have been observed in different types of tumor cells. In some cancers demethylation activates a normally silenced gene (loss of imprinting, LOI), and in others methylation inactivates an expressed gene (gain of imprinting, GOI). Other kinds of changes to imprinted genes occur in tumor cells, such as the reversal of chromatin conformation from compacted (inactivation) to open (expression) or the reverse.

key terms

Angelman syndrome
antisense RNA
Beckwith–Wiedemann syndrome
biallelic expression
coding strand
CpG dinucleotide
CpG island
DNA methylation
dosage compensation
enhancer DNA element
epigenetic
erasure
establishment
gametogenesis
gene silencing
genomic imprinting
hemizygous
heterodisomy
housekeeping gene
hypermethylation
hypoacetylation
imprint
imprinting control region
isodisomy
LINE-1 (L1) element
maintenance
maternally expressed allele
maternally silenced allele
microdeletion
monoallelic expression
non-Mendelian inheritance
noncoding strand
oogenesis
parent-of-origin effect
paternally expressed allele
paternally silenced allele
Prader–Willi syndrome
primordial germ cell
reading
spermatogenesis
trisomy rescue
uniparental disomy
X chromosome inactivation
***XIST* gene**

summary

One of the stipulations of Mendelian inheritance is that reciprocal crosses with autosomal loci are functionally equivalent and the sex of the parent has no effect on the phenotype of the offspring. However, there are exceptions to this rule. A phenotype can be determined by the parental origin of an allele. In these cases of non-Mendelian inheritance, the transcriptional status of each allele is established during sex cell formation. For some loci, the allele that is acquired from the father will be transcribed, that is, paternally expressed and the other allele from the mother is maternally silenced. Other genes are maternally expressed and paternally silenced. The phenomenon of inactivation of an allele in one parent and activation of the other allele in the other parent is called genomic imprinting.

Many imprinted genes are arranged in clusters that often contain both paternally and maternally expressed alleles. Generally, imprinted loci control fetal growth or transfer of nutrients across the placenta or influence fetal brain development. Paternally expressed alleles usually stimulate fetal growth, and maternally expressed alleles constrain it.

The precise mechanisms that establish a gamete-specific imprint are unknown. Nevertheless, an important aspect of allele silencing is DNA methylation. This chemical modification entails the addition of a methyl group to the 5-position of the cytosine of a CpG dinucleotide in DNA. There are a number of ways that DNA methylation can inactivate an imprinted allele. The most direct strategy is methylation of the promoter region of an allele during sex cell formation. Other inactivation schemes are more complex. For example, some imprinted genes are controlled by an imprinting control region (ICR). For example, on one chromosome, a nonmethylated ICR combines with a enhancer DNA element that facilitates transcription of a nearby gene(s), whereas on the homologous chromosome, the enhancer DNA element cannot bind to a methylated ICR, which enables it to activate the transcription of another set of genes.

The transcriptional status of an imprinted allele is usually maintained through all somatic cell divisions. However, in some cases, allele-specific expression is confined to one type of tissue and biallelic expression occurs in other cell types. Notwithstanding these intricacies,

each imprint from the previous generation is normally erased in primordial germ cells before a new imprint is established for the next generation.

An imprinted locus is effectively hemizygous, because one of the alleles is routinely silenced. Consequently, if the active allele is removed by a deletion, inactivated by mutation, or not present because two chromosomes from the same parent, that is, uniparental disomy (UPD), carry silenced genes, then no gene product is formed. A disorder of fetal growth and brain development is likely with any of these conditions.

The foremost examples of imprinted gene clusters in humans are located at 15q11–q13 and 11p15.5. An excessive appetite and overeating disorder called Prader–Willi syndrome (PWS) occurs when there is a large deletion of the 15q11–q13 region that is inherited from the father, maternal UPD of chromosome 15, or microdeletions within the 15q11–q13 region that cause silencing of genes that otherwise would be marked for expression during spermatogenesis. The absence of one or more paternally expressed gene products of the 15q11–q13 cluster leads to PWS.

Another disorder called Angelman syndrome (AS) occurs when the enzyme ubiquitin ligase 3A that is normally synthesized from a maternally expressed allele of the *UBE3A* locus of the 15q11–q13 cluster is not produced. Among other features, the AS phenotype includes uncontrolled outbursts of laughter, a happy disposition, and severe mental handicap. The genetic conditions that most often cause AS are deletion of the *UBE3A* gene on a maternally derived chromosome, paternal UPD for chromosome 15, or inactivating mutations of the *UBE3A* gene.

An imbalance of imprinted genes from the 11p15.5 cluster causes Beckwith–Wiedemann syndrome (BWS). The BWS phenotype primarily entails organ overgrowth during fetal development and a predisposition to childhood tumors. There are at least two imprinting control regions within the 11p15.5 region. One of these ICRs regulates a group of genes including the *IGF2* gene. The other ICR controls $p57^{KIP2}$ and some additional genes. Individuals with BWS commonly have ICR mutation(s) or paternal UPD of chromosome 11 that engender biallelic expression of the *IGF2* gene and no transcription from the $p57^{KIP2}$ gene. In sum, epigenetic processes, such as genomic imprinting, add versatility to regulatory systems by creating transient modifications at the DNA level.

References

Avner, P. and E. Heard. 2001. X-chromosome inactivation: Counting, choice and initiation. *Nat Rev Genet* **2**:59–67.

Bailey, J. A. et al. 2000. Molecular evidence for a relationship between LINE-1 elements and X chromosome inactivation: the Lyon repeat hypothesis. *Proc Natl Acad Sci USA* **97**:6634–6639.

Bell, A. C. and G. Felsenfeld. 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. *Nature* **405**:482–485.

Brannan, C. I. and M. S. Bartolomei. 1999. Mechanisms of genomic imprinting. *Curr Opin Genet Dev* **9**:164–170.

Carrel, L. et al. 1999. A first-generation X-inactivation profile of the human X chromosome. *Proc Natl Acad Sci USA* **96**:14440–14444.

Chao, W. et al. 2002. CTCF, a candidate *trans*-acting factor for X-inactivation choice. *Science* **295**:345–347.

Constância, M. et al. 1998. Imprinting mechanisms. *Genome Res* **8**:881–900.

Costanzi, C. and J. R. Pehrson. 1998. Histone macroH2A is concentrated in the inactive X chromosome of female mammals. *Nature* **393**:599–601.

Davis, T. L. et al. 2000. The *H19* methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. *Hum Mol Genet* **9**:2885–2894.

Falls, J. G. et al. 1999. Genomic imprinting: Implications for human disease. *Am J Pathol* **154**:635–647.

Feil, R. and S. Khosla. 1999. Genomic imprinting in mammals: an interplay between chromatin and DNA methylation? *Trends Genet* **15**:431–435.

Ferguson-Smith, A. et al. 2003. Genomic imprinting—insights from studies in mice. *Semin Cell Dev Biol* **14**:43–49.

Gilbert, S. L. and P. A. Sharp. 1999. Promoter-specific hypoacetylation of X-inactivated genes. *Proc Natl Acad Sci USA* **96**:13825–13830.

Hark, A. T. et al. 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the *H19/Igf2* locus. *Nature* **405**:486–489.

Hatada, I. and T. Mukai. 2000. Genomic imprinting and Beckwith-Wiedemann syndrome. *Histol Histopathol* **15**:309–312.

Horike, S. et al. 2000. Targeted disruption of the human LIT1 locus defines a putative imprinting control element playing an essential role in beckwith-wiedemann syndrome. *Hum Mol Genet* **9**:2075–2083.

Jirtle, R. L. 1999. Genomic imprinting and cancer. *Exp Cell Res* **248**:18–24.

Lyon, M. F. 1998. X-chromosome inactivation spreads itself: Effects in autosomes. *Am J Hum Genet* **63**:17–19.

Lyon, M. F. 2000. LINE-1 elements and X chromosome inactivation: A function for "junk" DNA? *Proc Natl Acad Sci USA* **97**:6248–6249.

Lyon, M. F. 2003. The Lyon and the LINE hypothesis. *Semin Cell Dev Biol* **14**:313–318.

Mannens, M. and M. Alders. 1999. Genomic imprinting: concept and clinical consequences. *Ann Med* **31**:4–11.

Murphy, S. K. and R. L. Jirtle. 2003. Imprinting evolution and the price of silence. *Bioessays* **25**:577–588.

Nicholls, R. D. and J. L. Knepper. 2001. Genome organization, function, and imprinting in Prader-Willi and Angelman syndromes. *Annu Rev Genomics Hum Genet* **2**:153–175.

Peterson, K. and C. Sapienza. 1993. Imprinting the genome: Imprinted genes, imprinting genes, and a hypothesis for their interaction. *Annu Rev Genet* **27**:7–31.

Plath, K. et al. 2002. *Xist* RNA and the mechanism of X chromosome inactivation. *Annu Rev Genet* **36**:233–278.

Ray, P. F., R. M. Winston, and A. H. Handyside. 1997. *XIST* expression from the maternal X chromosome in human male preimplantation embryos at the blastocyst stage. *Hum Mol Genet* **6**:1323–1327.

Razin, A. and R. Shemer. 1995. DNA methylation in early development. *Hum Mol Genet* **4**:1751–1755.

Reik, W. and J. Walter. 2001. Genomic imprinting: Parental influence on the genome. *Nat Rev Genet* **2**:21–32.

Schwienbacher, C. et al. 2000. Gain of imprinting at chromosome 11p15: A pathogenetic mechanism identified in human hepatocarcinomas. *Proc Natl Acad Sci USA* **97**:5445–5449.

Tilghman, S. M. 1999. The sins of the fathers and mothers: Genomic imprinting in mammalian development. *Cell* **96**: 185–193.

Verona, R. I., M. R. Mann, and M. S. Bartolomei. 2003. Genomic imprinting: Intricacies of epigenetic regulation in clusters. *Annu Rev Cell Dev Biol* **19**:237–259.

White, W. M. et al. 1998. The spreading of X inactivation into autosomal material of an X;autosome translocation: Evidence for a difference between autosomal and X-chromosomal DNA. *Am J Hum Genet* **63**:20–28.

Wolffe, A. P. and M. A. Matzke. 1999. Epigenetics: Regulation through repression. *Science* **286**:481–486.

review questions

1. Describe the major features of genomic imprinting.
2. Specify the genotypes, transcriptional status of the alleles under consideration, and parental origins of the chromosomes of I-1, II-5, III-4, III-10, and IV-4 in both Figure 11.7A and B.
3. Give a complete interpretation of the cytogenetic designation: 46, XX, upd(12)mat.
4. How is uniparental disomy used to discover and map imprinted genes?
5. Describe three different ways of silencing an imprinted allele.
6. Describe the life cycle of an imprinted gene.
7. A female with two 15 chromosomes, both with intact 15q11–q13 gene clusters, has a phenotypically normal husband who is heterozygous for a deletion of the entire 15q11–q13 gene cluster. What are the parental origins of the 15 chromosomes and the transcriptional status of the imprinted genes of the 15q11–q13 cluster in each of these individuals? What is the probability that this couple will have a child with Prader–Willi syndrome?
8. Which parent transmits a mutated *UBE3A* gene to a daughter with Angelman syndrome? Explain your answer.
9. Construct a diagram showing the transcriptional status of the *IGF2* and $p57^{KIP2}$ genes of an individual with maternal uniparental disomy of chromosome 11.
10. What is the *XIST* gene and how does it function?

chapter 12

Molecular Genetics of Mitochondrial Disorders

The Living fabrick now in pieces take,
Of every part due observation make.

RICHARD BLACKMORE (1650–1729)

We find great things are made of little things.

ROBERT BROWNING (1812–1889)

. . . much interest has been aroused in recent years by cytological studies on the mitochondria or chondriosomes, cytoplasmic structural elements now widely believed to play an important part in the chemical activities of cells . . .

E. B. WILSON (1856–1939)

Mitochondria and Oxidative Phosphorylation

Virtually all biological processes require chemical energy in the form of adenosine triphosphate (ATP). In humans and other eukaryotic nonplant organisms, about 90% of the cellular ATP is generated by oxidative phosphorylation (OXPHOS) in cytoplasmic organelles called mitochondria (sing., mitochondrion). In Greek, *mito* means thread, and *chondrion* means granule. Mitochondria are spherical, rod-shaped, or long filamentous (threadlike) structures found in almost every type of human cell. The average width of a mitochondrion is about 0.5 μm, and the length is about 2 μm or longer. The cells of the brain, skeletal muscle, heart, kidney, and liver have high energy demands and, consequently, contain thousands of mitochondria, whereas cells with low energy requirements have only between 10 and 100 mitochondria. Structurally, a mitochondrion has an outer and inner membrane that partitions it into two compartments. One compartment consists of the space (intermembrane space) lying between the two membranes, and the other (matrix) includes the entire internal area bounded by the inner membrane (Figure 12.1). The inner membrane has a large number of folds (cristae, crests) that create a large internal surface area. Regions where the outer and inner membranes meet (contact sites, contact points) facilitate the entry of proteins into the matrix.

Five major multiprotein complexes (I to V) embedded in the inner mitochondrial membrane are responsible for OXPHOS (Figure 12.2). Complex I has ~46 proteins, complex II has 4, complex III has 11, complex IV has 13,

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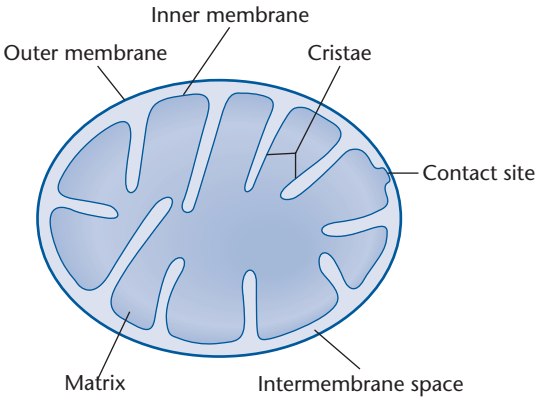

Figure 12.1 Schematic representation of a cross section of a mitochondrion.

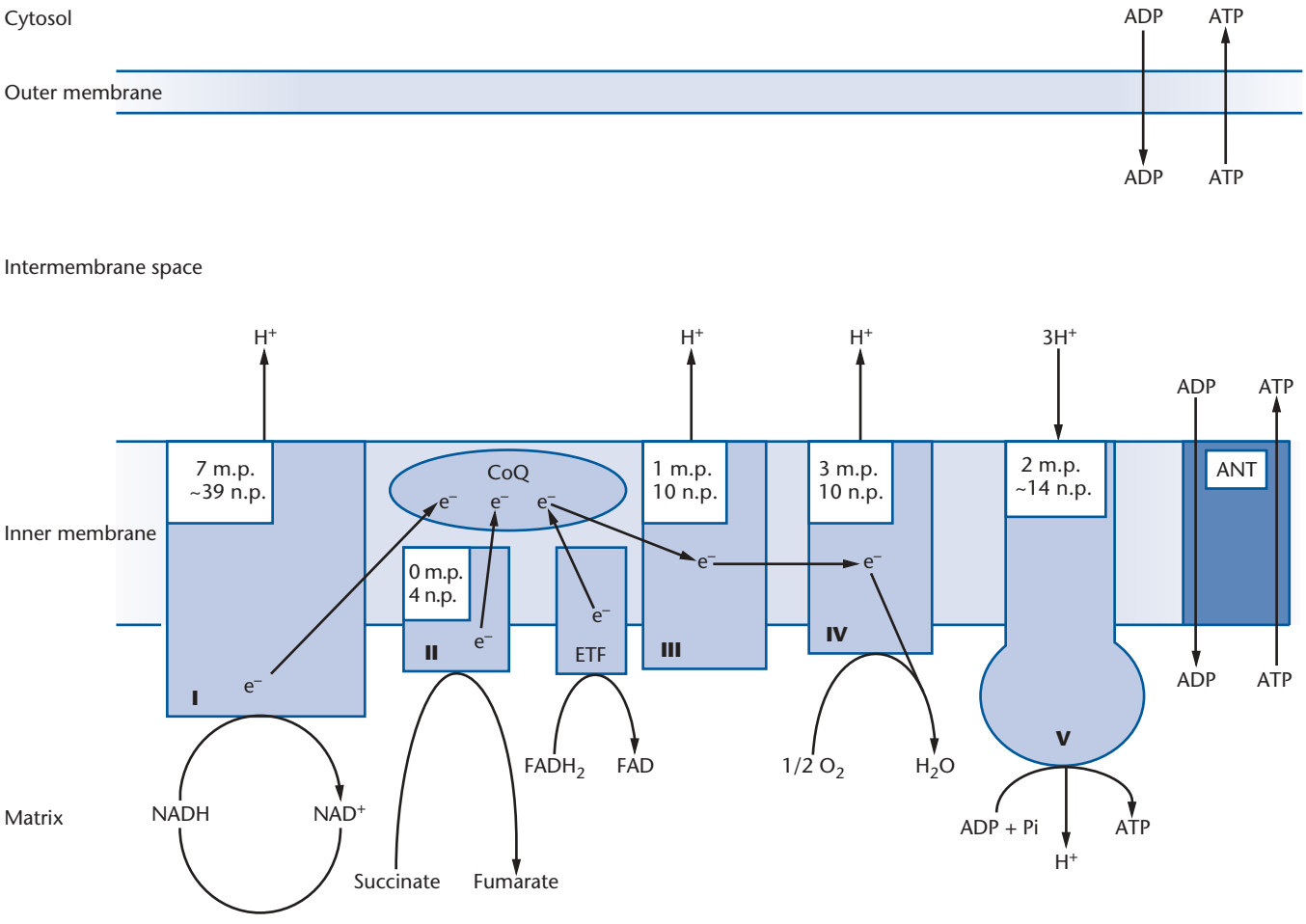

Figure 12.2 Schematic representation of oxidative phosphorylation (OXPHOS). Electrons (e^-) produced by oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD) by complex I, of succinate to fumarate by complex II, and of reduced flavin adenine nucleotide ($FADH_2$) to flavin adenine nucleotide (FAD) by electron transport factors (ETF) are passed to coenzyme Q (CoQ) and then to complex III and complex IV. Complex IV catalyzes the production of H_2O. Protons (H^+) are pumped into the intermembrane space by complexes I, III, and IV and are used by complex V to synthesize ATP. Adenine nucleotide translocator (ANT) facilitates the passage of ADP into and ATP out of the matrix. Each box in the upper left corner of the representations of complexes I to V contains the number of mitochondrial (m.p.) and nuclear (n.p.) subunits encoded by mitochondrial and nuclear genes, respectively.

and complex V has ~16. Briefly, complex I (NADH : ubiquinone oxidoreductase) converts NADH (reduced nicotinamide adenine nucleotide) to NAD^+ (nicotinamide adenine nucleotide) and complex II (succinate : ubiquinone oxidoreductase, succinate dehydrogenase) catalyzes the formation of fumarate from succinate. Both of these reactions generate electrons that are transferred to CoQ (coenzyme Q, ubiquinone). Similarly, after the oxidation of reduced flavin adenine nucleotide ($FADH_2$) to flavin adenine nucleotide (FAD) by various electron transport factors (ETFs), electrons are passed to CoQ. The reduced substrates oxidized by complexes I and II and the ETFs are formed during the metabolism of fatty acids and pyruvate by enzyme systems in the mitochondrial matrix. The electrons stored by CoQ are conveyed to complex III (ubiquinol : cytochrome *c* oxidoreductase) and from complex III to complex IV (cytochrome *c* oxidase). Complex IV catalyzes the production of H_2O. An additional feature of the oxidation reactions conducted by complexes II, III, and IV is the release of protons (H^+) into the intermembrane space.

In sum, complexes I to IV, in concert with other components, act together as an electron transport chain. The protons released into the intermembrane space form an electrochemical gradient, which provides potential energy used by complex V (ATP synthase) for the phosphorylation of ADP to ATP. The ATP in the matrix is transferred to the intermembrane space by an inner membrane channel protein, adenine nucleotide translocator (ANT). For each ATP molecule passed into the intermembrane space by ANT, one ADP molecule is carried into the matrix. Both ADP and ATP freely move through the mitochondrial outer membrane, which is considerably more permeable than the inner membrane.

In contrast to other cytoplasmic organelles, such as lysosomes and peroxisomes that are highly specialized membrane vesicles, mitochondria are semiautonomous replicating entities with their own genetic apparatus. Mitochondrial DNA, which has been called the 25th chromosome by some and chromosome M by others, is circular with 16,568 bp. (The correct number of base pairs is 16,568, but, based on the original sequencing data, the value 16,569 is usually cited and position 3106 is considered a gap.) Mitochondrial DNA carries the genetic information for 2 ribosomal RNAs, 22 transfer RNAs, and 13 proteins (Figure 12.3). The complete nucleotide sequence of human mitochondrial DNA is known. The 13 proteins encoded by the mitochondrial genome and translated within the matrix of the mitochondria are involved in OXPHOS functions. Seven of these mitochondrial proteins (ND1, ND2, ND3, ND4, ND4L, ND5, ND6) are subunits of complex I of the electron transport chain, one (cyt b) of complex III, three (COX I, COX II, COX III) of complex IV, and two [ATPase 6 (A6), ATPase 8 (A8)] of complex V. Nuclear genes encode about 70 additional proteins that make up the OXPHOS system, and about 1000 other mitochondrial proteins promote replication, transcription, and translation, facilitate the transport of various compounds through mitochondrial membranes, and metabolize fatty acids and pyruvate. In other words, a mitochondrion is the joint product of two different genetic systems.

There are from 2 to 10 separate mitochondrial genomes in the matrix of each mitochondrion. After replication and during mitochondrial propagation, each mitochondrial genome is randomly distributed to a daughter mitochon-

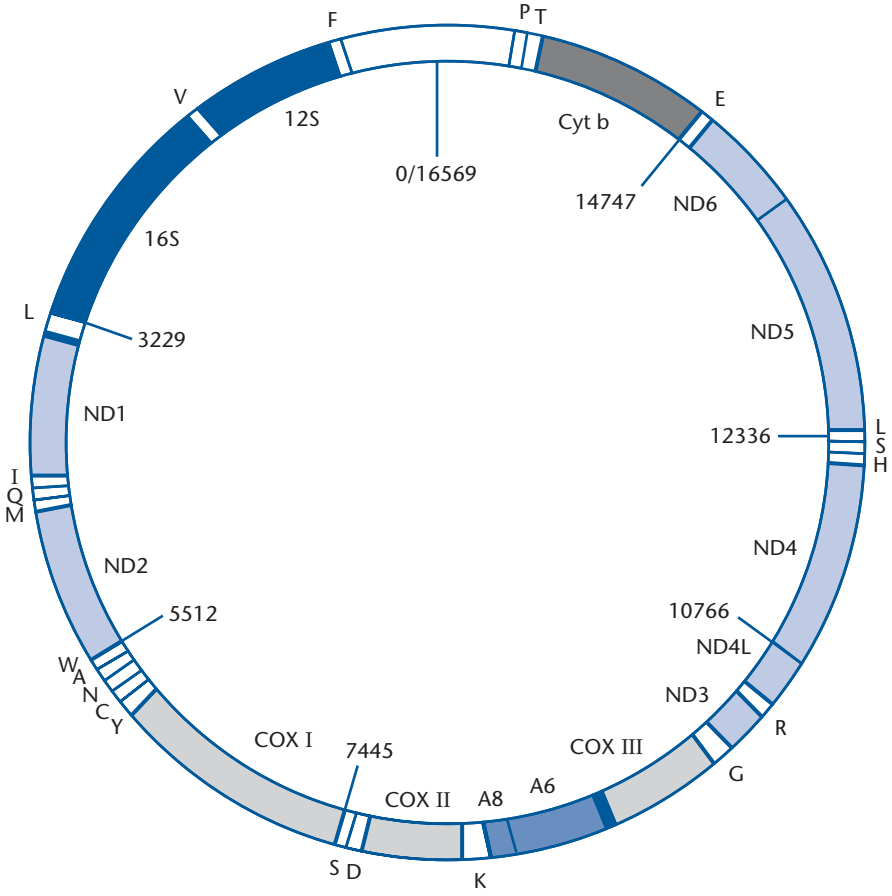

Figure 12.3 Human mitochondrial genome. The nucleotide positions (numbers) are marked at various intervals in a counterclockwise direction. The tRNA loci are indicated by single-letter amino acid codes. The ribosomal RNA sites are 12S and 16S. The loci for mitochondrial proteins that contribute to complex I (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), complex III (Cyt b), complex IV (COX I, COX II, COX III), and complex V (A6, A8) are identified by an abridged form of the name of the encoded protein. The numbering system is based on the original sequencing results. Adapted from Schon, E. A., E. Bonilla, and S. DiMauro. 1997. Mitochondrial DNA mutations and pathogenesis. *J Bioenerg Biomembr* **29**:131–149.

drion. The details of mitochondrial reproduction are not known. The pattern of inheritance of mutant mitochondrial genes does not conform to Mendelian principles because mitochondrial DNA is not part of the chromosomal system. Consequently, a number of questions arise. What are the principal features of mitochondrial inheritance in humans? Do mutations of the various mitochondrial genes produce a common "energy deficiency" phenotype? Alternatively, do mutations of different mitochondrial genes give rise to distinct syndromes? Does the ratio of mitochondria with a mitochondrial gene mutation to normal (wild type) mitochondria in the cells of a tissue have to exceed a certain value to cause a clinical condition? Are there any examples of disorders that result from mutations of nuclear genes that encode mitochondrial proteins?

Mitochondrial Genetics

In humans, the mitochondria of the zygote come from the oocyte, that is, from the mother and almost never from the sperm, that is, from the father. This form of transmission is called maternal inheritance (Figure 12.4). Thus, if the same set of symptoms in a number of different multigenerational families is inherited only from affected females and almost never from affected males, then there is a strong likelihood that the condition is caused by a mutation in

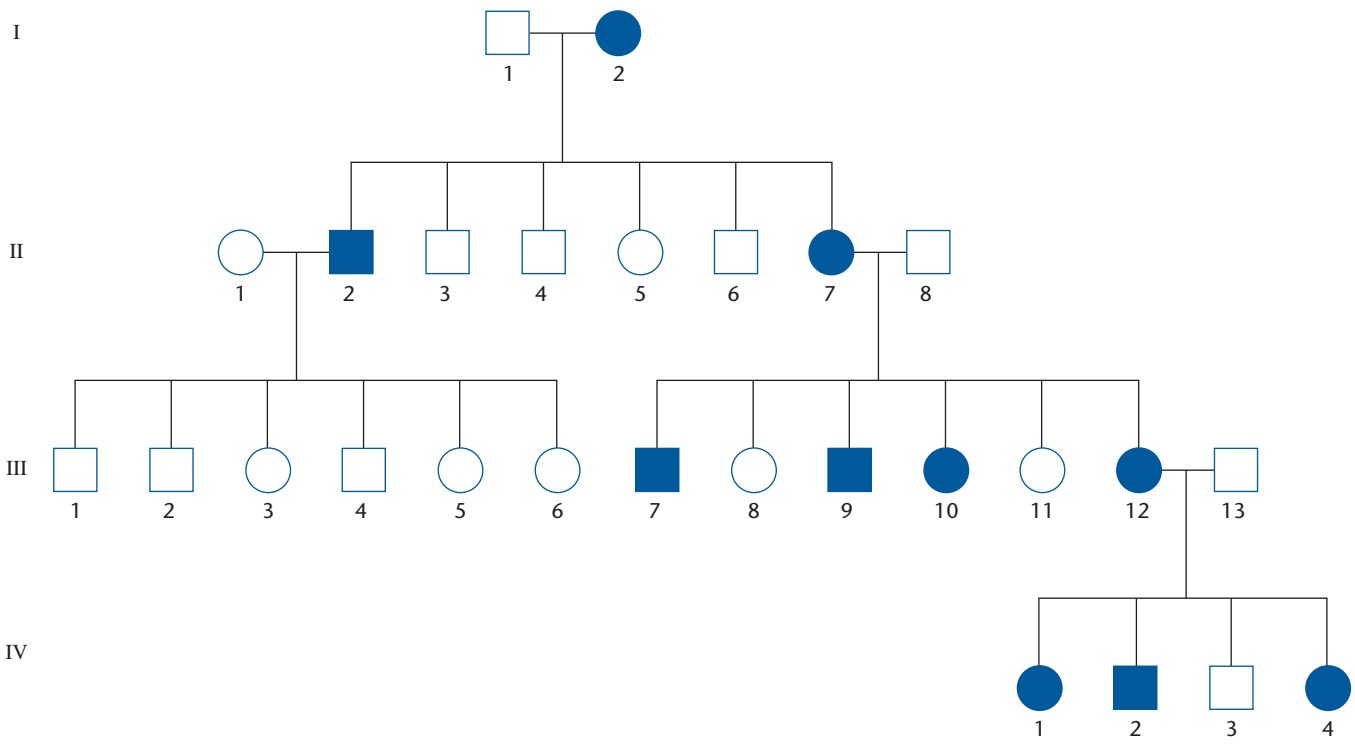

Figure 12.4 Pedigree illustrating maternal inheritance. Solid symbols indicate affected individuals. Key indicators are that affected females have affected offspring and that affected males almost never have affected offspring.

one of the genes of the mitochondrial DNA. Sequence analysis of mitochondrial DNA is used to determine which gene is mutated. A mutant nuclear gene that encodes a mitochondrial protein may be inherited as an AR, AD, or XL trait and can be localized and characterized with conventional protocols.

A human oocyte has approximately 100,000 mitochondria, but, as it matures, vast numbers of mitochondria are lost. The actual number of mitochondria that remain after maturation is not known, but estimates range from less than 10 to not more than 100. During the first few days of embryonic cell division, mitochondrial propagation builds up the number of mitochondria per cell to about 10,000 or more. The process of reducing the number of mitochondria from 100,000 to less than 100 has been called a genetic bottleneck. If one of the mitochondria that survives the bottleneck happens to carry a mutated gene, then this genome will be well represented in the ensuing mitochondrial population and, after development is completed, will populate the tissues of an individual. Moreover, during cell divisions of both embryogenesis and tissue formation, mitochondria are randomly distributed, that is, replicatively segregated, into daughter cells. Thus, by chance alone, some progenitor cells might receive a large number of mitochondria that carry a mutant mitochondrial gene, which, subsequently, would result in some adult tissues having a high proportion of mitochondria with a mutant gene and other tissues with, more or less, only wild-type mitochondria. Regardless of how a disparity in the distribution of different types of mitochondria occurs, a lowered level of energy production in a tissue where mitochondria with a defective OXPHOS system outnumber wild-type mitochondria will affect tissue func-

tions, especially if the tissue requires large amounts of energy. The ratio of mutated to normal mitochondrial DNA is called the mitochondrial mutation load.

The term homoplasmy describes the situation in which all the mitochondria of a cell or tissue have the same genome, which may be either the wild-type sequence or one with a gene mutation. Heteroplasmy denotes a cell or tissue containing both mutant and wild-type mitochondrial genomes. If a mitochondrial gene mutation reduces the production of ATP, then cells with a high energy demand that are homoplasmic for the mutant mitochondrial DNA will be seriously damaged. On the other hand, homoplasmy for mutant mitochondrial DNA would have little impact on cells with low energy requirements. In heteroplasmic cells, the proportion of mutant to wild-type mitochondrial DNA determines whether an energy shortage occurs. Clearly, if the population of mitochondria with mutant genomes is small, then the output of energy would not be affected significantly. Alternatively, in the cells of a tissue with large numbers of mutated mitochondrial genomes, energy production would not be sufficient to maintain cellular functions and would lead to an abnormal condition of some sort. In other words, for mitochondrial disorders, there is a threshold for phenotypic expression. The threshold at which the deleterious effects of a mitochondrial gene mutation become apparent depends on the energy needs of a particular cell or tissue. Thus, the brain, skeletal muscles, heart, and liver, all of which have considerable energy requirements, are highly susceptible to mitochondrial gene mutations. In this context, as expected, mutant nuclear genes that encode mitochondrial proteins have a significant impact on tissues with a high energy demand.

Mitochondrial Disorders

More than 50 single nucleotide pair mutations in the mitochondrial genome have been associated with various multisystem disorders. The overall incidence of mitochondrial disorders is about 1 in 10,000 live births. Some of the biological consequences of mitochondrial mutations are myopathy, cardiomyopathy, dementia, sudden uncontrolled muscle contractions (myoclonus epilepsy), deafness, blindness, anemia, diabetes, and loss of cerebral blood supply (stroke). The combination of defects associated with a particular mitochondrial disorder depends on a number of factors such as which mitochondrial gene is mutated, the extent of replicative segregation of the mutant mitochondrial genome during the early stages of embryonic development, the abundance of the mutant mitochondrial gene in a particular tissue, and the threshold level of mutant mitochondrial DNA required in a tissue before an abnormality is evident clinically. All of these confounding circumstances make the determination of a mitochondrial disorder a complicated process. However, a good clue that a disorder is mitochondrial is a consistent pattern of maternal inheritance in a number of families. Generally, point mutations in mitochondrial genes are inherited maternally. On the other hand, mitochondrial genomes with large deletions and other structural alterations usually do not pass through the genetic bottleneck and, therefore, do not show a pattern of maternal inheritance. These sporadic cases are identified when a group of unrelated individu-

als share the same set of defects that appear to be the result of impaired energy production. Once a mitochondrial abnormality is inferred, molecular analysis of the mitochondrial DNA of affected tissues determines whether there is a consistent structural change.

Myoclonus Epilepsy and Ragged Red Fibers

Myclonus epilepsy and ragged red fibers (MERRF) is a rare, maternally inherited, heteroplasmic, debilitating multisystem disorder that includes transient seizures (myoclonus epilepsy), failure to coordinate muscle movement (ataxia), loss of muscle cells (myopathy), and, to a lesser extent, dementia, deafness, and degeneration of spinal nerves. The term "ragged red fibers" refers to large clumps of abnormal mitochondria that accumulate mostly in muscle cells and are stained red by a dye that is specific for complex II of the electron transport chain. In general terms, MERRF is a member of a group of disorders called mitochondrial encephalomyopathies that feature mitochondrial defects with altered brain and muscle functions. In patients with severe forms of MERRF, neuronal loss occurs in the olivary and dentate nuclei of the brain and in regions of the cerebellum, brain stem, and spinal cord. Onset of MERRF is usually during childhood, with the condition often persisting for many years.

The majority of MERRF cases are the result of a point mutation (A8344G) in the transfer RNA^{Lys} gene of the mitochondrial genome. The official designation for this mutation is *MTTK*MERRF*8344G. The nomenclature for mitochondrial base substitution diseases has three elements. First, the locus is designated. The *MT* of *MTTK* denotes that a mitochondrial gene is mutated, the second *T* signifies a transfer RNA gene, and *K* represents the single-letter amino acid designation for lysine, thus indicating that the mutation is in the mitochondrial $tRNA^{Lys}$ gene. Second, after the asterisk, instead of a designation for the mutant allele as is used for nuclear genes, an acronym is used for the clinical features most often associated with the base substitution at the specific nucleotide site, which, in this case, is *MERRF*. Third, the term 8344G indicates that the mutant nucleotide is guanine (G) at nucleotide position 8344. Although not as common, a second MERRF mutation has been observed in the same gene but at a different site, namely, *MTTK*MERRF*8356C.

If 90% of the mitochondria in nerve and muscle cells carry the *MTTK*MERRF*8344G mutation, then the defining symptoms of MERRF are present. When the mitochondrial mutation load is lower, there are fewer MERRF symptoms and the condition is less severe. Both of the MERRF mutations diminish overall mitochondrial protein synthesis by interfering with aminoacylation and/or the codon-anticodon interaction. In addition, a set of MERRF-specific translation products are produced and the amounts of all the OXPHOS components except for complex II are decreased. It is not clear whether the primary cause of MERRF is lowered levels of some electron transport chain subunits or one or more of the MERRF-specific proteins.

Mitochondrial Encephalomyopathy with Lactic Acidosis and Strokelike Episodes

Mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes (MELAS) is the most common maternally inherited mitochondrial disease.

The clinical features include recurrent strokes that begin before 40 years of age, myopathy, ataxia, muscle twitching (myoclonus), dementia, and deafness. To a lesser extent, recurring vomiting, migrainelike headaches, diabetes, weakness, or paralysis of the external muscles of the eye that prevents side-to-side movement (progressive external ophthalmoplegia, PEO), droopy eyelids (ptosis), muscle weakness, and short stature are also associated with MELAS. Lactic acidosis is an increase of lactic acid concentration that decreases the pH and lowers the buffering capacity of body fluids. With MELAS, abnormal mitochondria presumably do not metabolize pyruvate and the excess pyruvate is reduced to lactic acid, which accumulates in the blood and other body fluids. A characteristic pathological feature in MELAS patients is the accumulation of large clumps of abnormal mitochondria in the walls of the small arteries and capillaries of the brain and muscles, although it is not clear whether these obstructions are responsible for either recurring strokes or other MELAS symptoms. Despite some similarities with MERRF, MELAS has a significantly different array of clinical manifestations.

The *MTTL1*MELAS*3243G mutation accounts for more than 80% of the cases of MELAS. This base substitution is in one of the two mitochondrial transfer RNA^{Leu} genes. Specifically, the A3243G mutation occurs in the $tRNA^{Leu(UUR)}$ gene, where UUR indicates that the codon for this leucine transfer RNA has uridine (U) in the first two positions and a purine (R), either guanine or adenine, in the third position. Generally, *MTTL1*MELAS*3243G is heteroplasmic. When this mutation is present in ≥90% of the mitochondrial DNA of muscle tissue, there is an increased likelihood of recurrent strokes, dementia, epilepsy, and ataxia. When heteroplasmy for the A3243G mutation is ~40% to 50%, chronic progressive external ophthalmoplegia (CPEO), myopathy, and deafness are likely to occur. Other MELAS mutations occur at sites 3252, 3271, and 3291 within the $tRNA^{Leu(UUR)}$ gene and in the mitochondrial $tRNA^{Val}$ (*MTTV*) and COX III (*MTCO3*) genes.

When studied in cultured cells, the MELAS mutations at sites 3243 and 3271 of the $tRNA^{Leu(UUR)}$ gene reduce mitochondrial protein synthesis and, unlike MERRF, neither of these mutations produces any unusual proteins. The A3243G mutation decreases aminoacylation, reduces tRNA half-life, and impairs pre-tRNA processing. And, based on codon usage, the *ND3* and *ND6* genes with 8.8% and 8.1% UUR codons, respectively, are more likely to be affected by the A3243G mutation than the other mitochondrial structural genes. The biochemical consequences of the *MTTV* and *MTCO3* mutations are not known. It is unlikely that merely lowering the amounts of the OXPHOS components is sufficient to account for the specific spectrum of MELAS symptoms.

At present, the clinical variability of different mitochondrial mutations is perplexing and inexplicable. A striking illustration of this aspect of mitochondrial genetics is the diverse phenotypes, other than MELAS, produced by various single mutations of the *MTTL1* gene (Figure 12.5). In some individuals with the A3243G mutation, the only phenotypic features are diabetes mellitus (DM) and deafness (DF). Myopathy predominates with mutations at sites 3250, 3251, 3302, 3303, and 3260. Cardiomyopathy is the main defect associated with base substitutions at sites 3260 and 3303. In one patient, a deletion

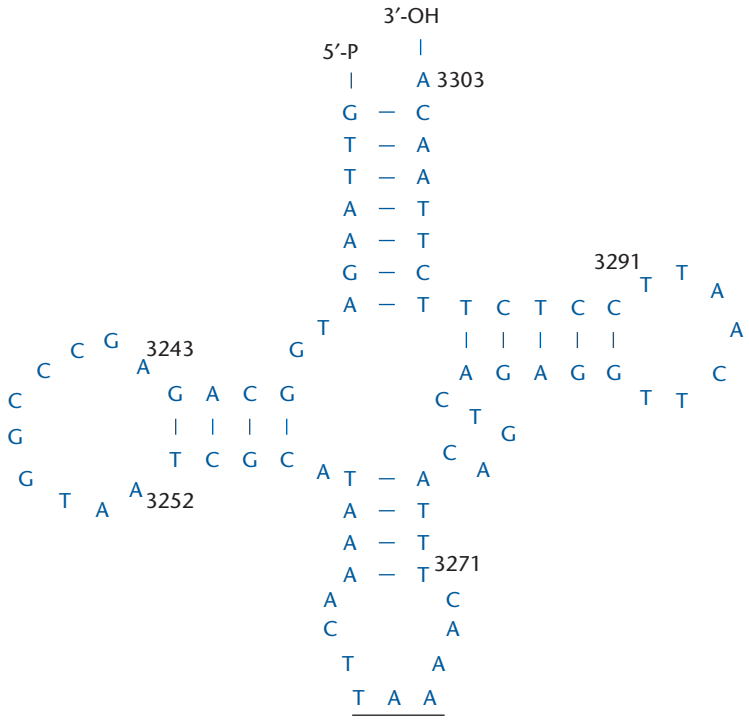

Figure 12.5 Mitochondrial $tRNA^{Leu(UUR)}$ (*MTTL1*) gene. The transcribed DNA strand of the *MTTL1* gene is shown in the shape of the $tRNA^{Leu(UUR)}$ molecule. The numbers denote the nucleotide positions within the mitochondrial genome. The anticodon is underlined.

of the thymidine residue at 3271 was responsible for deafness by the age of 5, followed by degenerative eye disease, renal failure, seizures, cerebral damage, and, ultimately, death. One patient with a C3256T mutation had both MELAS and MERRF symptoms. In sum, mutations of different mitochondrial tRNA genes can produce similar clinical features, whereas different mutations of the same tRNA gene lead to very different phenotypes.

Leber Hereditary Optic Neuropathy

Leber hereditary optic neuropathy (LHON) is a rare mitochondrial disorder of the eye. Typically, the first sign of LHON is a blurring of vision, followed within a few months by a painless, complete, or near-complete, loss of sight. Often, both eyes are affected simultaneously. If not, blindness in the second eye occurs very shortly after the loss of vision in the first. Degeneration of the optic nerve and neurons of the retina are the principal pathological features of LHON. Additional abnormalities, such as deterioration of peripheral nerves, tremors, heart conduction defects, and diminished muscle tonicity (dystonia) sometimes accompany the bilateral blindness. The onset of LHON is usually when patients are in their mid-20s, but can range from childhood to adults older than 70 years. There is generally a sex bias, with approximately five times more males than females showing the disorder. The reason for this difference has not been determined.

At least 18 different missense mutations in nine genes (*ND1*, *ND2*, *CO1*, *ATP6*, *CO3*, *ND4*, *ND5*, *ND6*, *CYTB*) that encode mitochondrial proteins are responsible directly or indirectly for the LHON phenotype. Five of these mutations are sufficient, each on their own, to cause LHON. The remaining so-

called LHON mutations require additional circumstances, such as a second mitochondrial mutation, the presence of certain nuclear genes, or nongenetic factors, to produce the LHON phenotype. On this basis, cases of LHON fall into two categories. Primary LHON occurs when a single mitochondrial mutation suffices to produce the phenotype. Secondary LHON, which is very rare, requires a second mutation or other conditions to generate the phenotype. The biological basis of secondary LHON is controversial and uncertain. However, with primary LHON, three mutations (*MTND1*LHON*3460A, *MTND4*LHON*11778A, *MTND6*LHON*14484C) account for more than 90% of all cases and the 11778A mutation is found in 50% to 70% of these patients. Homoplasmy is a common occurrence among many primary LHON families. The threshold level for mutant mitochondrial DNA in the heteroplasmic LHON families is ≥70%.

Although subunits (ND1, ND4, ND6) of complex I of the respiratory chain are altered in most LHON patients, a number of studies have failed to pinpoint the precise basis of the biochemical defect. The 11778A mutation lowers the rate of oxidation of NAD-linked substrates, 3460A reduces the activity of complex I by about 80%, and 14484C also may decrease the activity of complex I. Each of these three major LHON mutations affects the respiratory chain to some extent. However, it is not apparent why these LHON mutations preferentially disturb the optic nerve and ganglion cell layer of the retina. Complex I may have a critical role in light-induced nerve transmission that is independent of its overall role in the production of ATP.

Neuropathy, Ataxia, and Retinitis Pigmentosa

Neuropathy, ataxia, and retinitis pigmentosa (NARP) is a rare heteroplasmic mitochondrial disorder characterized by delayed development, muscle weakness, dementia, seizures, retinitis pigmentosa, and diminished sensory functions. Mutations at site 8993 in the mitochondrial *ATP6* gene (*MTATP6*NARP*8993G and *MTATP6*NARP*8993C) are associated with NARP when the level of heteroplasmy of the mutant gene ranges from ~70% to 90%. When heteroplasmy for either 8993 mutation is greater than 90%, a fatal infancy disorder called Leigh syndrome (LS) occurs. The major pathological features of LS are degeneration of the neurons of the basal ganglia and brain stem. A moderately benign form of LS, called familial bilateral striatal necrosis (FBSN), is the result of mutations at sites 8851 (*MTATP6*FBSN*8851C) and 9176 (*MTATP6*FBSN*9176C) of the *ATP6* gene. In vitro cell studies have indicated that the 8993G mutation prevents the translocation of protons by complex V, but the actual relationship between the activity of ATP synthase and the altered ATP6 subunit is not known.

Kearns–Sayre Syndrome

The common clinical features of Kearns–Sayre syndrome (KSS) are progressive external ophthalmoplegia (PEO) and degeneration of the pigment layer of the retina. Often the KSS phenotype includes cardiac conduction abnormalities, ataxia, and, to a lesser extent, deafness, dementia, and diabetes mellitus. Onset begins before 20 years of age, and most patients die a few years after diagnosis.

KSS does not show a consistent pattern that would indicate either maternal or nuclear gene inheritance. However, the symptoms suggest that it might represent a mitochondrial disorder. Analysis of muscle mitochondria DNA of KSS patients has revealed DNA structural changes including large deletions (>1000 bp) and duplications. Mitochondrion genome abnormalities are often detected by Southern blotting of DNA extracts hybridized with a mitochondrion-specific DNA probe. If, in addition to the normal 16.5-kb mitochondrial DNA band, the probe hybridizes to larger or smaller DNA molecules, then it is likely that either a mitochondrial duplication or a deletion is present in some of the mitochondria of affected individuals. Sequence analysis is then used to determine precisely the nature and extent of the structural abnormality. About one-third of the cases of KSS are associated with a 4977-bp deletion ("common" deletion) that has breakpoints in the *ATP8* and *ND5* genes with the loss of the intervening structural and tRNA genes (Figure 12.3). Although most cases of KSS are sporadic, the rare occurrence of inheritance from an asymptomatic mother cannot be precluded.

The severity of KSS depends on the extent of heteroplasmy and the tissue distribution of structurally altered mitochondrial genomes. An extreme form of the KSS phenotype occurs when the frequency of deleted mitochondrial genomes in muscle cells is >85%. At lower levels of heteroplasmy, PEO is often the major characteristic. When deleted and/or duplicated mitochondrial genomes are abundant in blood-forming cells, a fatal early-onset disorder called Pearson syndrome (PS) develops. The principal feature of PS is the inability of blood cells to use iron for hemoglobin synthesis, causing sideroblastic anemia. The few patients who survive PS invariably acquire KSS later in life.

The pathogenic basis of either deleted or duplicated mitochondrial genomes has not been elucidated. Obviously, when the proportion of deleted mitochondrial DNA molecules is very high in a tissue, energy production is dramatically reduced as a result of the loss of mitochondrial structural and tRNA genes. Alternatively, when the proportion of duplicated mitochondrial genomes is elevated, overproduction of mitochondrial structural and tRNA genes could upset the balance of OXPHOS subunits and interfere with the assembly of protein complexes in the electron transport chain. Despite our ability to make these obvious generalizations, it is a mystery why structurally abnormal mitochondrial genomes seem to produce a phenotypically limited set of disorders.

Nuclear-Encoded Mitochondrial Disorders

Each nuclear-encoded mitochondrial protein usually has a sequence of 15 to 30 amino acids (target sequence) at its N-terminus. The target sequence binds to a receptor on the surface of the mitochondrial outer membrane. Mitochondrial receptors facilitate the entry of proteins through the outer membrane into the intermembrane space or through a contact site into the matrix. After importation, among a myriad of functions, the nuclear-encoded proteins transport molecules between the intermembrane space and the matrix, metabolize substrates, produce ATP by means of oxidative phosphorylation, regulate the mitochondrial uptake of iron, control mitochondrial DNA replication, and maintain the structural integrity of mitochondrial DNA. Although mutations

of nuclear genes that impair mitochondrial functions are generally rare, the catalog of such defects is increasing.

Mitochondrial Protein Importation Defects

Pyruvate dehydrogenase complex (PDHC) is a mitochondrial matrix multienzyme complex with pyruvate decarboxylase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3) activities. The E1 component of PDHC is a tetramer with two E1α subunits and two E1β subunits. The *PHDA1* gene encodes the E1α subunit and is located at Xp22.1. Mutational analysis of one patient with low levels of pyruvate dehydrogenase activity revealed a base substitution in both alleles of the *PHDA1* gene. The amino acid change was within the E1α target sequence and probably prevented the importation of E1α into the mitochondrial matrix. The phenotype associated with this fatal X-linked recessive disorder is heterogeneous, with ocular abnormalities and CNS degeneration predominating.

Ornithine aminotransferase (OAT) is a monomeric mitochondrial matrix enzyme that catalyzes the conversion of ornithine to glutamic γ-semialdehyde and vice versa. A deficiency in OAT activity is associated with blindness in young adults as the result of degeneration of the choroid and retina. This condition is called gyrate atrophy of the choroid and retina (GACR) and is inherited as an autosomal recessive trait. The *OAT* gene has been mapped to chromosome 10q26. Mutation screening of an adult male with mild GACR-like symptoms, including ocular defects, revealed a homozygous missense mutation of the *OAT* gene. Biochemical studies indicate that, although this mutation does not affect an amino acid of the target sequence, it probably changes the configuration of the OAT protein and prevents it from passing into the mitochondrial matrix.

Substrate Transport Defects

The oxidation of fatty acids in mitochondria is important for energy production, especially in heart and skeletal muscle during exercise. Genetic defects have been identified in a number of the genes that encode the fatty acid oxidation genes. The phenotypes for these mutations are similar and include abnormalities of the liver and both heart and skeletal muscles. For example, when the fatty acid oxidation enzyme carnitine-acylcarnitine translocase, encoded by the *CACT* gene, is defective and long-chain fatty acids are not transported through the inner mitochondrial membrane, multisystem abnormalities are evident and death occurs within days or a few months after birth.

Substrate Utilization Defects

Mutations of the *CPT2* gene at chromosome 1p32 that encodes carnitine palmitoyl transferase produce two phenotypes. Muscle weakness and soreness after strenuous activity or fasting are common features of the adult-onset form. The infant-onset form, which is severe and usually fatal, affects the liver, heart muscles, and skeletal muscles. In both of these cases, oxidation of long-chain fatty acids by CPTII, which is bound to the inner mitochondrial membrane, is impaired.

A multienzyme complex that participates in the β-oxidation of fatty acids and has 3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and 3-ketoacyl-CoA thiolase activities provides another example of a nuclear-encoded mitochondrial disorder. This trifunctional protein (TP) has four α (HADHA) and four β (HADHB) subunits. The 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase active sites are part of the HADHA cluster, and the four HADHB subunits constitute the 3-ketoacyl-CoA thiolase domain. The clinical features of TP deficiency are sudden infant death, coma, low blood sugar, liver failure, degeneration of skeletal muscles, and cardiomyopathy. Most mutations in the *HADHA* and *HADHB* genes, which are both located at chromosome 2p23, cause loss of the entire TP complex, whereas with some *HADHA* gene mutations only the 3-hydroxyacyl-CoA dehydrogenase activity is abolished. Interestingly, the phenotype is the same whether the loss of TP activity is partial or complete.

Mutations of genes that encode enzymes of the Krebs cycle are very rare. However, a deficiency of mitochondrial fumarase (fumarate hydratase, FH) causes infant death with many of the symptoms of a mitochondrial encephalomyopathy. This condition is inherited as an autosomal recessive trait and is the result of mutations in the *FH* gene located at chromosome 1q42.1.

Iron Transport Defect

Friedreich ataxia (*FRDA*) is a fatal trinucleotide repeat expansion disorder inherited as an autosomal recessive trait (see Table 14.3 in Chapter 14 on Molecular Genetics of Neurological Disorders). Clinical features include progressive failure of limb movement and, to a lesser extent, blindness, deafness, and diabetes mellitus. Death before early adulthood is often from hypertrophic cardiomyopathy. Many of these defects resemble the constellation of abnormalities that accompany mitochondrial disorders. Studies of the FRDA gene product, frataxin, in mice and the frataxin homolog in yeast localized this protein to either the inner mitochondrial membrane or the mitochondrial matrix. Biochemical examination of some FRDA patients showed that the uptake of iron-sulfur (Fe-S) clusters that form part of the electron transport function of OXPHOS complexes I, II, and III and aconitase, which is a Krebs cycle enzyme, is not properly regulated when the mutant form of frataxin is present. As a result, excess iron accumulates in the mitochondria, which either directly or indirectly disrupts mitochondrial functions. How frataxin controls iron transport into the mitochondrial matrix is not known.

Electron Transport Chain Defects

The cDNAs for at least 18 of the ~39 nuclear-encoded complex I proteins have been isolated and sequenced. Of these, about 14 have been localized to chromosome locations by in situ hybridization. The *NDUFS4* gene at 5q11.1 that encodes one of the subunits (AQDQ) of complex I was screened for mutations in 20 patients, each of whom had a deficiency in complex I activity. One of these patients was discovered to have a homozygous 5-bp duplication. This patient was admitted to a hospital at 8 months of age for treatment of severe vomiting, extremely stretched muscles (hypotonia), and a failure to thrive. The child died from cardiorespiratory failure 8 months later. Other complex I

from the HUMAN GENETICS files

Revelations From the Grave: Using Mitochondrial DNA Analysis to Resolve Historical Mysteries

Groucho Marx (1895–1977) often asked losing contestants on his 1950s television program "Who's buried in Grant's tomb?" just to ensure that they would go home with some money. Although almost everyone knows the answer to this specific question, there are a number of ongoing historical disputes about whether this man or that woman were truly who they claimed to be when they were alive. In other instances, there may be doubts about who is actually buried in a particular grave. With the advent of PCR assays for short tandem repeat (STR) polymorphisms and highly polymorphic regions of the mitochondrial genome, it became possible to address controversies about alleged relationships among the living and the dead.

Generally, DNA can be extracted from bones, teeth, or hair of interred bodies, subjected to PCR, and, if necessary, sequenced. However, quite often nuclear DNA is so severely degraded that STR polymorphism analyses are impossible to perform. By contrast, human remains, especially bones and teeth, consistently retain some intact mitochondrial DNA molecules that can be assayed. For resolving paternity disputes and accumulating evidentiary information in criminal cases, STR polymorphism analyses usually are conducted because fresh samples often are available. For studying the DNA of human remains, the samples must be authenticated and strict measures are taken to make sure that they are definitely not contaminated with DNA from other sources.

Until he died in 1845, Carl Wilhelm Naundorff insisted he was the son of Louis XVI, King of France, and Marie Antoinette, both beheaded in 1793 during the French Revolution. According to Naundorff, he was the legitimate successor to the Bourbon monarchy. Official documents, however, recorded that the son of Louis XVI, who became Louis XVII after the execution of his father, had died of tuberculosis in captivity in 1795. But some monarchists were so anxious to perpetuate the Bourbon dynasty that they accepted Naundorff's story. Although generally discounted, Naundorff maintained his right to the throne until his death.

In the 1990s, mitochondrial DNA from samples of hair and bone from Naundorff's remains, nonexhumed hair from Marie Antoinette, two of her sisters, and other dead relatives; and donated DNA samples from living descendants were amplified and sequenced. The results indicated that Naundorff could not have been Marie Antoinette's son. In other words, Naundorff was, as many had suspected, a convincing imposter.

There is more to this story. After his death in 1795, the heart of Louis XVII was apparently placed in a crystal urn and stored in the crypt of a Paris church. The authenticity of this relic has been questioned over the last two hundred years. However, mitochondrial DNA sequence analyses showed that samples from the desiccated heart and from living and dead maternal relatives of Louis XVII were the same, making it unlikely that the relic was from someone other than Louis XVII.

Anna Anderson first declared in the 1920s that she was Anastasia, the youngest daughter of Tsar Nicholas II and Tsarina Alexandra of the Russian Romanov royal family. She maintained this contention until her death in 1984. On the other hand, the revolutionary government in Russia reported in 1918 that the entire Russian royal family, including Nicholas and Alexandra and their five children, had been executed. Moreover, according to an investigation of the murders in 1919, all the bodies of the royal family, along with some servants and the family physician, had been burned. However, at the time, there were widespread rumors that some or all of the royal family had managed to survive the slaughter. Although most exiled Russian aristocrats did not believe Anna Anderson's story, some, for various reasons, found it compelling.

The issue of Anna Anderson's biological status as a Romanov was resolved by comparing DNA sequence data derived from some of her hospital tissue samples, not material from her remains, with mitochondrial DNA samples from the Duke of Edinburgh (Prince Philip), who is the husband of the Queen of England and whose grandmother was Tsarina Alexandra's sister. The data did not support Anna Anderson's lifelong assertion that she was the only surviving member of the Romanov royal family.

In 1991, human remains discovered in an unmarked grave near the site where Nicholas and Alexandra and their family were supposed to have been burned were examined extensively by Russian forensic pathologists. The scientists concluded that the skeletal fragments represented three servants, the family physician, three daughters of the royal family, Alexandra, and Nicholas. Although the weight of this evidence was substantial and convincing, DNA analyses were conducted to satisfy skeptics and to provide what is believed to be definitive proof of identity. Samples from each of the reconstructed skeletons that had been exhumed from the mass grave, from the remains of Nicholas's brother, who died of tuberculosis in 1899 and was entombed in St. Peter and Paul Cathedral in St. Petersburg, and from living descendants of Alexandra were examined for mitochondrial DNA polymorphisms. The results confirmed that the remains of five individuals from the mass grave were from members of the Romanov royal family. The two missing family members, including the son (the tsarevitch) and one of the daughters, are assumed to have been burned.

Mitochondrial DNA analysis has also been used in an attempt to resolve a historical conundrum dating back more than 50,000 years. In this case, the basic question was whether Neandertals and modern humans interbred. Neandertals lived in

Europe and western Asia from about 300,000 to 30,000 years ago before disappearing from the paleontological record. During the last 50,000 to 100,000 years of their existence, Neandertals occupied the same geographic space as modern humans. Sequences of a PCR-amplified segment of Neandertal mitochondrial DNA from at least five individuals are similar and significantly different from both early and recent modern humans. Despite the possibilities that Neandertal mitochondrial DNA may have been lost because of genetic drift or swamped out by the mitochondrial DNA contributions of modern humans, it seems likely that there was little interbreeding, if any, between these two groups.

subunit genes (*NDUFS1*, 2q23–q34; *NDUFS2*, 1q23; *NDUFS7*, 19p13; *NDUFS8*, 11q13; *NDUFV1*, 11q13) have been identified. Generally, homozygous recessive individuals with mutations of these genes typically develop Leigh syndrome with cardiomyopathy.

A deficiency of complex II (succinate dehydrogenase) in two siblings of a consanguineous marriage was associated with a homozygous mutation of the *SDHA* nuclear gene at chromosome 5p15 that encodes subunit A of the succinate dehydrogenase complex. In these individuals, the onset of the disorder was in early childhood. Computed tomography scans showed a pattern of degeneration of the substantia nigra and basal ganglia that resembled Leigh syndrome. Similarly, individuals with mutations of the genes (*SDHB*, 1p36.13; *SDHC*, 1q21; *SDHD*, 11q23) for the other three subunits of complex II suffer from Leigh syndrome. And, mutations of the *UQCRB* gene at 8q22 that encodes a complex III, subunit cause, among other defects, hypoglycemia and lactic acidosis.

There are about 60 nuclear proteins (ancillary proteins) that participate in the assembly and functioning of the OXPHOS complexes. Mutations of the *BCS1L* gene at 2q33 affect the assembly of complex III, with the overall phenotype being either Leigh or GRACILE (growth retardation, amino aciduria, lactic acidosis, and early death) syndromes accompanied by kidney and liver defects. Complex IV is altered by mutations at five loci (*COX10*, 17p12–p11.2; *COX15*, 10q24; *SCO1*, 17p12–p13; *SCO2*, 22q13; *SURF1*, 9q34), and Leigh syndrome is the primary clinical outcome with specific secondary abnormalities in some individuals. For example, infant cardiomyopathy is common with *SCO2* and *COX15* mutations and kidney and liver dysfunction with *SCO1* and *COX10* mutations, respectively.

Mitochondrial DNA Defects

A number of kindreds have been found with autosomal dominant inheritance of progressive external ophthalmoplegia (adPEO). In these cases, the phenotype also includes ptosis, skeletal muscle weakness, sudden death, and, to varying degrees, ataxia, deafness, and depression. Onset of the disorder frequently occurs within the fourth decade of life. Analysis of the mitochondrial DNA of affected individuals from these families indicated that large numbers of mitochondrial genomes have deletions of different lengths. By contrast, individuals with mitochondrion based KSS, PEO, or PS have mitochondrial deletions of equal lengths.

At least three nuclear genes have been associated with adPEO and large multiple mitochondrial DNA deletions. First, the *ANT1* gene at 4q35 encodes adenine nucleotide translocator 1 that is embedded in the inner membrane of

heart and skeletal muscle mitochondria and shuttles ADP into the matrix and ATP into the intermembrane space. The relationship between adPEO and *ANT1* mutations is not known. Possibly, alteration of the adenine nucleotide pool affects replication and increases the frequency of errors, including deletions. Second, linkage studies of pedigrees with adPEO family members localized a gene to 10q24. A computer search of the coding sequences in this region identified *C10orf2* (chromosome 10 open reading frame 2) as the most likely candidate gene. After it was confirmed that *C10orf2* was responsible for adPEO in these families, its designation was changed to *PEO1*. A BLAST analysis indicated that the *PEO1* gene shared sequence similarity with gene 4 of T7 bacteriophage. Gene 4 encodes a primase/helicase that participates in T7 DNA replication. The PEO1 protein, which was dubbed Twinkle because of its immunostaining pattern within mitochondria, binds to mitochondrial DNA. If Twinkle is a mitochondrial helicase, then mutant forms likely impair DNA replication, repair, or recombination that might produce structural alterations. Third, mutations of mitochondrial DNA polymerase gene (*POLG*, 15q26.1), the enzyme that is responsible for mitochondrial DNA replication and repair, were found in individuals with adPEO. Defective mitochondrial DNA polymerase activity could give rise to multiple deletions.

Multiple mitochondrial DNA deletions have also been found in a rare autosomal recessive disorder called mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) that has PEO, other physical abnormalities indicative of a mitochondrial disorder, and OXPHOS deficiency as features of a multisystem phenotype. The MNGIE locus has been mapped to chromosome 22q13.32–qter. Mutation analyses of MNGIE patients established that the thymidine phosphorylase gene (*TP*), which falls within 22q13.32–qter, has nucleotide changes in both alleles. In these cases, the level of thymidine phosphorylase was reduced by about 95%. Under normal conditions, thymidine phosphorylase, which catalyzes thymidine to thymine and vice versa (thymidine + P $\leftrightarrow$ thymine + 2-deoxy-D-ribose 1-phosphate), is expressed in the brain, peripheral nerves, gastrointestinal system, bladder, and lungs. In addition to its potential role in controlling cellular and, possibly, mitochondrial thymidine levels, thymidine phosphorylase stimulates blood vessel formation and inhibits glial cell proliferation. It is not clear how lowered levels of thymidine phosphorylase lead to multiple mitochondrial deletions. Perhaps mitochondrial DNA replication, which is continuous in most cells, is unusually susceptible to a thymidine imbalance. Interestingly, a frequent site for one of the MNGIE-deletion breakpoints is within the *ND5* gene.

The loss of all of the DNA of the mitochondria of specific tissues is a major feature of a group of rare, fatal autosomal recessive disorders that have been designated as mitochondrial DNA-depletion syndromes (MDSs). When DNA depletion affects >88% of the mitochondria in a tissue, infants usually die within the first three years after birth. When 66% to 88% of the mitochondria lose their DNA, the onset of progressive mitochondrial encephalomyopathy, hypotonia, and lactic acidosis begins during late childhood. Two MDS genes have been mapped and characterized. First, mutations of the *DGUOK* gene at 2p13 that encodes mitochondrial deoxyguanosine kinase (dGK) cause mitochondrial DNA depletion of liver cells, liver and neurological dysfunction,

increased lactate concentration, and hypoglycemia. Second, mutations of the mitochondrial thymidine kinase gene (*TK2*, 16q22.1) are responsible for severe loss of muscle mitochondrial DNA. The production of mitochondrial dNTPs depends on both of these kinases. Consequently, mutations that limit the supply of dNTPs constrain DNA replication and bring about DNA depletion. Presumably, under these conditions, there are too few active mitochondria to enable high-energy-demand tissues to function properly. At present, there is no explanation for the apparent tissue specificity of the different mutant kinases.

key terms

ATP
cristae
electron transport chain
genetic bottleneck
GRACILE
helicase
heteroplasmy
homoplasmy
intermembrane space
Kearns–Sayre syndrome
Leigh syndrome
LHON
maternal inheritance
matrix
MDS
MELAS
MERRF
mitochondrion
mitochondrial DNA depletion
MNGIE
mutation load
NARP
oxidative phosphorylation
OXPHOS
PEO
ragged red fibers
replicative segregation
threshold effect

summary

Mitochondria are semiautonomous, self-propagating, DNA-containing cytoplasmic organelles that are centers for production of ATP. Human mitochondrial DNA encodes 13 mitochondrial proteins, 22 tRNAs, and 2 rRNAs. The 13 mitochondrial proteins are synthesized in the mitochondria and contribute subunits to complexes I, III, IV and V of the oxidative phosphorylation (OXPHOS) system that generates ATP. The remaining subunits of these OXPHOS complexes, other proteins of the OXPHOS system, and about 1000 additional mitochondrial proteins are encoded by nuclear genes. The nuclear-encoded mitochondrial proteins participate in a myriad of mitochondrial functions.

Mutations that affect the production of ATP occur in various mitochondrial genes. For a mitochondrion with such a mutated gene to have a biological impact, it usually must be present in 70% or more of the mitochondrial genomes of a tissue. Tissues such as the brain, heart, muscles, and liver, which require large amounts of chemical energy, are likely to be damaged when there is a shortfall of ATP. A mixture of mitochondria with different genomes in a cell or tissue is called heteroplasmy. Homoplasmy is when a cell or tissue has only one type of mitochondrial genome. Homoplasmy or a high level of heteroplasmy with a mitochondrial gene mutation is the result of chance events. First, from the time of maturation of an oocyte to the early stages of embryogenesis there is a loss of thousands of mitochondria until there are 100 or fewer mitochondria per cell. Thus, if the mitochondrial population of an oocyte is heteroplasmic and a mitochondrion with a gene mutation survives the bottleneck, then an early embryonic cell and all of its descendants will be populated with mitochondria that carry the mutant gene. Second, the distribution of mitochondria during cell division cycles is a random process. Thus, when heteroplasmy is present, it is possible that a cell will receive a disproportionate number of mitochondria with a gene mutation. There is then a good probability that a high level of mutant mitochondrial genomes will be maintained through successive cell division cycles.

The identification of a mitochondrial disorder is aided by observing the mode of acquisition of the syndrome in multigeneration kindreds. Under these circumstances, affected mothers have affected offspring, but affected fathers almost never have affected offspring. When this

continued

pattern of maternal inheritance is consistently observed, it is probable that the root cause of the disorder is a mutation of a mitochondrial gene.

More than 50 different point mutations of various mitochondrial genes have been discovered. For example, a clinical condition called myoclonus epilepsy and ragged red fibers (MERRF) has a gene mutation in the mitochondrial $tRNA^{Lys}$ gene in the majority of cases. Mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes (MELAS) is the most common maternally inherited mitochondrial disorder. Most of the MELAS mutations occur in one of the two mitochondrial leucine transfer RNAs (i.e., $tRNA^{Leu(UUR)}$). Interestingly and currently inexplicably, mutations at various sites on the $tRNA^{Leu(UUR)}$ gene produce very different phenotypes. Mutations at some sites give rise to cardiomyopathy as the major phenotype, whereas at other sites the main clinical features are diabetes and deafness. In addition, mutations in two other mitochondrial genes give rise to the MELAS phenotype. Leber hereditary optic neuropathy (LHON) is the result of nucleotide changes in mitochondrial genes that encode subunits of OXPHOS complex I. And neuropathy, ataxia, and retinitis pigmentosa (NARP) occurs when mitochondrial genes that specify ATPase 6 of complex V are mutated.

Mitochondrial genomes with structural abnormalities, such as deletions or duplications, usually do not pass through the mitochondrial bottleneck. However, a mitochondrial genome with a specific deletion can accumulate in the tissues. When heteroplasmy for a mitochondrial genome with a deletion of 4977 bp of mitochondrial DNA, with breakpoints in the *ATP8* and *ND5* genes, is >85% in muscles of the eye and the retinal pigment layer, a distinct set of clinical features known as Kearns–Sayre syndrome occurs, including progressive external ophthalmoplegia and degeneration of the retinal pigment layer.

Mutations of nuclear-encoded mitochondrial genes affect importation of a protein into mitochondria, the transport of compounds between compartments of a mitochondrion, substrate conversions by enzymes of mitochondrial metabolic pathways, transport of iron into mitochondria, and the functioning of OXPHOS complexes. Dominant mutations of three nuclear-encoded mitochondrial genes (*ANT1*, *PEO1*, *POLG*) cause multiple deletions in mitochondrial genomes, with the main clinical feature being progressive external ophthalmoplegia. A multisystem syndrome called mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is associated with mutations of the thymidine phosphorylase (*TP*) gene at 22q13.32–qter. Mutations of mitochondrial thymidine and deoxyguanosine kinases are often responsible for fatal mitochondrial DNA depletion of muscle and liver cells, respectively.

References

Bourgeron, T. et al. 1995. Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency. *Nat Genet* **11**:144–149.

Chinnery, P. F. et al. 1997. Molecular pathology of MELAS and MERRF. The relationship between mutation load and clinical phenotypes. *Brain* **120**:1713–1721.

Chomyn, A. 1998. The myoclonic epilepsy and ragged-red fiber mutation provides new insights into human mitochondrial function and genetics. *Am J Hum Genet* **62**:745–751.

de Lonlay, P. et al. 2001. A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure. *Nat Genet* **29**:57–60.

De Meirleir, L. et al. 2003. Clinical and diagnostic characteristics of complex III deficiency due to mutations in the *BCS1L* gene. *Am J Med Genet* **121A**:126–131.

DiMauro, S. and E. A. Schon. 2003. Mitochondrial respiratory-chain diseases. *N Engl J Med* **348**:2656–2668.

Fromenty, B. et al. 1997. High proportions of mtDNA duplications in patients with Kearns-Sayre syndrome occur in the heart. *Am J Med Genet* **71**:443–452.

Gill, P. et al. 1994. Identification of the remains of the Romanov family by DNA analysis. *Nat Genet* **6**:130–135.

Gill, P. et al. 1995. Establishing the identity of Anna Anderson Manahan. *Nat Genet* **9**:9–10.

Goto, Y., I. Nonaka, and S. Horai. 1990. A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature* **348**:651–653.

Haut, S. et al. 2003. A deletion in the human QP-C gene causes a complex III deficiency resulting in hypoglycaemia and lactic acidosis. *Hum Genet* **113**:118–122.

Hirano, M. et al. 1998. Mitochondrial neurogastrointestinal encephalomyopathy syndrome maps to chromosome 22q13.32–qter. *Am J Hum Genet* **63**:526–533.

Holt, I. J. et al. 1990. A new mitochondrial disease associated with mitochondrial DNA heteroplasmy. *Am J Hum Genet* **46**:428–433.

Howell, N. 1998. Leber hereditary optic neuropathy: respiratory chain dysfunction and degeneration of the optic nerve. *Vision Res* **38**:1495–1504.

Ivanov, P. L. et al. 1996. Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of Tsar Nicholas II. *Nat Genet* **12**:417–420.

Jehaes, E. et al. 1998. Mitochondrial DNA analysis on remains of a putative son of Louis XVI, King of France and Marie-Antoinette. *Eur J Hum Genet* **6**: 383–395.

Kaukonen, J. et al. 2000. Role of adenine nucleotide translocator 1 in mtDNA maintenance. *Science* **289**:782–785.

Kobayashi, T. et al. 1995. A single amino acid substitution within the mature sequence of ornithine aminotransferase obstructs mitochondrial entry of the precursor. *Am J Hum Genet* **57**:284–291.

Krings, M. et al. 1997. Neandertal DNA sequences and the origin of modern humans. *Cell* **90**:19–30.

Mandel, H. et al. 2001. The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nat Genet* **29**:337–341.

Moraes, C. T. et al. 1989. Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns-Sayre syndrome. *N Engl J Med* **320**:1293–1299.

Nishigaki, Y., R. Marti, and M. Hirano. 2004. ND5 is a hot-spot for multiple atypical mitochondrial DNA deletions in mitochondrial neurogastrointestinal encephalomyopathy. *Hum Mol Genet* **13**:91–101.

Nishino, I., A. Spinazzola, and M. Hirano. 1999. Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science* **283**:689–692.

Olsson, C. et al. 1998. Level of heteroplasmy for the mitochondrial mutation A3243G correlates with age at onset of diabetes and deafness. *Hum Mutat* **12**:52–58.

Orii, K. E. et al. 1997. Genomic and mutational analysis of the mitochondrial trifunctional protein β-subunit (HADHB) gene in patients with trifunctional protein deficiency. *Hum Mol Genet* **6**:1215–1224.

Rötig, A. et al. 1997. Aconitase and mitochondrial iron-sulphur protein deficiency in Friedreich ataxia. *Nat Genet* **17**:215–217.

Saada, A. et al. 2001. Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nat Genet* **29**: 342–344.

Schon, E. A., E. Bonilla, and S. DiMauro. 1997. Mitochondrial DNA mutations and pathogenesis. *J Bioenerg Biomembr* **29**: 131–149.

Schweitzer, R. R. 1995. Anastasia and Anna Anderson. *Nat Genet* **9**:345.

Serre, D. et al. 2004. No evidence of Neandertal mtDNA contribution to early modern humans. *PLoS Biol* **2**:0313–0317.

Shoffner, J. M. et al. 1990. Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA tRNA(Lys) mutation. *Cell* **61**:931–937.

Smeitink, J. A. et al. 1998. Nuclear genes of human complex I of the mitochondrial electron transport chain: state of the art. *Hum Mol Genet* **7**:1573–1579.

Spelbrink, J. N. et al. 2001. Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat Genet* **28**:223–231.

Taanman, J. W. et al. 1997. Molecular mechanisms in mitochondrial DNA depletion syndrome. *Hum Mol Genet* **6**: 935–942.

Takakubo, F. et al. 1995. An amino acid substitution in the pyruvate dehydrogenase E1α gene, affecting mitochondrial import of the precursor protein. *Am J Hum Genet* **57**:772–780.

van den Heuvel, L. et al. 1998. Demonstration of a new pathogenic mutation in human complex I deficiency: a 5-bp duplication in the nuclear gene encoding the 18-kD (AQDQ) subunit. *Am J Hum Genet* **62**:262–268.

Van Goethem, G. et al. 2001. Mutation of *POLG* is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nat Genet* **28**:211–212.

von Kleist-Retzow, J. C. et al. 2003. Mitochondrial diseases—an expanding spectrum of disorders and affected genes. *Exp Physiol* **88**:155–166.

Wallace, D. C. 1992. Diseases of the mitochondrial DNA. *Annu Rev Biochem* **61**: 1175–1212.

Wallace, D. C. and M. T. Lott. 2004. "MITOMAP: A Human Mitochondrial Genome Database." http://www.mitomap.org

Wallace, D. C. et al. 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science* **242**: 1427–1430.

Wolpoff, M. H. 1998. Neandertals: not so fast. *Science* **282**:1991.

review questions

1. What is OXPHOS? What are the key features of this system?
2. What does the mitochondrial genome contribute to a mitochondrion?
3. What do nuclear genes contribute to a mitochondrion?
4. Compare the patterns of inheritance of a mitochondrial gene disorder and an X-linked recessive trait in multigeneration kindreds.
5. What is the mitochondrial genetic bottleneck? What is its positive feature? What is its negative feature?
6. What is heteroplasmy? What role does heteroplasmy play in various mitochondrial disorders?
7. How does homoplasmy for a mitochondrial gene mutation occur?
8. What are ragged red fibers?
9. Why don't different mutated mitochondrial genes produce the same phenotype?
10. What are some of the clinical features that might form part of a newly discovered mitochondrial disorder?
11. What is replicative segregation?
12. What is a mitochondrial mutational load?
13. What is a threshold effect?

continued

14. What are the two major features of mitochondrial inheritance?

15. How would you test for MERRF?

16. What are the key diagnostic features of MELAS?

17. What does *MTTF*MELAS*583A signify?

18. How would you distinguish between defects due to mitochondrial and nuclear-encoded mitochondrial gene mutations?

19. What is mitochondrial DNA-depletion syndrome (MDS)? What are the likely causes of MDS?

20. Draw a pedigree showing the relationship between the Duke of Edinburgh (Prince Philip) and Anastasia. Why was this analysis sufficient to discredit Anna Anderson's claim that she was Anastasia?

chapter 13

Molecular Genetics of Muscle Disorders

Aches contract and starve your supple joints.

WILLIAM SHAKESPEARE (1564–1616)

It changes your whole life. It affects everything, every decision, every activity. . . . It is relentless, it is not that you have a level of disability and adjust to that. He gets worse. We see him changing daily. The demands don't ease up and there's never a break or a day off.

Mother of a boy with Duchenne muscular dystrophy.

SMILING, WRITING, RUNNING, drawing, walking, gripping, chewing, kissing, swallowing, breathing, and virtually any other activity that involves movement depend on one or more of the 650 different muscles in the human body. In general terms, muscles are responsible for locomotion, upright posture, balancing on two legs, support of internal organs, controlling valves and body openings, production of heat, and movement of materials, including blood, along internal tubes. There are three types of muscle tissue: skeletal, cardiac, and smooth. Skeletal muscles, which make up about 40% of our body mass, are attached to the skeleton. They are responsible mainly for locomotion, and both contraction and relaxation are controlled voluntarily (i.e., consciously). Cardiac muscle, found only in the heart, pumps blood and functions involuntarily. Smooth muscle, which lines the walls of internal systems, is involuntary and propels material through internal passageways. The elasticity of muscle tissue enables it to return to its original length after either contracting or extending. Both voluntary and involuntary muscle contractions are stimulated by nerve impulses.

Structure of Skeletal Muscle

Skeletal muscles are composed of clusters of muscle cells, which are also called muscle fibers, myofibers, or myocytes. A muscle cell is long and spindle-shaped and contains many nuclei, that is, multinucleate. The plasma membrane (cell membrane) of a muscle cell is called the sarcolemma, and the cytoplasm is called the sarcoplasm. Some muscle cells can be >30 cm in length, whereas

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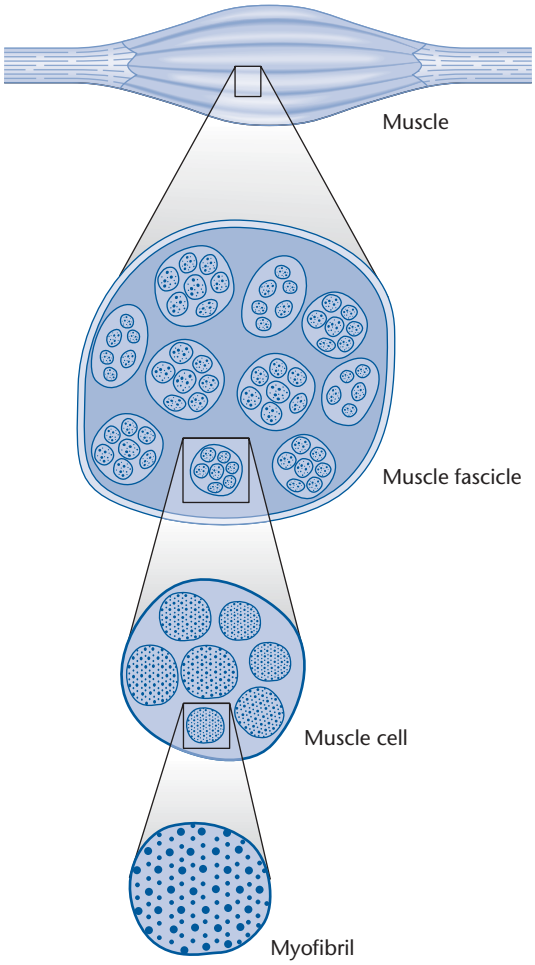


Figure 13.1 Schematic representation of skeletal muscle. A muscle is composed of separate fascicles that each contain a number of muscle cells. Each muscle cell has myofibrils, and each myofibril consists of an array of thick and thin myofilaments.

others are only 0.1 cm long. The diameter of skeletal muscle cells ranges from 0.001 to 0.01 cm. A muscle consists of packages of muscle cells called fascicles (Figure 13.1). Within each muscle cell are elongated protein molecules (myofibrils) aligned in parallel arrays and extending along the full length of the cell. The myofibrils consist of protein chains (myofilaments) that, when histologically stained and viewed in cross section, show a symmetrical, alternating pattern of thick and thin elements. The thick myofilament consists of a large number of bundled myosin molecules aligned in overlapping arrays. A myosin molecule often is represented diagrammatically with a bulbous head and a hinged connecting rod (Figure 13.2). Skeletal muscle myosin is a hexameric protein with two identical heavy chains and two pairs of different light chains. One of the pairs of the light chains is designated as the regulatory light chain (RLC) and the other as the essential light chain (ELC). The thin myofilament (F-actin, filamentous actin) is made up of two helically intertwined chains of G-actin (globular actin) units. Some of the other proteins that attach to the actin molecules are tropomyosin and the three members of the troponin protein complex (Figure 13.2). The thick and thin myofilaments, with accessory myofibril proteins, are responsible for muscle contraction.

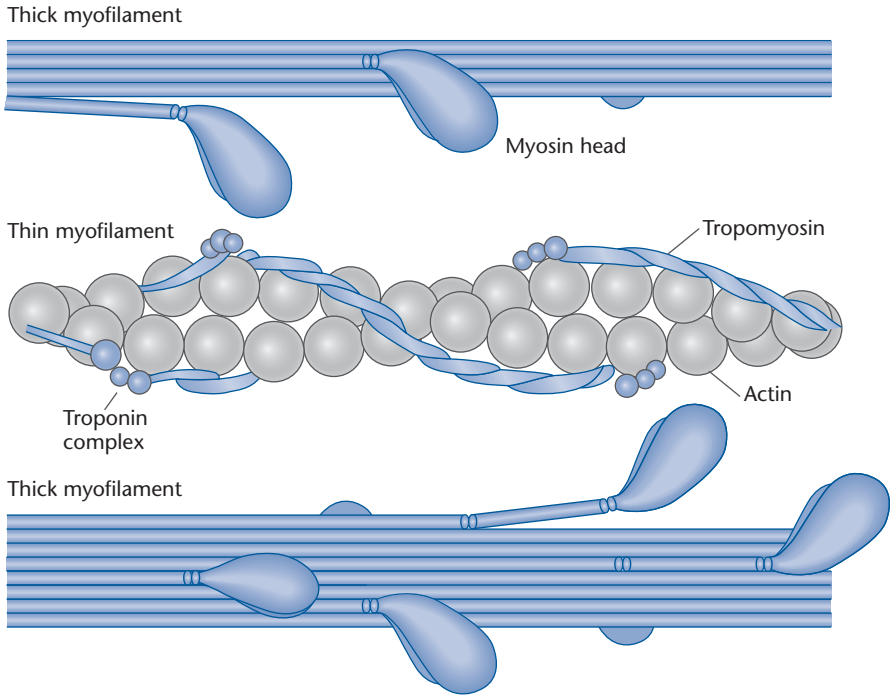


Figure 13.2 Schematic representation of the thick and thin myofilaments of skeletal muscle cells. The thick myofilament is composed of myosin molecules. A single myosin molecule has two heavy chains and two pairs of different light chains (not shown). The thin myofilament is made up of actin, tropomyosin, and troponin molecules. Three different troponin molecules form the troponin complex. Other accessory myofilament proteins are not depicted.

As a result of a nerve impulse, calcium ions are taken into the muscle cell. The influx of calcium induces the troponin complex to pull tropomyosin molecules away from the G-actin subunits, thereby unblocking myosin-binding sites. The heads of myosin molecules then can bind to actin subunits and form cross bridges. The formation of myosin-actin connections occurs when an active site in each myosin head, part of the heavy myosin chain, disrupts the high-energy bond of adenosine triphosphate (ATP) molecules stored in the head of the heavy myosin chain. This release of energy moves the myosin heads toward the F-actin, and, when contact is made with the actin subunits, the F-actin is pulled along, causing the myofilament to contract. The coordinated contraction of all of the myofilaments of all of the muscle cells of a muscle causes the entire muscle to contract. During relaxation, calcium ions are carried away from the myofilaments, the myosin-actin linkage loosens, the troponin complex and tropomyosin bind to the myosin-binding sites on the F-actin subunits, and both the myosin and F-actin myofilaments return to their original positions.

Dystrophin and Associated Muscle Proteins

A set of noncovalently associated proteins forms an interconnected system extending from the F-actin myofilaments and the cytoskeleton in the sarcoplasm to the matrix that surrounds each muscle cell (Figure 13.3). The N-terminal end of the muscle protein dystrophin is attached to F-actin, and the region near the C-terminus of dystrophin binds to β-dystroglycan embedded in the sarcolemma. In addition, α-dystrobrevin, a sarcoplasm protein, binds to

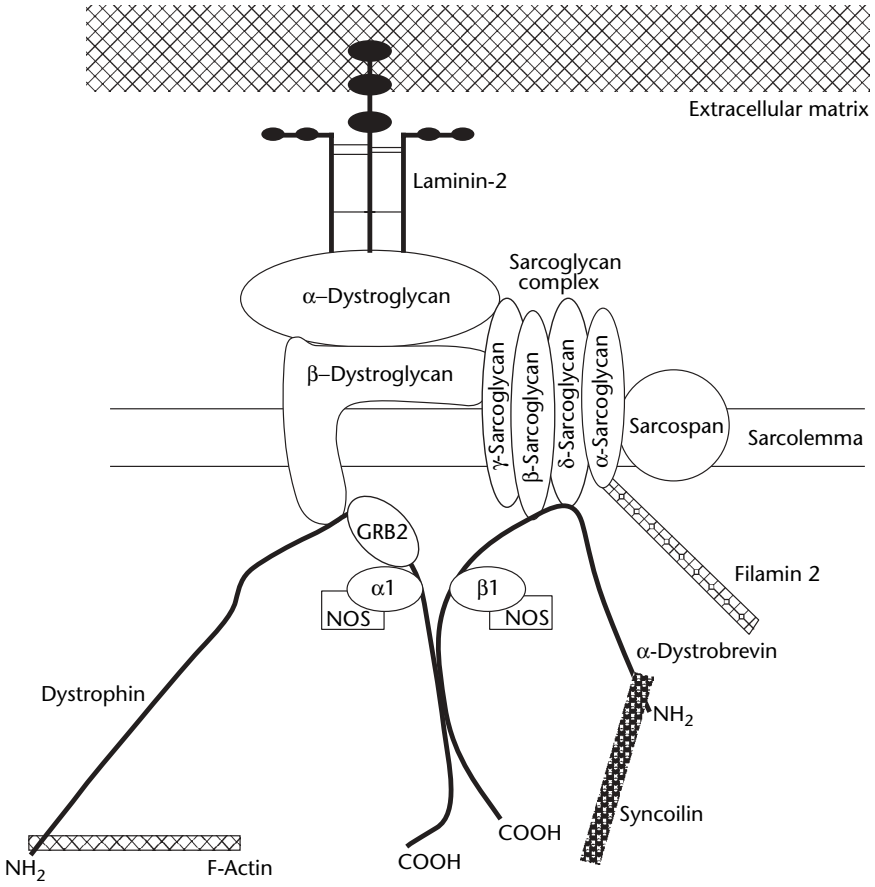


Figure 13.3 Schematic representation of the dystrophin-glycoprotein complex (DGC) of skeletal muscle cells. The α1- and β1-syntrophins are marked as α1 and β1, growth factor receptor-bound protein 2 as GRB2, and neuronal nitric oxide synthase as NOS. The proteins, protein domains, and protein associations are not drawn to scale.

dystrophin and to members (β-sarcoglycan, δ-sarcoglycan) of the sarcoglycan complex that are embedded in the sarcolemma. Other proteins bind to dystrophin and α-dystrobrevin. On the outside of the muscle cell, the protein α-dystroglycan binds to both β-dystroglycan and to a heterotrimeric protein called laminin-2. Laminin-2 binds to the extracellular matrix surrounding the muscle cell. In addition, transmembrane proteins called integrins attach cells to extracellular matrix proteins. The extracellular matrix is a meshwork of fibers, mostly collagen, and other components that provide a structural framework for muscle cells. The dystrophin-glycoprotein complex (DGC) is a multifunctional structure. On the one hand, dystrophin and α-dystrobrevin and their associated proteins form a bridge between the internal cytoskeleton and the extracellular matrix to prevent stress-induced fractures of the sarcolemma during muscle cell contraction. On the other hand, components of the DGC, for example, growth factor receptor-bound protein 2 (GRB2) and neuronal nitric oxide synthase (NOS), are instrumental in intracellular signaling transduction pathways that maintain cell viability and regulate cell functions such as cytoskeletal organization (See Figure 16.2 in Chapter 16 on Molecular Genetics of Cancer Syndromes for an example of a signal transduction pathway).

Cardiac and Smooth Muscle

The muscle cells of the heart (cardiocytes) are about 10 to 20 μm in diameter and 50 to 100 μm in length. Each cardiocyte has a single nucleus. The myofibrils and accessory muscle contraction proteins are similar to those found in skeletal muscle cells. However, many of these proteins are encoded by genes specifically expressed in cardiocytes.

Smooth muscle cells are long and spindle-shaped. They encircle blood vessels, the digestive tract, and organs such as the stomach, bladder, and uterus. The myofilaments of smooth muscle cells are not organized in extensive parallel arrays, as in skeletal and cardiac muscle. Therefore, a symmetrical pattern of thick and thin filaments is not evident. In addition, in comparison to skeletal and cardiac muscle, smooth muscle cells do not have a troponin complex. Although the contractile mechanism of smooth muscle cells is similar to the other muscle types, it has its own distinctive features.

Studying Inherited Muscle Disorders

There are probably more than 1000 different nuclear genes specifically required for the proper formation and functioning of muscles. Microarray analyses of skeletal muscle cells have detected about 3500 expressed genes. Most of the clinically important inherited muscle conditions are the result of mutations of genes that encode (a) enzymes that break down glycogen or fatty acids for energy, (b) proteins that maintain the structural integrity and mechanical properties of a muscle cell, and (c) proteins that either regulate or participate in the contractile process. Clearly, mutations of genes that are expressed primarily in muscle cells will lead to disorders that, for the most part, are confined to muscles. There are also a number of different gene mutations, especially those of genes expressed in nervous tissue, with impacts on multiple organ systems, including the muscles. However, the focus in this chapter is on those genes that principally affect muscle activity.

A number of inherited recessive metabolic disorders of muscle cells have been identified (Table 13.1). For example, mutations of the muscle-specific glycogen phosphorylase gene (*PYGM*), located in region 11q13.1, are responsible for phophorylase deficiency (McArdle disease), with symptoms that include muscle stiffness, cramps, and muscle deterioration (myopathy) after exercise, especially prolonged exercise. Other muscle-specific enzyme defects that impair glycogen utilization produce a similar phenotype. Enzyme deficiencies that cause muscle deterioration as a result of reduced energy supply have not been studied extensively because they are rare and easily subject to misdiagnosis.

There is at least one example of an inherited defect of a sarcolemma protein that affects the flow of calcium ions in and out of muscle cells. Linkage analysis of families with an autosomal dominant disorder called hypokalemic periodic paralysis (hypoKPP) localized the site of the gene to 1q31–q32. Patients with hypoKPP have episodes of extreme muscle weakness and/or paralysis lasting from 4 to 24 h. Physiologically, the level of potassium in their serum is low and the intracellular concentration of calcium is high. Screening for mutations of candidate genes showed that the gene encoding the dihydropyridine

Table 13.1 Muscle cell enzyme deficiency disorders.

Disorder	Gene	Site	Protein activity	Onset (yr)	Symptoms
Phosphorylase deficiency; McArdle disease	*PYGM*	11q13.1	Glycogen phosphorylase; catalyzes and regulates conversion of glycogen to glucose-1-phosphate	~5–16	Exercise-induced myopathy; cramps; muscle stiffness
Acid maltase deficiency; Pompe disease	*GAA*	17q25.3	α-Glucosidase; breakdown of glycogen to glucose-1-phosphate	~2–20	Weakness of upper leg and arm, trunk, and respiratory muscles; enlarged liver, heart, and tongue
Phosphofructokinase deficiency; Tarui disease	*PFKM*	12q13.3	Phosphofructokinase; converts fructose-6-phosphate to fructose 1,6-bisphosphate	~5	Muscle fatigue; severe cramps; nausea, vomiting; myopathy
Debrancher enzyme deficiency; Cori or Forbes disease	*AGL*	1p21.2	Amylo-1,6-glucosidase; 4-α-glucanotransferase; glycogen debranching enzyme	~5	Myopathy; muscle weakness; hypoglycemia; enlarged liver
Phosphoglycerate kinase deficiency	*PGK1* *PGK2*	Xq13 6p12.3	1,3-Bisphoshoglycerate + ADP to 3-phosphoglycerate + ATP	~5–18	Muscle pain and weakness; exercise-induced myopathy
Phosphoglycerate mutase deficiency	*PGAM2*	7p13	3-Phosphoglycerate to 2-phosphoglycerate	~5–20	Muscle pain and weakness; exercise-induced myopathy
Lactate dehydrogenase deficiency	*LDHA*	11p15.1	Lactate + NAD to pyruvate + NADH	~5–20	Exercise-induced myopathy
Myoadenylate deaminase deficiency	*AMPD1*	1p13.1	Adenosine monophosphate deaminase-1; deamination of adenosine monophosphate to inosine monophosphate	~18–45	Muscle fatigue and weakness; exercise-induced muscle cramping

receptor protein was mutated in many hypoKPP patients. This protein forms a channel in the sarcolemma and regulates the uptake of calcium into muscle cells. Thus, in these patients, a defective dihydropyridine receptor protein is responsible for increasing the intracellular level of calcium in muscle cells, preventing muscle relaxation and leading to paralysis.

To date, molecular genetics studies of muscle disorders have centered on those conditions that occur relatively frequently, are classified easily, and can be analyzed readily with candidate gene strategies. A considerable amount of information has been assembled about the genes that encode the structural and contractile proteins of skeletal and heart muscle cells. Very little is known about gene mutations that specifically affect smooth muscle cells.

Skeletal Muscle Disorders

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked recessive, progressive muscle-wasting disease that affects approximately 1 in 3300 live-born males. DMD derives its name from French neurologist Guillaume Duchenne (1806–1875), who studied this devastating and ultimately lethal condition. The onset of DMD is evident at about 4 to 5 years, when affected children show

muscular weakness of the hips, an inability to run, a characteristic set of maneuvers when rising from a kneeling position (Gower's sign), and, as a consequence of muscle cell degeneration, extremely enlarged calves (pseudohypertrophy) as a result of the accumulation of collagen (fibrosis) and fat. Usually, the muscles of the shoulders and pelvis (i.e., proximal muscles) weaken before those of the arms and legs (i.e., distal muscles). In most cases, DMD patients are confined to a wheelchair by their 10th year, and the mean age of death is 17 years. Extreme cardiac and smooth muscle failure occurs in approximately 20% of DMD patients. Usually, death results from weakened lung and heart muscles. Samples of skeletal muscle tissue from DMD males indicate a loss of muscle cells and a breakdown in the organization of the muscle, with the lengths of the remaining muscle fibers being variable. Although DMD is predominantly a skeletal muscle disorder, other tissues such as the heart, brain, and bladder are affected, to some extent, in DMD patients.

A characteristic biochemical feature of DMD is an elevated level of creatine phosphokinase (CPK) in the serum, ranging from 10 to 10,000 times normal levels. CPK is a muscle enzyme responsible for releasing the energy stored in creatine phosphate during muscle contraction. The presence of large amounts of CPK in the blood system reflects muscle degeneration but does not necessarily represent the severity of the damage to the muscles.

Females who are heterozygous (carriers) for the DMD gene usually show no significant muscle weakening, although slightly elevated levels of CPK are found in the serum. Some carriers, however, have muscle pain and cramps during exercise, some heart problems, and, in rare cases, enlarged calves. Severe DMD occurs in females who have a translocation between the X chromosome and an autosome. Invariably, in these individuals, one of the translocation breakpoints falls within the same band (Xp21.2) of the X chromosome. In addition, females with a single X chromosome (monosomy X) that carries a defective *DMD* gene suffer the full impact of DMD. Overall, the occurrence of DMD in females is rare.

Becker muscular dystrophy (BMD), an X-linked recessive disorder, is a mild form of DMD. Generally, BMD affects 1 in 30,000 live-born males. In these patients, muscle wasting (myopathy) becomes apparent later in life than in DMD, and the course of the disease is extremely variable. The life span is longer for individuals with BMD than for those with DMD. And, in some instances, BMD patients, despite very weak muscles, remain ambulatory for decades. Before the discovery of the DMD gene, it was not known whether DMD and BMD resulted from different mutations of the same gene (allelic heterogeneity) or mutations in different genes (genetic heterogeneity).

The *DMD* gene at Xp21.2 is one of the largest of all known genes. It occupies about 2400 kb of chromosome DNA and has 79 exons. The protein encoded by the *DMD* gene is called dystrophin and constitutes about 0.002% of the total protein of a muscle cell and about 5% of the proteins that are bound to the sarcolemma. Dystrophin has four functional domains. The N-terminal domain consists of approximately 240 amino acids and contains a binding site(s) for F-actin. The second domain has 24 repeating units that are each approximately 88 to 126 amino acids in length. This segment is the central rod domain, which is about 100 to 125 nm in length and includes about 3000 amino

acids. The third domain is a cysteine-rich, 280-amino acid segment that binds to β-dystroglycan. The fourth domain has approximately 420 amino acids and binding sites for syntrophin, α-dystrobrevin, and other proteins (Figure 13.3).

Mutation detection tests revealed that defects in the *DMD* gene are responsible for BMD. Thus, BMD is a less severe allelic form of DMD. Approximately 65% of all DMD males have deletions of the *DMD* gene. Another 5% of the DMD cases are the result of a duplication of one or more exons. Small nucleotide changes are probably responsible for the remaining DMD mutations. Although mutation rates are difficult to measure, generally the frequency of *DMD* gene mutations, which is about 7.6×10^{-5} genes per generation, is much higher than the rates for many other loci. The majority of genetic changes within the *DMD* gene, including single base pair mutations, cause protein truncation with little or none of the third or fourth domains of dystrophin being translated. These changes produce the DMD phenotype. In contrast, deletions of the *DMD* gene that produce a BMD phenotype have intact reading frames. In these cases, a partially functional dystrophin, lacking an internal segment, is synthesized. Of the few missense mutations found in DMD patients, one (C3304Y) prevents dystrophin from binding to β-dystroglycan. This single nucleotide mutation underscores the important role that dystrophin plays in linking F-actin to the sarcolemma.

The precise role(s) of dystrophin in muscle cells is not known. A common observation in patients with DMD is that, in addition to dystrophin deficiency, there are reduced levels of the proteins of the sarcolemma associated with dystrophin. In the absence of these proteins, there is no bridge between the internal contraction system and the extracellular matrix, which may lead to rupturing of the sarcolemma, muscle cell degeneration, and, eventually, deterioration of the whole muscle. As well, without the dystrophin binding sites for proteins that initiate signal transduction pathways, among many possibilities, the constraints on the programmed cell death system are relieved and, as a consequence, muscle cells are destroyed.

Transcription of the *DMD* gene is quite complex. In addition to the full-size dystrophin of skeletal muscles, there are several tissue-specific dystrophin molecules (isoforms). Full-size dystrophin isoforms in muscle (skeletal and cardiac), brain (cortex, hippocampus), and cerebellar Purkinje cells arise from different promoters, each with a unique first exon. For the less than full-size isoforms, transcripts are synthesized from four promoters within introns of the third and fourth dystrophin domains of the *DMD* gene. These smaller dystrophin isoforms are found in retinal, embryonic, and nerve cells. Exon skipping and exon scrambling also produce short dystrophin isoforms. Even though the functions of the various dystrophin isoforms have not been fully clarified, the *DMD* gene clearly has multiple biological capabilities in addition to its important role as part of the DGC.

Limb-Girdle Muscular Dystrophy

In addition to DMD and BMD, other muscular dystrophies are inherited as autosomal dominant and recessive traits. For the most part, although there is considerable phenotypic variability, the physical features of these progressive autosomal muscular dystrophies include initial weakening of the pelvic and

Table 13.2 Genetic heterogeneity of limb-girdle muscular dystrophy.

Type	Gene	Site	Protein	Function
LGMD1A	*TTID*	5q31	Titin immunoglobulin domain protein; myotilin	Thin filament-associated Z-disk protein; binds to α-actinin, filamin c, and F-actin; stabilizes and anchors thin filaments
LGMD1B	*LMNA*	1q21.2	Lamins A/C	Intermediate filament of inner nuclear membrane
LGMD1C	*CAV3*	3p25	Caveolin-3	Sarcolemma component; intracellular routing; protein assembly; mediates signal transduction pathways
LGMD2A	*CAPN3*	15q15.1	Calpain-3	Intracellular protease; binds to myofibrils
LGMD2B	*DYSF*	2p13.2	Dysferlin	Sarcolemma fusion and repair
LGMD2C	*SGCG*	13q12	γ-Sarcoglycan	Sarcoglycan complex
LGMD2D	*SGCA*	17q21	α-Sarcoglycan	Sarcoglycan complex
LGMD2E	*SGCB*	4q12	β-Sarcoglycan	Sarcoglycan complex
LGMD2F	*SGCD*	5q33.3	δ-Sarcoglycan	Sarcoglycan complex
LGMD2G	*TCAP*	17q12	Titin cap protein; telethionin	Thin filament-associated Z-disk protein; substrate for titin kinase
LGMD2H	*TRIM32*	9q33.2	Member of tripartite motif family	Possibly, enzymatic addition of ubiquitin to proteins (E3-ubiquitin ligase)
LGMD2I	*FKRP*	19q13.3	Fukutin-related protein	Glycosylation of α-dystroglycan; possibly phospholigand transferase
LGMD2J	*TTN*	2q31.2	Titin	Very large skeletal muscle protein (3700 kDa) with binding sites for many muscle proteins; component of thick filaments

shoulder muscles, followed by loss of the use of muscles in the upper arms and legs. The term limb-girdle muscular dystrophy (LGMD) has been given to this genetically heterogeneous set of disorders. The LGMDs have been divided into two categories, with LGMD1 and LGMD2 representing forms with dominant and recessive inheritance, respectively. To date, no X-linked examples of LGMD have been identified. The types of LGMD in each category are assigned an uppercase letter in order of discovery. Overall, at least 13 different LGMD genes have been identified (Table 13.2).

Linkage analyses with either large or consanguineous families with normal dystrophin have been used to assign gene loci for LGMD. In addition, some of the genes that encode the proteins associated with the DGC have been screened for LGMD mutations. To date, no mutations have been observed in α- or β-dystroglycan, syntrophins, or dystrobrevin genes of patients with LGMD. On the other hand, mutations in the genes that encode for the members of the sarcoglycan proteins are associated with LGMD (Table 13.2).

Because the components of the sarcoglycan subcomplex are bound to each other in the sarcolemma, one defective sarcoglycan protein probably prevents the others from assembling, which, in turn, presumably disrupts the "shock-

absorbing" mechanism that links F-actin to the extracellular matrix by means of dystrophin, dystrophin-associated proteins, and extracellular proteins. Also, the signaling functions associated with proteins of the DGC may be impaired. The end result is muscle cell degeneration and loss of muscle activity. The LGMD phenotype may occur in other ways, because some of the mutant LGMD genes encode muscle proteins that are apparently not associated with the DGC.

Generally, the LGMDs illustrate essential themes in human molecular genetics that are encountered a number of times in this book. First, mutations of a number of different genes produce the same phenotype. This phenomenon is likely when any member of a set of tissue-specific interacting gene products is defective. By analogy, one can think of an automobile that won't start (phenotype), which might be the consequence of any one (faulty gene) of a number of different problems such as ignition, fuel injection, electrical system, and so on. Second, mutations of one gene are, in some cases, responsible for dissimilar disorders. For example, in addition to LGMD1B, mutations of the *LMNA* gene occur with other muscle disorders such as dilated cardiomyopathy (CMD1A) and Emery–Dreifuss muscular dystrophy; familial partial lipodystrophy, which entails loss of fat cells (adipocytes) in adults; loss of axons of peripheral nerves (Charcot–Marie–Tooth disease, CMT2B1); and Hutchison–Gilford progeria syndrome, which is a premature aging disorder. It is not known how various mutant versions of lamins A/C, an intermediate filament of inner nuclear membrane, produce such diverse phenotypes.

Congenital Muscular Dystrophy

Congenital muscular dystrophy (CMD), also designated MDC for muscular dystrophy, congenital, is both clinically and genetically heterogeneous. Inherited usually as an autosomal recessive disorder, CMD severely affects the muscles of newborns and, in some children, also causes brain and nerve damage. The muscles are permanently stretched (hypotonia) and weakened. The joints are deformed as a result of prolonged and repeated contractures. Analysis of sectioned muscle tissue shows variations in the size of muscle cells and an accumulation of an excessive amount of extracellular matrix. On the basis of the latter observation, researchers examined the status of the laminin α2 subunit (formerly called merosin) of the muscle-specific protein called laminin-2 in CMD patients. Laminin-2 is a heterotrimer with one heavy chain (laminin α2) and two light chains (laminin β1 and laminin γ1). In muscle cells, laminin α2 binds to α-dystroglycan and the extracellular matrix (Figure 13.3). In addition, a transmembrane heterodimer, integrin α7β1, also binds to laminin-2 and α-dystroglycan. About half of those born with CMD lack, to some extent, the laminin α2 subunit. Homozygosity mapping and linkage studies of families with members who have laminin α2-deficient CMD localized a putative locus to 6q2. Mutation screening of the *LAMA2* gene, which had previously been assigned to 6q2–q23 and encodes laminin α2, established that it is responsible for laminin α2-deficient CMD. Most of the *LAMA2* mutations cause truncation of laminin α2.

The laminin α2 chain is synthesized in muscle and peripheral nerve cells, whereas the laminin β1 and laminin γ1 chains are synthesized in many differ-

Table 13.3 Genetic heterogeneity of congenital muscular dystrophy.

Disorder	Gene	Site	Protein	Function
Laminin α2 deficient-CMD (MDC1A)	*LAMA2*	6q22.33	α2 Subunit of laminin-2	Links α-dystroglycan to extracellular matrix
Fukuyama CMD	*FCMD*	9q31.1	Fukutin; phosphoryl ligand transferase	Glycosylation of α-dystrophin and possibly other muscle cell proteins
Integrin α7 deficiency	*ITGA7*	12q13.2	Integrin α7	Subunit of integrin α7β1; sarcolemma protein binds to extracellular matrix
Muscle-eye-brain disease	*POMGnT1*[a] (*MGAT1.2*)	1p34–p33	*O*-mannose β1,2-*N*-acetylglucosaminyltransferase	*O*-mannosylation
Walker Warburg syndrome	*POMT1*	9q34.13	Protein *O*-mannosyl transferase	*O*-mannosylation
Rigid spine syndrome	*SEPN1*	1p36.13	Selenoprotein N	Possibly prevents oxidant damage
MDC1C	*FKRP*	19q13.3	Fukutin-related protein; phosphoryl ligand transferase	Glycosylation of α-dystrophin and possibly other muscle cell proteins
MDC1D	*LARGE*	22q12.3	Glycosyltransferase	Glycosylation of muscle protein(s)
Ullrich syndrome 1	*COL6A1*	21q22.3	α1 collagen chain of collagen VI	Component of extracellular matrix
Ullrich syndrome 2	*COL6A2*	21q22.3	α2 collagen chain of collagen VI	Component of extracellular matrix
Ullrich syndrome 3	*COL6A3*	2q37.3	α3 collagen chain of collagen VI	Component of extracellular matrix

[a]Not an approved gene symbol.

ent cell types. It is likely, then, that mutations of the *LAMA2* gene cause either a muscle or nervous system disorder, whereas mutations of the genes that encode the ubiquitous laminin β1 and laminin γ1 proteins may be lethal during fetal development and preclude live births. How truncated laminin α2 subunits cause CMD is not known. Possibly, in the absence of a functional laminin α2 chain, laminin-2 does not bind to the extracellular matrix, disrupting the linkage between F-actin and the extracellular matrix and leading to stress fracturing of the sarcolemma and severe weakening of all skeletal muscles.

Linkage studies of families with CMD individuals who have normal levels of laminin α2 showed no linkage to 6q2, which substantiates the genetic heterogeneity of this disorder. Consistent with this observation has been the discovery of at least 10 additional CMD loci (Table 13.3). From a broad perspective, the proteins of the genes associated with the various forms of CMD fall into three categories. Some are involved in the binding of the sarcolemma and the extracellular matrix (*LAMA2*, *ITGA7*), others form part of the muscle cell extracellular matrix (*COL6A1*, *COL6A2*, *COL6A3*), and a number glycosylate α-dystroglycan and possibly other muscle cell proteins (*FCMD*, *POMGnT1*, *POMT*, *FKRP*, *LARGE*).

Fukuyama-type CMD (FCMD), which maps to 9q31.1, is characterized by severe CMD and nervous system disorders, including mental retardation and seizures. The life span of FCMD patients is seldom more than 20 years. The ridges (gyri, convolutions) and the six laminated layers of the cortical region of the brain do not form to any significant extent (micropolygyria, type II lissencephaly) in patients with FCMD. Because the neurons that embody the

cortex of the brain arrive by migration from deep within the brain, the FCMD defect may hinder neuronal movements.

At a frequency of 1 in 10,000 live births, FCMD is one of the most prevalent genetic disorders in Japan, whereas in the rest of the world it is seldom encountered. Linkage disequilibrium mapping indicated that about 80% of the Japanese population carry a mutated *FCMD* gene that can be traced back 2000 to 2500 years to a common ancestor. Labeled DNA from a cosmid contig that overlapped the *FCMD* locus was hybridized to restriction endonuclease-digested DNA from FCMD patients and non-FCMD individuals. An 8-kb fragment was present in the DNA samples from FCMD patients, whereas instead of this 8-kb fragment, a 5-kb fragment was observed in the DNA sample from normal control subjects. The difference between these fragments is a 3-kb insert within the 3′ untranslated region of the *FCMD* gene. For most FCMD individuals, both *FCMD* alleles have the 3-kb insert. However, there are some FCMD patients who are compound heterozygotes for this gene. In these cases, one of the alleles has the 3-kb insert and the other allele has a mutation that creates a premature stop codon. In one patient, the nucleotide change is a nonsense mutation in one allele and a 2-bp deletion in the other.

The distinctive features of the *FCMD* gene 3-kb insert are a hexanucleotide that is repeated 41 times, a tandem array of 27 copies of a 49-bp sequence, a short interspersed element (SINE)-type sequence, and a string of AT base pairs. In addition, the ends of the insert have 17-bp direct repeats, which indicates that the insert is a retrotransposon. Retrotransposons are genomic DNA sequences that retain some of the characteristics of integrated retroviruses. Specifically, retrotransposons can be transcribed, but they do not encode reverse transcriptase. However, there are other DNA elements in our genomes that produce reverse transcriptase. Occasionally, a retrotransposon transcript can act as a template for reverse transcriptase, and, as a consequence, a cDNA is produced. This retrotransposon cDNA is capable of integrating into various sites scattered throughout the genome. As part of the integration process, the target site is duplicated, and each end of the insert has a direct repeat of this sequence.

Disruption of gene function by a retrotransposon is uncommon, but some of these insertions have been associated with human disorders and cancers. The protein encoded by the *FCMD* gene is called fukutin. In normal individuals, fukutin mRNA is expressed in the brain, skeletal muscle, heart, and pancreas. In FCMD patients, intact fukutin mRNA is not found in these organs. The 3-kb insert either prevents transcription of the *FCMD* gene or makes the transcript extremely unstable. Fukutin is a Golgi apparatus protein that has glycosylation activity and probably modifies α-dystroglycan.

Facioscapulohumeral Muscular Dystrophy

Facioscapulohumeral muscular dystrophy (FSHD) initially affects the muscles of the face, shoulders, and upper arms, followed by weakening of the pelvic, abdominal, and lower limb muscles. As the loss of facial muscles progresses, some FSHD individuals are unable to close their eyes or smile. Many patients also have hearing loss and abnormalities of the capillaries of the retina. In most cases of FSHD, onset occurs when patients are 20 years of age or older. About

20% of those diagnosed with FSHD are confined to a wheelchair by the time they are 50 years old. In some families and in many sporadic cases, the onset of FSHD occurs in early childhood. This disorder is inherited as an autosomal dominant and has a frequency of 1 in 20,000.

Linkage studies of many FSHD families localized the *FSHD* locus to 4q35, which is close to the end (terminus; ter) of the chromosome. FSHD may be genetically heterogeneous, because a small number (~5%) of FSHD families do not show linkage with 4q35. In addition to being linked to 4q35, DNA analyses showed that the *FSHD* locus cosegregated with an *Eco*RI restriction fragment length polymorphism. In FSHD patients, this cosegregating DNA fragment usually ranged from 10 to ~30 kb and, invariably, was <35 kb, whereas in normal individuals the fragment was always 40 kb or longer. Genomic sequencing established that the cosegregating DNA region contained a set of repeating DNA units 3.3 kb in length. This array of repeats was designated as D4Z4. The genetic difference between FSHD patients and normal individuals in the 4q35 region is the number of intact 3.3-kb units lying between the putative *FSHD* locus and the end of the long arm of chromosome 4. FSHD pateints have fewer than 11 repeats, and normal individuals have from 11 to 100 repetitive units. The shorter the cosegregating DNA segment, the earlier the onset of FSHD, the more severe the symptoms, and the sooner a wheelchair is required.

The *FSHD* gene has not been identified. And it is not known how a reduced D4Z4 array leads to FSHD. Initially, it was postulated that the loss of 3.3-kb repeat units puts the terminus closer to the *FSHD* gene, which changes the chromosome configuration in this region and, consequently, blocks *FSHD* gene expression (Figure 13.4). This type of phenomenon, called position effect

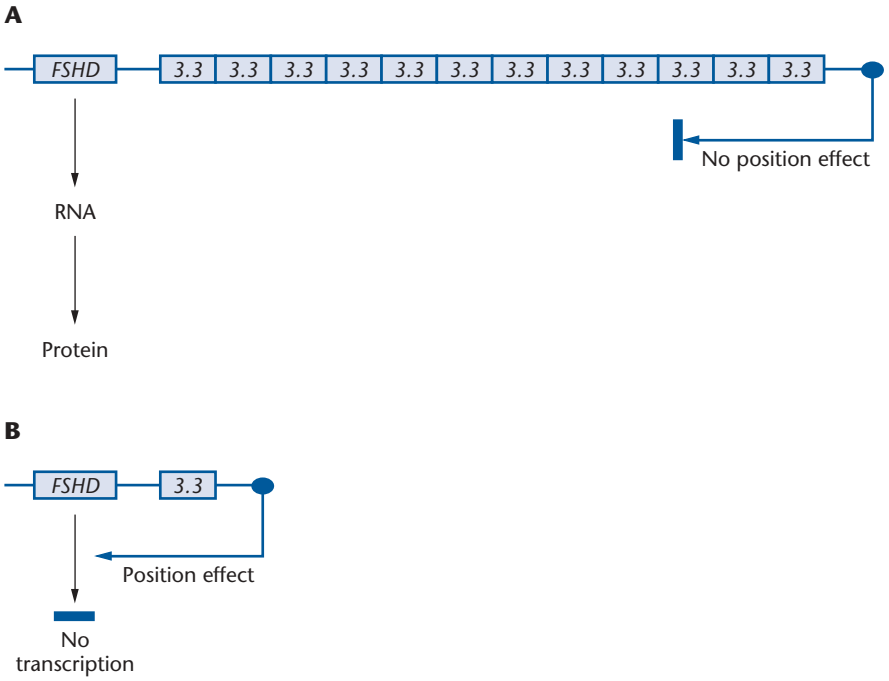


Figure 13.4 Schematic representation of how FSHD may be caused by position effect variegation. (A) In a normal individual the *FSHD* gene (*FSHD*) is separated from the end (solid knob) of the long arm of chromosome 4 by a stretch of DNA that contains a number of repeating 3.3-kb DNA units (3.3). Because the end of the chromosome is far away from the *FSHD* gene, it does not alter the conformation of the DNA region that contains the *FSHD* gene, and, consequently, the *FSHD* gene is expressed. (B) In an FSHD individual, a portion of the DNA segment containing the 3.3-kb repeating units has been deleted, which puts the end (solid knob) of the chromosome arm close to the *FSHD* gene. The region near the end of the chromosome arm alters the conformation of the chromosome segment containing the *FSHD* gene, and, as a result, it is not expressed.

variegation (PEV; position effect), has been observed in *Drosophila* and other organisms. However, this hypothesis has been discarded because various studies have not found inactivation of the genes proximal to the D4Z4 region in FSHD patients. In fact, the experimental data on this point are contradictory. On the one hand, DNA binding studies showed that the normal D4Z4 array combines with a multiprotein complex and the neighboring proximal genes are repressed. Thus, theoretically, repressor binding sites are lost with the removal of D4Z4 repeats, which leads to deleterious overexpression (derepression) of the genes of the 4q35 region. Alternatively, at variance with the latter model, overexpression of genes of the 4q35 region was not observed with either chromatin or microarray analyses of FSHD patients. Further studies suggest that FSHD may be the consequence of disruption of contact between widely separated regulatory and response elements.

Cardiac Muscle Disorders

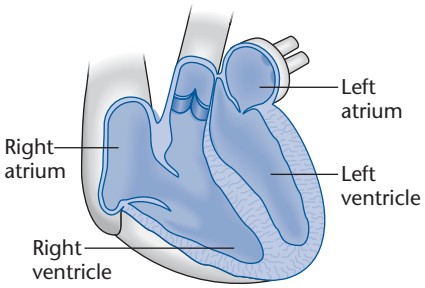
Dilated Cardiomyopathy

The human heart has four chambers: right atrium, right ventricle, left atrium, and left ventricle (Figure 13.5). In contrast to the atria, the walls of the ventricles are well-muscled, especially the left ventricle, which pumps blood to all parts of the body. One commonly observed disorder of the heart is dilated cardiomyopathy (DCM). Generally, cardiomyopathy is a disease of cardiac muscle (myocardium). The principal features of DCM include enlargement of the ventricular chambers, thinning of the ventricular walls, and loss of contractility of the heart muscle. This disease, which accounts for approximately 60% of all cardiomyopathies, is debilitating and often fatal, with death occurring suddenly. Approximately 4% of the population of the United States and probably the same percentage worldwide have DCM. In addition, it is the major condition leading to heart transplant.

DCM is the end result of a number of different conditions. About 50% of cases can be attributed to obstruction of the coronary arteries, viral infections, nutritional deficiencies, abnormalities of the immune system, or myocardial toxins, such as excessive alcohol consumption. The basis of the underlying pathogenesis in the remaining cases of DCM is unknown (i.e., idiopathic). Of these idiopathic occurrences, approximately 25% to 35% are familial. Both familial and sporadic DCM are clinically diverse. In some patients, DCM is accompanied by a defect in the electrical conduction system of the heart. The conduction system of the heart consists of specialized cardiac cells (sinuatrial node), which generate and distribute electric impulses to the heart muscle and ensure the coordinated contraction of the atria and ventricles. In another group, the phenotype is primarily ventricular dilation. In addition, DCM sometimes occurs with skeletal muscle degeneration. In other cases, DCM is accompanied by a large number of additional symptoms.

Studies of families with DCM have indicated autosomal recessive, autosomal dominant, and X-linked modes of inheritance. The autosomal recessive forms of DCM (ARDCM) are rare, with cardiomyopathy as a minor symptom of the overall phenotype. Mutations of nuclear genes that play a role in generating energy from fatty acids in mitochondria are responsible for some forms of ARDCM. Mitochondria are cytoplasmic organelles that oxidize pyruvate

Figure 13.5 Chambers of the human heart.



and fatty acids by way of acetyl-CoA to CO_2 and, concomitantly, by a chemiosmotic process produce chemical energy in the form of ATP. A shortage of ATP impairs the extent of muscle activity. Specifically, when carnitine palmoyltransferase II (CPTII), part of the complex that transfers long-chain fatty acids from the cytoplasm into mitochondria, is deficient, then cardiomyopathy occurs along with other complications including low plasma glucose concentration (hypoglycemia), vomiting, and coma. Severe cases with this enzyme deficiency result in early death. Also, individuals who lack the enzyme medium-chain (C4–C12) acyl-CoA dehydrogenase (MCACAD), which catalyzes the β-oxidation of fatty acids and leads to the production of acetyl CoA, occasionally have DCM as one of the symptoms (Table 13.4).

Table 13.4 Genetic heterogeneity of dilated cardiomyopathy.

Gene	Site	Protein	Function
ACADM	1p31.1	Medium-chain acyl-CoA dehydrogenase	Oxidation of stored medium-chain fatty acids
CPT2	1p32.3	Carnitine palmitoyltransferase II	Transports long-chain fatty acids to mitochondria
CMD1A	1p10–1q10	Unknown	Unknown
CMD1B	9q13–q22	Unknown	Unknown
CMD1C	10q21–q23	Unknown	Unknown
CMD1E	3p25–p22	Unknown	Unknown
CMD1F	6q23	Unknown	Unknown
CMD1H	2q14–q22	Unknown	Unknown
CMD1J	6q23–q24	Unknown	Unknown
CMD1K	6q12–q16	Unknown	Unknown
TTN (*CMD1G*)	2q31	Titin	Associated with myosin filaments and attached to Z disk
TAZ (*G4.5*, *CMD3A*)	Xq28	Tafazzin	Unknown
ACTC	15q14	Cardiac muscle α-actin	Thin filament
DMD	Xp21.2	Dystrophin	Dystrophin-glycoprotein complex
LMNA	1q22	Lamins A/C	Component of inner nuclear membrane
TNNT2 (*CMD1D*)	1q32	Cardiac troponin T	Thin filament
DES (*CMD1D*)	2q35	Desmin	Cytoskeleton
SGCD	5q33.3	δ-Sarcoglycan	Sarcolemma transmembrane glycoprotein
TPM1	15q22.2	α-Tropomyosin	Thin filament
MYBPC3	11p11	Cardiac myosin binding protein C	Thick filament
MYH7	14q12	β-Myosin heavy chain	Thick filament
TNNI3	19q13.42	Cardiac troponin I	Thin filament
CSRP3 (*CMD1M*)	11p15.1	Cardiac LIM protein	Cytoskeleton
LDB3	10q23	LIM domain binding protein 3	Z-disk protein
PLN	6q22.31	Phospholamban	Transmembrane regulator of sarcoplasmic reticulum Ca^{2+}-ATPase
ACTN2	1q43	α-Actinin-2	Z-disk protein
VCL	10q22.2	Metavinculin; isoform of vinculin	Links cytoskeleton to sarcolemma

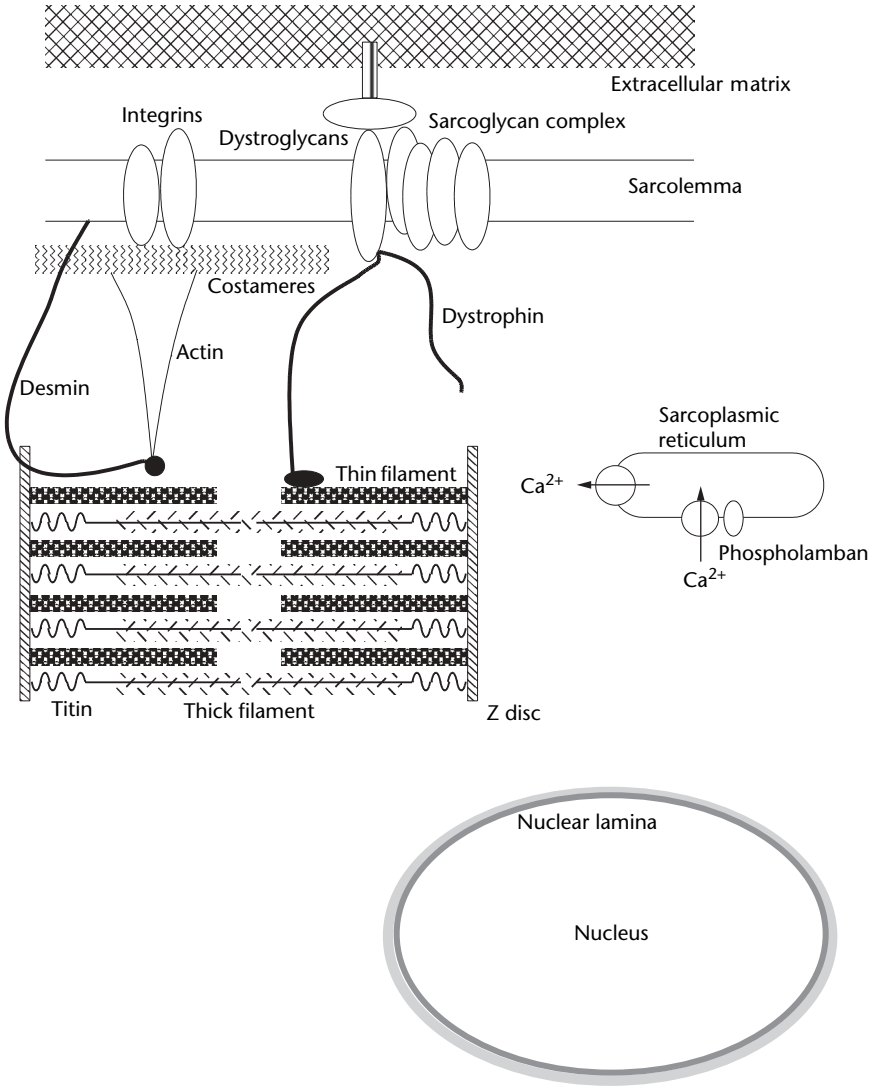


Figure 13.6 Schematic representation of components of a cardiac muscle cell.

On the other hand, DCM is the major phenotypic feature of the autosomal dominant and X-linked forms. Loci of the former group are designated as CMD1 and the latter CMD3, with CMD2 reserved for examples of autosomal recessive inheritance. Letters are assigned in order of discovery to the members of each group. Once a CMD locus is identified, the specific gene symbol replaces the CMD designation, for example, CMD3A became *TAZ*.

As shown in Table 13.4, there are a large number of loci, both known and unknown genes, that are responsible for DCM. The DCM genes encode proteins from nearly all components of cardiac muscle cells (cardiocytes) such as the sarcolemma, cytoskeleton, cardiac DGC, contractile complex (Z disk, thick and fibrils, titin), sarcoplasmic reticulum, and nuclear lamina (Figure 13.6).

The X-linked DCMs (XLCDMs) are of interest. Linkage studies of large families with XLDCM localized a putative gene to Xp21. This X-linked DCM is a progressive, fatal condition that initially appears in males between 15 and 20 years of age. Death frequently occurs a few years after diagnosis. Moreover,

there is no evidence of skeletal muscle abnormalities in affected individuals. Thus it was somewhat surprising when mutation screening revealed that deletions, an insertion, a splice site mutation, and at least one missense mutation of the *DMD* gene were responsible for XLDCM. Additional studies detected splice and missense *DMD* mutations in individuals with sporadic DCM. There is no apparent commonality among the known XLDCM *DMD* gene mutations that explains why cardiac muscle and not skeletal muscle cells is affected. In one case of *DMD* gene XLDCM, a deletion removed both the muscle-specific promoter (Pm) and muscle exon 1 of the *DMD* gene. In the skeletal muscle cells of these individuals, the brain-specific (Pb) and Purkinje cell (Pp) promoters, including brain exon 1 and Purkinje cell exon 1, respectively, are transcribed, and full-length dystrophin isoforms are synthesized. By contrast, these promoters are not active in the cardiac muscle cells of the same patients. Consequently, the skeletal muscles have full-length dystrophin isoforms that can perform acceptably as part of the skeletal muscle contraction system, whereas cardiac muscle cells have no full-length dystrophin molecules. It is not known why dystrophin promoters that are normally repressed in muscle cells are upregulated exclusively in skeletal muscle cells and not in cardiac muscle cells of individuals with a "Pm-muscle exon 1" deletion. In the other cases of XLDCM with *DMD* gene mutations, it is assumed that the defect in the dystrophin molecule is not severe enough to impair its function in skeletal muscle cells, whereas the mutated dystrophin is unable to maintain the integrity of the membranes of heart muscle cells that are continually contracting and relaxing. Overall, the different phenotypes, such as DMD, BDM, and XLDCM, that result from the various mutations of the *DMD* gene, provide a notable example of allelic heterogeneity.

Barth syndrome (BTHS), which maps to Xq28, is an XLDCM that is often fatal. The onset of BTHS occurs in infancy or early childhood and usually is accompanied by myopathy of skeletal muscles, retarded growth, decreased

from the HUMAN GENETICS files

Discovering the DMD *Gene*

The primary cause of DMD was unknown from the time it was first described in full in 1862 to the late 1980s. Because there was no biochemical evidence to suggest which muscle protein might be responsible for the disorder, the only way the *DMD* gene could be discovered was to use a series of experimental procedures that would lead from the most likely chromosome location to the identification of the target gene. This process was called positional gene cloning. Gene discovery by positional cloning became obsolete when the complete human genome was sequenced and sets of overlapping BACs for each chromosome were established.

Three different forms of genetic information were used to localize the *DMD* gene to a specific region of the X chromosome. First, chromosome analysis of females with DMD, each of whom had one X chromosome as part of a balanced translocation with an autosome, showed that the translocation breakpoint in the X chromosome always occurred in band Xp21.2. Second, cytogenetic analysis of the X chromosomes of a number of DMD males revealed deletions of the region containing Xp21.2. In these cases, despite other abnormalities, DMD was always associated with deletion of region Xp21.2, regardless of the length of the deletion. Third, studies with X chromosome DNA probes for polymorphic loci and families with DMD always showed linkage at or near Xp21.2. The closest linked site was about 8 map units (~8×10^6 bp) from the *DMD* gene. In sum, the translocation, deletion, and linkage data indicated that the *DMD* locus lay within or near the Xp21.2 region.

The next problem was to develop strategies to identify clones carrying the

continued

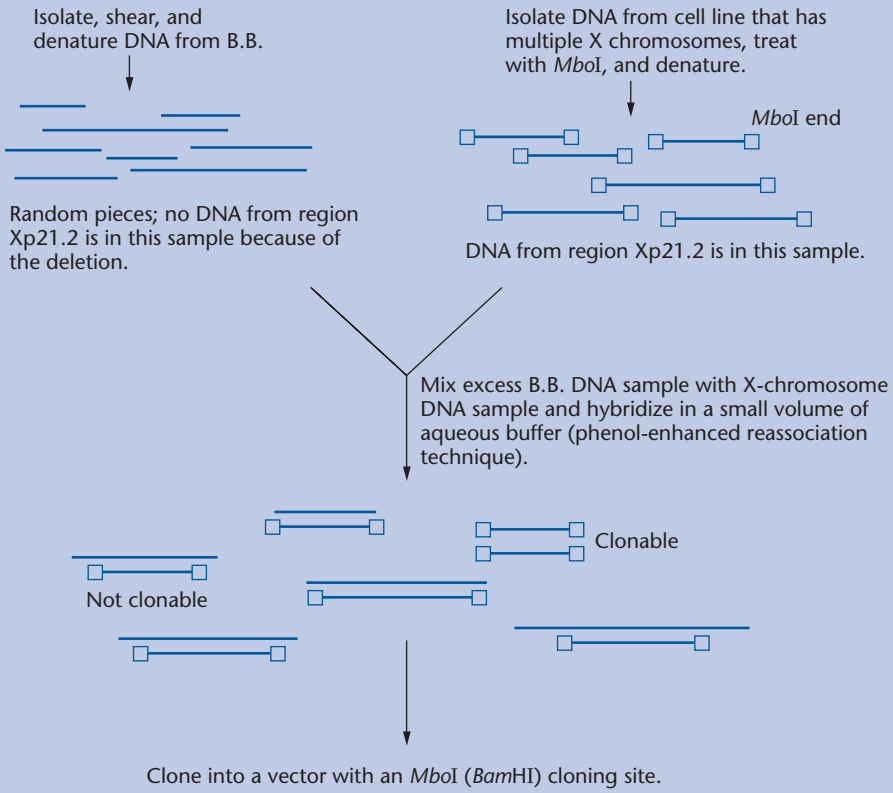
DMD gene or a part of it. However, in the mid-1980s, when the search for the *DMD* gene was initiated, resources such as BACs or X chromosome STSs were not available. Consequently, creative approaches were needed to identify DNA probes for sites closer to the *DMD* gene than the existing repertoire of DNA probes.

One of these strategies entailed isolating a DNA fragment that spanned the Xp21.2-autosome translocation junction point of the translocated chromosome in a female with DMD. In this instance, the translocation chromosome t(X;21)(p21.2;p12) was used because the breakpoint in chromosome 21 occurs within a ribosomal RNA tandem repeat. A library was constructed with DNA from an individual with t(X;21)(p21.2;p12) and screened with a ribosomal RNA DNA probe. Each "ribosome DNA-positive" clone was tested for hybridization with DNA from the X chromosome. One clone (XJ1.1) was found that contained both X chromosome and ribosomal RNA DNA sequences. In other words, XJ1.1 spanned the junction point of the translocation. The X chromosome portion of XJ1.1 was subcloned and characterized. It did not contain exons but was used as a starting point to search for clones carrying exons from the *DMD* gene because it very likely represented DNA residing within or close to the *DMD* gene.

A second strategy used the DNA from a patient (initials B.B.) with DMD who also had three other X-linked disorders. B.B.'s X chromosome had an extensive deletion that included part of Xp21.2. His genomic DNA was used to isolate DNA clones from the Xp21.2 region of the X chromosome (Figure A). DNA isolated from cultured cells derived from patient B.B. was sheared mechanically and denatured. As well, DNA was isolated from a cell line with multiple X chromosomes, cleaved with the restriction endonuclease *Mbo*I, and denatured. The two DNA samples were mixed and hybridized. The hybridization reaction was carried out in a small volume of aqueous buffer to enhance the rate of DNA strand hybridization. This hybridization protocol was called the phenol-enhanced reassociation technique (PERT) because phenol was used to reduce the volume of the DNA solution. For the hybridization reaction, the sheared DNA from patient B.B. was added in excess because these fragments hybridize with all the pieces of *Mbo*I-cut DNA that are shared between the two DNA samples. On the other hand, the *Mbo*I-DNA fragments that correspond to the region deleted in patient B.B. will hybridize only to each other. The hybridized DNA pieces with base-paired *Mbo*I sequences at both ends can be cloned into the appropriate site of a vector. None of the other hybridized DNA molecules can be cloned because they do not have pairs of *Mbo*I ends (Figure A). After the cloning step, nine clones carrying DNA that was not found in patient B.B.'s DNA were isolated and characterized. In other words, these clones carried DNA that occurred within the limits (breakpoints) defined by patient B.B.'s deletion. Because this deletion was so large and more than the *DMD* gene had been deleted, the potential DMD probes were tested by hybridization with DNA from both DMD females with Xp21.2 translocation breakpoints and DMD males who had small deletions (microdeletions). Based on these and other analyses, it was concluded that one of the clones, pERT87, in all likelihood, carried DNA either from within the *DMD* gene or very close to it.

The pERT87 clone had a 200-bp insert, which was too small to indicate whether the sequence represented an exon from the *DMD* gene. Consequently, clones with larger DNA inserts (about

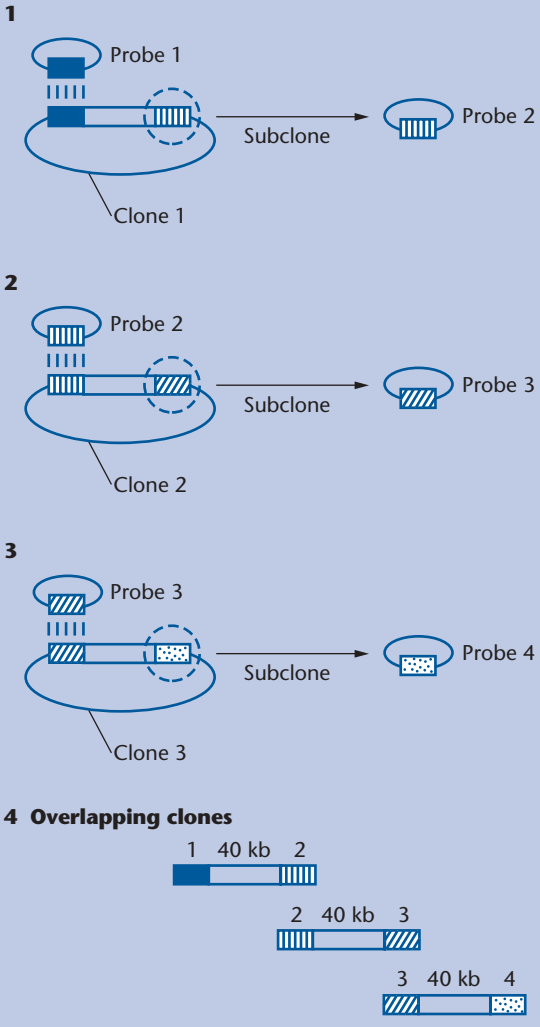
Figure A Phenol-enhanced reassociation technique (PERT) for cloning DNA fragments from region Xp21.2 of the human genome. Double-stranded DNA that is cut with the restriction endonuclease *Mbo*I can be cloned into either an *Mbo*I or a *Bam*HI site.



40 kb) that hybridized to pERT87 and additional clones that overlapped each other were isolated and characterized for exons. This strategy of assembling overlapping clones was called chromosome walking (Figure B). For this procedure, a cosmid human DNA library is screened with the original probe. The insert of a positive genomic clone(s) is mapped with restriction endonucleases and tested for exons by screening a muscle cDNA library. If the genomic clone does not contain a likely exon(s) for the target gene, then the ends of the 40 kb-insert are subcloned and used to screen the genomic library to isolate a second round of clones that are characterized for exons. If required, the ends of the insert(s) from the second round clone(s) are subcloned and used to screen the genomic library a third time. In this way, after a number of rounds of chromosome walking, a contiguous section of genomic DNA can be assembled.

With pERT87 as the initial clone, a nine-round chromosome walk assembled approximately 220 kb of juxtaposed DNA. One of these clones contained evolutionarily conserved sequences and detected a large mRNA transcript (about 14 kb) in muscle cells. It was used to isolate cDNA clones corresponding to the 14-kb transcript. Eventually, a set of cDNA clones encompassing the complete transcript was isolated and characterized. Sequence analysis established that this mRNA encoded a very large protein with 3685 amino acids. Moreover, hybridization studies showed that a full-sized transcript was present in normal muscle cells but not in muscle cells of patients with DMD, who had deletions of Xp21.2. This observation suggested that the 14-kb transcript was derived from the *DMD* locus. Additional chromosome walking was required before the entire *DMD* gene was available for analysis. And, as they say, the rest is history!

Figure B Chromosome walking. (**1**) A probe (Probe 1) hybridizes to a cloned 40-kb piece of DNA. After restriction endonuclease mapping and subcloning, the region farthest away from the original hybridizing sequence is used to form a second probe (Probe 2). (**2**) With Probe 2, another clone from the library (other than clone 1) is selected, and the region farthest away from the Probe 2 sequence is used to form a third probe (Probe 3). (**3**) Same as steps 1 and 2, but using Probe 3. With a third clone, the walk has advanced another 40 kb. (**4**) Three overlapping clones that encompass about 120 kb of contiguous chromosomal DNA. Chromosomal walking can be bidirectional, and restriction endonuclease mapping determines the direction of the successive walks.



numbers of granulocytic white blood cells (neutrophils), and, in some patients, increased levels of 3-methylglutaconic acid in the urine. The Xq28 region contains a large number of different genes, of which at least five are expressed in both skeletal and cardiac muscle cells. Mutation screening of BTHS family members established that gene *G4.5* was responsible for Xq28-linked

XLDCM. The protein encoded by gene *G4.5* was called tafazzin (after Tafazzi, a comic character on an Italian television program), and the approved gene symbol became *TAZ*. Because tafazzin does not share any significant sequence similarity with any known protein, it is impossible to deduce its function without further studies.

Some mutations of the *TAZ* gene cause a lethal neonatal condition called isolated noncompaction of the left ventricular myocardium (INVM). The major features of INVM are the presence of numerous fibrous bundles and deep clefts in the wall of the left ventricle, which cause the heart to malfunction. This condition is quite different phenotypically from the symptoms associated with BTHS and represents another striking example of allelic heterogeneity.

There are probably a number of different pathways that lead to DCM. Of these, a defective force contraction system seems to be the most common pathogenic mechanism. Because of its continual activity, heart muscle is particularly susceptible to altered structural proteins. Briefly, nonuniform contraction, caused by a faulty protein, damages cardiocytes to the extent that various cell death systems are invoked, including programmed cell death, which leads to thinning of the ventricular wall and, eventually, heart failure.

Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is a common lethal autosomal dominant heart disease affecting approximately 1 in 5000 or more individuals worldwide, with a prevalence of 1 in 500 among young adults. The hallmark of this disease is an unusually enlarged (hypertrophic) wall of the left ventricle, which greatly reduces the ventricular volume. In addition, the cardiac muscle cells of the left ventricle are enlarged and disorganized. HCM is responsible for approximately 35% of sudden deaths among athletes and is a major cause of sudden death in apparently healthy juveniles and young adults. About 60% of HCM cases are familial, with the remaining occurrences attributed to new mutations and nongenetic causes. The HCM phenotype is variable, with the disease showing different outcomes within the same family. For example, some members of a family may die suddenly at a young age from HCM, whereas others with the same mutation have mild symptoms and are long lived. On the other hand, sudden death from HCM may be rampant in some families (Figure 13.7).

A number of gene identification studies have established that HCM is genetically heterogeneous and includes, for the most part, genes that encode the proteins of the thick and thin filaments that are specific for cardiac muscle cells (Table 13.5). Because these genes are expressed exclusively in cardiac cells, the mutations have a preferential impact on the heart. In other words, hypertrophic cardiomyopathy is fundamentally a disorder of the cardiac sarcomere, that is, the contractile unit bounded by the Z disks (Z lines) (Figure 13.6). Mutations of the genes encoding the thick filament proteins, cardiac β-myosin heavy chain and cardiac myosin-binding protein C, and the thin filament component, cardiac troponin T, account for about 35%, 30%, and 15%, respectively, of all of the cases of HCM.

Familial HCM is usually inherited as an autosomal dominant disorder. This mode of inheritance suggests either that a single defective protein (“poison”

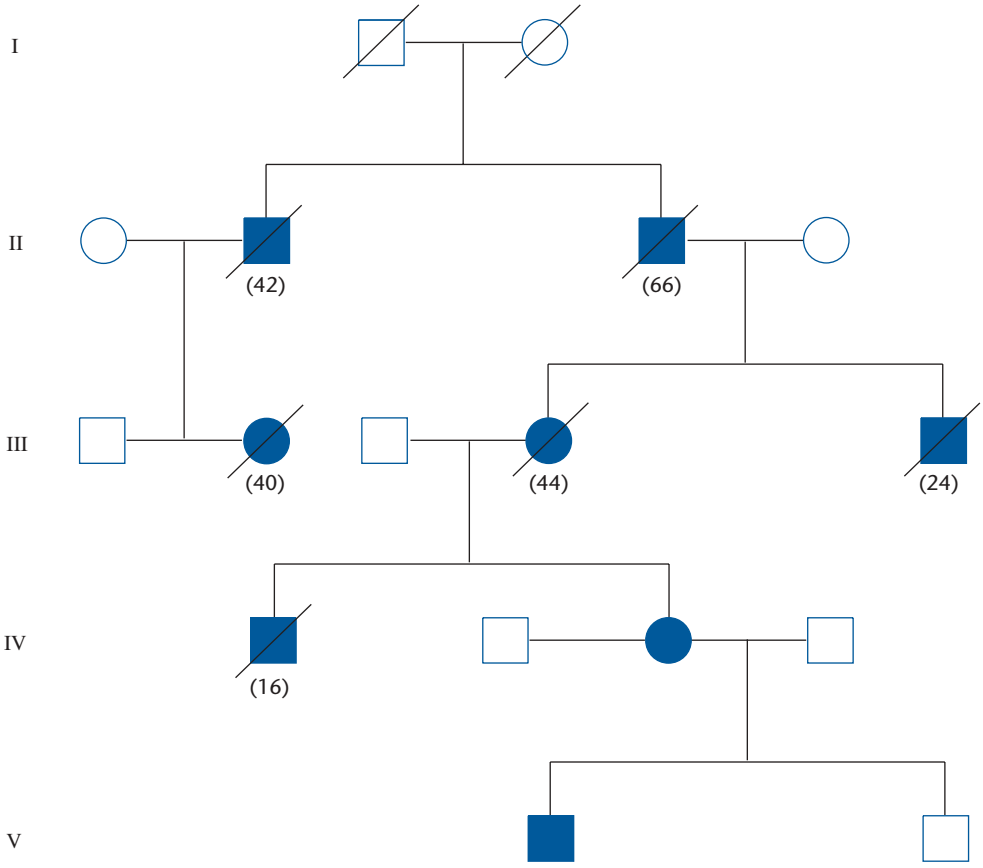


Figure 13.7 Pedigree with hypertrophic cardiomyopathy. Solid symbols represent affected individuals. Age at death in parenthesis. The mutation is R719Q of the cardiac β-myosin (*MYH7*) gene. Adapted from Figure 3 in Doolan, A., L. Nguyen, and C. Semsarian. 2004. Hypertrophic cardiomyopathy: From "heart tumour" to a complex molecular genetic disorder. *Heart Lung Circ* **13**:15–25.

Table 13.5 Genetic heterogeneity of hypertrophic cardiomyopathy.

Gene	Site	Protein	Function
MYH7	14q11.2	Cardiac β-myosin heavy chain	Thick filament
MYBPC3	11p11.2	Cardiac myosin-binding protein C	Thick filament
MYH6	14q11.2	Cardiac α-myosin heavy chain	Thick filament
MYL3	3p21.31	Essential myosin light chain, ventricular isoform	Thick filament
MYL2	12q24.11	Cardiac regulatory myosin light chain	Thick filament
TTN	2q31.2	Titin	Thick filament
ACTC	15q14	Cardiac actin	Thin filament
TNNT2	1q32.1	Cardiac troponin T	Thin filament
TPM1	15q22.2	α-Tropomyosin	Thin filament
TNNI3	19q13.42	Cardiac troponin I	Thin filament
CSRP3	11p15.1	Cardiac LIM protein	Cytoskeleton; sarcomere stabilization

polypeptide) prevents the proper aggregation of a filament or that there is not enough of a particular normal filament protein (haploinsufficiency) to form a functional contractile system. In either case, as a result of a defective contractile system, the cardiac muscle cells become disorganized, the myofibril loses its capacity to generate tension, and, for some unknown reason, possibly as a result of overcompensation, hypertrophy of the left ventricle occurs.

key terms

α-dystroglycan
β-dystroglycan
actin
allelic heterogeneity
Becker muscular dystrophy (BMD)
cardiac muscle
chromosome walking
congenital muscular dystrophy (CMD)
cytoskeleton
dilated cardiomyopathy (DCM)
Duchenne muscular dystrophy (DMD)
dystrobrevin
dystrophin
extracellular matrix
facioscapulohumeral muscular dystrophy (FSHD)
fascicle
Fukayama-type CMD (FCMD)
genetic heterogeneity
hypertrophic cardiomyopathy (HCM)
isoform
lamins A/C
laminin-2
limb-girdle muscular dystrophy (LGMD)
muscle cell
myofiber
myofibril
myofilament
myosin
PERT
positional gene cloning
position effect variegation
sarcoglycan complex
sarcolemma
sarcomere
sarcoplasm
skeletal muscle
smooth muscle
titin
tropomyosin
troponin complex
Z disk

summary

There are three kinds of muscle tissue in humans. Skeletal muscle is attached to the skeleton and contracts voluntarily. Cardiac muscle tissue is found only in the heart and contracts involuntarily. Smooth muscle is controlled involuntarily and is responsible for the contractility of the internal organs, including the stomach, intestines, and bladder, as well as the contractility of hollow tubes such as blood vessels. Contraction of muscle tissue is triggered by nerve impulses and accomplished by the interaction of thick and thin myofilaments. A thick myofilament consists of myosin molecules and associated proteins. A thin myofilament is a chain of actin units combined with tropomyosin and the three proteins of the troponin complex. There are no troponin proteins attached to the thin myofilaments of smooth muscle cells. A collection of myofilaments is called a myofibril, and a muscle cell (muscle fiber, myofiber) has a number of myofibrils. In skeletal muscle tissue, muscle cells are bundled together as a fascicle, and a number of fascicles comprise a muscle. The cells of cardiac muscle tissue are closely packed and joined end to end. Smooth muscle tissue often consists of sheets of cells.

In skeletal muscle cells, a group of sarcoplasm, sarcolemma, and extracellular proteins, including dystrophin, the syntrophins, dystrobrevin, the sarcoglycan complex, α-dystroglycan, β-dystroglycan, and laminin-2 are linked to one another. Presumably, this connected set of proteins enables the sarcolemma to withstand fractures, by absorbing the stress generated by repeated cycles of contraction and extension. Mutations of some genes that encode members of this protein complex are responsible for various types of muscle weakening and wasting disorders (muscular dystrophies). For example, Duchenne muscular dystrophy (DMD), a progressive and lethal muscular disorder, is caused by defective dystrophin.

The genes involved in other types of muscular dystrophies have been identified with various cloning strategies. Limb-girdle muscular dystrophy is genetically heterogeneous, with gene mutations of the sarcoglycan complex contributing to some cases. Congenital muscular dystrophy (CMD), which affects newborns, is also genetically heterogeneous. Mutations of the *LAMA2* gene are common among individuals with CMD.

Facioscapulohumeral muscular dystrophy (FSHD) results from a loss of 3.3-kb repeating units that lie between the mapped FSHD site and the end of the long arm of chromosome 4. How the shortening of the distance between the FSHD locus and the 4qter affects *FSHD* gene expression is not known. Initially, position effect variegation (PEV) appeared responsible for the disorder. Presumably, an altered chromosome configuration suppressed the transcription of the *FSHD* gene. Additional studies have not supported the PEV hypothesis. Although unresolved, loss of 3.3-kb repeats may remove silencer sequences, causing a deleterious overexpression of proximal genes, or the shortened DNA may prevent an essential physical interaction between DNA elements.

Genetic heterogeneity is a significant feature of the familial forms of dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). Some mutations of the *DMD* gene cause DCM with no manifestation of either DMD or BMD. This is a remarkable example of allelic heterogeneity. Many DCM genes are unknown, and the others encode diverse cardiac muscle proteins. By contrast, familial HCM is due primarily to mutations of genes that encode cardiac sacromere proteins.

References

Arad, M., J. G. Seidman, and C. E. Seidman. 2002. Phenotypic diversity in hypertrophic cardiomyopathy. *Hum Mol Genet* **11**:2499–2506.

Arimura, T. et al. 2004. A *Cypher/ZASP* mutation associated with dilated cardiomyopathy alters the binding affinity to protein kinase C. *J Biol Chem* **279**:6746–6752.

Bashyam, M. D. et al. 2003. Molecular genetics of familial hypertrophic cardiomyopathy (FHC). *J Hum Genet* **48**:55–64.

Blake, D. J. et al. 2002. Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol Rev* **82**:291–329.

Bonnefont, J.-P. et al. 1996. Molecular analysis of carnitine palmitoyltransferase II deficiency with hepatocardiomuscular expression. *Am J Hum Genet* **58**:971–978.

Camacho Vanegas, O. et al. 2001. Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. *Proc Natl Acad Sci USA* **98**:7516–7521.

Doolan, A., L. Nguyen, and C. Semsarian. 2004. Hypertrophic cardiomyopathy: From "heart tumour" to a complex molecular genetic disorder. *Heart Lung Circ* **13**:15–25.

Esapa, C. T. et al. 2002. Functional requirements for fukutin-related protein in the Golgi apparatus. *Hum Mol Genet* **11**:3319–3331.

Fatkin, D. and R. M. Graham. 2002. Molecular mechanisms of inherited cardiomyopathies. *Physiol Rev* **82**:945–980.

Feng, J. et al. 2002. Mutations in the dystrophin gene are associated with sporadic dilated cardiomyopathy. *Mol Genet Metab* **77**:119–126.

Feng, J. et al. 2002. Comprehensive mutation scanning of the dystrophin gene in patients with nonsyndromic X-linked dilated cardiomyopathy. *J Am Coll Cardiol* **40**:1120–1124.

Grewal, P. K. and J. E. Hewitt. 2003. Glycosylation defects: a new mechanism for muscular dystrophy? *Hum Mol Genet* **12**:R259–R264.

Hackman, P. et al. 2002. Tibial muscular dystrophy is a titinopathy caused by mutations in *TTN*, the gene encoding the giant skeletal-muscle protein titin. *Am J Hum Genet* **71**:492–500.

Itoh-Satoh, M. et al. 2002. Titin mutations as the molecular basis for dilated cardiomyopathy. *Biochem Biophys Res Commun* **291**:385–393.

Jiang, G. et al. 2003. Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. *Hum Mol Genet* **12**:2909–2921.

Ki, C. S. et al. 2002. Identification of lamin A/C (*LMNA*) gene mutations in Korean patients with autosomal dominant Emery-Dreifuss muscular dystrophy and limb-girdle muscular dystrophy 1B. *J Hum Genet* **47**:225–228.

Kirschner, J. and C. G. Bönnemann. 2004. The congenital and limb-girdle muscular dystrophies: sharpening the focus, blurring the boundaries. *Arch Neurol* **61**:189–199.

Lapidos, K. A., R. Kakkar, and E. M. McNally. 2004. The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circ Res* **94**:1023–1031.

Lenk, U. et al. 1996. A cysteine 3340 substitution in the dystroglycan-binding domain of dystrophin associated with Duchenne muscular dystrophy, mental retardation and ERG b-wave. *Hum Mol Genet* **5**:973–975.

Longman, C. et al. 2003. Mutations in the human *LARGE* gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan. *Hum Mol Genet* **12**:2853–2861.

Martin-Rendon, E. and D. J. Blake 2003. Protein glycosylation in disease: new insights into the congenital muscular dystrophies. *Trends Pharmacol Sci* **24**:178–183.

Michele, D. E. and K. P. Campbell. 2003. Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. *J Biol Chem* **278**:15457–15460.

Moghadaszadeh, B. et al. 2001. Mutations in *SEPN1* cause congenital muscular dystrophy with spinal rigidity and restrictive respiratory syndrome. *Nat Genet* **29**:17–18.

Monaco, A. P. and L. M. Kunkel. 1988. Cloning of the Duchenne/Becker muscular dystrophy locus. *Adv Hum Genet* **17**:61–98.

Muntoni, F., S. Torelli, and A. Ferlini. 2003. Dystrophin and mutations: one gene, several proteins, multiple phenotypes. *Lancet Neurol* **2**:731–740.

Noguchi, S. et al. 2003. cDNA microarray analysis of individual Duchenne muscular dystrophy patients. *Hum Mol Genet* **12**: 595–600.

Prior, T. W. et al. 1995. Spectrum of small mutations in the dystrophin coding region. *Am J Hum Genet* **57**:22–33.

Rando, T. A. 2001. The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. *Muscle Nerve* **24**:1575–1594.

Salmikangas, P. et al. 2003. Myotilin, the limb-girdle muscular dystrophy 1A (LGMD1A) protein, cross-links actin filaments and controls sarcomere assembly. *Hum Mol Genet* **12**:189–203.

Schmitt, J. P. et al. 2003. Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. *Science* **299**:1410–1413.

Smythe, G. M. et al. 2003. A caveolin-3 mutant that causes limb girdle muscular dystrophy type 1C disrupts Src localization and activity and induces apoptosis in skeletal myotubes. *J Cell Sci* **116**:4739–4749.

Tupler, R. and D. Gabellini. 2004. Molecular basis of facioscapulohumeral muscular dystrophy. *Cell Mol Life Sci* **61**:557–566.

Vatta, M. et al. 2003. Mutations in *Cypher/ZASP* in patients with dilated cardiomyopathy and left ventricular non-compaction. *J Am Coll Cardiol* **42**:2014–2027.

Winokur, S. T. et al. 2003. Expression profiling of FSHD muscle supports a defect in specific stages of myogenic differentiation. *Hum Mol Genet* **12**:2895–2907.

Yang, F. et al. 2004. Cytogenetic and immuno-FISH analysis of the 4q subtelomeric region, which is associated with facioscapulohumeral muscular dystrophy. *Chromosoma* **112**:350–359.

Zatz, M. et al. 2003. The 10 autosomal recessive limb-girdle muscular dystrophies. *Neuromuscul Disord* **13**:532–544.

review questions

1. Describe the structural organization of skeletal muscle.
2. What are the macromolecular components of thick and thin myofilaments?
3. What is a Z disk (Z line)? Cite some of the disorders that arise from the genes that encode Z disk proteins.
4. Describe the structural proteins of skeletal muscle that probably maintain the integrity of the sarcolemma.
5. How was the *DMD* gene originally discovered? How would the *DMD* gene be determined today?
6. What is an isoform? Describe how isoforms are generated from the *DMD* gene.
7. Discuss the nature of *DMD* gene mutations and the changes to dystrophin that lead to DMD, BDM, and XLDCM.
8. What is the sarcoglycan complex? Discuss the clinical features of sarcoglycanopathies.
9. What is the genetic basis of congenital muscular dystrophy?
10. Discuss the molecular genetics of FCMD.
11. Schematically represent the left ventricle of the human heart under normal conditions, DCM, and HCM.
12. With OMIM as a primary resource, determine whether genes have been identified for the unknown forms of CMD1.
13. Discuss the molecular genetics of familial hypertrophic cardiomyopathy.
14. Account for the FSHD phenotype. What is PEV?
15. What is titin? What are the clinical features of titin gene mutations?
16. With OMIM, PubMed, GeneClinics reviews, and other Internet resources, write brief essays on the clinical features, inheritance, and molecular genetics of nemaline myopathy, Emery–Dreifuss muscular dystrophy, and myotubular myopathy.
17. Has the function of tafazzin been determined? If so, what does it do and how do *TAZ* mutations cause DCM?

chapter 14

Molecular Genetics of Neurological Disorders

Men ought to know that from nothing else but the brain come joys, delights, laughter and sports, and sorrows, griefs, despondency, and lamentations. . . . And by the same organ we become mad and delirious, and fears and terrors assail us, some by night, and some by day, and dreams and untimely wanderings, and cares that are not suitable, and ignorance of present circumstances, desuetude, and unskilfulness. All these things we endure from the brain when it is not healthy. . . .

HIPPOCRATES (~460–377 BCE)

Oh the nerves, the nerves; the mystery of this machine called man! Oh the little that unhinges it: poor creatures that we are.

CHARLES DICKENS (1812–1870)

A piece of brain tissue is an intricate network that responds to electrical and chemical signals in three-dimensional space and in time. It sends out dynamic patterns and receives and responds to such patterns. These patterns affect each other and, through other nerve connections, the action of other organs of the body—the heart, kidneys, lungs, muscles, and glands. The brain is the master controller and its rhythmic patterns alter how you breathe, pump blood, digest your food, and move.

GERALD M. EDELMAN (b. 1929)

THE NERVOUS SYSTEM IS A COMPLEX, extensive cellular communication network that not only controls and coordinates bodily functions but also formulates novel responses based on stored information. It is divided into two parts: the central (CNS) and peripheral (PNS) nervous systems. The CNS consists of the brain and spinal cord. All the nerves extending from and going to the CNS comprise the PNS. Information from internal and external sources is detected and transmitted by impulses along nerves to the CNS, where these input data are processed and interpreted. Response signals then are conducted back via nerves to nerve, muscle, or gland cells. The complete nervous system has more than 100 billion (1×10^{11}) individual nerve cells (neurons) and perhaps 5 to 10 times as many nonneuronal cells that are closely associated with neurons.

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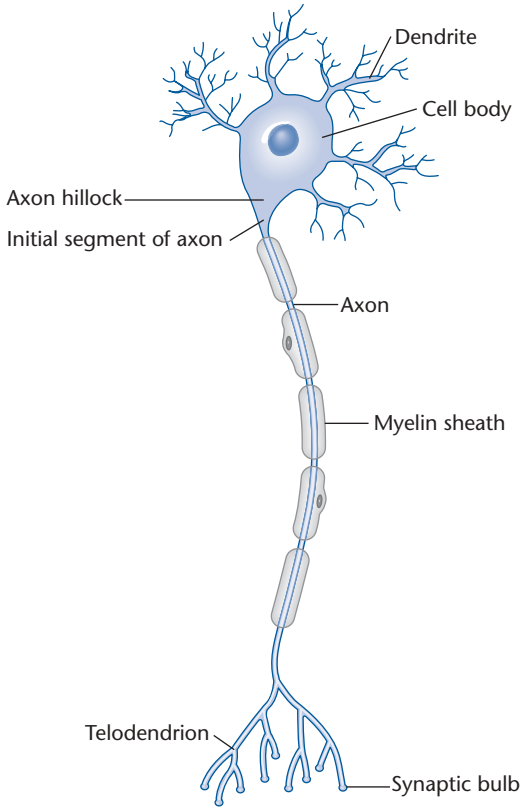


Figure 14.1 Schematic representation of a motor neuron. Motor neurons transmit impulses from the spinal cord to muscles.

Neurons

Neurons are highly specialized cells that transmit electrical impulses and have a variety of shapes, sizes, and roles. They are also biochemically differentiated. Some neurons synthesize acetylcholine (ACh), dopamine, serotonin, gamma (γ)-aminobutyric acid (GABA), somatostatin, or other compounds important for the transmission of nerve impulses from one neuron to another neuron, muscle, or gland cell. A neuron has three basic structural elements: cell body, dendrites, and an axon (Figure 14.1). The cell body (soma) is an enlarged portion of the neuron, containing the nucleus, the machinery for protein biosynthesis, metabolic pathways, and protein strands called neurofibrils. Dendrites are branched elements extending from the cell body. Most neurons have large numbers of multibranched arrays of dendrites. In some cases, dendrites have thousands of minute extensions (spines). Dendrites receive stimuli and transmit nerve impulses toward the cell body. In contrast to the large number of dendrites, each neuron has only one axon (nerve fiber) extending from the cell body. Axonal side branching (at right angles) can occur from a main axon. An axon always carries a nerve impulse away from the cell body. The cone-shaped region of the cell body where an axon originates is called the axon hillock. The thin region of the axon adjacent to the axon hillock is the initial segment. Axons vary in length from a few millimeters in the brain to a meter or more in nerves running from the spinal cord to the muscles of the toes. Many axons form branches near their ends. The tip of each terminal axon

branch is a specialized structure called the synaptic bulb (synaptic knob, end bulb), which makes close contact with a dendrite, cell body, or, occasionally, an axon of another neuron, or with a muscle or gland cell. Each region of contact between a synaptic bulb and the cell receiving a nerve impulse forms a specific junction called a synapse. The space between two cells at a synapse is approximately 20 μm. Overall, the dendrites and cell body of a neuron may have from 10^2 to 10^4 synapses.

Approximately 500 membrane-bound spheres (synaptic vesicles, vesicles) accumulate within each synaptic bulb. The population of synaptic vesicles in a neuron usually contains one kind of molecule, such as acetylcholine, dopamine, serotonin, GABA, glutamic acid, or endorphin. Each of these molecules is synthesized and packaged into a vesicle in the neuronal cell body and transported down the axon to the synaptic bulb. These molecules are called neurotransmitters (Figure 14.2) and are essential for the transmission of a nerve impulse from one neuron to another, or to muscle or gland cells. The neuron that transmits an impulse to another cell is called the presynaptic neuron (presynaptic sender element), and the neuron or other entity that receives and, most of the time, carries the impulse away from a synapse is the postsynaptic neuron or postsynaptic receiver element.

There are three classes of neurons: afferent neurons, efferent neurons, and interneurons. Afferent neurons do not have dendrites. They transmit impulses from specialized structural elements at their peripheral ends to the CNS. Only a small segment of an axon of an afferent neuron lies within the CNS. Efferent neurons conduct electrical signals from the CNS to muscles or gland cells. In these cases, most of the axon is outside the CNS. Interneurons reside entirely within the CNS and make up about 99% of all neurons.

Neuroscience terminology distinguishes between similar neuronal structures in the CNS and PNS. A nerve is actually a bundle of axons (nerve fibers) that are outside of the CNS, whereas a similar axonal aggregation within the CNS is called a tract or pathway. A commissure is a collection of nerve fibers linking the right and left halves of the CNS. Cell bodies of many neurons are often grouped together as discrete clusters at precise locations. When these assemblages are in the CNS, they are called nuclei (sing., nucleus); in the PNS, they are called ganglia (sing., ganglion).

Acetylcholine (Ach)
Aspartic acid
Dopamine (DA)
Endorphin
Epinephrine (Epi)
Gamma (γ)-aminobutyric acid (GABA)
Glutamic acid (glutamate)
Glycine
Histamine
Norepinephrine (NE)
Serotonin (5-hydroxytryptamine, 5-HT)
Somatostatin
Substance P

Figure 14.2 Some examples of neurotransmitters.

Nonneuronal Cells of the Nervous System

The nonneuronal cells (neuroglia, glial cells, glia) of the nervous system have important functions, even though they are not capable of generating and transmitting nerve impulses (signals). There are four kinds of neuroglia in the CNS. First, astrocytes, which get their name because they are star-shaped, provide neurons with nutrients and physical support. Membrane extensions from astrocytes encase the outer surface of blood vessels in the brain and form a barrier (blood-brain barrier) that restricts the passage of certain substances from the blood to the brain. Second, oligodendrocytes put out cell membrane extensions that wrap many times around sections of different axons. These multilayered, lipid-rich cell membranes are called myelin sheaths. Third, microglia are small cells that scavenge cell debris and remove pathogens from the brain. Fourth,

ependymal cells, which line the hollow cavities (ventricles) and tubes in the CNS, secrete cerebrospinal fluid and facilitate its circulation in the ventricles of the brain and the central canal of the spinal cord. Cerebrospinal fluid acts as a shock absorber and distributes nutrients to neurons.

In contrast to the CNS, the PNS has only two kinds of neuroglia. First, satellite cells (capsule cells) surround and protect the cell bodies of the ganglia. Second, each Schwann cell (neurolemnocyte) forms a myelin sheath by wrapping its cell membrane many times around a segment of one axon. The rate of transmission of the nerve impulse is enhanced through a myelinated axonal segment. However, if an axon were ensheathed completely in myelin there would be no conduction of a nerve impulse. Consequently, a myelinated axon has myelinated segments interspersed with nonmyelinated regions. Cell bodies, dendrites, and some CNS and PNS axons are never myelinated.

Resting Membrane Potential

Electrical signals, which are the basis of information processing and neuronal responses to internal and external changes, are generated and propagated by special features of the neuronal cell membrane that convey these impulses from presynaptic neurons to postsynaptic cells. When a neuron is not conducting an impulse, the surface on the outside of the cell membrane is positively charged and the inside face of the cell membrane is negatively charged. Under these conditions, the cell membrane is polarized and the difference in the charge at any point across the cell membrane is called the resting membrane potential. The resting membrane potential is, for the most part, established by concentration gradients of various ions from the inside to the outside of the cell membrane. For example, the extracellular concentration of sodium ions (Na^+) is greater than the intracellular concentration, whereas the reverse holds true for potassium ions (K^+). These resting gradients are maintained, in part, by the cell membrane protein Na^+-K^+ ATPase, which pumps three sodium ions out of the cell at the same time as it brings in two potassium ions. This net difference leads to a build up of a positive charge on the outside of the cell. The negative charge inside the cell, for the most part, is the result of negatively charged proteins that cannot pass through the cell membrane.

In addition to the Na^+-K^+ pump, other neuronal membrane proteins act as ports of entry or exit for specific ions, including Na^+ and K^+. These protein conduits are called channel proteins, ion channels or, simply, channels. Some channels remain open continuously and cannot be closed by extrinsic factors. Others are opened or closed depending on local conditions and/or the binding of a specific low-molecular-weight compound to the channel. In other words, they are gated channels. Because the neuronal Na^+ and K^+ channels are opened and closed by the nature of the charge on the inside of the cell membrane, they are voltage-gated channels. When a Na^+ channel is opened, Na^+ enters the cell, whereas when a K^+ channel is opened, K^+ leaves the cell. Ions do not move through a channel when it is closed. The opened and closed states are the consequence of changes in the conformation of the channel protein. By analogy, one can think of the closing and opening of a channel as the clenching and unclenching of a fist.

Initiation, Propagation, and Synaptic Transmission of a Nerve Impulse

A nerve impulse (excitation, signal) is initiated when a stimulus reverses (depolarizes) the resting membrane potential of a neuronal cell membrane. Generally, a change in the resting membrane potential occurs when Na^+ and K^+ channels, normally closed, are opened and Na^+ rushes into the cell and K^+ rushes out. As a consequence, the resting membrane potential of the portion of the cell membrane with the opened Na^+ and K^+ channels is depolarized. Moreover, this alteration of the charge spreads to the adjacent segment of the cell membrane, which opens the Na^+ and K^+ channels and causes depolarization of this region of the cell membrane. Meanwhile, after 1 to 2 milliseconds (ms), the Na^+ and K^+ channels of the previously depolarized segment close, the Na^+-K^+ ATPase starts pumping K^+ and Na^+ into and out of the cell, respectively, and a resting membrane potential is restored (repolarization) to this section of the cell membrane. If the initial stimulus is neither sufficiently strong nor frequent, the depolarization event is confined to a small area of the cell membrane. If, on the other hand, the initial stimulus is very strong or is repeated frequently, the impulse is propagated along the cell membrane.

Because the threshold level for depolarization in the initial segment of an axon is low, a moderate-strength nerve impulse can trigger a powerful depolarization, leading to a succession of rapid depolarizations that proceed down the axon. The laboratory-determined units for a transmembrane potential are millivolts (mV). The resting membrane potential (resting potential) for neurons is –70 mV, the threshold potential is about –50 mV, and the maximum response for opening the voltage-gated Na^+ and K^+ channels is +30 mV and is called the action potential (Figure 14.3). If the threshold potential is reached, then an action potential will be initiated. Once established, an action potential will continue down an axon unabated. The current flow is unidirectional, because, after the Na^+ and K^+ channels in a sector of an axon have opened and closed, there is a period of time during which they cannot respond to depo-

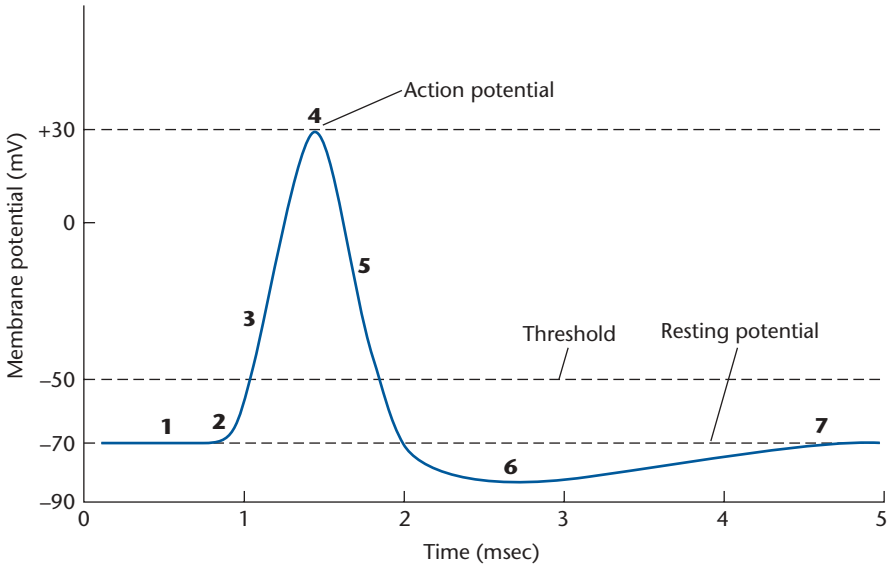


Figure 14.3 Time course of an action potential. The states of the Na^+ and K^+ channels at various times (1–7) during the formation and recession of an action potential are described. (1) Both Na^+ and K^+ channels are closed and openable. (2) The Na^+ channels are open, and sodium ions rush into the neuron. The K^+ channels are closed and openable. (3) The Na^+ channels are open, and sodium ions rush into the neuron. The K^+ channels open, and potassium ions rush out of the neuron. (4 and 5) The Na^+ channels are closed and unopenable. The K^+ channels are open, and potassium ions rush out of the neuron. (6) Both Na^+ and K^+ channels are closed and unopenable. (7) Both Na^+ and K^+ channels are closed and openable.

larizing conditions (refractory period). When these channels are eventually capable of responding, not only has the resting potential been restored, but the region experiencing an action potential has moved too far down the axon to affect these channels.

When the action potential reaches a region of the cell membrane near the synaptic bulb, the depolarization opens voltage-gated calcium ion (Ca^{2+}) channels that are confined to this portion of the axon, and Ca^{2+} rushes into the synaptic bulb (Figure 14.4). The introduction of Ca^{2+} causes synaptic vesicles to fuse with the presynaptic cell membrane and the vesicular contents (i.e, neurotransmitter molecules) to be dumped into the synaptic space (synaptic cleft). Receptor protein molecules embedded in the cell membrane of the postsynaptic receiver element specifically bind the released neurotransmitter molecules. Some receptor proteins are closed ion channels that open after the binding of a neurotransmitter. If, for example, the receptor protein in a synapse is a closed Na^+ channel, then, after the attachment of the neurotransmitter, the channel opens and Na^+ rushes into the postsynaptic neuron. The membrane is depolarized, and an impulse is generated. In some cases, the receptor protein is not a closed ion channel, but instead is a protein that, after the binding of a neurotransmitter, initiates a series of activations by phosphorylation of various cytoplasmic proteins (signal transduction pathway). One of these activated proteins opens the Na^+ channels, generating an impulse. This indirect process of generating a nerve impulse in a postsynaptic neuron is called the second-messenger system.

Not all receptor membrane channel proteins initiate depolarization of the cell membrane of the postsynaptic neuron. In some instances, the neurotransmitter binds to a closed chloride (Cl^-) channel, which, after opening, causes Cl^- to rush into the postsynaptic neuron and K^+ to pour out. Under these conditions at these synapses, the negative charge inside the cell is increased and the cell membrane becomes hyperpolarized, preventing the formation of an action potential. In other words, neurotransmission is inhibited. The vast majority of the more than 50 known neurotransmitters initiate depolarization; that is, they are excitatory. However, the GABA and glycine neurotransmitters both bind to receptors that inhibit neurotransmission in the CNS.

A neurotransmitter, either in the synaptic cleft or bound to its receptor protein, is short lived. Otherwise, either the excitatory or inhibitory state of the postsynaptic cell would be maintained for too long a period. For example, continual excitation of a muscle would cause prolonged and severe contraction. Neurotransmitter molecules are removed by a number of processes, including rapid diffusion, active reuptake by the presynaptic neuron, and enzymatic breakdown. Some presynaptic neurons have specific membrane proteins (transporter proteins) that facilitate the reuptake of released neurotransmitter molecules.

In addition to chemical synapses that use neurotransmitters and receptor proteins, some neurons transmit a nerve impulse by electrical coupling. In these cases, the space between two cells that form an electrical synapse is bridged by barrel-shaped hexameric transmembrane protein complexes called connexons. One connexon complex in the membrane of a presynaptic cell joins with a connexon complex in the membrane of a postsynaptic cell. An electrical synapse

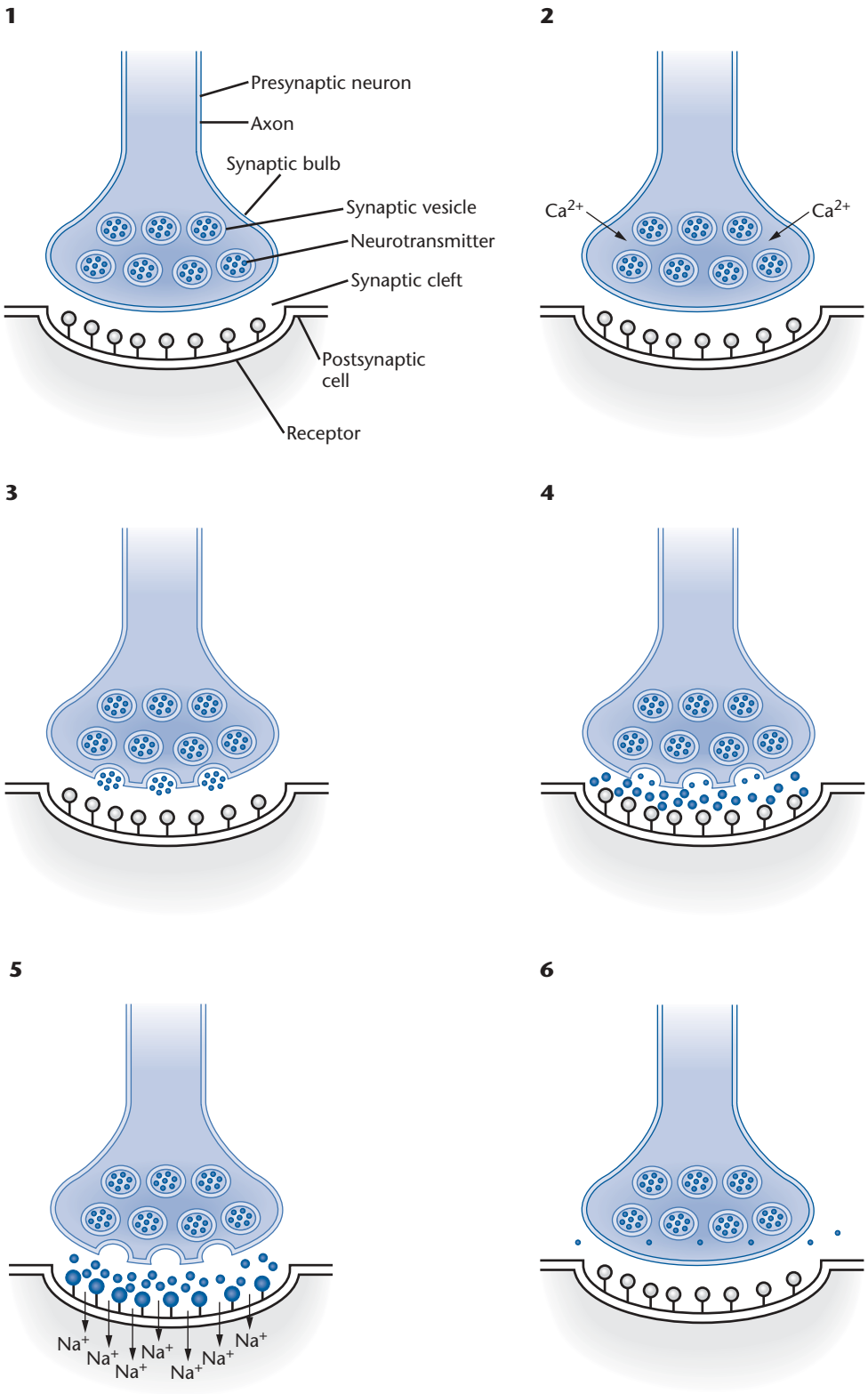


Figure 14.4 Schematic representation of the events during chemical synaptic transmission. (1) The synapse before the arrival of an action potential. (2) An action potential opens voltage-gated Ca^{2+} channels, and Ca^{2+} rushes into the presynaptic bulb. (3) Synaptic vesicles fuse with the presynaptic membrane. (4) Released neurotransmitter molecules flood the synaptic cleft. (5) Neurotransmitter molecules bind to receptors embedded in the postsynaptic membrane, opening Na^+ channels. Sodium ions rush into the postsynaptic cell and initiate a nerve impulse. (6) Neurotransmitter molecules are cleared from the synaptic cleft by diffusion, reuptake by the presynaptic neuron, and enzymatic breakdown.

between two cells has hundreds of these one-to-one connections in a symmetrical array. Each pair of connexons has a central tube that allows ions and other small molecules to flow readily from one cell to the other. When an action potential reaches an electrical synapse, ions flow from the presynaptic cell into the postsynaptic cell, and an action potential is generated without delay in the postsynaptic cell membrane. Electrical synapses increase the synchrony of action of heart muscle and smooth muscle of the intestine. In comparison to chemical synapses, however, electrical synaptic transmission is rarely used in the CNS.

Parts of the Brain

Despite the frequency with which we hear the assertion that "the brain is the most complex structure in the known universe," it remains a valid description of this extraordinary organ. The brain can be subdivided into six main regions: the cerebrum, diencephalon, midbrain, pons, medulla, and cerebellum (Figure 14.5). The midbrain, pons, and medulla form the brain stem connecting the mass of the brain to the spinal cord. These three structures contain neurons that relay signals from the spinal cord to the cerebrum and cerebellum. Among the clusters of cell bodies (nuclei) within the midbrain, and possibly in part of the cerebrum, the red nucleus controls muscle coordination and maintains posture, and the substantia nigra inhibits forced involuntary movements. Neurons that relay signals for chewing, sensations to the head and face, certain respiratory functions, eye movement, taste, salivation, facial expression, and equilibrium are located in the pons. The medulla contains all of the tracts that travel in both directions between the brain and spinal cord. In addition, various nuclei of the medulla transmit nerve impulses that control the heart rate, constriction and dilation of blood vessels, blood pressure, swallowing, sneezing, and other functions. The cerebellum is located in the lower posterior portion

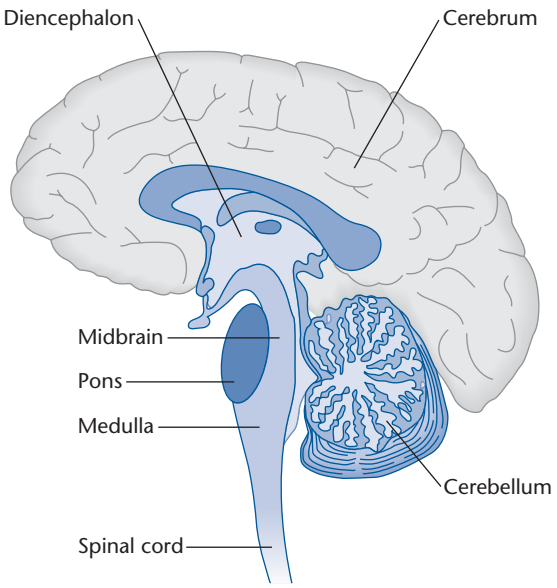


Figure 14.5 Sagittal section showing some of the major regions of the human brain. The front of the brain is at the left.

of the brain and is responsible for responding to signals from muscles, tendons, joints, and some sense organs, as well as controlling skeletal muscle contractions and maintaining coordination, muscle tone, balance, and posture.

The thalamus and hypothalamus are major components of the diencephalon. The thalamus is a complex of nuclei that facilitate hearing, taste, vision, sensation, wakefulness, and voluntary motor control. The hypothalamus is also a collection of nuclei that control, among other things, the autonomic nervous system, secretion from various glands, heart rate, movement of food in the intestine, rage, aggression, water balance, body temperature, hunger, satiety, thirst, wakefulness, and sleep.

The cerebrum, with two hemispheres, accounts for approximately 80% of the total mass of the brain. The uppermost 2- to 4-mm layer of the cerebrum is crammed with cell bodies and neuroglia and is called the cerebral cortex. Human consciousness, motor skills, awareness of subtle variations in the external environment, language, reasoning, and imagination are among some of the many vital functions of the cerebral cortex. The cerebral cortex is composed of regions with specific neuronal responsibilities. Within each hemisphere beneath the cerebral cortex there are a number of nuclei. Of these, the basal ganglia (cerebral nuclei) contain a number of different nuclei and subdivisions within some of these nuclei, such as the caudate nucleus, putamen, globus pallidus, and subthalamic nucleus (Figure 14.6). For the most part, these nuclei interconnect with neurons of the cerebral cortex, thalamus, and hypothalamus and control involuntary skeletal muscle movements and other motor functions. The limbic system is another set of neuronal structures that lies deep within the cerebrum near the diencephalon and midbrain (Figure 14.7). The hippocampus, one of the components of the limbic system, contributes to emotional states, such as fear, anger, rage, pleasure, and sorrow, and is also associated with learning and memory capabilities.

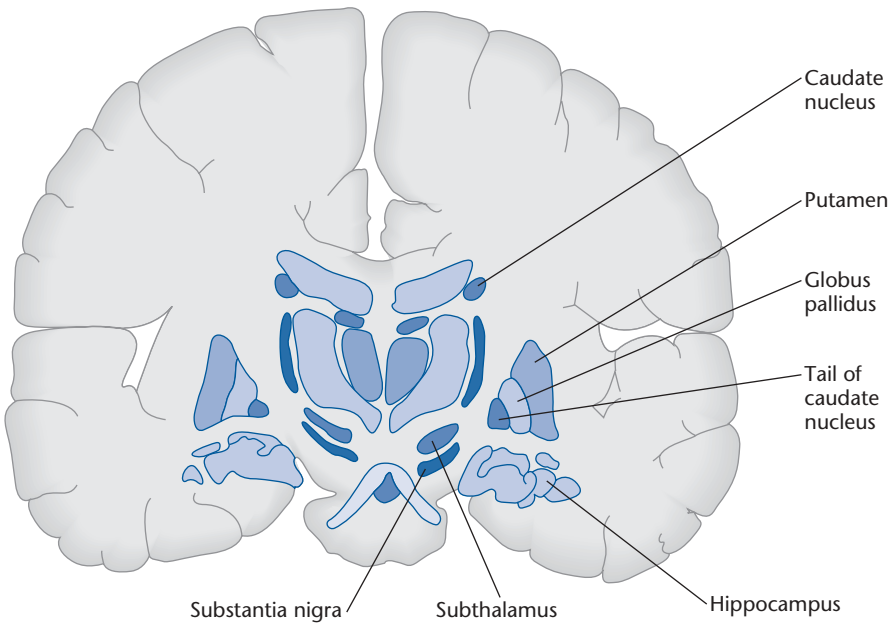


Figure 14.6 Coronal section through the anterior portion of the cerebrum of the human brain showing some of the elements of the basal ganglia and limbic system. Adapted from Carola, R., J. P. Harley, and C. R. Noback. 1992. *Human Anatomy and Physiology*. 2nd ed. McGraw-Hill, New York.

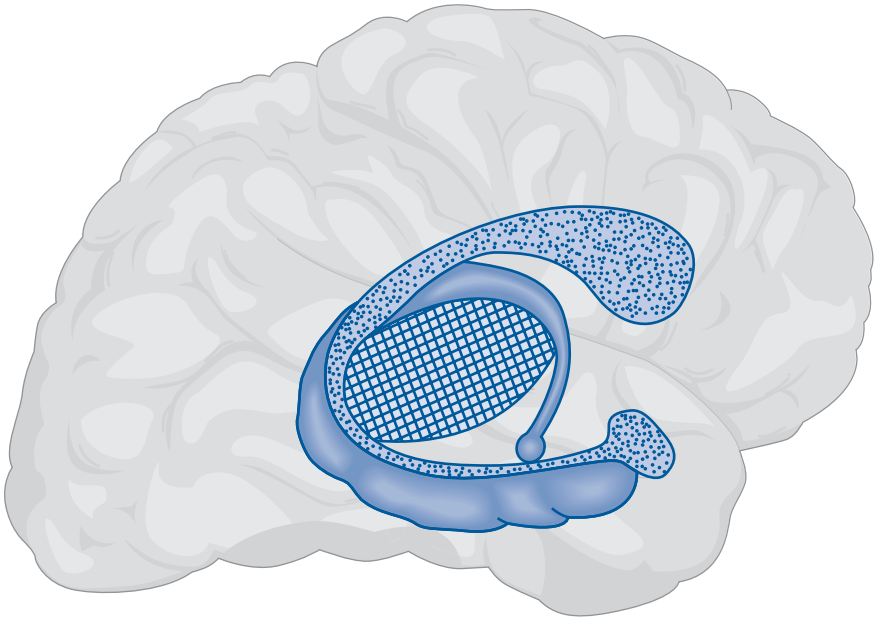


Figure 14.7 Spatial relationship among components of the basal ganglia (stippled), limbic system (solid), and thalamus (cross-hatched) within the cerebrum of the human brain. The front of the brain is at the right. Adapted from Carola, R., J. P. Harley, and C. R. Noback. 1992. *Human Anatomy and Physiology*. 2nd ed. McGraw-Hill, New York.

Neuronal Channelopathies

Channel proteins, in many cases, facilitate and regulate the flow of specific ions and small charged molecules in and out of a cell. The process of opening and closing (gating) of a channel is finely tuned. The channels that handle ions (ion channels) respond to the electric potential across the membrane (voltage gated) and other channels to signaling molecules (ligand-gated). The voltage-gated channels are part of an extensive family of more than 400 related proteins. The ion channels in excitable tissues such as neurons, muscles, and heart muscle are highly selective for sodium, potassium, calcium, or chloride ions that, depending on the conditions, generate, repress, or propagate action potentials. Structurally, the K^+ channels consist of four individual subunits (α subunits), each with six transmembrane segments (Figure 14.8A). By contrast, the channels for other ions are often a single polypeptide with four membrane-embedded domains, each with six transmembrane segments (Figure 14.8B). In addition, accessory proteins (β subunits) are required for the activity of many channels.

Disorders caused by mutations of channel-encoding genes are called channelopathies. Of these, neuronal channelopathies tend to feature intermittent loss of brain function (seizures, convulsions, epilepsy), uncontrolled muscle movement (ataxia), and severe headaches with vomiting, nausea, and extreme sensitivity to light (migraine). The genes for a number of neuronal channelopathies have been identified (Table 14.1). In each case, the ion current is either enhanced or reduced. However, the relationship(s) between an altered electrophysiological effect and the final phenotype is not well understood. Moreover, the molecular genetics of channelopathies can be confusing. In some instances, alleles of a gene produce quite different clinical symptoms. Alternatively, mutations in different genes produce similar disorders. By way of illustration, mutations of the *KCNQ2* and *KCNQ3* genes that encode K^+

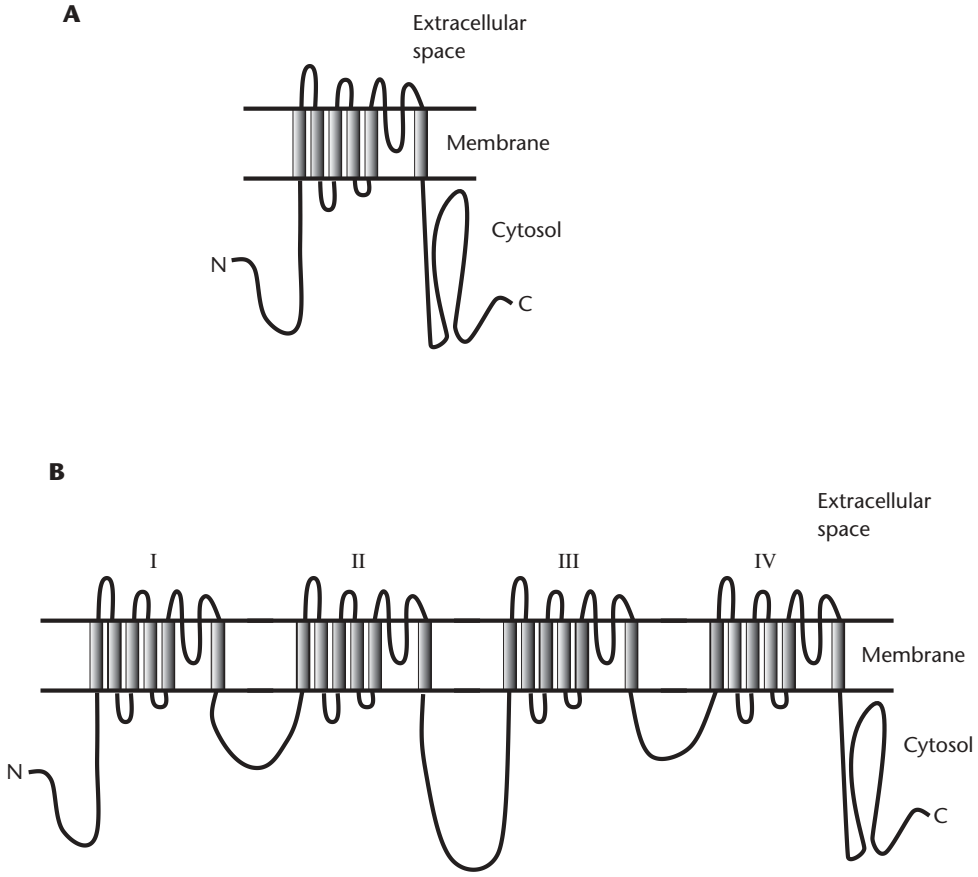


Figure 14.8 Schematic representations of ion channels. (A) K^+ channel α subunit. (B) Na^+ channel α subunit. The regions marked I, II, III, and IV are transmembrane domains.

channels are responsible for benign familial neonatal convulsions (BFNC), whereas mutations of the *SCNA2* Na^+ channel gene lead to benign familial neonatal-infantile seizures (BFNIS). The features of these two syndromes overlap. With BFNC seizures are usually observed in the first month after birth, whereas with BFNIS the onset is often at 6 months of age. In addition, 85% of individuals with BFNC cease having seizures after their first year, and those with BFNIS, apparently, never experience seizures after 12 months of age. It is not known why BFNC and BFNIS seizures do not occur in children and adults.

Alzheimer Disease

Dementia is the deterioration of intellectual capabilities, memory, judgment, and personality to the extent that daily functioning and quality of life are seriously impaired. Generally, dementia affects the elderly but is not confined to this age group. A number of different circumstances including Alzheimer disease (AD), Huntington disease (HD), Parkinson disease (PD), Pick disease, Creutzfeldt–Jakob disease (CJD), adrenoleukodystrophy, head injury, multiple strokes (multi-infarct dementia), boxing (dementia pugilistica), alcoholism, and viral infections impair brain function and cause dementia.

Table 14.1 Neuronal channelopathies.

Channels	Locus	Location	Subunit	Function	Clinical features (ion current effect)
Voltage-gated					
Potassium	*KCNA1*	12p13	$K_V1.1\alpha$	Repolarization of axons	Episodic ataxia; neuromyotonia (decreased)
	KCNQ2	20q13.3	KCNQ2α	Low threshold current modulated by muscarinic receptors	Benign familial neonatal convulsions (decreased)
	KCNQ3	8q24	KCNQ3α	Low threshold current modulated by muscarinic receptors	Benign familial neonatal convulsions (decreased)
Calcium	*CACNA1A*	19p13	$Ca_V2.1\alpha$	Current in Purkinje and granule cells; presynaptic terminals	Unilateral migraine; episodic ataxia; spinocerebellar ataxia (decreased)
Sodium	*SCN1A*	2q24	$Na_V1.1\alpha$	Somatodendritic Na^+ influx	Seizures with high fever; infant epilepsy (increased)
	SCN2A	2q23–q24.3	$Na_V1.2\alpha$	Axonal fast Na^+ influx	Benign familial neonatal-infantile seizures (increased)
	SCN1B	19q13.1	Brain β_1	Accessory protein	Seizures with high fever (increased)
Ligand-gated					
γ-Aminobutryic acid ($GABA_A$) receptors	*GABRG2*	5q31.1–q33.1	γ_2	Fast inhibition	Seizures with high fever (decreased Cl^-)
Nicotinic acetylcholine (ACh) receptors	*CHRNA4*	20q13.2–q13.3	α_4	Presynaptic transmitter release	Nocturnal frontal lobe epilepsy (increased Na^+)
	CHRNB2	1p21	β_2	Presynaptic transmitter release	Nocturnal frontal lobe epilepsy (increased Na^+)
Glycine receptors	*GLRA1*	5q32	α_1	Fast inhibition	Extreme startle response, i.e., hyperekplexia (decreased Cl^-)

Adapted from Table 1 in Kullmann, D. M. 2002. The neuronal channelopathies. *Brain* **125**:1177–1195.

Alzheimer disease was named for the German neurologist Alois Alzheimer (1864–1915), who described its clinical and neuropathological features in 1907. Today, AD accounts for two-thirds or more of all diagnosed cases of dementia. There are more than 4 million people with AD in the United States. As the population ages, this number will increase. About 100,000 people die from AD each year in the United States, which makes this disease the fourth leading cause of death after heart disease, cancer, and stroke. Alzheimer disease affects approximately 3%, 19%, and 47% of those in age ranges between 65 and 74, 75 and 85, and older than 85, respectively.

Many different clinical features are associated with AD. Among the first symptoms are the inability to create new memories and the loss of short-term memory. The ability to concentrate and recall past events worsens as the disease progresses. Alzheimer disease patients often have no accurate sense of time and are unable to correctly identify the day, month, or year. They may be disoriented, wander away, and become hopelessly lost. Although standard speech patterns and clichés are retained in the early stages, both sentence formation and coherent verbal communication diminish and are eventually lost. Invariably, these losses are accompanied by personality changes. Some AD patients become extremely passive, others very hostile, and some abnormally suspicious.

Delusions are prevalent in 50% of the cases. In the end, AD patients are mute, immobilized, and uncomprehending. Death is often the result of respiratory failure. For AD patients diagnosed at 65 years or older, the disease lasts from about 8 to 20 years. In cases of onset before 65 years of age, the course of the disease is rapid and death occurs within 5 to 10 years from the time of diagnosis.

Histological analysis of autopsied brains of AD patients shows three distinctive neuropathological features. First, there are devastating losses of synapses and neurons within the hippocampus and the entorhinal cortex, which is the region of the cerebral cortex (neocortex) beneath the hippocampus. One of the distinguishing general characteristics among neurodegenerative disorders is neuronal loss and often nervous system dysfunction that is localized, at least initially, within a certain region of either the CNS or PNS. As part of AD, many cerebral cortex neurons that connect with other cortical neurons also degenerate. By the final stage of the disease, the overall width of most of the neocortex is dramatically reduced. Second, dense spherical structures (20–200 μm in diameter), called senile plaques (SPs), are prevalent outside the neurons of the hippocampus and other regions of the brain. When senile plaques are surrounded by cellular debris from disintegrated axons and dendrites, they are called neuritic plaques (NPs). Third, aggregations of fibrils (neurofibrillary tangles, NFTs) accumulate within the cell bodies and dendritic processes of the neurons of the hippocampus, the neocortex including the entorhinal region, the amygdala, and other parts of the brain.

Biochemistry of Senile Plaques and Neurofibrillary Tangles

The core of a senile plaque is a densely packed fibrous structure that historically has been described as an amyloid body. Originally, amyloid bodies were thought to be made up of carbohydrates. More definitive chemical analyses established that the major component(s) was protein. The label "amyloid," however, has never been changed. The principal protein of AD amyloid bodies is a 4-kDa peptide (Aβ protein; β-protein; A4, β/A4). Within AD amyloid bodies, the Aβ protein (amyloid β-protein) consists of isoforms that range in amino acid number from 39 to 43. Of these, the two main isoforms contain 40 and 42 amino acids and are designated Aβ1–40 (Aβ40) and Aβ1–42 (Aβ42), respectively. When an experimental protocol cannot distinguish between Aβ isoforms with 42 or 43 amino acids, the expression Aβ1–42 (43) or Aβ42/43 is used.

There are at least 13 other peptides or proteins that form amyloid bodies and are associated with diseases that do not in any way resemble AD (Table 14.2). The common feature among all of these disorders is that the amyloid bodies, despite different core proteins, share a very similar structural organization with more or less the same microscopic appearance and spectral response to certain histological dyes. The production of an amyloid body is called amyloidosis. Proteins capable of forming the core of an amyloid body are considered to be fibrillogenic. The fibrillogenic proteins of the various amyloid bodies do not share any amino acid sequence similarity and, in most instances, are either abnormal breakdown products or mutated versions of a source (precursor) protein. Amyloid fibrils are straight, smooth, and approximately 10

Table 14.2 Source proteins for various amyloid diseases.

Source protein	Disease
Amyloid precursor protein	Alzheimer disease
Immunoglobin light chain	Primary systemic amyloidosis
Serum amyloid A	Secondary systemic amyloidosis
Transthyretin	Familial amyloid polyneuropathy (FAPI)
Cystatin C	Hereditary cerebral amyloid angiopathy
β_2-Microglobin	Hemodialysis-related amyloidosis
Apolipoprotein A-1	Familial amyloid polyneuropathy (FAPIII)
Gelsolin	Finnish hereditary systemic amyloidosis (FAPIV)
Islet of Langerhans amyloid protein	Type II diabetes
Calcitonin	Thyroid medullary cancer
Prion	Spongioform encephalopathy
Atrial nutriuretic factor	Atrial amyloidosis
Lysozyme	Hereditary systemic amyloidosis
Fibrinogen	Nonneuropathic amyloidosis (Ostertag type)

nanometers (nm) in diameter. Dense clusters of these fibers bind the dye Congo red. When viewed with polarizing light under a microscope, the Congo red-stained amyloid bodies emit a characteristic birefringence. In addition, the different amyloid bodies produce very similar X-ray diffraction patterns. The basic molecular organization of all types of amyloid bodies is a set of stacked sheets of antiparallel amyloid protein chains running perpendicular to the axis of each fiber. Each sheet is a cross-β structure with the distance between sheets being 0.5 to 1.0 nm.

In addition to the principal fibrillary peptide, an amyloid body has non-fibrillar components. Some of these accessory molecules are proteins, such as apolipoprotein E and serum amyloid β-component, that are found in all types of amyloid bodies. Other proteins bind to specific amyloid bodies such as α-antichymotrypsin and α-synuclein, which associate only with AD amyloid bodies.

Both cDNA and genomic libraries were screened with a probe based on the Aβ40 sequence. Sequence analysis of the isolated clones revealed that the Aβ isoforms of senile plaques are derived by proteolytic cleavage from a larger protein that was called amyloid precursor protein (APP). The chromosome site for the *APP* gene is 21q21.2. The amyloid precursor protein has a single transmembrane domain and commonly occurs as three isoforms with 695, 751, and 770 amino acids. The 695-amino acid APP isoform is found in neurons throughout the CNS. Some of these APP molecules are found in the membrane at the terminal ends of axons and others in intracellular membranes including the Golgi network. The 751- and 770-amino acid isoforms are produced, for the most part, by glial cells. Some complete versions and processed segments of the 751- and 770-APP isoforms are secreted. Amyloid precursor protein is also synthesized by other cell types, but to a lesser extent.

For convenience, the amino acid numbering system for APP and Aβ isoforms is based on the 770-amino acid isoform. The Aβ43 isoform encompasses amino acid sites 672 to 714 of the APP molecule. Under normal conditions, APP molecules are processed by proteolytic enzymes (Figure 14.9). An enzyme called α-secretase cleaves the APP molecule after the amino acid at site 687,

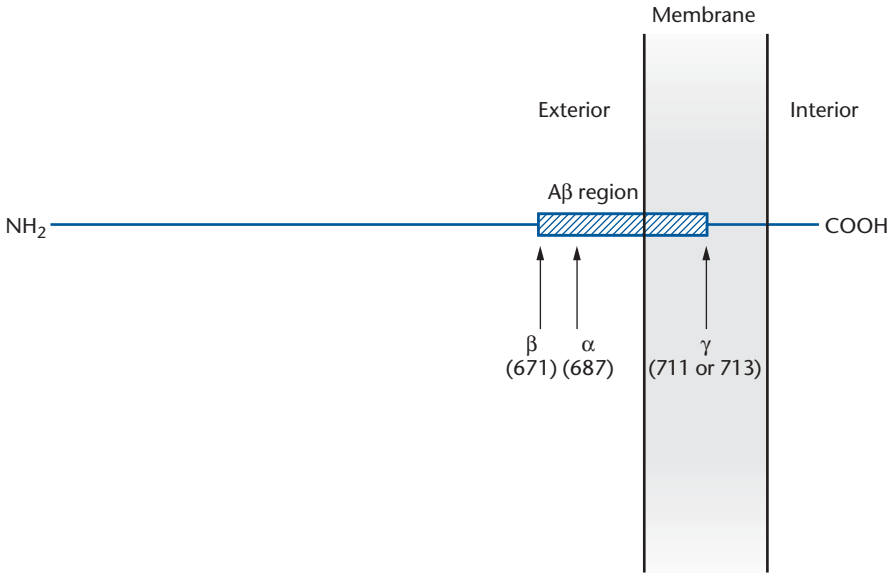


Figure 14.9 The Aβ region and major secretase cleavage sites of the amyloid precursor protein. The Aβ region is hatched. The marked arrows and numbers in parentheses indicate the major sites of proteolytic cleavage by α-, β-, and γ-secretases. The numbering of amino acid sites is based on the 770-amino acid APP isoform. The APP molecule is not drawn to scale.

which lies within the Aβ region. Neither of the fragments produced by this cleavage are fibrillogenic (amyloidogenic). Cleavage by β-secretase (BACE-1, β-site APP-cleaving enzyme) after amino acid site 671 also produces two non-amyloidogenic fragments. Both α- and β-secretase cleavages of APP are normal processes and occur in almost all cells When some APP molecules are doubly cleaved by β-secretase and by γ-secretase, which cuts after either site 711 or site 713, then Aβ40 and Aβ42 isoforms are released. In vitro studies have shown that large amounts of both Aβ40 and Aβ42 are amyloidogenic and Aβ42 readily forms fibrils at low concentrations. Cleavage of APP by both α- and γ-secretases releases an internal piece of the APP protein that has 24 amino acids (Aβ17-40; p3) and is not amyloidogenic. Because each of the APP secretases lacks absolute specificity and cleaves peptide bonds on either side of its major site, small amounts of other low-molecular-weight Aβ isoforms are formed.

The functional roles of APP and the various fragments of APP in the nervous system are not well understood. The cell membrane-bound 695-amino acid APP isoform may facilitate cell-to-cell contact, adhesion to the extraneuronal matrix, and/or synaptic stability. Intracellular APP may be associated with the cytoskeletal system, which is composed of filaments and microtubules conveying vesicles and other components from one part of a cell to another. The two types of secreted APP fragments [APP 1–671 (βAPPs); APP 1–687 (αAPPs)] may protect neurons from damage and modulate events at synapses. The amounts of Aβ40 and Aβ42 isoforms under normal conditions are inconsequential because most of these molecules are degraded. It is unlikely that these Aβ isoforms play a significant role in the CNS. On the other hand, as discussed later, atypical processing of APP that leads to an excess of the Aβ42 isoform is probably responsible for the destruction of synapses during the early stages of AD, the formation of senile plaques, and, eventually, degradation of neurons.

The main fibrous elements of NFTs are numerous paired helical filaments (PHFs). A PHF consists of two cross-linked, intertwined protein strands with each strand made up of a number of joined tau (τ) protein molecules that form a tau filament. The *MAPT* gene encodes the tau protein and is located at 17q21. The tau protein binds to tubulin molecules and facilitates the assembly of tubulin into microtubules. And, in combination with other microtubule-associated proteins (MAPs), tau protein maintains the stability of microtubules. Microtubules form part of the cytoskeletal system and are responsible for transporting cellular materials, such as vesicles, within a cell. In neurons, they transport vesicles and other components up and down axons.

In contrast to tau molecules under normal conditions, those that make up NFTs are hyperphosphorylated (HP-tau). In vitro studies have shown that HP-tau does not bind to tubulin but attaches to other MAPs. Consequently, by extrapolation, it is likely that in vivo HP-tau molecules block the formation of microtubules and undermine the stability of existing microtubules. Moreover, under in vitro conditions, HP-tau binds to normal tau molecules and forms long, intertwined filaments. Thus HP-tau probably disassembles microtubules and acts as an aggregation center for the formation of PHFs and NFTs. The dissolution of the neuronal microtubule system would prevent axonal transport, which in turn would cause the loss of synapses and lead eventually to the degeneration of synaptic bulbs.

The formation of NFTs is not unique to AD. They are found in a number of neurodegenerative disorders with dementias (tauopathies) affecting various parts of the brain such as Pick disease, familial presenile dementia, progressive supranuclear palsy, and dementia pugilistica. A number of these conditions, despite phenotypic heterogeneity, map to 17q21–22. All the "17q21–22" disorders have been designated frontotemporal dementia with parkinsonism, chromosome 17-type (FTDP-17). The phenotype for the FTDP-17 disorders is broad-ranging, with both neurological and movement abnormalities. CNS defects include diminished ability to speak, personality changes, and dementia. The problems with movement include periods of uncontrolled rigidity, tremors, extremely slow movements (bradykinesia), and, occasionally, myopathy. Generally, these changes resemble the characteristics that accompany Parkinson disease (PD), hence the designation "parkinsonism." Onset of these disorders is from middle to late age. Mutation screening of some FTDP-17 families revealed nucleotide substitutions in the *MAPT* gene. It is likely that the FTDP-17 dementias are the result of faulty microtubule formation.

About a decade ago, there was a rigorous debate about whether Aβ amyloid or NFTs triggered the pathogenesis of AD. Much research indicates that the oligomeric form of Aβ42 and/or Aβ amyloid bodies are the primary cause of AD, with the development of NFTs occurring as the direct consequence of Aβ amyloid or from neuronal damage produced by Aβ amyloid. The researchers who support this prevailing viewpoint, namely, the amyloid cascade theory, have been called bap-tists, where "bap" stands for "beta amyloid protein." On the other hand, those who hold the minority view that the tau protein is instrumental in initiating the series of changes leading to AD have been called tau-ists.

Genetics of Alzheimer Disease

Alzheimer disease is a complicated genetic disorder. Only about 10% of cases occur as well-defined, fully penetrant autosomal dominant inheritance. In these families, the onset of the disease is between 55 and 60 years of age, which is considered early for a condition that usually affects much older people. The original patient examined by Alois Alzheimer was only 51 years old when she first had serious problems with memory and geographic orientation. Because of the relatively young age of occurrence of these symptoms, the condition was originally designated as "presenile dementia." Patients with early-onset AD (EOAD) show the same clinical and neuropathological features as those with late-onset AD (LOAD), that is, >65 years, except that the time course of EOAD is usually more rapid than LOAD.

Approximately 90% of AD cases are late onset, and many of these are non-familial (sporadic). However, 25% to 40% of those with LOAD have at least one close relative with the disorder, which suggests the involvement of a genetic component(s). Because there is no obvious pattern of inheritance among families with two or more members who have LOAD, considerable genetic heterogeneity may account for these cases.

Mutations of the Amyloid Precursor Protein Gene

Mutation screening of the *APP* gene in members of a large family with early-onset AD revealed a missense mutation changing the amino acid at APP site 717 (V717I). This site is three amino acids downstream from amino acid 43 of the Aβ protein. The V717I and two other missense mutations at the same site (V717G, V717F) have been found in AD individuals in various families in different parts of the world. One individual with AD in a single family had an *APP* gene mutation that affected site 716 (I716V) of APP. In another family, those with AD had a double mutation that altered APP sites 670 (K670N) and 671 (M671L). This double mutation is adjacent to the γ-secretase cleavage site. The age of onset of AD in families with *APP* gene mutations is between 45 and 60 years. Except for the difference in time of onset, AD individuals with *APP* gene mutations have the same phenotype as those with late-onset AD. Exhaustive mutation screening has failed to detect APP mutations in control populations or, to any significant extent, among those with late-onset AD.

The biochemical consequences of the AD-causing *APP* mutations have been studied in various ways. First, cDNAs with the single AD *APP* mutations and the known double mutation were cloned into vectors under the control of an active promoter and introduced (transfected) into cells in culture. Then the production of Aβ40 and Aβ42/43 was monitored. Second, cultured cells and plasma from individuals with an AD *APP* mutation were assayed for relative amounts of Aβ40 and Aβ1-42/43. Third, the presence of Aβ40 and Aβ42/43 was scored in transgenic mice that had been genetically engineered to carry a mutant *APP* gene. Although these approaches were indirect, similar results were observed in each case. Transfected cells with *APP* mutant sequences produced more Aβ40 and Aβ42/43 than cells with the wild-type *APP* gene sequence. Similarly, increased amounts of Aβ42/43 were observed both in the cells and plasma of AD patients with *APP* mutations and in transgenic mice that carried a mutant *APP* transgene.

Depending on the AD *APP* gene mutation, either β- or γ-secretase activity is altered. Mutations close to APP716 and APP717 may both enhance and shift the site of proteolytic cleavage by γ-secretase to yield more Aβ42/43. The double mutation for APP sites 670 and 671 may stimulate cleavage at the β-secretase site or decrease α-secretase cleavage. Because Aβ42/43 is amyloidogenic, increasing its production might trigger the formation of amyloid bodies. On the whole, these results are consistent with the "amyloid cascade" hypothesis, which postulates that the primary cause of AD is the overproduction of Aβ, especially Aβ42. An excess of Aβ42 likely causes neurotoxicity and the initiation of a stepwise sequence of biochemical and structural events including amyloid body formation that lead to AD. Although this hypothesis is attractive, the AD *APP* mutations represent only a small percentage of all of the occurrences of EOAD and about 0.5% of all cases of AD. However, there are other defects that lead to the accumulation of neurotoxic Aβ42 over time and the formation of amyloid bodies.

An interesting familial mutation has been observed that affects the Aβ region of the *APP* gene and causes an amino acid substitution in APP site 693 (E693Q). Although amyloid bodies are formed, none of the features of AD is present. There are no NFTs, and neither the neocortex nor hippocampus undergoes any significant neuronal loss. Instead, the phenotype associated with APP(E693Q) is severe rupturing (hemorrhaging) of the small blood vessels of the cortex and cerebellum as the result of an accumulation of diffuse Aβ amyloid bodies. This condition is called cerebral amyloid angiopathy (CAA) and is quite rare. The CAA *APP*(E693Q) mutation is inherited as an autosomal dominant trait. Onset occurs between 45 and 55 years of age. Approximately 50% of those with CAA *APP*(E693Q) die within 12 months after the first serious hemorrhage. Those who live longer experience a series of debilitating strokes. Transfection studies with mutant *APP*(E693Q) cDNA indicated that there was no increase in the production of either Aβ40 or Aβ42/43. It is not known why this mutation does not produce the AD phenotype. Perhaps, in this case, an Aβ isoform is produced that is specifically amyloidogenic in the cerebral vascular system and not in the CNS.

Mutations in the Presenilin Genes

Linkage studies mapped a locus for AD in six large families with early-onset AD to 14q24.3. Nineteen transcripts were identified in a YAC contig that spans 14q24.3. Each transcript was screened for mutations by RT-PCR with postmortem brain tissue, and one of them had a single mutation difference in comparison to the sequence from non-AD family members. On this basis, both the gene and the protein it encodes were identified. Because of the early onset of AD in these families, the gene is designated as presenilin 1 and assigned gene symbol *PSEN1*. The protein is called presenilin-1 (PSEN1, ps-1, PS1). *PSEN1* mutations account for approximately 40% of all cases of familial early-onset AD, with the first clinical signs of the disorder ranging from 30 to 55 years of age.

Another site for familial early-onset AD was localized to 1q32-q42 shortly after the *PSEN1* gene was discovered. With a probe derived from *PSEN1*, the AD gene on chromosome 1 was isolated and shown to carry a mutation in

those family members who had AD. This gene is called presenilin 2 (*PSEN2*), and the protein is presenilin-2 (PSEN2, ps-2, PS2). *PSEN2* mutations occur in less than 1% of families with early-onset AD, and commencement of AD ranges from 40 to 75 years of age. The presenilins participate in γ-secretase cleavage of APP. Mutant PSEN1 and PSEN2 proteins likely increase the production of the Aβ42 peptide and, in addition, may have deleterious effects on neurons. Together the *APP*, *PSEN1*, and *PSEN2* mutations represent approximately 50% of all familial early-onset AD. Thus, other genes that lead to early-onset AD remain to be discovered. Moreover, *APP*, *PSEN1*, and *PSEN2* mutations are not prevalent in individuals with late-onset AD.

Genetic Risk Factor for Alzheimer Disease

Linkage between AD and 19q13.2 was observed in a group of families with late-onset AD. Because the gene (*APOE*) for apolipoprotein E is in this region, it became a candidate for familial late-onset AD. The *APOE* gene is polymorphic for three common alleles, *APOE*2*, *APOE*3*, and *APOE*4*, that occur in most populations with frequencies of about 8%, 78%, and 14%, respectively. Each *APOE* allele encodes a distinctive isoform: ApoE2, ApoE3, and ApoE4. Apolipoprotein is synthesized primarily in the brain by astrocytes. Among other functions, apolipoprotein E sequesters cholesterol and triglycerides from cellular debris and transports these molecules into neurons, where they are used for the formation of synaptic membranes.

After a number of extensive studies of clinical and autopsy material from AD individuals and large samples of individuals with late-onset AD, it became clear the *APOE*4* allele was significantly associated with the occurrence of AD. This allele may account for approximately 50% of the genetic component of AD. However, it is neither sufficient nor necessary for AD, because many individuals with either one or two *APOE*4* alleles never show any signs of AD. For example, about 50% of persons homozygous for *APOE*4* who survive to be 80 years old never develop any signs of AD. In genetic parlance, the *APOE*4* allele is a risk factor that, in combination with other genes and/or environmental factors, significantly increases the likelihood that AD will occur. Generally, the presence of one or two *APOE*4* alleles increases the risk of AD by 2 to 5- or 5 to 10-fold, respectively, in European populations. The odds ratios for AD vary in different populations, probably because of various genetic and environmental factors (Table 14.3).

Other than AD, *APOE*4* is not significantly associated with any neurological disorder. However, the presence of an *APOE*4* allele exacerbates nervous tissue damage from head injuries such as concussions by increasing the severity of the response and lengthening the recovery period and, in the long term, increases the likelihood that presenile dementia of the Alzheimer type will occur.

How ApoE4 contributes to AD is not known. Possibly, because ApoE4, in contrast to the other ApoE proteins, does not facilitate either neuronal repair or the growth of axons, damaged neurons in the presence of ApoE4 may evoke the overproduction of either amyloid β-protein or HP-tau protein. Alternatively, ApoE4 may neither effectively remove Aβ42 peptide that is produced normally nor prevent fibril formation. Another APOE polymorphism signifi-

Table 14.3 Odds ratios for developing Alzheimer disease with *APOE* genotypes in different populations.

APOE genotype	Odds ratio			
	European	African American	Hispanic	Japanese
*APOE*3/APOE*3*	1.0[a]	1.0	1.0	1.0
*APOE*2/APOE*2*	0.9	2.4	2.6	1.1
*APOE*2/APOE*3*	0.6	0.6	0.6	0.9
*APOE*2/APOE*4*	1.2	1.9	3.2	2.4
*APOE*3/APOE*4*	2.7	1.1	2.2	5.6
*APOE*4/APOE*4*	12.5	5.7	2.2	33.1

[a]The *APOE*3/APOE*3* genotype is the reference genotype (referent). Adapted from Table 3 in Farrer, L. A., L. A. Cupples, J. L. Haines, B. Hyman, W. A. Kukull, R. Mayeux, R. H. Myers, M. A. Pericak-Vance, N. Risch, and C. M. van Duijn. 1997. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. *JAMA* **278**:1349–1356.

cantly associated with AD, but not to the extent of *APOE*4*, is a single-nucleotide change in the regulatory region that increases transcription of the *APOE* gene. Evidence for other AD genetic risk factors is sparse despite more than 100 association studies and over 10 complete genomic scans with affected individuals and families. Based on limited replicability, three chromosome regions (6q21, 10q24, 11q23) with nine possible candidate genes have been identified.

Huntington Disease and Other Trinucleotide Repeat Expansion Diseases

Huntington disease (HD) is a catastrophic disorder affecting about 1 in 10,000 people worldwide. Its first signs usually begin at about 35 years of age, although onset can occur anytime during the human life span. In families with a history of HD, there is a marked tendency for the disorder to occur earlier and earlier in successive generations. Initially, muscle coordination is slightly impaired, and forgetfulness, cognitive disorganization, and personality changes are evident. These symptoms steadily worsen. Eventually, both involuntary and voluntary movements become uncontrolled with jerking and writhing, speech is slurred, thought processes diminish, and severe psychiatric conditions appear, including depression, paranoid delusions, and uncontrolled rage. In the later stages, HD patients are mute, cognitively nonfunctional, and immobilized in contorted positions as a result of rigid joints and severe contractions. HD can last more than 30 years from inception to death, if the patient is placed on a life-support system. However, half of all patients usually die from pneumonia, choking, or heart failure within 15 to 20 years of the appearance of the disorder. Suicide is very common in the early stages of HD.

The pathological features of HD occur entirely within the brain. There is selective neuronal degeneration throughout the cerebral cortex, caudate nucleus, and putamen, with lesser damage to the ventrolateral nucleus, globus pallidus, and substantia nigra (Figure 14.10). AD-like NFTs and SPs are not found in or near affected neurons. However, a combination of granular and fil-

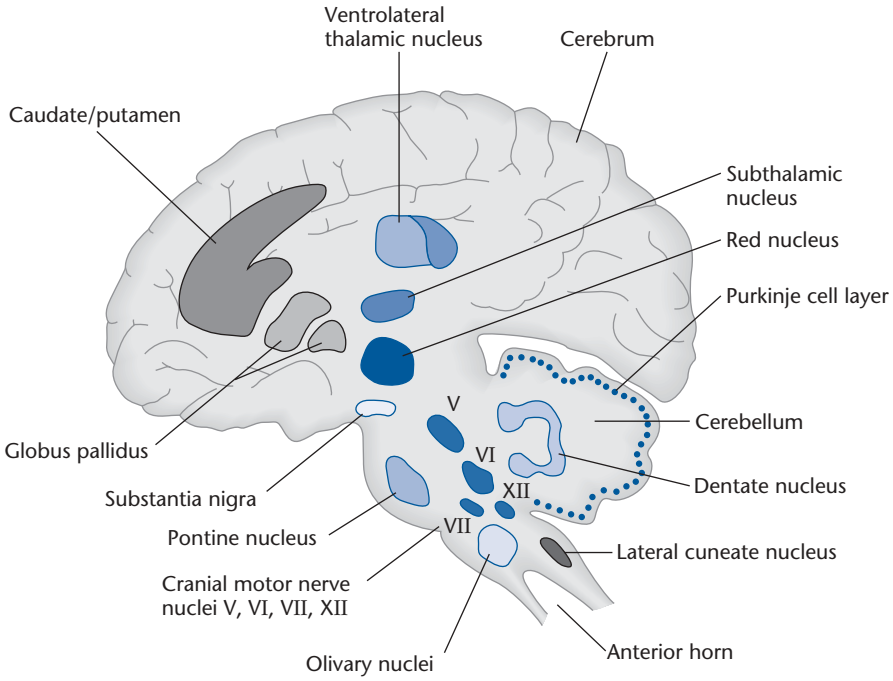


Figure 14.10 Some of the regions of the human brain affected by various trinucleotide repeat expansion disorders. See Table 14.4 for the relationships between degeneration of specific regions of the brain and a particular trinucleotide repeat expansion disorder.

amentous material (neuronal intranuclear inclusions, NIIs) accumulates in the nuclei of neurons in the regions of the brain affected by HD. By the final stages of HD, the brain loses about 25% of its initial weight. The pronounced changes in movement accompanying HD are probably the consequence of disruption of neurons interconnecting the cerebral cortex, basal ganglia, and thalamus. Dementia results from a loss of neurons within the cerebral cortex.

HD is inherited as an autosomal dominant trait. In 1983, HD was the first genetic disease to be localized to a chromosome location (4p16.3) with RFLP linkage analysis. The analysis was performed on a very large pedigree from Venezuela. With probes that were closely linked to the Venezuelan HD site, studies of large numbers of HD families from around the world also showed linkage to 4p16.3. Thus, HD is genetically homogeneous. If, by any chance, genetic heterogeneity exists for HD, the other HD-causing genes are extremely rare.

The HD gene was isolated in 1993 after a decade of intense collaborative efforts among many laboratories from various countries and officially designated *HD*. The interesting feature of this gene is the presence of an uninterrupted stretch of CAG/GTC repeats in the first exon. Generally, individuals who never develop HD have between 6 and 35 CAG/GTC repeats, whereas those with HD have 40 or more repeats in one *HD* allele. In rare cases of HD, there may be as many as 250 CAG/GTC repeats. Alleles with 36 to 39 CAG/GTC repeats represent a phenotypic "twilight zone," with some healthy individuals and others with HD.

In mRNA, CAG is the codon for glutamine, and a series of CAG codons in an exon produces a polyglutamine tract during translation. A set of contiguous trinucleotides is called a trinucleotide repeat. In other words, an *HD* mutation is the result of an increase in the length of a trinucleotide repeat. The

HD gene has 67 exons, covers about 200 kb of genomic DNA, and is expressed in a wide range of cell types, including neurons. Within neurons, the HD protein, which is called huntingtin, is found in the cytoplasm and in association with membranes, vesicles, and the cytoskeleton. It is also concentrated in synaptic bulbs.

Both before and after the discovery of the genetic basis of HD, other trinulceotide repeat expansion diseases (TREDs) were noted (Table 14.4). To date, at least, 16 TREDs have been identified and tend to fall into two categories. For the Type 1 TREDs, the trinucleotide repeat is part of the coding region, and, in all cases so far, the trinucleotide expansion codes for a stretch of glutamine residues (polyglutamine, polyGln, polyQ). On the other hand, the expanded trinucleotide repeat of the Type 2 TREDs is in or close to a noncoding portion of the affected gene.

Unlike other mutations, trinucleotide repeats are unstable, especially when they are near the high end of the normal range, and there is an increased likelihood of additional expansion. The term dynamic mutation has been used to describe this genetic instability. For some time, researchers had been aware that certain genetic diseases, including HD, become more severe and, in some affected families, begin earlier and earlier in successive generations. This phenomenon was called genetic anticipation (anticipation, Sherman paradox). It was later discovered that genetic anticipation commonly occurs in TREDs and is the consequence of the expansion of trinucleotide repeats from one generation to the next (Figure 14.11). Generally, the longer the length of a trinucleotide repeat within the range that causes the disease, the earlier the onset and the greater the severity of the disorder. Another interesting feature of TREDs, for which there is no satisfactory explanation, is parental bias. In some TREDs, fathers are more likely to produce and transmit longer expansions than mothers, whereas the reverse holds for other TREDs (Table 14.4).

The molecular basis for the susceptibility of expansion of a trinucleotide repeat is not fully understood, but it is likely to depend on the formation of an intrastrand loop (hairpin) involving nucleotides of the trinucleotide repeat during DNA synthesis (Figure 14.12). The process of DNA replication is different for each of the newly synthesized strands. One of the new strands is synthesized continuously by one DNA polymerase complex (continuous strand synthesis; leading strand synthesis). The other new strand is synthesized discontinuously (lagging strand synthesis) with a number of successive initiations of DNA synthesis (Figure 14.13). The replicated fragments produced by lagging strand synthesis are ligated together to form an intact strand. One model that explains trinucleotide expansion involves the formation of a hairpin structure in a newly synthesized, lagging strand fragment containing the trinucleotide repeat sequence (Figure 14.14). During its formation, the hairpin structure drags the 5′ portion of the fragment forward, leaving the segment of the repeat region of the template strand that had been replicated available for replication a second time by a trailing DNA polymerase. After ligation and a second complete round of DNA replication, a chromosome is formed with an expanded trinucleotide region (Figure 14.14).

The consequences of Type 1 trinucleotide repeat expansions are difficult to explain. Under normal conditions, a polyglutamine (polyQ) tract probably

Table 14.4 Trinucleotide Repeat Expansion Disorders.

	Huntington disease (HD)	Dentatorubral-pallidoluysian atrophy (DRPLA)
Clinical phenotype	Spasmodic, rapid, and involuntary control of limb movement; limb rigidity; impaired cognition; extreme psychiatric disturbances	Inability to coordinate muscles; abnormal, involuntary movements; slow movements; psychiatric disturbances
Sites of neuropathology	Caudate nucleus; putamen; cerebral cortical neurons; ventrolateral thalamic and subthalamic nuclei; globus pallidus; substantia nigra	Dentate nucleus; subthalamic nucleus; red nucleus; globus pallidus; caudate nucleus; putamen; cerebellar Purkinje cell layer
Onset	<10 to 60 yr; usually ~35 yr	10 to 70 yr; usually ~30 yr
Inheritance	Autosomal dominant	Autosomal dominant
Chromosome location	4p16.3	12p13.31
Trinucleotide repeat	CAG	CAG
Gene	*HD*	*DRPLA*
Location of repeat	Coding region; polyglutamine	Coding region; polyglutamine
Normal repeat range	9 to 35	7 to 23
Affected repeat range	40 to 121	49 to 75
Anticipation	Yes	Yes
Gender and repeat expansion	Paternal > maternal	Paternal > maternal
Protein	Huntingtin	Atrophin-1
Possible molecular effect of mutation	Disruption of vesicle trafficking; induction of apoptosis; alteration of transcription; overproduction of reactive oxygen species	Cytotoxic to neurons; abnormal nuclear interactions

	Spinobulbar muscular atrophy (SBMA; Kennedy disease)	Spinocerebellar ataxia type 1 (SCA1)
Clinical phenotype	Progressive muscle weakness; muscle loss; reduced male fertility	Uncoordinated walking and limb movements; slurred speech; inability to swallow
Sites of neuropathology	Motor nerve nuclei of the brain; anterior horns; dorsal root ganglia	Cerebellar Purkinje cell layer; dentate, pontine, and lateral cuneate nuclei
Onset	30 to 50 yr	~30 to 50 yr
Inheritance	X-linked	Autosomal dominant
Chromosome location	Xq11–q12	6p23
Trinucleotide repeat	CAG	CAG
Gene	*AR*	*SCA1*
Location of repeat	Coding region; polyglutamine	Coding region; polyglutamine
Normal repeat range	12 to 30	6 to 39
Affected repeat range	40 to 72	41 to 81
Anticipation	No	Yes
Gender and repeat expansion	Paternal > maternal	Paternal > maternal
Protein	Androgen receptor	Ataxin-1 (possibly a transcription factor)
Possible molecular effect of mutation	Toxic to motor neurons	Transcriptional dysregulation

Table 14.4 Trinucleotide Repeat Expansion Disorders (*continued*)

	Spinocerebellar ataxia type 2 (SCA2)	Spinocerebellar ataxia type 3 [SCA3; Machado–Joseph disease (MJD)]
Clinical phenotype	Uncoordinated movements; speech dysfunction; loss of sensorimotor responses; impaired ocular tracking	Uncoordinated walking; tremors; limited eye movement; speech dysfunction; loss of reflexes to lower limbs
Sites of neuropathology	Cerebellar Purkinje cell layer; pontine and olivary nuclei; substantia nigra	Dentate and pontine nuclei; substantia nigra; globus pallidus
Onset	2 to 65 yr; usually <25 yr	10 to 60 yr; usually >40 yr
Inheritance	Autosomal dominant	Autosomal dominant
Chromosome location	12q24.1	14q24.3–q31
Trinucleotide repeat	CAG	CAG
Gene	*SCA2*	*MJD1*
Location of repeat	Coding region; polyglutamine	Coding region; polyglutamine
Normal repeat range	15 to 29; usually 22	14 to 40
Affected repeat range	35 to 59; usually 37	68 to 82
Anticipation	Yes	Yes
Gender and repeat expansion	Paternal = maternal	Paternal = maternal
Protein	Ataxin-2	Ataxin-3 (possibly a cysteine protease)
Possible molecular effect of mutation	Disruption of Golgi apparatus; induction of cell death	Disruption of normal protein degradation pathway

	Spinocerebellar ataxia type 6 (SCA6)	Spinocerebellar ataxia type 7 (SCA7)
Clinical phenotype	Uncoordinated walking and limb movements; speech dysfunction; rapid eye movement; loss of sensorimotor responses; wooziness after quick movements	Uncoordinated movements; speech dysfunction; retinal degeneration; inability to swallow; sphincter dysfunction; impaired eye movement
Sites of neuropathology	Cerebellar Purkinje cell layer; olivary nucleus; brain stem	Cerebellar Purkinje cell layer; pontine nucleus; olivary nucleus
Onset	~24 to 65 yr; usually >50 yr	1 to 70 yr; usually ~30 yr
Inheritance	Autosomal dominant	Autosomal dominant
Chromosome location	19p13	3p21.1–p12
Trinucleotide repeat	CAG	CAG
Gene	*CACNL1A4*	*SCA7*
Location of repeat	Coding region; polyglutamine	Coding region; polyglutamine
Normal repeat range	6 to 17	7 to 18
Affected repeat range	21 to 30	38 to >200
Anticipation	Yes	Yes
Gender and repeat expansion	Paternal ≥ maternal	Paternal > maternal
Protein	α_{1A}-voltage-dependent calcium channel subunit	Ataxin-7 (possibly a member of the histone acetylation complex)
Possible molecular effect of mutation	Altered calcium influx in Purkinje cells leads to cell death	Transcriptional dysregulation

Table 14.4 Trinucleotide Repeat Expansion Disorders (*continued*)

	Fragile X site A (FRAXA)	Fragile X site E (FRAXE)
Clinical phenotype	Severe mental retardation; epilepsy; enlarged genitalia; protruding jaw; enlarged ears	Mental retardation
Sites of neuropathology	Hippocampus, putamen, globus pallidus, thalamus, cerebral cortex	Hypothalamus, amygdala
Onset	At birth	At birth
Inheritance	X-linked	X-linked
Chromosome location	Xq27.3	Xq28
Trinucleotide repeat	CCG	CCG
Gene	*FMR1*	*FMR2*
Location of repeat	5′-untranslated region (5′-UTR)	5′-untranslated region (5′-UTR)
Normal repeat range	6 to 54	6 to 35
Affected repeat range	200 to 2000	>200
Anticipation	Yes	Yes
Gender and repeat expansion	Maternal	Maternal
Protein	FMR1	FMR2
Possible molecular effect of mutation	Export of mRNA from nucleus; translation	Transcription factor

	Myotonic dystrophy (dystrophia myotonica, DM)	Friedreich ataxia
Clinical phenotype	Muscle rigidity; muscle loss; reduced gonads; heart irregularities; mental impairment	Uncoordinated gait; paralysis; disorientation, slurred speech
Sites of neuropathology	Motor and sensory nerves; cerebral cortex	Dorsal root ganglia; sensory nerves; spinocerebellar tracts; peripheral nerves
Onset	<10 to 50 yr; usually ~20 yr	1 to 40 yr; usually ~10 to 15 yr
Inheritance	Autosomal dominant	Autosomal recessive
Chromosome location	19q13.3	9q13–q21.1
Trinucleotide repeat	CTG	GAA
Gene	*DMPK*	*FRDA*
Location of repeat	3′-UTR	Intron
Normal repeat range	5 to 37	7 to 22
Affected repeat range	>2000	200 to ~1200
Anticipation	Yes	No
Gender and repeat expansion	Maternal = paternal; sperm with long repeats are not viable	Maternal > paternal
Protein	Dystophia myotonia protein kinase (DMPK)	Frataxin
Possible molecular effect of mutation	Inappropriate binding of an essential protein(s) to the expanded CUG tract of *DMPK* RNA	Nuclear-encoded mitochondrial protein that regulates the uptake of iron-sulfur clusters into the mitochondrial matrix

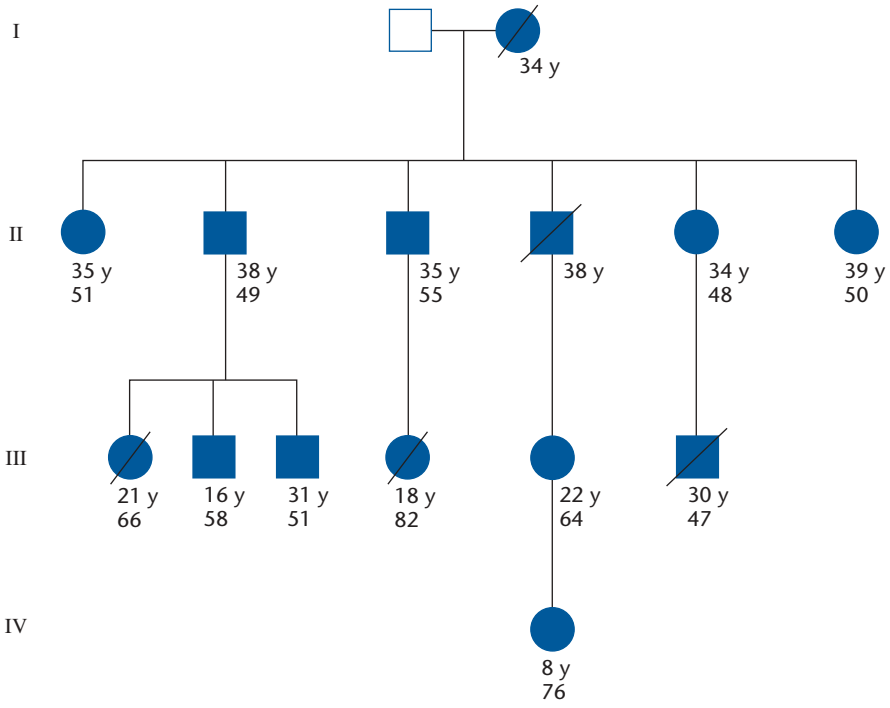


Figure 14.11 Genetic anticipation. Pedigree with affected HD members (closed symbols) and one unaffected individual (open symbol). The year of onset of HD (e.g., 34 yr for I-2) and, where known, the number of CAG repeats in the *HD* gene (e.g., 76 for IV-1) are shown. Slashes mark deceased individuals. Adapted from Ross, C. A., R. L. Margolis, A. Rosenblatt, N. G. Ranen, M. W. Becher, and E. Aylward. 1997. Huntington disease and the related disorder, dentatorubral-pallidoluysian atrophy (DRPLA). *Medicine* **76**:305–338.

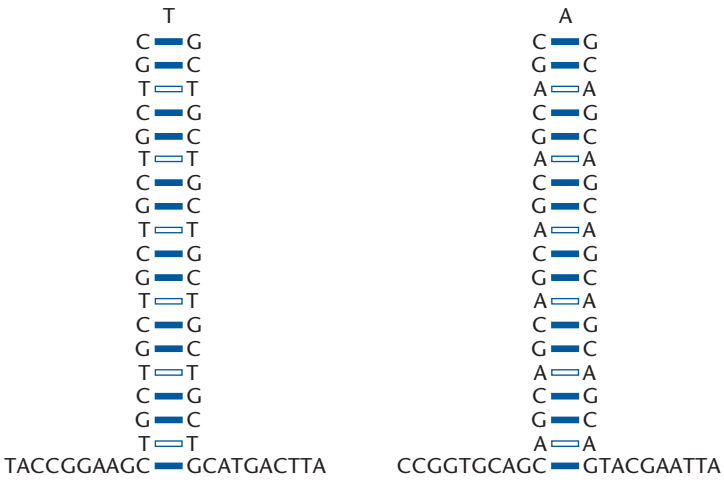


Figure 14.12 Hairpin structures formed by trinucleotide repeats in single-stranded DNA. The CTG hairpin (left) is more stable than the CAG hairpin (right). Most of the T-T and A-A mismatched bases (open bars) are stabilized by hydrogen bonds. The solid bars represent conventional Watson–Crick G-C base pairs.

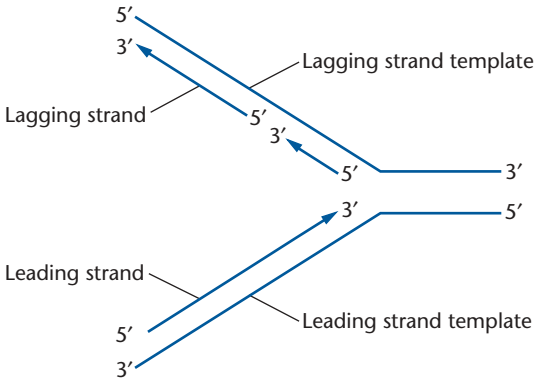


Figure 14.13 Strand synthesis during DNA replication. One new strand is synthesized continuously (leading strand) and the other discontinuously (lagging strand). The DNA fragments of the lagging strand are joined by DNA ligase.

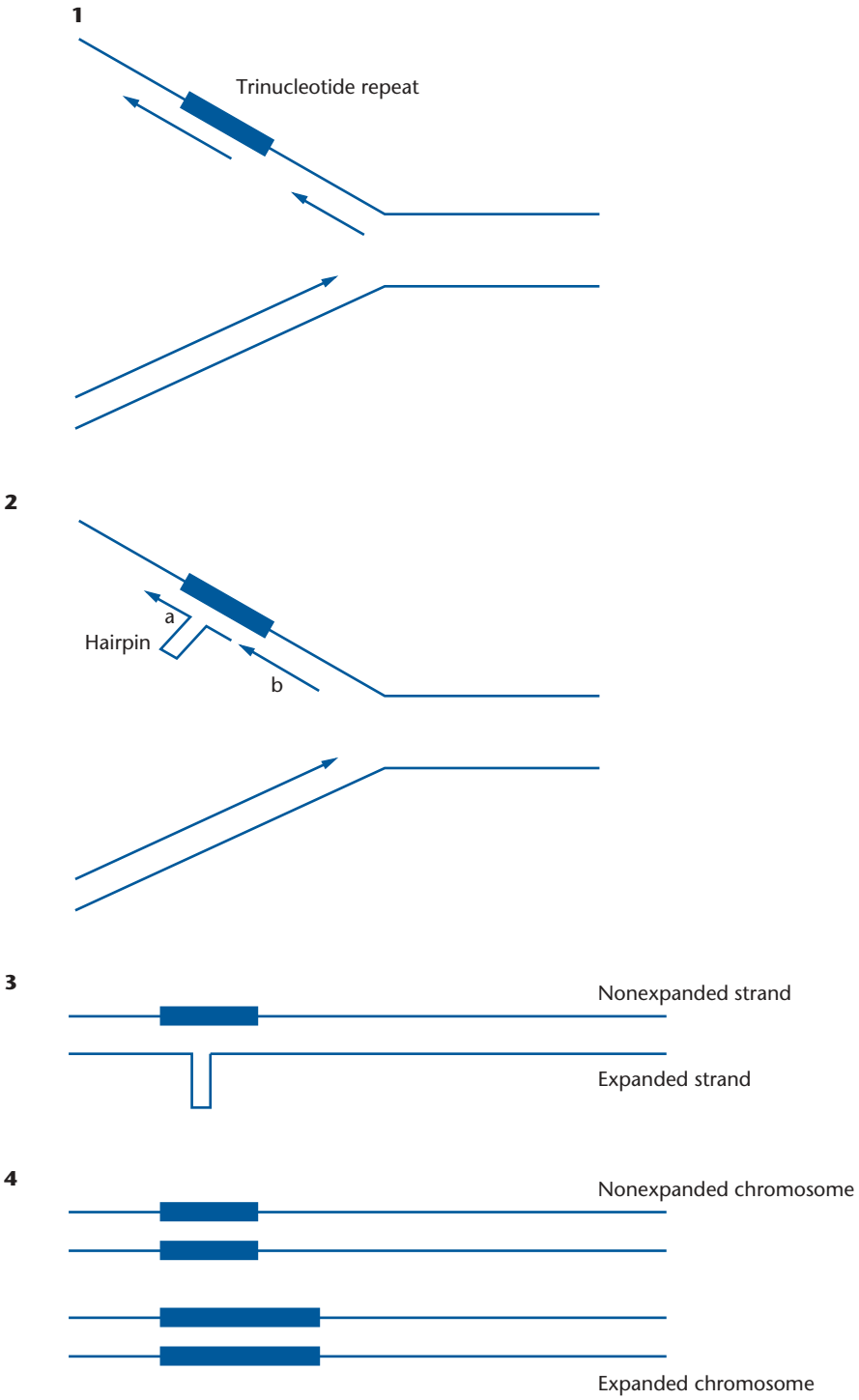


Figure 14.14 Schematic representation of the expansion of a tract of trinucleotide repeats. (1) Replication through the region containing a trinucleotide repeat. The trinucleotide repeats are shown as a solid box on the lagging strand template, and the complementary sequence on the leading strand template is not demarcated. The arrows point in the direction of replication. (2) A hairpin loop of trinucleotide repeats is formed within a fragment (a) on the lagging strand. Replication of the trailing fragment (b) extends into the trinucleotide repeat region. (3) After completion of replication and ligation of the fragments of the lagging strand, the chromosome has one strand with a nonexpanded set of trinucleotide repeats and the other strand has an expanded trinucleotide repeat region. The expanded trinucleotide repeats are part of a hairpin structure. (4) After DNA replication of the expanded : nonexpanded DNA molecule depicted in 3, two chromosomes are produced. One of the daughter chromosomes contains a nonexpanded trinucleotide repeat region in both strands (nonexpanded chromosome) and the other an expanded trinucleotide repeat region in both strands (expanded chromosome).

facilitates the binding of a number of proteins to one another. On the other hand, extended polyglutamine tracts misfold and form aggregates (inclusions). Although not established experimentally, the inclusions themselves may be neurotoxic. In addition, the inclusions inhibit the proteolytic degradation

pathway, induce programmed cell death (apoptosis), and cause an overproduction of reactive oxygen species (ROS). This combination of cellular abnormalities would have a devastating effect on neurons over time.

There are other possibilities that might explain the Type 1 TRED neuropathology. For example, the production of expanded polyglutamine tracts could deplete the neuronal reservoir (pool) of free glutamine, which, in turn, would decrease the amount of glutamate synthesized from glutamine by glutaminase. A glutamate deficiency could cause inactivation and loss of neurons with glutamate receptors. Because approximately 35% of all CNS neurons use glutamate as a neurotransmitter, the lack of glutamate would have a major neurological impact.

A key question is why specific sets of neurons are affected by an expanded polyglutamine region, whereas other neurons and cell types that synthesize the mutated protein escape observable damage. The answer may be that in some neurons there are specific proteins that combine to excess with an expanded polyQ tract and cause various dysfunctions, whereas other neurons and cell types that do not synthesize these particular proteins are spared. In this context, huntingtin, with a normal-length polyglutamine sequence, binds at least three different proteins, huntingtin-associated protein 1 (HAP1), huntingtin-interacting protein 1 (HIP-I), and cystathionine β-synthase. Both HAP1 and HIP-I associate with proteins of the cytoskeleton and vesicle transport system. Consequently, expansion of the polyglutamine sequence may remove either one of these proteins from its normal site of action and lead to disassembly of the cytoskeleton, disruption of vesicle traffic, and ultimately, neuronal degeneration. Sequestering of neuron-specific proteins may also account for the other Type 1 TRED clinical phenotypes.

The mode of action of Type 2 trinucleotide repeat expansions has not been fully delineated. However, extreme expansions in noncoding regions could interfere with transcription of the gene, and thereby diminish the amount of an essential protein. The impact of these mutations is most likely at the level of DNA and/or RNA. In the former case, transcription of genes close to an extended repeat may be affected by a change in the organization of the chromatin. With the latter, the formation of unusual RNA structures would not only interfere with splicing but sequester other proteins including transcription factors. As a result, different cellular functions and cell-specific translation of diverse genes would be affected. In this way, trinucleotide expansion in a noncoding region can cause a multisystem disorder.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis [ALS; motor neuron disease (MND); Lou Gehrig disease] is a fatal, progressive neurodegenerative disease that affects about 1 in 10,000 people worldwide. In the majority of cases, onset occurs at about 56 years of age and is marked by the loss of movement in one leg and/or arm. The condition spreads, and, within a year or two, patients experience extreme muscle atrophy of the arms, hands, and legs; impaired speech; difficulty in chewing and swallowing; excessive drooling; and problems in breathing. Mental capabilities are not affected. By the time of death, usually 3 to 5

Table 14.5 Genetic heterogeneity of amyotrophic lateral sclerosis.

Designation	Inheritance	Onset	Location
ALS1	AD	Adult; ~46 yr	21q22.1
	AR	~13 to 94 yr	
ALS2	AR	Juvenile; 3 to 23 yr	2q33.2
ALS3	AD	Juvenile	18q21
ALS4	AD	Juvenile; ~17 yr	9q34
ALS5	AD	Juvenile	15q15.1–q21.1

years after initial diagnosis, patients often are completely immobile, mute, and confined to bed. Motor neurons in the cerebral cortex and medulla oblongata (upper motor neurons) and in the spinal cord (lower motor neurons) that convey signals to skeletal muscles are selectively destroyed. A consistent neuropathological sign of ALS is the accumulation of neurofilaments in affected motor neurons. Axon terminals are lost, and, as a consequence of denervation, muscles atrophy. Approximately 90% of ALS cases are sporadic, and the rest are familial (FALS). There is no significant difference in the clinical phenotype between sporadic and familial ALS. To date, there are five designated genes that give rise to FALS (Table 14.5) with, at least, one more autosomal dominant adult-onset FALS at 16q12.1–q12.2 and possible other FALS loci in the centromere region of chromosome X and within 20p. Of these, the autosomal dominant form (ALS1), located at 21q21.1, accounts for about 20% of FALS occurrences, with the remaining known ALS genes comprising less than 5% of the familial cases. Clearly, other ALS-causing genes await discovery.

A candidate gene for the ALS locus at chromosome 21q21.1 was the *SOD1* gene that encodes Cu,Zn superoxide dismutase (SOD1). Mutation analysis of *SOD1* genes of ALS family members established that this gene was responsible for ALS. Greater than 95% of the more than 100 different ALS *SOD1* gene nucleotide substitutions are missense mutations. In most families, ALS is inherited as an autosomal dominant trait with symptoms beginning at 46 years of age, 10 years earlier than the onset of sporadic ALS. In the few families with autosomal recessive ALS, the affected individuals were homozygous for *SOD1* mutations.

Cu,Zn superoxide dismutase is a widely distributed enzyme and is especially abundant in motor neurons. It converts superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and water. Superoxide anion is formed as a normal by-product of aerobic metabolism and is a reactive free radical that causes oxidative damage to proteins, nucleic acids, membranes, and other cell constituents. Hydrogen peroxide, also a toxic compound, is converted rapidly to water by catalase or glutathione peroxidase. Superoxide dismutase has limited peroxidase activity. Despite the protective role that SOD1 plays in the dismutation of $O_2^{\cdot-}$, when it breaks down H_2O_2, hydroxyl radicals ($^{\cdot}OH$) are produced. Hydroxyl radicals react readily with any type of macromolecule and, consequently, are very toxic. In addition, a decrease in SOD1 activity has an impact on the neurotransmitter nitric oxide. When superoxide anions accumulate, they combine with nitric oxide to form peroxynitrate, which is highly reactive and indiscriminately adds nitrate to tyrosine and other cell constituents.

Initially, it was thought that autosomal dominant ALS *SOD1* mutations lowered the level of superoxide dismutase activity, which increased the amount of reactive oxygen species such as superoxide anion. The resulting cellular damage then would be responsible for ALS. However, after a number of studies, it became clear that many of the ALS *SOD1* mutations did not diminish SOD1 activity. In addition, transgenic mice carrying a mutated human *SOD1* DNA sequence developed symptoms similar to ALS, despite producing normal levels of SOD from their own superoxide dismutase genes. Thus, the autosomal dominant *SOD1* gene base substitutions probably create injurious "gain-of-function" mutations rather than ones that cause "loss of function." Currently, it is thought that ALS SOD1 might result from a combination of toxicity of oxygen radicals (oxidative stress), a buildup of copper that cannot bind to mutant SOD1 molecules (copper toxicity), misfolding of mutant SOD1 and aggregate formation, and/or sequestering to SOD1 aggregates of various proteins including those that normally prevent programmed cell death.

The genes for two juvenile forms of ALS, namely, *ALS2* and *-4*, have been characterized. The *ALS2* gene at 2q33.2 encodes a protein that has significant homology with proteins that play a role in intracellular signaling processes (GTPase regulatory proteins). The *ALS4* gene at 9q34 is equivalent to the *SETX* (*Senataxin*) gene. The SEXT protein is probably a DNA/RNA helicase that participates in RNA processing, DNA repair, transcript stabilization, translation initiation, and other activities. Mutations of both these genes have been found in individuals with neurological disorders that are distinct from ALS. How *ALS2* and *SEXT* gene mutations lead to neuronal degeneration has not been determined.

Charcot–Marie–Tooth Disease

In 1886, French neurologists J. M. Charcot (1825–1893) and P. Marie (1853–1940) and, independently, H. H. Tooth (1856–1925), a British physician, published detailed descriptions of a disease characterized by atrophy of the muscles on the outer side (peroneal) and distal portion of the legs, progressing over time to the feet, hands, and forearms. This disorder originally was known as peroneal muscular atrophy, but, eventually, Charcot–Marie–Tooth disease (CMT) became the more accepted name. CMT is a commonly inherited condition, affecting about 1 in 2500 people, that is both genetically and clinically heterogeneous. Because both motor and sensory nerves are primarily affected by CMT, a more specific designation—hereditary motor and sensory neuropathy (HMSN)—has been suggested as an alternative name for CMT.

The initial signs of CMT are a stumbling gait, awkwardness while running, and ankle weakness in childhood. This is followed by atrophy of the muscles of the feet, and then by weakening of the muscles of the hand and forearms. Later, in many adult patients, sensory responses to touch, pain, temperature, and vibration in the feet and hands decrease and often are lost. In some instances, learning or breathing may be adversely affected. Although debilitating, most clinical forms of CMT are not fatal and are compatible with a long, albeit arduous, life. In some cases, the onset of the disease occurs very early in life and then progresses rapidly.

On the basis of neuropathology and electrical conduction behavior of motor neurons as well as other features including genetics, four clinical types of CMT have been delineated. Generally, demyelination is the primary defect with CMT Types 1, 3, and 4. Axonal loss highlights CMT Type 2. When the features of CMT do not fall clearly into one or the other of the major classes, the condition is often designated as intermediate CMT, for example, dominant intermediate type C (DI-CMTC). Additional groupings for other genes that cause CMT are based on whether the mode of inheritance is autosomal recessive or X-linked. With CMT Type 1 (CMT1), motor nerve conduction values (MNCVs) are reduced from the normal range of 48 to 53 meters per second (m/s) to less than 38 m/s. Histological examination shows two identifying features. First, there is extensive demyelination of motor nerves. Second, axons of motor nerves are surrounded by Schwann cells that do not produce myelin sheaths but instead form bulbous outcroppings called "onion bulbs." By contrast, with CMT2 (axonal CMT), the MNVCs are within the normal range and, although there is no obvious demyelination, considerable selective loss of motor nerve axons occurs. The age of onset of CMT1 is in the first to second decade of life and about 10 years earlier than the onset of CMT2. Of all the cases of CMT, 80% undergo demyelination and the remainder have loss of axons.

For the most part, CMT is inherited as an autosomal dominant trait, although there are examples of autosomal recessive, X-linked recessive, and X-linked dominant forms. At least 15 genes have been identified that are associated with CMT (Table 14.6). As well, a number of CMT loci have been mapped to various chromosome regions, for example, 2q14, 19q13.3, 12q24, 10q24.1–q25.1, 3q13–q22, and 1p34–p35. The CMT1 and CMT2 classes are subdivided according to different gene loci that make up each class. In the CMT1 category, about 60% to 80% of the cases are CMT1A and have a mutation of the *PMP22* gene at 17p12–p11.2 that encodes peripheral myelin protein 22 (PMP22). The mutation in about 75% of these individuals is a duplication of one of the two *PMP22* genes. The remaining affected individuals generally have *PMP22* point mutations. Peripheral myelin protein 22 is found in a wide range of tissues, including Schwann cells, where it makes up about 5% of the total cell protein. PMP22 has four transmembrane domains and is a component of PNS myelin. The *PMP22* gene duplication frequently occurs by interchromosomal exchange during spermatogenesis and, to a lesser extent, by intrachromosomal exchange during oogenesis. Overexpression of multiple copies of the *PMP22* gene in transgenic rodents produces the same CMT1A phenotype found in humans. A severe form of CMT1A occurs in individuals with duplicated *PMP22* genes on homologous chromosomes.

A deletion of a *PMP22* gene on one of the two homologous chromosomes, that is, haploinsufficiency, causes a phenotypically distinct peripheral neuropathy called hereditary neuropathy with liability to pressure palsies (HNPP). These patients have recurring bouts of paralysis (palsy) of varying duration in both the legs and arms. Many HNPP patients experience a loss of sensory responses. MNCVs are below normal, and myelin sheaths form sausagelike structures between apparently normally myelinated regions. In addition, some

Table 14.6 Genetic heterogeneity of Charcot–Marie–Tooth (CMT) disease.

CMT type[a]	Gene	Location	Protein	Function
CMT1A	*PMP22*	17p12–p11.2	Peripheral myelin protein 22	Compact myelin protein
CMT1B	*MPZ*	1q22.1	Myelin protein zero	Peripheral myelin protein
CMT1C	*LITAF*	16p13.3–p12	Lipopolysaccharide-induced TNF-α factor	Possibly trafficking between late endosomes and lysosomes
CMT1X	*GJB1*	Xq13.1	Connexin 32	Gap junctions between myelin sheaths
CMT2A	*MFN2*	1p36.22	GPTase mitofusin 2	Mitochondrial membrane protein; mitochondrial assembly regulator
CMT2B	*RAB7*	3q21.3	RAS-related GTP-binding protein	Late endocytic pathway
CMT2B1	*LMNA*	1q22	Lamin A/C	Nuclear lamina matrix protein
CMT2D	*GARS*	7p15	Glycyl-tRNA synthetase	Charging Gly-tRNA
CMT2E	*NEFL*	8p21	Neurofilament light polypeptide	Cytoplasmic fiber network
CMT4A	*GDAP1*	8q21.11	Ganglioside-induced differentiation-associated-protein 1	Possibly glutathione transferase
CMT4B1	*MTMR2*	11q22	Myotubularin-related-protein-2	Possibly tyrosine phosphatase
CMT4B2	*CMT4B2*	11p15.3	SET binding factor 2	Pseudophosphatase
CMT4D	*NDRG1*	8q24.3	N-myc downstream regulated gene 1 protein	Possibly α/β hydrolase
CMT4E	*EGR2*	10q21.1	Early growth response protein 2	Transcription factor
CMT4F	*PRX*	19q13.2	Periaxin	Possibly stabilizes myelin sheath

[a] See http://www.neuro.wustl.edu/neuromuscular/time/hmsn.html for more information about the genetics and neuropathology of CMT and other motor sensory neuropathies.

cases of Dejerine–Sottas disease (DSD), a very severe form of CMT1, have *PMP22* point mutations.

Mutations in the *MPZ* gene at chromosome 1q22.1, which encodes myelin protein zero (P_0, MPZ), produce the same clinical characteristics as *PMP22* gene mutations and have been classified as CMT1B. Myelin protein zero is expressed in Schwann cells and comprises about half the total protein of the myelin sheath of peripheral nerves. It has adhesive properties and probably links layers of the myelin sheath to each other. Interestingly, not only *PMP22* mutations but some *MPZ* mutations also produce Dejerine–Sottas disease. In other words, mutations of different genes for myelin sheath proteins produce a very similar clinical phenotype. Overall, *MPZ* mutations account for approximately 8% of all cases of autosomal dominant CMT.

Mutations of the *GJB1* gene at Xp13.1, which encodes the gap junction protein connexin 32, are responsible for CMT, specifically CMT1X. Connexin

32 (Cx32) is a member of a family of proteins that span membranes. Generally, six connexin molecules form a circular, doughnutlike aggregate (hemichannel) in the membrane of one cell that combines with a connexin hemichannel in the membrane of another cell to form a channel (gap junction) that connects the two cells. Gap junctions allow ions and small molecules to flow from one cell to another. Cx32 may form gap junctions that connect the folds of myelin sheaths of the peripheral nerves and enable nutrients and small molecules to be distributed throughout the myelin sheath. The phenotype associated with *GJB1* mutations is a demyelinating peripheral neuropathy that closely resembles CMT1, although axon loss has been noted in some affected individuals.

How mutations of the remaining CMT genes, which are rare, contribute to PNS neuropathy is unresolved. Interestingly, the *MPZ*, *PMP22*, and *GJB1* genes, which each play an important role in the myelination process of motor neurons, are not expressed in oligodendrocytes and their protein products are not part of the myelin sheath of CNS neurons. In other words, inherited hypomyelination disorders of CNS nerves, which are infrequent, involve a different set of genes.

Inherited Prion Diseases

Transmissible spongioform encephalopathies (TSEs) are fatal neurodegenerative disorders affecting livestock (sheep, goats, and cattle), other animals (mules, deer, elk, mink, cats, mice, and hamsters), and humans. The neuropathology is more or less similar in all species and involves the formation of large spongelike perforations in the cerebral cortex (hence the term spongioform), excessive proliferation of glia cells (gliosis), selective loss of neuronal cells, and, although somewhat variable in extent and location in the brain, the formation of rod-shaped extracellular amyloid plaques. The effects of spongioform encephalopathy were first noted in sheep in the 1700s. Because affected animals tended to rub (scrape) themselves raw against fence posts and trees, the condition was called scrapie. In 1946, by chance, scrapie was found to be a transmissible disease that could be passed from the tissue of infected sheep to unaffected sheep. The pathogenic agent was called scrapie. Subsequently, many of the other spongioform encephalopathies were shown to be infectious.

In the late 1950s, a disease strikingly similar to animal TSEs was observed in the Fore tribe of Papua-New Guinea. The Fore called this lethal disease kuru, which in their language means "trembling" and corresponds to the involuntary muscle tremors of the initial stages of the disease. After the jerking and twitching phase, those afflicted with kuru become demented and then stuporous. Death usually occurs about one year after the onset of symptoms. Postmortem studies of brains from kuru patients revealed a neuropathology that resembled scrapie. Kuru probably was transmitted among the Fore, especially to women and children, by the consumption of the brains of those who had died from the disease. The eating of parts of the body of a dead person was part of a celebratory mortuary feast. Since the Fore abandoned this form of ritual cannibalism, the occurrence of kuru is nonexistent. The infectious nature of kuru was established when spongioform encephalopathy was produced in

from the HUMAN GENETICS files

Genetically Engineered Animal Models for Studying Inherited Human Disorders

Biomedical research relies on the use of laboratory animals for the study of the biochemistry and pathogenesis of many human disorders. In the past, chemical and physical treatments were used to simulate the effects of inherited human disorders. This approach is intrinsically unsatisfactory because the inducing agents may cause adverse responses that are unrelated to the syndrome being mimicked. Occasionally, animals with naturally occurring mutations for a disease of interest have been identified and studied. Clearly, if it were possible to genetically manipulate laboratory animals at will, then models for almost every human single-gene disorder could be created.

In the early 1980s, genetically engineered mice were produced by injecting an identifiable gene into fertilized mouse eggs and implanting the inoculated eggs into surrogate mother mice. In these experiments, a few offspring carried the cloned gene in some of their cells. Moreover, genetic lines that perpetuated the cloned gene were established from some of the mice. The process of introducing DNA into the genome of a multicellular organism is called transgenesis.

Now mouse transgenesis is used routinely to create models for human genetic diseases and for other applications including the study of the temporal regulation of gene expression. Mice were chosen for these studies because they are manipulated easily in experiments, breed readily, can be maintained in an animal facility, are exceptionally well known genetically, and because, for many purposes, mouse and human anatomy and physiology closely resemble each other. However, despite the importance of mouse models for human diseases, a mouse is, after all, not a human.

Transgenic mouse models can be divided into two broad categories. In the first, structural genes (transgenes) are added to the mouse genome. In these cases, the inserted coding sequences often are driven by strong, tissue-specific promoters for overexpression of the transgene. Depending on the purpose of the model, a transgene may have a wild-type or a mutant sequence. Transgenes have different formats, including full-length or portions of cDNAs, synthetic genes, parts of genes, complete genes, multiple genes on YACs, and coding sequences with point mutations or inserted in-frame codons. In the second category, mouse genes are incapacitated (knocked out) by either homologous recombination or tissue-specific induced removal of part of a gene. The major purpose of gene disruption or ablation is to determine the biological consequences of the loss of a specified mouse gene. In addition, the availability of a knockout mouse provides the opportunity to study the effect of a transgene without the confounding influence of expression of a homologous mouse gene.

DNA constructs may be introduced into mice by retroviral vectors that infect cells of an early-stage embryo, by microinjection in the enlarged sperm nucleus (male pronucleus) of a fertilized egg, or by the addition of genetically engineered embryonic stem (ES) cells into an early-stage embryo. The retroviral vector method is used rarely for creating models of human disorders, because the input DNA integrates randomly in the mouse genome, retroviral vectors carry small amounts of cloned DNA, and transgene expression is modest.

DNA microinjection is a standard way of producing transgenic mice. The transgene, usually free of vector DNA, is injected into the male pronucleus of a fertilized egg. In mice, after entry of the sperm into the egg, both the sperm nucleus (male pronucleus) and female nucleus exist separately. The female nucleus completes its meiotic division after fertilization to become the female pronucleus, and then nuclear fusion (karyogamy) occurs. The male pronucleus tends to be larger than the female pronucleus. The transgenesis procedure uses a dissecting microscope and a fine-movement mechanical manipulator for aligning each fertilized egg, inserting the tip of a very fine glass pipette directly into the male pronucleus, and then injecting the input DNA. Several hundred male pronuclei can be inoculated in a single day.

After microinjection, 25 to 40 eggs are implanted microsurgically into a foster mother, who has been made pseudopregnant by being mated to a vasectomized male. In mice, copulation is the only known way to prepare the uterus for implantation. In this case, because the male mate lacks sperm, none of the eggs of the foster mother is fertilized. Delivery of pups occurs about 3 weeks after implantation.

To identify transgenic animals, DNA from a small piece of the tail can be assayed by either Southern blot hybridization or the polymerase chain reaction (PCR) for the presence of the transgene. A transgenic mouse can be mated to another mouse to determine whether the transgene is in the germ line of the founder animal. Subsequently, progeny can be bred to generate pure (homozygous) transgenic lines. If a homozygous genotype causes death during embryogenesis, then lines are maintained as heterozygotes.

The transgenesis procedure, although apparently simple, requires the coordination of a number of experimental steps. Even a highly trained practitioner can expect, at best, only 5% of inoculated eggs to develop into live transgenic animals. None of the steps in the procedure is 100% efficient. Consequently, large numbers of microinjected fertilized eggs must be used. With the mouse system, about 66% of the fertilized eggs survive the injection procedure, about 25% of the implanted eggs develop into pups, and about 25% of the pups are transgenic. Thus, from 1000 inoculated fertilized eggs, 30 to 50 transgenic pups are produced. Furthermore, with this method, the injected DNA often integrates at random sites within the genome, and

multiple copies of the injected DNA are incorporated at one site. Moreover, not all of the transgenic pups will have the appropriate phenotype. In some animals, the transgene may not be expressed because of the location of the chromosome site of integration, and in others the copy number may be excessive and lead to overexpression, disrupting the physiology of the animal. Despite the overall inefficiency, it has become routine to use DNA microinjection to generate lines of mice carrying functional transgenes.

By using ES cells instead of pronuclear microinjection to generate transgenic mice, researchers can craft highly specific gene modifications and be confident that the transgenic animals will carry these predefined changes at specified chromosome locations. This methodology depends on an interesting biological phenomenon. Cells from an early stage (blastocyst) of a developing mouse embryo can proliferate in cell culture and still retain the capability of differentiating into all other cell types—including germ line cells—after they are reintroduced into another blastocyst embryo. Such cells are called pluripotent embryonic stem (ES) cells. Moreover, ES cells can be readily engineered genetically without altering their pluripotency. With this system, a functional transgene is integrated at a specific site in the genome of ES cells. Then the genetically engineered cells are selected, grown, and used to generate transgenic animals. In this way, the randomness of integration inherent with the DNA microinjection system is avoided.

Monitoring the consequences of incapacitating (knocking out) a mouse gene has become a commonplace and essential research procedure. The effectiveness of the knockout process depends on selecting cells that have a DNA construct integrated at a targeted chromosome site. To this end, a procedure called positive-negative selection (PNS) was devised. For PNS, the targeting DNA construct has a Neo^r gene inserted within a cloned exon of the target gene and a gene (*TK*) that specifies thymidine kinase (Figure A).

There are three possible outcomes after ES cells are treated (transfected) with a PNS targeting vector. First, in some cells the vector will integrate at the target (correct) site as a result of homologous recombination between the exon sequences of the construct and the chromosome (Figure A). The target gene is inactivated by the insertion of the Neo^r gene into its coding region. In this case, the *TK* gene is not included as part of the integrated DNA. Second, in other cells, integration will occur at nontarget (spurious) sites by nonhomologous recombination, and the entire DNA construct including the *TK* gene is integrated into the chromosome (Figure B). Third, the majority of the cells do not have any integrated input DNA.

The PNS scheme, which depends on the expression of the Neo^r and *TK* genes, permits only cells with DNA that is integrated by homologous recombination to survive. After the introduction of DNA into cells in culture, the ES cells are washed free of unincorporated DNA and

Figure A. Positive-negative selection and homologous recombination of a targeting DNA construct. The target gene is disrupted by the insertion of cloned DNA carrying a Neo^r gene. Under these circumstances, the *TK* gene is not integrated. Cells with this genetic organization survive treatment with G418 and ganciclovir because the Neo^r gene product confers resistance to G418 and, in the absence of the *TK* gene, the lethal phosphorylated derivative of ganciclovir is not produced. The A′ and A″ designations denote coding sequences from exon A of the target gene.

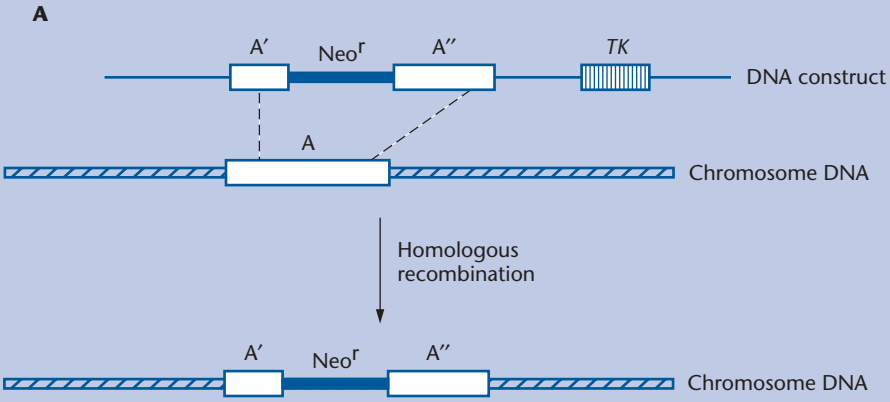
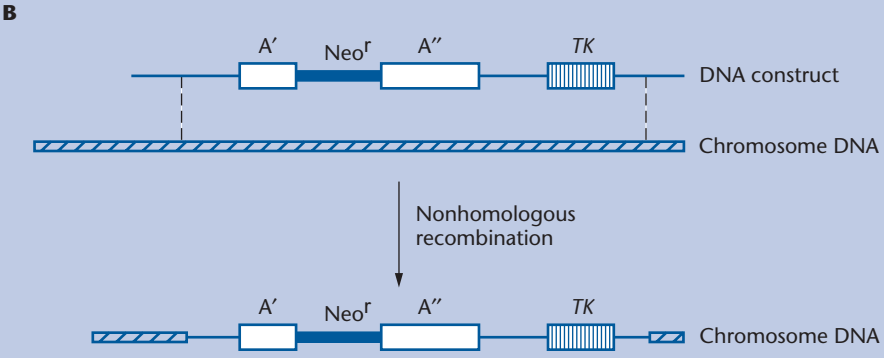


Figure B. Positive-negative selection and nonhomologous recombination of a targeting DNA construct. The targeting DNA construct with both the Neo^r and *TK* genes integrates somewhere in the genome of an ES cell. Cells with this insert are resistant to G418 because they produce the product of the Neo^r gene. However, they are killed by phosphorylated ganciclovir that is formed by the thymidine kinase encoded by the *TK* gene.



continued

placed in a culture medium containing the chemical compounds G418 and ganciclovir. All cells without a functional Neo^r gene are killed by G418. As a result, cells with an integrated Neo^r gene are spared, that is, they are positively selected. All cells that synthesize thymidine kinase encoded by the *TK* gene borne by the DNA construct are killed by phosphorylated ganciclovir, that is, these cells are negatively selected. The thymidine kinase specified by the inserted *TK* gene phosphorylates ganciclovir inside cells. And, because phosphorylated ganciclovir is not secreted into the culture medium, other cells are not affected. Virtually all the cells that remain after this dual selection strategy have a Neo^r gene disrupting the target gene. Once it is confirmed that the selected ES cells have the desired genetic modification, they are cultured and placed inside blastocyst stage embryos, and the mixed-cell embryos are implanted into pseudopregnant foster mothers. Pups with the genetic modification in their germ line cells are used for breeding and establishing homozygous or, if necessary, heterozygous lines.

Thousands of different types of transgenic and knockout mice have been created to study the pathogenesis of a myriad of human disorders. Many of these transgenics have been generated as models for neurodegenerative diseases, such as AD, HD, ALS, and CJD. Because the creation of knockin (transgenic) and knockout mice is pervasive and the worldwide repertoire is extensive, databases have been established to keep track of the lines. Although designed for researchers, the Transgenic/Targeted Mutation Database (TBASE), which can be accessed at http://tbase.jax.org, provides an informative view of these indispensable research tools.

primates after the administration of tissue samples from the brains of those who had died from kuru.

Initially, the TSEs appeared to be caused by viruses, presumably slow-acting varieties. However, extensive research has yet to reveal a virus or viruslike entity that could be responsible for any of these disorders. Surprisingly, much of the research data point to the pathogenic agent being an abnormal protein, which in some way generates more abnormal protein from the normal form of the protein. As nearly as can be determined, no extrachromosomal polynucleotide plays a role in this protein-based replicative process. This concept has been dubbed the "protein-only" hypothesis. The term prion, which is derived from the phrase "proteinaceous infectious particles," was coined by S. Prusiner (b. 1942) to emphasize that the infective agent of a TSE is a protein. Prions have been extensively studied in animal systems.

The protein-only hypothesis maintains that a prion is a single protein (prion protein, PrP) with a stable altered topology and that it produces more prions from normal protein molecules. The abnormal conformation isoform(s) is usually resistant to proteolytic degradation, highly insoluble, and readily forms a β-sheet. The normal isoform is sensitive to proteolytic breakdown and soluble and has a low propensity to form a β-sheet. The infective PrP has been designated PrP^{Sc} (scrapie prion protein), and the normal isoform is designated as PrP^{C} (cellular prion protein). Some workers in this field of study use the notation PrPres and PrPsen, instead of PrP^{Sc} and PrP^{C}, respectively, where res and sen refer to resistance and sensitivity to proteolytic degradation. The mechanism of conversion of PrP^{C} to PrP^{Sc} is not known. Various models postulate that a PrP^{Sc} molecule combines with a PrP^{C} molecule and reshapes it into a PrP^{Sc} molecule. The conversion process requires the presence of PrP^{C}. Without PrP^{C}, no neurodegeneration occurs. Another intriguing and fundamental question is how the PrP^{Sc} conformation is formed initially.

The cause of neurodegeneration by PrP^{Sc} is problematic. PrP^{Sc} may be neurotoxic or it may deplete the amount of PrP^{C}, which may be an essential protein in neurons. Although PrP^{Sc} molecules aggregate and form amyloid bodies, these structures do not seem to be the primary cause of the neuropathology of prion diseases. The deleterious effects of PrP^{Sc} will become easier to under-

stand when the function of PrP^C is clarified. PrP^C is a copper-binding brain protein anchored to the outer cell membrane of neurons and nonneuronal cells. Its possible functions include regulation of copper concentration in the synaptic region, copper-dependent superoxide dismutase activity, acting as a neurotransmitter, maintenance of cell viability, and/or activation of an intraneuronal signal pathway. PrP^C also interacts with membrane-bound receptors, heat shock proteins, and cell surface molecules.

In addition to kuru, at least three other human prion diseases have been identified. These are Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), and fatal familial insomnia (FFI). These disorders are quite rare and together affect about one person per million worldwide. The majority of cases of human prion diseases are CJD, which, for the most part, occurs sporadically, although there are also examples of familial CJD. In addition, some individuals with CJD acquired PrP^{Sc} by accidental transmission from medical treatments. These medically caused (iatrogenic) cases result from the inadvertent introduction of prion-infected material during surgical procedures or, in the past, from the administration of human growth hormone prepared from human pituitary glands that contained prions. Precautions are now in place to prevent iatrogenic CJD. And, although the origin of the outbreak of kuru in Papua-New Guinea is unknown, it is likely that it initiated from a single person who died from sporadic CJD. Finally, some cases of CJD, called variant CJD (vCJD), have occurred by acquisition of bovine spongiform encephalitis (BSE, mad cow disease) from eating contaminated meat products. Considerable effort has gone into establishing that the human food chain is free of bovine prions.

Familial CJD represents from 10% to 15% of cases of human spongioform encephalopathies. The clinical phenotypes for familial and sporadic CJD are, for the most part, the same. Onset is usually between the ages of 60 and 65 years and is rarely earlier than 45 or later than 75 years of age. The progression of the disease is rapid, and death occurs within 6 months in 70% of cases. The initial symptoms are tremors, seizures, and a loss of muscle coordination, followed by dementia, immobility, and stupor. It is assumed that a number of years are required before the pathogenic effects emerge. Iatrogenic CJD has occurred in children and vCJD in young adults, and the progress of the disease, in both instances, is exceptionally rapid.

Both GSS and FFI are, for the most part, familial disorders. Families with CJD, GSS, and FFI show autosomal dominant inheritance. Clinical features of GSS include onset at about 46 years of age, with a stumbling gait and leg weakness followed by dementia and death after about 5 years. Numerous prion protein amyloid plaques are present throughout the CNS, although the extent of cerebral spongiosis is considerably less than with CJD. Fatal familial insomnia, which is quite rare, begins in adults older than 50 years and involves both uncoordinated movements and an inability to sleep that does not respond to any known treatment. The course of the disease usually ends within 12 to 15 months in stupor and coma. In the brains of individuals with FFI, there is a significant selective loss of neurons of the thalamus, mild cerebral gliosis, moderate spongiosis of the cerebrum, and very few prion protein amyloid plaques. In addition to the apparent distinctive pathological features of CJD, GSS, and

FFI, there is considerable phenotypic variation within individual families. Transmission studies with test animals established that familial CJD, GSS, and FFI are TSEs.

From a genetic perspective, the main question is whether the familial forms of CJD, GSS, and FFI are the result of different autosomal genes or whether, despite the neuropathological differences, they are the result of mutations of the same gene. A good candidate for one or more of these inherited human prion diseases clearly would be the gene that encodes the prion protein. With amino acid sequence data from purified prion protein, DNA probes were generated, and the human PrP (*PRNP*) gene was isolated, characterized, and localized to 20pter–p12. Linkage studies with CJD, GSS, and FFI families placed the putative gene, in each case, on the short arm of chromosome 20. And, for each of these inherited prion diseases, mutations were found in the *PRNP* gene of affected individuals.

Over 20 different pathogenic *PRNP* mutations have been recorded. The most common CJD mutation is a glutamic acid to lysine change at codon 200, that is, E200K. Some forms of familial CJD have an insert of 1, 2, or 4 to 9 extra units of an eight-amino acid segment that is normally repeated five times in PrP^{C}. There are at least seven *PRNP* point mutations associated with GSS, of which P102L is the most frequent. Fatal familial insomnia only occurs with the missense mutation D178N and a methionine codon at site 129 (129M). Codon 129 in the normal *PRNP* gene is polymorphic, and the encoded amino acid can be either methionine or valine. Interestingly, the CJD phenotype results with the missense mutation D178N and valine at site 129.

No germline *PRNP* mutations have been found among the sporadic cases of CJD. However, homozygosity for either methionine or valine at site 129 is more frequent than expected among those with sporadic and iatrogenic CJD. The nature of the factors directly responsible for sporadic CJD or that indirectly increase the susceptibility for CJD are not known. Perhaps somatic mutation of the *PRNP* gene and/or spontaneous change of PrP^{C} to a conformation that leads to an accumulation of PrP^{Sc} account for sporadic CJD.

On the basis of the protein-only hypothesis, it is possible that the CJD, GSS, and FFI *PRNP* gene mutations encode different PrP^{Sc} isoforms that assume distinct topologies and cause specific neuropathological damage. Moreover, the PrP^{Sc} isoforms may be either intact molecules or, in some cases, proteolytic fragments of the mutated prion protein. In this context, the GSS *PRNP* mutation A117V produces a transmissible pathogenic fragment of the prion protein (^{Ctm}PrP, C-terminal transmembrane prion protein). The induction of neurodegeneration by either an abnormally folded intact PrP^{Sc} or a PrP^{Sc} fragment is a fascinating phenomenon that is biologically unique and is beginning to be understood at the molecular level. And, among other things, it remains to be determined how different conformations of PrP^{Sc} affect predominantly the cerebellum in GSS, the cerebral cortex in CJD, and the thalamus in FFI.

Schizophrenia

Schizophrenia is a psychiatric disorder that causes a major derangement of personality marked by significant loss of perception of reality. Generally,

schizophrenic symptoms begin during late adolescence, at about 19 to 21 years of age. An estimated 1% of the worldwide population has this debilitating condition. At any particular time, approximately 20% of hospital beds in the United States are occupied by schizophrenic patients. Patients with schizophrenia in its acute form cease to develop socially and often regress. Schizophrenics are unable to finish work tasks and are reluctant to associate with others. They lose interest in personal needs, including hygiene, and are aptly described as "withdrawn into themselves." In addition, they experience tastes, smells, touch, vision, and especially sounds that have no apparent bases in fact. These hallucinations are often auditory and take the form of "hearing voices," a condition that can persist for several days. In addition, schizophrenics often hold firmly to delusional beliefs and viewpoints, despite convincing, obvious, and concrete evidence to the contrary. The conversation of a schizophrenic is generally incoherent and chaotic.

After onset, the extent of schizophrenic symptoms varies considerably in different individuals. For some, the severe form is lifelong. Others have periodic episodes that may last up to six months, interspersed with periods of little or no disturbances. Some patients may have only a few episodes during their lifetime.

Despite several attempts to formulate a unified description of schizophrenia, clinicians have created at least three major categories that offer very broad, broad, and narrow definitions of the disorder. Moreover, five subtypes, including catatonic, paranoid, disorganized, residual, and undifferentiated schizophrenia, have been created with reference to the prevailing features. It is not known whether schizophrenia is a single biological disorder or many different conditions with more or less the same clinical characteristics. Currently, the diagnosis of schizophrenia is based on a multiple-item test that scores what clinicians call the "positive" (delusions, suspiciousness, and disorganized thinking) and "negative" (social withdrawal, apathy, and loss of emotional response) features of the disorder, as well as physical tests that determine responses to light (eye tracking) and auditory stimuli.

Neuropathological changes in schizophrenics are not obvious, but may include enlargement of two broad C-shaped cavities (lateral ventricles), one in each cerebral hemisphere, that are part of the cerebrospinal fluid system. In addition, brain mass often decreases. No specific region of the brain is affected by lesions. Schizophrenia is probably the result of errors during brain development, although there is no information as to what aspect of this process goes awry. More than likely, specific tracts of neurons are not positioned properly when they are first laid down, which later prevents communication between parts of the developed brain.

Schizophrenia has a significant genetic component. It occurs in families, although there is no obvious mode of inheritance. However, the closer the genetic relationship to a schizophrenic individual, the greater the likelihood of developing schizophrenia. Studies with one-egg twins (monozygotic, MZ) in comparison with same-sex, two-egg twins (dizygotic, DZ) indicate that if one member of an MZ pair has schizophrenia, then there is a 58% chance that the other member also will have schizophrenia. By contrast, the agreement (concordance) between same-sex DZ twins for schizophrenia is about 13%. Theo-

retically, if schizophrenia were a fully penetrant genetic disorder, there should be 100% agreement between MZ twins. But if the trait were completely nongenetic, that is, environmental in origin, there would be no difference in the frequency of agreement between MZ and same-sex DZ twins. Additional circumstantial support for a genetic component for schizophrenia comes from adoption studies. For example, newborns of schizophrenic mothers who are adopted into nonschizophrenic families have a higher frequency of schizophrenia than adopted newborns of nonschizophrenic mothers. However, these studies do not preclude an intrauterine effect on the newborn from schizophrenic mothers. In some studies, schizophrenia appears to be a genetic condition with very low penetrance, and in others, it very well may be the result of nongenetic factors. Complications during delivery, intrauterine viral infections, and family interactions have been suggested as nongenetic agents that might contribute to schizophrenia.

The task of unraveling the genetic features of schizophrenia has been difficult, elusive, and often very frustrating. Although not particularly rewarding, a large number of candidate genes including some major members of the dopamine transmitter (dopaminergic) system except, possibly, the dopamine receptor D3 (*DRD3*) and released-dopamine catabolic enzyme (*COMT*; catechol-*O*-methyltransferase) genes have been eliminated as likely causes of schizophrenia. In a more positive vein, despite the many false positives from a myriad of linkage and association studies, some genes that contribute to schizophrenia susceptibility have been identified.

Based on genomic scans and other information, a number of chromosome regions with possible schizophrenia genes have been subjected to higher-resolution analyses. In this way, the dystrobrevin-binding protein 1 or dysbindin (*DTNBP1*) gene at 6p22.3 was associated with schizophrenia. Dysbindin is present in the brain and other tissues. It binds to β-dystrobrevin of the dystrophin protein complex (DPC). In addition to maintaining the integrity of neuromuscular synapses, the DPC facilitates neural activity in parts of the brain. Another probable schizophrenia gene is neuregulin 1 (*NRG1*) at 8p12–p21. Neuregulin 1 regulates *N*-methyl-D-aspartate (NMDA), acetylcholine, and γ-aminobutyric acid (GABA) neurotransmitter receptors. It also participates in the myelination process. Moreover, *Ngr1*-knockout mice have behavioral and structural features that parallel aspects of schizophrenia.

A balanced translocation, t(1;11)(q42.1;q21), was found in individuals with schizophrenia and in others with behavioral problems in a large family. Molecular analysis of the breakpoints indicated that the 11q21 region hardly had any genes, whereas 1q42.1 was gene rich. Specifically, the two genes that were disrupted by the 1q42.1 breakpoint were designated *DISC1* and *DISC2* for "disrupted in schizophrenia." The *DISC1* gene encodes an 854-amino acid protein, and the *DISC2* gene expresses a noncoding RNA that is complementary to the *DISC1* sequence. The *DISC1* gene is actively transcribed during the development of the cerebral cortex and in other parts of the brain. The DISC-1 protein is multifunctional and interacts with a number of brain proteins that are present during formation of the cerebral cortex.

Alleles of other genes that have been implicated in increasing the risk for schizophrenia include D-amino acid oxidase activator (*DAOA*) at 13q33.2,

D-amino acid oxidase (*DAO*) at 12q24, and regulator of G protein signaling 4 (*RGS4*) at 1q23.2. Briefly, D-amino acid oxidase activator interacts with D-amino acid oxidase, which is a detoxifying enzyme, and regulator of G protein signaling-4 affects neurotransmission and intraneuronal signaling. At a less definitive level, there is suggestive evidence that 13q, 22q, 2q, and 10q22.3 contain schizophrenia genes. To date, there are no functional relationships among the schizophrenia genes that would account for neurological basis of the phenotype. On the other hand, the discovery of a number of disparate genes is not unexpected for a complex trait such as schizophrenia.

key terms

Aβ42/43
acetylcholine
action potential
Alzheimer disease (AD)
amyloid body
amyloid precursor protein (APP)
amyotrophic lateral sclerosis (ALS)
apolipoprotein E4
association test
astrocyte
brain stem
cerebellum
cerebral cortex
cerebrum
Charcot–Marie–Tooth disease
cholinergic neuron
Creutzfeldt–Jakob disease (CJD)
dendrite
depolarization
diencephalon
entorhinal cortex
ependymal cell
familial amyotrophic lateral sclerosis (FALS)
fatal familial insomnia (FFI)
γ-aminobutyric acid
gap junction
genetic anticipation
genetic risk factor
Gerstmann–Sträussler–Scheinker disease (GSS)
glial cell
glutamate
hairpin structure
hippocampus
Huntington disease (HD)
hyperpolarization
kuru
lagging strand
leading strand
midbrain
myelin sheath
nerve impulse
neurofibrillary tangles (NFTs)
neuron
neurotransmitter
nonneuronal cell
oligodendrocyte
oligogenic inheritance
polygenic inheritance
polyglutamine
postsynaptic neuron
putamen
resting membrane potential
satellite cell
schizophrenia
Schwann cell
secretases
senile dementia
senile plaques (SPs)
spongioform encephalopathy
synaptic bulb
synaptic cleft
trinucleotide repeat expansion disorders (TREDs)
voltage-gated channel

summary

The complexity of the human nervous system is mind boggling. Neurons are the fundamental signaling elements of the central (CNS) and peripheral (PNS) nervous systems. Neuronal dendrites receive nerve impulses or sensory stimuli. If the signals are sufficiently strong or frequent, they are passed along the cell membrane to the initial segment of the single axon, where an action potential is initiated and a nerve impulse travels to the terminal end of the axon. The propagation of a nerve impulse depends on a series of membrane depolarizations, which entail the successive opening and closing of voltage-gated Na^+ and K^+ channels. When these channels are opened, sodium ions rush into the neuron, and potassium ions flow out. This exchange of ions reverses the resting membrane potential in this section of the cell membrane and opens the Na^+ and K^+ channels in the adjacent section of the neuronal membrane. Near the synaptic bulb, an action potential opens voltage-gated Ca^{2+} channels and calcium ions rush into the synaptic bulb and trigger the release of neurotransmitter molecules into the synaptic cleft of a synapse. Closed channel receptors of postsynaptic receiver elements are opened by the binding of neurotransmitters, and a nerve impulse is initiated. In some cases, the opening of certain receptors allows chloride ions to rush into the postsynaptic neuron, which hyperpolarizes the cell membrane and inhibits the

continued

initiation of a nerve signal. Of the more common neurotransmitters, γ-aminobutyric acid is inhibitory, acetylcholine is usually excitatory, and glutamate, serotonin, and dopamine are excitatory.

At the structural level, neurons enter the brain from the spinal cord, go from the brain to the spinal cord, interconnect parts of the brain, travel from the brain or spinal cord to muscles and glands, and go from various organs to the spinal cord or brain. The cerebrum, cerebellum, midbrain, diencephalon, and brain stem are major regions of the brain and contain clusters of cell bodies (nuclei) with specialized functions that send out numerous axons to various components of the nervous system. The cerebral cortex, which is packed with neurons, has undemarcated regions that control many vital activities, such as consciousness. The nonneuronal cells of the CNS are glial cells, astrocytes, oligodendrocytes, and ependymal cells, and those of the PNS are satellite and Schwann cells.

Mutations of some of the many genes expressed more or less exclusively in cells of the nervous system cause neurological disorders. In this context, the genes for a number of neuronal channelopathies that feature intermittent loss of brain function (seizures, convulsions, epilepsy), uncontrolled muscle movement (ataxia), and severe headaches with vomiting, nausea, and extreme sensitivity to light (migraine) have been identified.

Alzheimer disease (AD) is the major form of senile dementia. After onset, impairment of memory and loss of intellectual functioning are followed by muteness and an inability to respond to any external stimuli. Within the brain, there is an initial loss of neurons that rely on acetylcholine as the neurotransmitter (cholinergic neurons) within the hippocampus and entorhinal cortex. As AD progresses, neurodegeneration spreads throughout the cerebral cortex and to other parts of the brain. The two major neuropathological features of AD are senile plaques (SPs) on the outside of neurons and neurofibrillary tangles (NFTs) within the cell bodies of neurons. The core of an SP is an amyloid body consisting primarily of Aβ40 and Aβ42/43 isoforms that are derived by aberrant processing of the amyloid precursor protein (APP). NFTs are made up of many intertwined strands of hyperphosphorylated-tau protein molecules.

Genetic studies with familial AD (FAD), which tends to have an early onset (younger than 60 years of age), have revealed three causative genes, *APP*, *PSEN1*, and *PSEN2*. Base substitutions in these genes account for approximately 50% of all cases of FAD and only about 10% of all occurrences of AD. Very few individuals with sporadic AD, which has a late onset (older than 65 years), have mutations in the *APP*, *PSEN1*, and *PSEN2* genes. However, the *APOE*4* allele increases the likelihood that AD will develop, although it is neither necessary nor sufficient for AD. Alleles that enhance the vulnerability for a disease are called genetic risk factors. Increased production of Aβ42, by whatever means, seems to be the critical step in the development of AD.

Huntington disease (HD) is an autosomal dominant trait that results from an expansion of a repeat of 40 or more CAG units in the coding region of the *HD* gene at 4p16.3. The CAG repeat number in a normal *HD* gene ranges from 9 to 35. The CAG repeat encodes a polyglutamine tract within huntingtin, the name given to the protein of the *HD* gene. Neurodegeneration in HD patients is centered in the cerebral cortex, caudate nucleus, and putamen and, to a lesser extent, in some of the other nuclei of the cerebrum. Clinical aspects of HD include uncontrolled rapid motions and mental deterioration leading to both physical and mental incapacitation and, eventually, death.

A trinucleotide repeat considerably beyond its normal length tends to be unstable and increases from one generation to the next. This expansion is probably attributable to the formation of a hairpin structure involving nucleotides of the repeat during lagging strand replication. Generally, as a result of expansion of the trinucleotide repeat from one generation to the next, HD and other trinucleotide repeat expansion disorders (TREDs) show increased severity and earlier onset in successive generations. This phenomenon is called genetic anticipation.

In Type 1 TREDs, the repeat is in the coding region and encodes a longer than normal polyglutamine tract. These long tracts may form neurotoxic aggregates and/or bind, and thereby effectively remove, essential neuronal proteins. As well, during synthesis they may deplete neurons of glutamine, which, in turn, causes a shortage of the neurotransmitter glutamate. In Type 2 TREDs, the abnormal expansion occurs in a noncoding region of the affected gene and may interfere with mRNA processing and/or translation.

Amyotrophic lateral sclerosis (ALS) is a disorder of the neurons of the CNS and PNS that control body movements. The condition starts with a loss of movement in a leg or arm and continues until the patient is fully immobilized. Familial ALS (FALS) represents only about 10% of all cases of ALS and is genetically heterogeneous, with at least five loci already identified. Adult onset ALS1, which has been studied extensively, entails

mutations in the *SOD1* gene at 21q22.1 that encodes Cu,Zn superoxide dismutase. The accumulation of reactive oxygen species, such as hydroxyl ion as a result of toxic gain-of-function *SOD1* gene mutations, likely contributes to the deterioration of motor neurons in some ALS1 patients. The basis of the neurological degeneration due to *SOD1* mutations with dismutase activity is unknown.

Charcot–Marie–Tooth disease (CMT), which occurs with a prevalence of 1 in 2500, is both clinically and genetically heterogeneous. Generally, CMT is not fatal. The neuropathology involves loss of PNS motor and sensory neurons that are connected to muscles and sensory receptors. There is a weakening of the muscles of the arms and legs and a decrease in responses to touch, pain, temperature, and vibration in the hands and feet. A large number of CMT genes have been identified, which in most cases cause demyelination, in some cases axonal loss, and in a few cases an intermediate phenotype.

Mutations of the prion protein *PRNP* gene in humans cause three phenotypically different fatal spongioform encephalopathies: Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), and fatal familial insomnia (FFI). In these disorders, an abnormal prion protein converts normal prion molecules into abnormal forms by a process that is not yet fully understood. Either the accumulation of prion protein with an abnormal conformation or the loss of normal prion protein causes neurodegeneration. Familial CJD accounts for about 15% of all cases of human spongioform encephalopathies. The clinical features of CJD are first recognized between the ages of 60 and 65 and include tremors. Patients later become stuporous, and death occurs 6 to 12 months from the inception of the disease. The cerebral cortex is the primary site of neuronal loss in CJD patients. Both GSS and FFI are very rare, and neurodegeneration occurs mainly in the cerebellum and thalamus, respectively.

Complex psychiatric disorders are probably the consequence of either a few different genes (oligogenic inheritance) or many different genes (polygenic inheritance) acting in concert with environmental factors to produce the phenotype. To date, some of the genes associated with schizophrenia have been identified.

References

Abalkhail, H. et al. 2003. A new familial amyotrophic lateral sclerosis locus on chromosome 16q12.1–16q12.2. *Am J Hum Genet* **73**:383–389.

Aguzzi, A. and C. Haass. 2003. Games played by rogue proteins in prion disorders and Alzheimer's disease. *Science* **302**: 814–818.

Bales, K. R. et al. 1999. Apolipoprotein E is essential for amyloid deposition in the *APP*(V717F) transgenic mouse model of Alzheimer's disease. *Proc Natl Acad Sci USA* **96**:15233–15238.

Berger, P., P. Young, and U. Suter. 2002. Molecular cell biology of Charcot-Marie-Tooth disease. *Neurogenetics* **4**:1–15.

Berkovic, S. F. et al. 2004. Benign familial neonatal-infantile seizures: characterization of a new sodium channelopathy. *Ann Neurol* **55**:550–557.

Bertram, L. and R. E. Tanzi. 2004. Alzheimer's disease: one disorder, too many genes? *Hum Mol Genet* **13**:R135–141.

Buxbaum, J. N. and C. E. Tagoe. 2000. The genetics of the amyloidoses. *Annu Rev Med* **51**:543–569.

Carola, R., J. P. Harley, and C. R. Noback. 1992. *Human Anatomy and Physiology*. 2nd ed. McGraw-Hill, New York.

Chen, Y. Z. et al. 2004. DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am J Hum Genet* **74**:1128–1135.

Chowdari, K. V. et al. 2002. Association and linkage analyses of *RGS4* polymorphisms in schizophrenia. *Hum Mol Genet* **11**:1373–1380.

Chumakov, I. et al. 2002. Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc Natl Acad Sci USA* **99**:13675–13680.

Cooper, E. C. and L. Y. Jan. 1999. Ion channel genes and human neurological disease: recent progress, prospects, and challenges. *Proc Natl Acad Sci USA* **96**:4759–4766.

Dickson, D. W. 1997. The pathogenesis of senile plaques. *J Neuropathol Exp Neurol* **56**:321–339.

Ebralidze, A. et al. 2004. RNA leaching of transcription factors disrupts transcription in myotonic dystrophy. *Science* **303**: 383–387.

Egan, M. F. et al. 2001. Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc Natl Acad Sci USA* **98**:6917–6922.

Ekelund, J. et al. 2001. Chromosome 1 loci in Finnish schizophrenia families. *Hum Mol Genet* **10**:1611–1617.

Fallin, M. D. et al. 2003. Genomewide linkage scan for schizophrenia susceptibility loci among Ashkenazi Jewish families shows evidence of linkage on chromosome 10q22. *Am J Hum Genet* **73**:601–611.

Farrer, L. A. et al. 1997. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA* **278**:1349–1356.

Finckh, U. 2003. The future of genetic association studies in Alzheimer disease. *J Neural Transm* **110**:253–266.

Friedhoff, P. et al. 2000. Structure of tau protein and assembly into paired helical filaments. *Biochim Biophys Acta* **1502**: 122–132.

Gambetti, P. et al. 2003. Sporadic and familial CJD: classification and characterisation. *Br Med Bull* **66**:213–239.

Gargus, J. J. 2003. Unraveling monogenic channelopathies and their implications for complex polygenic disease. *Am J Hum Genet* **72**:785–803.

Goedert, M. and M. G. Spillantini. 2000. Tau mutations in frontotemporal dementia FTDP-17 and their relevance for Alzheimer's disease. *Biochim Biophys Acta* **1502**:110–121.

Haass, C. 2004. Take five-BACE and the γ-secretase quartet conduct Alzheimer's amyloid β-peptide generation. *EMBO J* **23**:483–488.

Haass, C. and H. Steiner. 2002. Alzheimer disease γ-secretase: a complex story of GxGD-type presenilin proteases. *Trends Cell Biol* **12**:556–562.

Hand, C. K. and G. A. Rouleau. 2002. Familial amyotrophic lateral sclerosis. *Muscle Nerve* **25**:135–159.

Hardy, J. and D. J. Selkoe. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**:353–356.

Harris, D. A. 2003. Trafficking, turnover and membrane topology of PrP. *Br Med Bull* **66**:71–85.

Hashimoto, Y. et al. 2004. Amino- and carboxyl-terminal mutants of presenilin 1 cause neuronal cell death through distinct toxic mechanisms: Study of 27 different presenilin 1 mutants. *J Neurosci Res* **75**: 417–428.

Hegde, R. S. et al. 1998. A transmembrane form of the prion protein in neurodegenerative disease. *Science* **279**:827–834.

Hübner, C. A. and T. J. Jentsch. 2002. Ion channel diseases. *Hum Mol Genet* **11**:2435–2445.

Huntington's Disease Collaborative Research Group. 1993. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* **72**:971–983.

Huxley, C. et al. 1998. Correlation between varying levels of PMP22 expression and the degree of demyelination and reduction in nerve conduction velocity in transgenic mice. *Hum Mol Genet* **7**:449–458.

Jasinska, A. et al. 2003. Structures of trinucleotide repeats in human transcripts and their functional implications. *Nucleic Acids Res* **31**:5463–5468.

Jonsson, P. A. et al. 2004. Minute quantities of misfolded mutant superoxide dismutase-1 cause amyotrophic lateral sclerosis. *Brain* **127**:73–88.

Kullmann, D. M. 2002. The neuronal channelopathies. *Brain* **125**:1177–1195.

Lasmézas, C. I. 2003. Putative functions of PrP^{C}. *Br Med Bull* **66**:61–70.

Lehmann-Horn, F. and K. Jurkat-Rott. 1999. Voltage-gated ion channels and hereditary disease. *Physiol Rev* **79**:1317–1372.

Lewis, J. et al. 2001. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. *Science* **293**:1487–1491.

Michalik, A. and C. Van Broeckhoven. 2003. Pathogenesis of polyglutamine disorders: aggregation revisited. *Hum Mol Genet* **12**:173–186.

Millar, J. K. et al. 2000. Disruption of two novel genes by a translocation cosegregating with schizophrenia. *Hum Mol Genet* **9**:1415–1423.

Montagna, P. et al. 2003. Familial and sporadic fatal insomnia. *Lancet Neurol* **2**: 167–176.

Morris, J. A. et al. 2003. DISC1 (Disrupted-In-Schizophrenia 1) is a centrosome-associated protein that interacts with MAP1A, MIPT3, ATF4/5 and NUDEL: regulation and loss of interaction with mutation. *Hum Mol Genet* **12**:1591–1608.

Morris, J. C. 1996. Classification of dementia and Alzheimer's disease. *Acta Neurol Scand Suppl* **165**:41–50.

Murphy, R. M. 2002. Peptide aggregation in neurodegenerative disease. *Annu Rev Biomed Eng* **4**:155–174.

Murrell, J. R. et al. 1997. Familial multiple-system tauopathy with presenile dementia is localized to chromosome 17. *Am J Hum Genet* **61**:1131–1138.

O'Donovan, M. C., N. M. Williams, and M. J. Owen. 2003. Recent advances in the genetics of schizophrenia. *Hum Mol Genet* **12**:125–133.

Okado-Matsumoto, A. and I. Fridovich. 2002. Amyotrophic lateral sclerosis: a proposed mechanism. *Proc Natl Acad Sci USA* **99**:9010–9014.

Ozeki, Y. et al. 2003. Disrupted-in-Schizophrenia-1 (DISC-1): mutant truncation prevents binding to NudE-like (NUDEL) and inhibits neurite outgrowth. *Proc Natl Acad Sci USA* **100**:289–294.

Plomin, R. and P. McGuffin. 2003. Psychopathology in the postgenomic era. *Annu Rev Psychol* **54**:205–228.

Poorkaj, P. et al. 1998. Tau is a candidate gene for chromosome 17 frontotemporal dementia. *Ann Neurol* **43**:815–825.

Potter, S. Z. and J. S. Valentine. 2003. The perplexing role of copper-zinc superoxide dismutase in amyotrophic lateral sclerosis (Lou Gehrig's disease). *J Biol Inorg Chem* **8**:373–380.

Prusiner, S. B. and M. R. Scott. 1997. Genetics of prions. *Annu Rev Genet* **31**: 139–175.

Reichert, H. 1992. *Introduction to Neurobiology*. Oxford University Press, New York.

Riesner, D. 2003. Biochemistry and structure of PrP^{C} and PrP^{Sc}. *Br Med Bull* **66**: 21–33.

Ross, C. A. et al. 1997. Huntington disease and the related disorder, dentatorubral-pallidoluysian atrophy (DRPLA). *Medicine* **76**:305–338.

Rubinsztein, D. C. and J. Carmichael. 2003. Huntington's disease: molecular basis of neurodegeneration. *Expert Rev Mol Med* **5**:1–21.

Ruddy, D. M. et al. 2003. Two families with familial amyotrophic lateral sclerosis are linked to a novel locus on chromosome 16q. *Am J Hum Genet* **73**:390–396.

Rudnicki, D. D. and R. L. Margolis. 2003. Repeat expansion and autosomal dominant neurodegenerative disorders: consensus and controversy. *Expert Rev Mol Med* **5**: 1–24.

Sapp, P. C. et al. 2003. Identification of two novel loci for dominantly inherited familial amyotrophic lateral sclerosis. *Am J Hum Genet* **73**:397–403.

Scheel, H., S. Tomiuk, and K. Hofmann. 2003. Elucidation of ataxin-3 and ataxin-7 function by integrative bioinformatics. *Hum Mol Genet* **12**:2845–2852.

Schweitzer, J. K. and D. M. Livingston. 1998. Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation. *Hum Mol Genet* **7**:69–74.

Selkoe, D. J. 2002. Alzheimer's disease is a synaptic failure. *Science* **298**:789–791.

Selkoe, D. J. 2002. Deciphering the genesis and fate of amyloid beta-protein yields novel therapies for Alzheimer disease. *J Clin Invest* **110**:1375–1381.

Selkoe, D. J. and M. B. Podlisny. 2002. Deciphering the genetic basis of Alzheimer's disease. *Annu Rev Genomics Hum Genet* **3**:67–99.

Selkoe, D. J. and D. Schenk. 2003. Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics. *Annu Rev Pharmacol Toxicol* **43**:545–584.

Serpell, L. C. 2000. Alzheimer's amyloid fibrils: structure and assembly. *Biochim Biophys Acta* **1502**:16–30.

Sharp, A. H. and C. A. Ross. 1996. Neurobiology of Huntington's disease. *Neurobiol Dis* **3**:3–15.

Shifman, S. et al. 2002. A highly significant association between a COMT haplotype and schizophrenia. *Am J Hum Genet* **71**:1296–1302.

Smith, M. A. 1998. Alzheimer disease. *Int Rev Neurobiol* **42**:1–54.

Spillantini, M. G. et al. 1998. Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc Natl Acad Sci USA* **95**:7737–7741.

Stathopulos, P. B. et al. 2003. Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis show enhanced formation of aggregates *in vitro*. *Proc Natl Acad Sci USA* **100**:7021–7026.

Stefansson, H. et al. 2003. Association of neuregulin 1 with schizophrenia confirmed in a Scottish population. *Am J Hum Genet* **72**:83–87.

Stefansson, H. et al. 2002. *Neuregulin 1* and susceptibility to schizophrenia. *Am J Hum Genet* **71**:877–892.

Straub, R. E. et al. 2002. Genetic variation in the 6p22.3 gene *DTNBP1*, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. *Am J Hum Genet* **71**:337–348.

Suh, Y. H. and F. Checler. 2002. Amyloid precursor protein, presenilins, and α-synuclein: molecular pathogenesis and pharmacological applications in Alzheimer's disease. *Pharmacol Rev* **54**:469–525.

Tandon, A. and P. Fraser. 2002. The presenilins. *Genome Biol* **3**:1–9.

Thompson, L. K. 2003. Unraveling the secrets of Alzheimer's β-amyloid fibrils. *Proc Natl Acad Sci USA* **100**:383–385.

Thompson, R. F. 1985. *The Brain: An Introduction to Neuroscience*. W. H. Freeman and Company, New York.

Tsai, C. C. et al. 2004. Ataxin 1, a SCA1 neurodegenerative disorder protein, is functionally linked to the silencing mediator of retinoid and thyroid hormone receptors. *Proc Natl Acad Sci USA* **101**:4047–4052.

Wadsworth, J. D. et al. 2003. Molecular and clinical classification of human prion disease. *Br Med Bull* **66**:241–254.

Weissmann, C. and E. Flechsig. 2003. PrP knock-out and PrP transgenic mice in prion research. *Br Med Bull* **66**:43–60.

Wexler, A. 1996. *Mapping Fate: a memoir of Family, Risk and Genetic Research*. University of California Press, Berkeley, CA.

Wolfe, M. S. 2002. APP, Notch, and presenilin: molecular pieces in the puzzle of Alzheimer's disease. *Int Immunopharmacol* **2**:1919–1929.

Young, P. and U. Suter. 2003. The causes of Charcot-Marie-Tooth disease. *Cell Mol Life Sci* **60**:2547–2560.

Züchner, S. et al. 2004. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. *Nat Genet* **36**:449–451.

review questions

1. What is a neuron, a dendrite, an axon, a neurotransmitter, and a synapse?
2. Describe how an action potential is initiated and propagated down an axon.
3. What are the chief functions of the cerebrum, cerebellum, and caudate nucleus?
4. What are channelopathies?
5. Discuss the genetic aspects of a neuronal channelopathy.
6. What is the amyloid cascade theory?
7. Describe the genetic basis of early-onset Alzheimer disease.
8. Discuss the concept of a genetic risk factor using specific information from studies of Alzheimer disease.
9. Describe various ways that neurodegeneration might occur with a Type 1 TRED.
10. What is genetic anticipation?
11. Discuss the effects of Type 2 TREDS on cellular functions.
12. What are toxic gain-of-function and loss-of-function mutations?
13. Discuss the genetic basis of ALS. Check OMIM and other sources to determine whether any additional ALS genes been identified.
14. Account for the genetic heterogeneity of CMT.
15. What is a prion?
16. Describe the genetic and molecular aspects of human prion diseases.
17. What is the protein-only hypothesis?
18. Write a brief review on "The Molecular Genetics of Schizophrenia." Check OMIM and other sources to determine whether any additional schizophrenia genes have been identified.
19. What is the purpose(s) of an animal model for the study of a human genetic disease?
20. Describe the PNS protocol for developing transgenic mice.

chapter 15

Molecular Genetics of the Eye

Sight is almost infinitely delightful and immensely useful, therefore do I enjoy explaining it to the full and with particular care, because without it one cannot learn anything marvellous . . . sight shows us the variety of all things, and opens the way to a knowledge of everything, as experience teaches.

ROGER BACON (1214?–1294?)

The eye, which is called the window of the soul, is the chief means whereby the understanding may most fully and abundantly appreciate the infinite works of Nature.

LEONARDO DA VINCI (1452–1510)

THE ABILITY TO SEE, for the most part, transcends all of our other senses. The visual system conveys details about size, brightness, texture, distance, motion, color, and spatial relationships of objects in the environment. There is a considerable biological investment in human visual perception. The photoreceptors of the retina of the eye comprise about 70% of the total complement of human sensory receptors, and about 30% of all nerve fibers going to the central nervous system are contained in the optic nerves. In general, vision consists of a series of successive events. Initially, the light entering the eye is refracted, and the image is focused on the retina. The photoreceptor cells of the retina absorb light within specific ranges of wavelengths. The absorbed light initiates a series of protein activations, which lead to the formation of neuronal signals. These impulses are transmitted from the ganglia of the retina through the fibers of the optic nerves that travel to the visual cortex of the brain, where the signals are processed and a conscious interpretation of the retinal image is created.

Although injury, infections, and nonhereditary diseases account for much of the blindness that causes considerable human suffering, a large number of inherited conditions, some of which are common, also disrupt ocular function. Genetic conditions of the eye tend to fall into two categories. Some genetic disorders affect different tissue and organ systems, including the eye, at the same time. For example, mutations of a fibrillin gene (Marfan syndrome, *FBN1*, 15q21.1) are responsible for an assortment of heart, vascular, ocular, skeletal, and connective tissue defects. These multisystem diseases are called

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systemic (syndromic). On the other hand, there are a number of genetically determined, eye-specific (nonsystemic, nonsyndromic) diseases that modify a single ocular structure and/or adjacent components within the eye. A significant amount of information has been gathered about gene mutations that affect only the structures and functioning of the eye.

Human Visual System

Structure of the Eye

The adult human eyeball is a nearly spherical structure, with a diameter of about one inch (25 mm). The globe has three layers in the posterior portion, and, of these, the outer and middle layers form specialized components of the anterior (frontal) structures of the eye (Figure 15.1). The outer supporting layer (fibrous tunic) has two contiguous components. An opaque, white, tough fibrous layer, called the sclera, covers the posterior five-sixths of the eyeball. The cornea, a transparent convex band, forms the frontal portion of the outer layer. The middle layer (vascular layer) of the eye is also differentiated into contiguous sections. The choroid is a thin membrane containing blood vessels and occupying about two-thirds of the posterior portion of the eye. The middle layer expands into the ciliary body, which is a muscle-containing ring, near the junction of the sclera and cornea of the outer layer. Protrusions from the ciliary body attach to suspensory ligaments, which in turn are bound to the margin of a thin, clear capsule that encloses the biconvex lens. During the development of the eye, the lens, which is flexible and transparent, is formed independently of the three principal ocular layers. The iris extends from the ciliary body over the front of the lens, leaving a circular opening (pupil). The extent of the pupillary aperture is controlled by circular and radial smooth muscles of the iris. The innermost layer of the eye is the retina (nervous tunic), which lies in the posterior portion of the eye. The retina is a layered network of specialized nerve cells (Figure 15.2). A membrane (Bruch's membrane) lies between

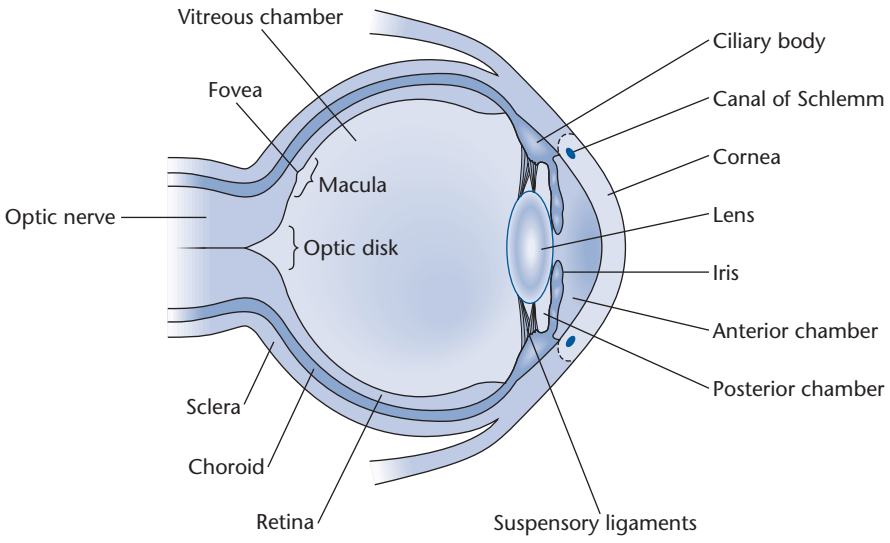


Figure 15.1 Structure of the human eye.

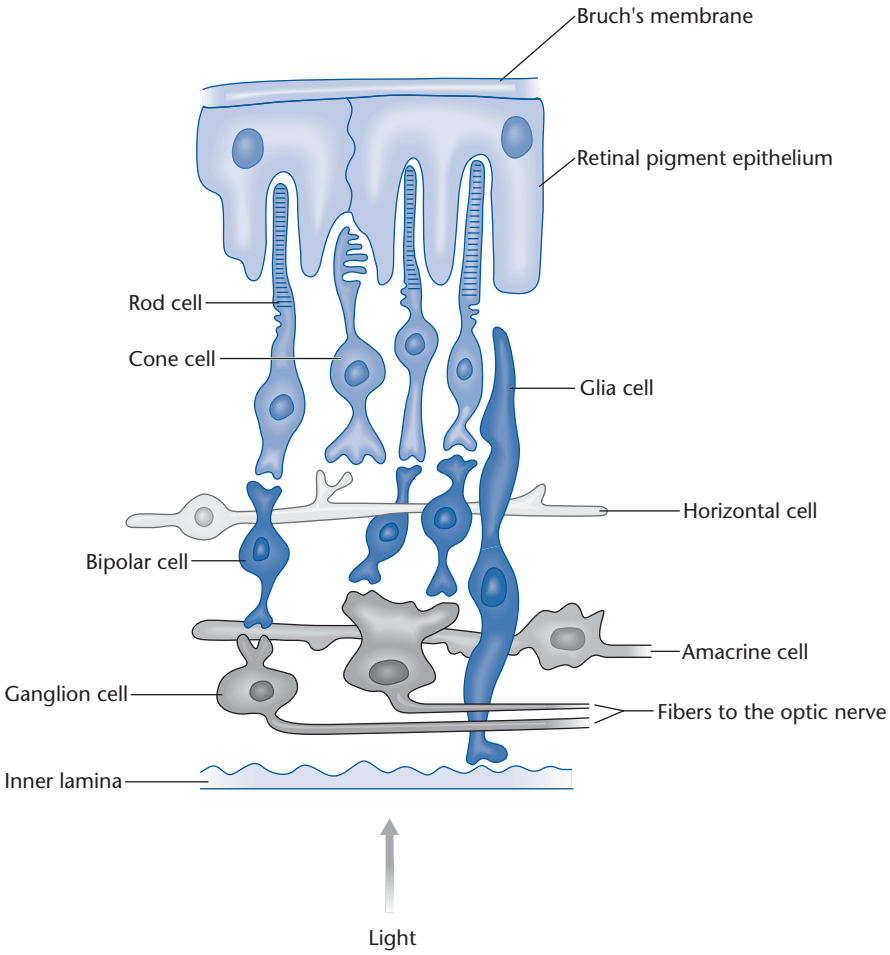


Figure 15.2 Schematic representation of the retina. The structural relationships among the retinal pigment epithelial cells, rods, cones, and neuronal cells of the retina are depicted. The arrow marks the direction of incoming light.

the choroid and a thin layer of pigmented cells (retina pigment epithelium, RPE). The RPE prevents back scattering of the light that passes through the layers of the retina. The ends of the two types of photoreceptors called rods and cones are surrounded by elongated extensions of RPE cells. A rod (rod photoreceptor cell, rod cell) consists of an outer segment with 700 to 1000 stacked free plasma membrane disks, each surrounded by cytoplasm, and an inner segment containing the cell nucleus and terminating with a synaptic process (Figure 15.3). A cone (cone photoreceptor cell, cone cell) has disks in its cone-shaped outer segment that are continuous with the plasma membrane (Figure 15.3). The inner segment of each cone ends with a synaptic process.

The interconnections of the neural cells of the retina are complex. Briefly, rods and cones synapse with bipolar cells, bipolar cells with ganglion cells, horizontal cells with rods, cones, and bipolar cells, and amacrine cells with bipolar and ganglion cells. After illumination, many rod cells pass signals to bipolar cells, which, in turn, stimulate a single ganglion. Signals are often transmitted from one cone cell through a bipolar cell to one ganglion cell. The difference in the numbers of stimulated rods and cones that activate a ganglion cell accounts in part for the general sensitivity of rods to light and the visual acuity of cone cells. The axons of all of the retinal ganglion neurons extend along the

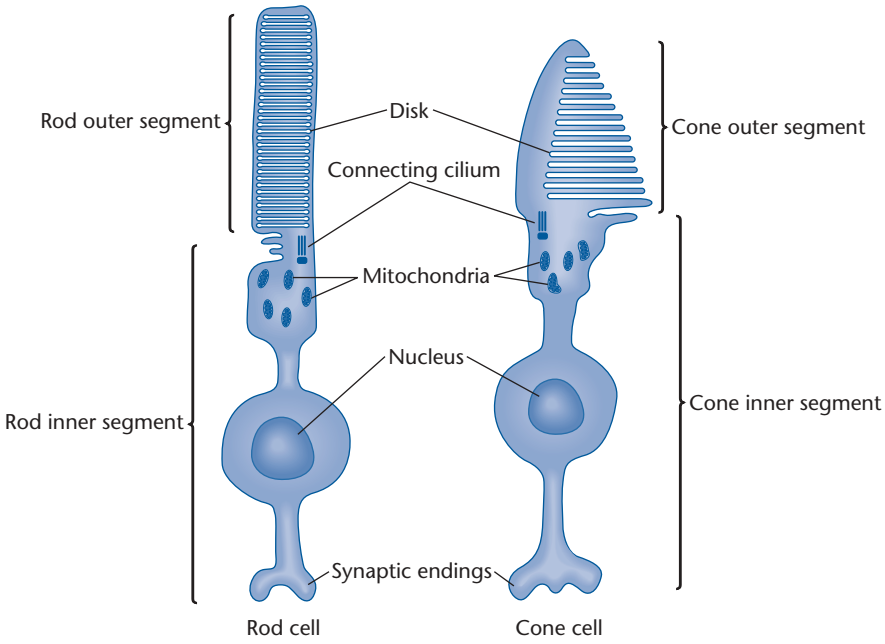


Figure 15.3 Schematic representations of a rod and a cone cell. Not drawn to scale.

front of the retina and exit at the back of the eyeball through a small opening, the optic disk, to form the optic nerve. There are approximately 120 million rod photoreceptor cells and 6 million cone cells in the human eye. These cells are not evenly distributed throughout the retina. Cones, which are responsible for daylight (color) vision and visual acuity, are concentrated in an area at the back of the eye called the macula (5 mm in diameter), which lies directly behind the lens. Within the macular region (macula lutea) is a depression (fovea) that is especially rich in cone cells. Color vision and sharpness of the visual image are optimal in the fovea of the macula. The remainder of the retina, which contains rods and some cones, is called the peripheral retina. The rods are extremely sensitive photosensors responsible for dim-light vision.

The interior of the eye consists of three chambers (Figure 15.1). The anterior chamber lies between the cornea and the frontal surfaces of the lens and iris. The posterior chamber is the small space between the iris and the lens. The watery fluid (aqueous humor) that fills these two chambers is produced continuously and is drained through the canal of Schlemm (scleral venous sinus), which lies in the outer layer of the eye near the junction of the sclera and cornea. The largest chamber of the eye, the vitreous chamber (vitreous body), occupies the volume bounded by the back of the lens, a portion of the ciliary body, and the retina. The material (vitreous humor) in the vitreous chamber has a jellylike (gelatinous) consistency and is a transparent meshwork of collagen fibers and mucopolysaccharides.

Phototransduction: Conversion of Radiant Energy into Nerve Impulses

Light waves enter the eye and are refracted onto the lens by the cornea. In turn, the shape of the lens, which can be adjusted by the suspensory ligaments, is used to focus light on the retina. The amount of light passing through the

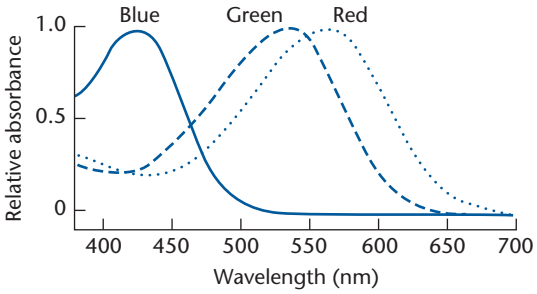


Figure 15.4 Absorption spectra of the human cone visual protein pigments.

lens is controlled by the pupil, which constricts in bright light and dilates in dim light. Light (radiant energy) in the visual spectrum ranges in wavelength from about 370 to 760 nm; that is, from violet to red, respectively. The photoreceptors of the retina, which surprisingly are not located at the front of the retina and do not have outer segments oriented toward the incoming light, each contain a visual pigment, embedded in the membrane of the disks, capable of absorbing light over a limited wavelength range. The visual pigment of the rods is the protein rhodopsin, which is localized in the upper and lower membranes of the disks of the outer segments. Rhodopsin maximally absorbs light with a wavelength of about 500 nm. There are three kinds of cones in the human retina, and each contains a discrete visual protein pigment with a distinctive spectral sensitivity (Figure 15.4). Blue cones contain a visual protein pigment (blue pigment, blue opsin, blue-sensitive pigment) that absorbs light optimally at about 426 nm. The visual protein pigments of the green and red cones (green opsin, red opsin) absorb light at about 530 and 555 nm, respectively.

The biochemistry of the conversion of radiant energy of light into a nerve impulse (phototransduction) has been studied extensively in rod cells. It consists of a cascade process (Figure 15.5) that includes successive activations of enzymatic proteins and ultimately prevents cations from entering the outer segment of the photoreceptor cell, which, as a result, generates an electrical nerve impulse. Rhodopsin, which plays the pivotal role in the process, is a transmembrane protein with seven domains embedded in the membrane of the disks of the rod outer segment. In the dark, 11-*cis*-retinal is bound to lysine 296 (K296) of the rhodopsin molecule. The energy from light at about 500 nm activates rhodopsin by photoisomerization of the 11-*cis*-retinal to all-*trans*-retinal (Figure 15.6). This conversion causes a conformational change in rhodopsin that enables an activated rhodopsin intermediate (metarhodopsin II) to activate a guanine nucleotide-binding protein (transducin, T). In its inactive form, transducin has three protein subunits (α, β, γ). Guanosine diphosphate (GDP) is bound to the α chain. The activated rhodopsin replaces the GDP moiety with GTP, causing the release of the β and γ chains, leaving an active α chain-GTP complex (Tα-GTP). One activated rhodopsin molecule can activate thousands of transducin molecules, thereby amplifying the effect of a single light-activated rhodopsin molecule. Next, the Tα-GTP complex, which also lies in the plane of the disk membrane, activates a cytoplasmic cyclic guanosine monophosphate phosphodiesterase (cGMP-PDE). In its inactive form, cGMP-PDE consists of two catalytic (α, β) and two regulatory (γ) subunits. The successive removal of the two γ chains by the activated transducin acti-

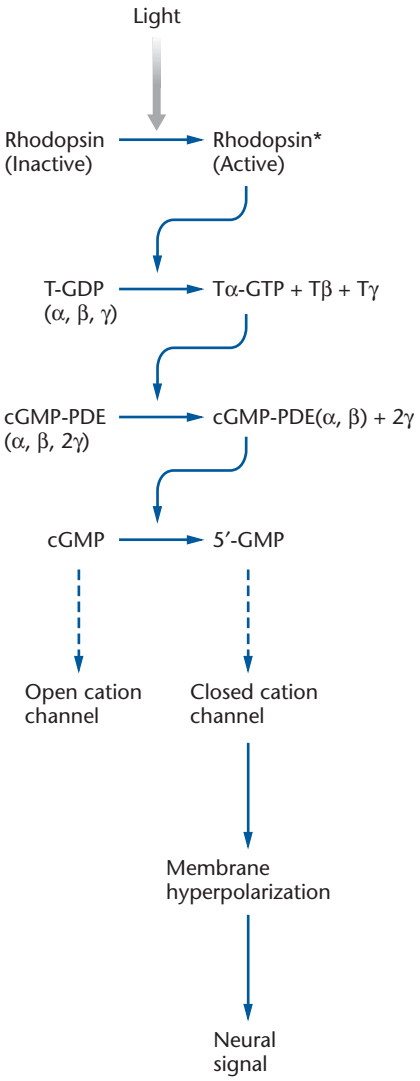


Figure 15.5 Schematic representation of the phototransduction cascade in the rod outer segment. Rhodopsin is activated (Rhodopsin*) by light. Inactive transducin (T-GDP), which consists of three subunits (α, β, γ), is converted by activated rhodopsin to its active form (Tα-GTP), with the concomitant release of Tβ and Tγ subunits. Tα-GTP converts inactive cyclic guanosine monophosphate phosphodiesterase (cGMP-PDE), which has four subunits (α, β, and 2γ), to its active form [cGMP-PDE(α, β)] by releasing the two γ subunits (2γ). cGMP-PDE(α, β) hydrolyzes cGMP to 5′-guanosine monophosphate (5′-GMP). Hydrolysis of cation channel protein-bound cGMP to 5′-GMP closes the channel, which causes membrane hyperpolarization and, subsequently, the formation of an electric (neural) signal.

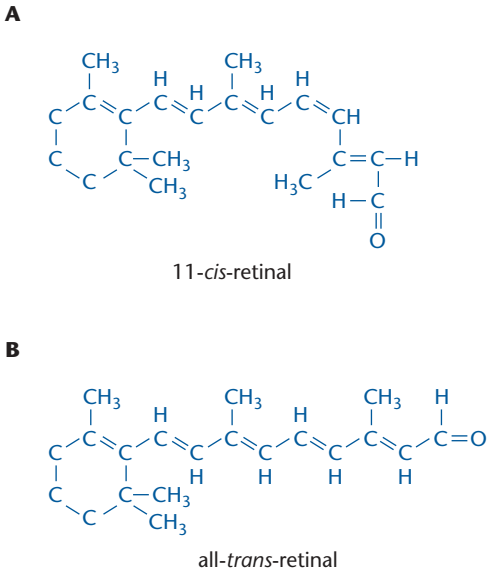


Figure 15.6 Molecular structures of 11-*cis*-retinal (A) and all-*trans*-retinal (B).

vates cGMP-PDE. The activated form of cGMP-PDE rapidly and efficiently converts cGMP to 5′-GMP. In the dark, cGMP binds to a cation channel protein in the plasma membrane of the rod outer segment. The presence of cGMP keeps the channel open and allows calcium and sodium ions (cations) to enter the rod outer segment. In the light, as a consequence of the cascade of protein activations, the cGMP is hydrolyzed to 5′-GMP, removing it from the cation channel protein and closing the channel. The absence of cation flow into the rod outer segment as the result of a closed channel induces hyperpolarization of the plasma membrane, creating a nerve signal. This signal is transmitted from the rod photoreceptor cell through the bipolar neurons and ganglion cells to the optic nerves and, eventually, to specific areas of the brain, where, with concurrent signals, the visual information is formulated.

The phototransduction mechanism in cones, each with a color-sensitive opsin, is presumably similar to that in rods, although cones are about 100 times less sensitive to light than rods. In all photoreceptor cells, the return to the dark state entails the deactivation of the light-activated proteins, synthesis of 11-*cis*-retinal, and, among other processes, the enzymatic regeneration of cGMP. The turning on and turning off of light-induced activities in the outer segments of photoreceptors are sensitive, rapid, and exquisitely coordinated processes.

Studying the Molecular Basis of Inherited Disorders of the Human Eye

Human vision involves a large number of different genes that contribute both to the formation and maintenance of the structures of the eye and to the phototransduction process. It is not surprising that many different ocular genetic disorders have been documented. The eye is a transparent organ that is readily accessible to diverse noninvasive examinations by ophthalmologists and

optometrists using a wide range of instruments. Consequently, there is considerable descriptive knowledge about pathological eye conditions. In fact, there may be more than 600 recorded inherited eye diseases, including both multisystem and nonsyndromic conditions. In all likelihood, many of the clinical descriptions for eye-specific disorders encompass phenotypic variations caused by different mutations of single genes (clinical heterogeneity, allelic heterogeneity). Linkage studies and the identification of mutant genes in individuals with inherited eye disorders help distinguish between clinical heterogeneity and those disorders that arise from mutations of different genes (genetic heterogeneity). The molecular genetics of a small but growing number of inherited eye diseases has been examined in detail.

These studies have been aided by a number of features that are distinctive to disorders of the eye. Most ocular conditions can be readily determined. Individuals with eye-specific disorders are commonly mentally competent, not disfigured, fully fertile, socially productive, and long-lived, and they sometimes produce large families that can be used for linkage studies. In addition, many of the genes that encode ocular proteins have been isolated, characterized, and placed at chromosome sites by in situ hybridization and other techniques. Thus, once a gene for a particular eye disorder has been mapped to a chromosome site, the choice of a potential candidate gene is often straightforward, because of the large number of known mapped ocular genes. Finally, mutant genes that affect the eyes in mice have been thoroughly studied and used as DNA probes for isolating evolutionarily conserved DNA sequences serving similar functions in humans. Conversely, an isolated human DNA sequence suspected of being the source of an ocular disorder can be tested to determine whether it is a version (homolog) of a mouse gene known to be associated with a specific inherited eye defect.

Genetic Disorders of the Human Eye

Corneal Dystrophies

The cornea has three major layers, which, from front to back, are the epithelium, the stroma, and the endothelium. The stroma makes up about 90% of the thickness of the cornea and contains a series of sublayers (lamellae) that each contain bundles of collagen fibrils embedded in a group of proteoglycans. The lamellae are organized alternatively, more or less at right angles to each other.

Corneal dystrophies are often classified according to the formation, pattern, location, and extent of accumulation of deposits. A number of autosomal disorders of the stroma have been identified with these criteria. In one category, the stromal deposits resemble grains of sugar or breadcrumbs and form white opaque spots and are designated granular corneal dystrophies (GCDs). Three GCD subtypes (types I to III) have been characterized. In the second category, which is designated as lattice corneal dystrophy (LCD), accumulations of dense spots with overlapping radiating filaments in the portion of the stroma beneath the epithelium are commonly observed. There are at least four LCD subtypes. The onset with GCD type I (Groenouw type 1 corneal dystrophy) and GCD type II (Avellino corneal dystrophy) is usually in the second decade

after birth, and with LCD type I and GCD type III (Reis–Bücklers corneal dystrophy) the opacities occur earlier in life, recur more often, and are more painful. With these corneal dystrophies, there is a significant loss of vision, often necessitating a corneal transplant to restore sight.

Linkage analysis of families with a number of these granular and lattice corneal dystrophies mapped the disease-causing gene in each case to chromosome 5q31. Positional cloning and mutation analysis established that a single gene, originally designated as *BIGH3* and now *TGFBI* (transforming growth factor, β-induced protein gene), was responsible for these inherited corneal disorders. The *TGFBI* gene encodes keratoepithelin, which is synthesized exclusively in corneal cells. Its cellular function is not known, although it may be involved in binding corneal stromal cells to one another and/or linking stromal cells to the extracellular matrix.

Mutational analyses of kindreds with four corneal dystrophies revealed an intriguing relationship between a particular phenotype and the mutated site in the *TGFBI* gene. Among the families studied, GCD type I is usually associated with a keratoepithelin R555W mutation, GCD type III with R555Q, LCD type I with R124C, and GCD type II with R124H. Additional *TGFBI* gene mutations have been observed for these corneal dystrophies; as well, mutations at other sites are responsible for different corneal disorders. For example, P501T occurs frequently with LCD type IIIA, in which the defects are not evident until the patients are more than 70 years old. Mutations in *TGFBI* likely cause conformational changes and/or abnormal proteolytic cleavage of keratoepithelin, which, in turn, facilitate aggregate (amyloid) formation. The rates of accumulation and patterns of deposition of opacities depend on specific mutations. For example, four mutations at site 124 of keratoepithelin, namely, R124H, R124C, R124L, and R124S, bring about different clinical corneal dystrophies.

There are a number of other inherited corneal dystrophies. For example, mutations of the carbohydrate sulfotransferase gene (*CHST6*) at 16q23.1 cause macular corneal dystrophy. The characteristics of this autosomal recessive condition are clouding of the stroma along with irregularly shaped stromal spots. *N*-acetyl-6-*O*-sulfotransferase, encoded by the *CHST6* gene, adds sulfur to the proteoglycans that are responsible for corneal transparency. Thus diminished sulfation of proteoglycans and the accumulation of nonsulfated glycosaminoglycans between the stromal lamellae probably lead to corneal opacity. Another corneal disorder is gelatinous droplike corneal dystrophy, a very rare autosomal recessive condition featuring deposition of amyloid material beneath the epithelial layer during early childhood. Over time, these initial agglomerations develop into large yellow-gray masses that obscure vision. Homozygosity mapping placed this gene on chromosome 1p, and further studies identified mutations in the *TACSTD2* gene (tumor-associated calcium signal transducer 2; formerly *M1S1*) at 1p32.1. The *TACSTD2* gene product is a transmembrane protein, and mutations may lead to increased epithelial permeability. Meesmann epithelial corneal dystrophy (MECD) is an autosomal dominant trait that is characterized by the early onset of many small vesicles in the corneal epithelium. Linkage studies with different families localized MECD sites to 12q and 17q, which were later refined to 12q13.13 and 17q12, respectively.

The former locus corresponds to the keratin 3 (*KRT3*) gene and the latter to the keratin 12 (*KRT12*) gene. Both KRT3 and KRT12 proteins contribute to the intermediate filament cytoskeleton of corneal epithelium. Mutation studies confirmed that affected individuals in MECD kindreds have mutations in one or the other of these two *KRT* genes. KRT12 and KRT3 combine to form a heterodimer that, in turn, becomes part of a meshwork that maintains the integrity of the corneal epithelium. Thus a mutation of either the *KRT12* or the *KRT3* gene would disrupt intermediate filament function and produce the same phenotype.

Glaucoma

Glaucoma, cataracts (lens opacity), and age-related macular degeneration (maculopathy) are the three leading causes of blindness in North America. Glaucoma, generally a painless disease, is a group of disorders featuring erosion of the optic disk (the exit site for the retinal ganglion axons at the back of the eye) and a progressive loss of visual sensitivity as the major clinical consequences. About 66×10^6 people worldwide have glaucoma. Of these, 90% are sporadic cases and the remainder are bilateral and, presumably, familial. Sporadic glaucoma is a progressive disease, with the first clinical signs appearing when patients are older than 50 years. Surgery, laser therapy, and drug treatment often are used to curtail the impact of the disorder and prevent blindness.

A common, but not necessary, feature of glaucoma is the elevation of pressure within the eyeball (intraocular pressure, IOP). Virtually nothing is known about the kinds of molecular changes that cause increased IOP, although there are a large number of conceivable defects that could account for this kind of change. Among other possibilities, IOP could be increased by a modification of the composition of the aqueous humor, structural abnormality of the aqueous humor drainage system (trabecular meshwork) that lies adjacent to the canal of Schlemm, blockage of the canal of Schlemm, loss of control of the intraocular fluid balance, or excessive discharge of aqueous humor from the ciliary body.

Clinically, the glaucomas are divided routinely into two types, designated as closed-angle (angle-closure) and open-angle glaucoma (Figure 15.7). In the former category, the angle between the iris and cornea (iridocorneal angle), near the canal of Schlemm, is not as wide as in persons without glaucoma. The closing of the iridocorneal angle may be the result of an abnormal extension of the iris or of other tissue filling the apex of the anterior chamber. It is assumed that, in cases of closed-angle glaucoma, IOP is elevated because drainage of the aqueous humor through the spongy trabecular meshwork to the canal of Schlemm is physically blocked. Open-angle glaucoma is more enigmatic than closed-angle glaucoma and accounts for about two-thirds of all glaucomas worldwide. With open-angle glaucoma, the iridocorneal angle is normal, with no obvious anatomic obstacles to the outflow of the aqueous humor through the trabecular meshwork and no sign of blockage of the canal of Schlemm.

In addition to the status of the iridocorneal angle, other features are often used to further classify glaucomas. First, it is important to distinguish between

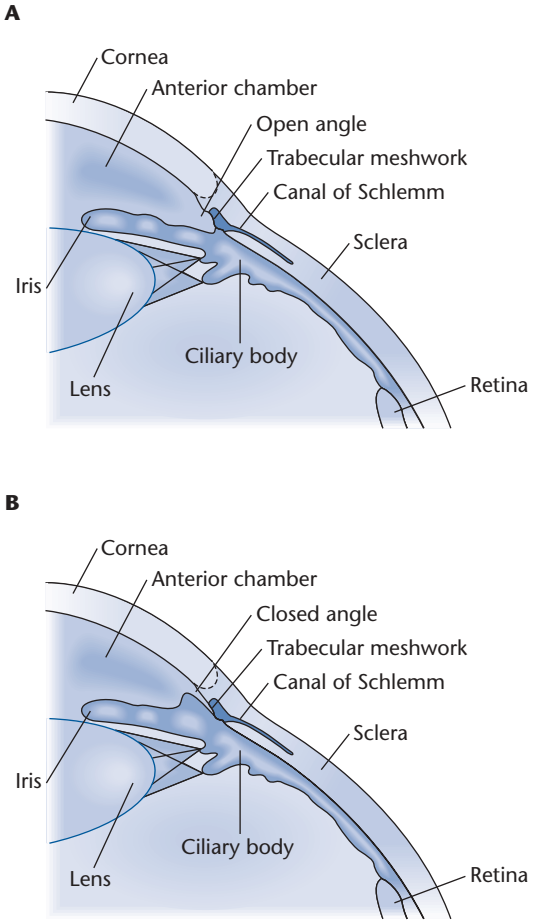


Figure 15.7 Section through an anterior segment of the eye showing the open angle (A) and closed angle (angle-closure) (B) of the anterior chamber.

glaucomas that are the exclusive defect (i.e., primary) from those that are part of a multisystem disorder (i.e., secondary). Second, age of onset has been used to differentiate among open-angle glaucomas. Specifically, pediatric glaucoma (primary congenital glaucoma, PCG) occurs from birth to 3 years of age; juvenile glaucoma (juvenile primary open-angle glaucoma, JPOAG) occurs from 3 to 30 years of age; and adult glaucoma (adult primary open-angle glaucoma, APOAG) occurs at 40 years or older. Finally, there is primary acute closed-angle glaucoma (PACG). Most of the molecular genetic studies have focused on juvenile and adult POAG and PCG.

At least 10 loci for POAG have been discovered with linkage studies and genomic scans. Of these, five genes with glaucoma-causing mutations have been characterized (Table 15.1). The *MYOC* gene is expressed in cells of the trabecular meshwork and other ocular tissues. About 5% of all POAG patients and about 15% with JOAG have a *MYOC* gene mutation. Neither the mode of action of myocilin nor the mechanism of pathogenesis has been elucidated. On the one hand, abnormal myocilin likely alters the flow of fluid between cells of the trabecular meshwork and the aqueous humor, which, in turn, increases the IOP and over time initiates degeneration of the optic nerve. In this case, glaucoma is the consequence of a loss of myocilin function. On the

Table 15.1 Glaucoma-causing genes.

Disorder	Gene	Site	Protein
Primary open-angle glaucoma with increased intraocular pressure	*MYOC*	1q24.3	Myocilin
Primary open-angle glaucoma with normal intraocular pressure	*OPTN*	10p13	Optineurin
Primary congenital glaucoma	*CYP1B1*	2p21	Cytochrome P4501B1; monooxygenase
Axenfeld–Regier syndrome with glaucoma	*PITX2*	4q25	Bicoid-like homeobox transcription factor
Axenfeld–Regier syndrome with glaucoma	*FOXC1*	6p25.3	Forkhead transcription factor

other hand, in vitro studies with trabecular meshwork cells showed that mutant myocilin misfolds and forms aggregates in the endoplasmic reticulum that cause cell death. The latter data suggest that the pathogenic mechanism results from gain-of-function *MYOC* gene mutations rather than defective myocilin activity.

The *OPTN* gene at 10p13 that encodes optineurin is mutated in individuals with adult-onset POAG and normal IOP. The cellular function of optineurin, a Golgi apparatus component in trabecular meshwork cells, is not known.

The *CYP1B1* gene, which is expressed in cells of the trabecular meshwork, ciliary body, retina, iris, and many other tissues and specifies the protein cytochrome P4501B1 (CYP1B1; cytochrome P450, family 1, subfamily B, polypeptide 1), is commonly mutated in individuals with PCG. There are more than 300 different members of the P450 gene family. Generally, P450 proteins are single-chain enzymes (monooxygenases) that insert an atom of atmospheric oxygen into a substrate to produce hydroxyl, carboxyl, or other types of oxygen-based functional groups. The incidence of PCG ranges from 1 in 1250 in the Roma population of Slovakia to 1 in 22,000 in western Europe. The mode of action of CYP1B1 in ocular cells is not known, and why its loss causes PCG is a mystery. Possibly, mutant CYP1B1 is incapable of metabolizing a specific target molecule in trabecular meshwork cells.

Both the *PITX2* and *FOXC1* genes encode transcription factors that are active during the development of the anterior portion of the eye, including the cornea, iris, lens, and drainage structures. Mutations of these genes cause, to varying degrees, defects of these components, with glaucoma as part of the phenotype in 50% to 75% of the cases. This combination of abnormalities along with teeth and umbilicus malformations is referred to as Axenfeld–Rieger syndrome. Little is known about the relationship between either *PITX2* or *FOXC1* gene mutations and glaucoma.

A familial form of angle-closure glaucoma (closed-angle glaucoma) that is secondary and associated with a genetically heterogeneous disorder is called nanophthalmos (*NNO1*, 11p13). This autosomal dominant condition features very small eyes with a short distance from the front to the back of the eye (axial length), which produces extreme farsightedness because the lens focuses the incoming light beyond the retina. The basis for the obstruction of the irido-

corneal angle in this case is not known, but it may result from the reduced size of the eye compressing cellular material into the apex of the anterior chamber and blocking the outflow of aqueous humor.

Aniridia

The absence of part or all of an iris (aniridia) is usually bilateral and occurs only in about 1 in 80,000 live births. In two-thirds of the cases of aniridia, one of the parents is affected, suggesting that the disorder is inherited as an autosomal dominant trait. The aniridia phenotype is extremely heterogeneous and usually includes a number of other ocular abnormalities. For example, alteration of the iris may be accompanied by impaired visual acuity, uncontrolled rolling or jerking of the eyes (nystagmus), underdevelopment of both the fovea and optic nerve, opacity of the lens (cataract), early-onset glaucoma, granulation of the cornea, crossed eyes, retinal abnormalities, and a permanent downward shift of the lens.

In many instances, aniridia occurs with childhood kidney tumors (Wilms tumor), abnormalities of both the genitalia and urinary tract, and mental retardation. This constellation of disorders has been called the WAGR syndrome. Cytogenetic studies revealed that many children with this combination of abnormalities have a deletion of chromosome 11p13. Consequently, researchers surmised that a gene that caused aniridia lay within this region. This hypothesis was supported when translocations and, in one case, a paracentric inversion involving 11p13 were found in individuals who had only aniridia and none of the other features of the WAGR syndrome.

The cloning of the 11p13 translocation breakpoint from one individual with aniridia provided a DNA sequence that was used to initiate a search for the aniridia gene. Eventually, with various strategies, a cDNA sequence was isolated and characterized. On the basis of several criteria, including expression, chromosome location, and protein sequence, this cDNA sequence was considered to be an excellent candidate for the human aniridia gene. Moreover, studies with the mouse *Pax6* gene, responsible for an eyeless phenotype, established that this mouse gene and the putative human aniridia gene, designated *PAX6*, were homologs. To date, no other gene has been found that causes aniridia in humans.

The human PAX6 protein, which is identical to the mouse Pax-6 protein, contains two DNA-binding regions. One of these regions is called a paired domain and the other a homeodomain. A third region at the carboxyl terminus is rich in proline, serine, and threonine (PST-rich region) and probably acts as a transcriptional activating domain (Figure 15.8). The terms paired domain and homeodomain are used because these DNA motifs are quite similar to DNA sequences originally characterized from the fruit fly *Drosophila melanogaster*. Proteins containing a paired domain, a homeodomain, or both control the expression of other genes during the development and embryoge-

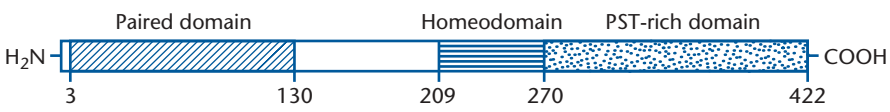


Figure 15.8 Schematic representation of the PAX6 protein. The paired domain, homeodomain, and PST-rich domain are labeled. The numbers denote the amino acid sites.

nesis of *Drosophila*. There are large numbers of closely-related paired box (pax) and homeobox (hox) sequences in the genomes of invertebrates, vertebrates, plants, and fungi. The pax and hox genes regulate many facets of development, including the determination of cell fates and the establishment of anterioposterior and dorsoventral axes in multicellular organisms.

Studies of familial and sporadic cases of aniridia provided definitive proof that *PAX6* gene mutations were responsible for aniridia. For example, of the first 15 *PAX6* genes to be screened for mutations, 14 had mutations that caused protein truncation. Now, of the more than 200 different *PAX6* gene mutations that have been observed, about 90% are truncating and are scattered throughout the coding region. The *PAX6* missense aniridia mutations tend to occur both in the paired DNA-binding domain and in the part of the PAX6 protein that facilitates its entry into the nucleus. On the basis of so many truncating mutations, aniridia is likely the result of either a dominant-negative effect or haploinsufficiency. Truncated protein molecules may either interact with normal protein chains to give inactive multimeric complexes or compete with intact chains for DNA-binding sites and prevent them from functioning properly. On the other hand, as is often the case with regulatory genes, the full extent of normal function is not achieved unless a specified amount of protein is produced. In other words, the loss of a functional allele, which would occur more readily with a termination rather than a missense mutation, causes the cellular concentration of the normal protein to be diminished by 50%, and, as a result of this shortfall, only some of the target genes will be activated.

Additional insight into the impact of the *PAX6* gene as a developmental regulator was gained from a small family with members who carried two different *PAX6* gene mutations. In this family of four, one family member had normal vision, one had mild opacity of the lens (cataracts), one had aniridia, and the fourth individual had severe central nervous system abnormalities, microcephaly (abnormally small head), and no eye tissue (anophthalmia). Single-strand conformation polymorphism (SSCP) of the complete *PAX6* gene revealed two different mutations in this family. One of the mutations was within the paired domain, and the other was in the PST-rich region. DNA sequencing established that both mutations caused protein truncation. The person with normal irises had neither of these mutations. The one with aniridia had the truncating mutation within the paired box domain, and the individual with mild ocular alterations had the PST-rich region mutation. The most severe defects, affecting the central nervous system, restricting the size and development of the brain, and blocking the formation of all eye tissue, was observed in the family member who had inherited both *PAX6* gene mutations. On the basis of the phenotypes and genotypes in this family, it can be concluded that PAX6 is essential for the development of the entire eye and plays a significant role in the differentiation of both the central nervous system and the brain.

Molecular examination of another family with a very rare ocular disorder called Peters anomaly showed that, despite the deletion of the *PAX6* gene or the presence of a missense mutation in the paired box coding region (R26G), the development of the iris was normal. However, each of these mutations led to abnormalities of both the anterior chamber of the eye and the cornea. It is

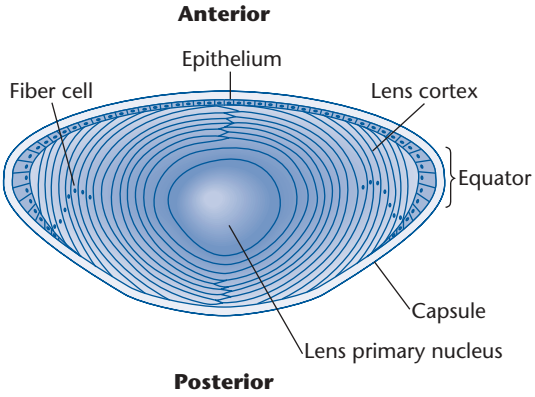


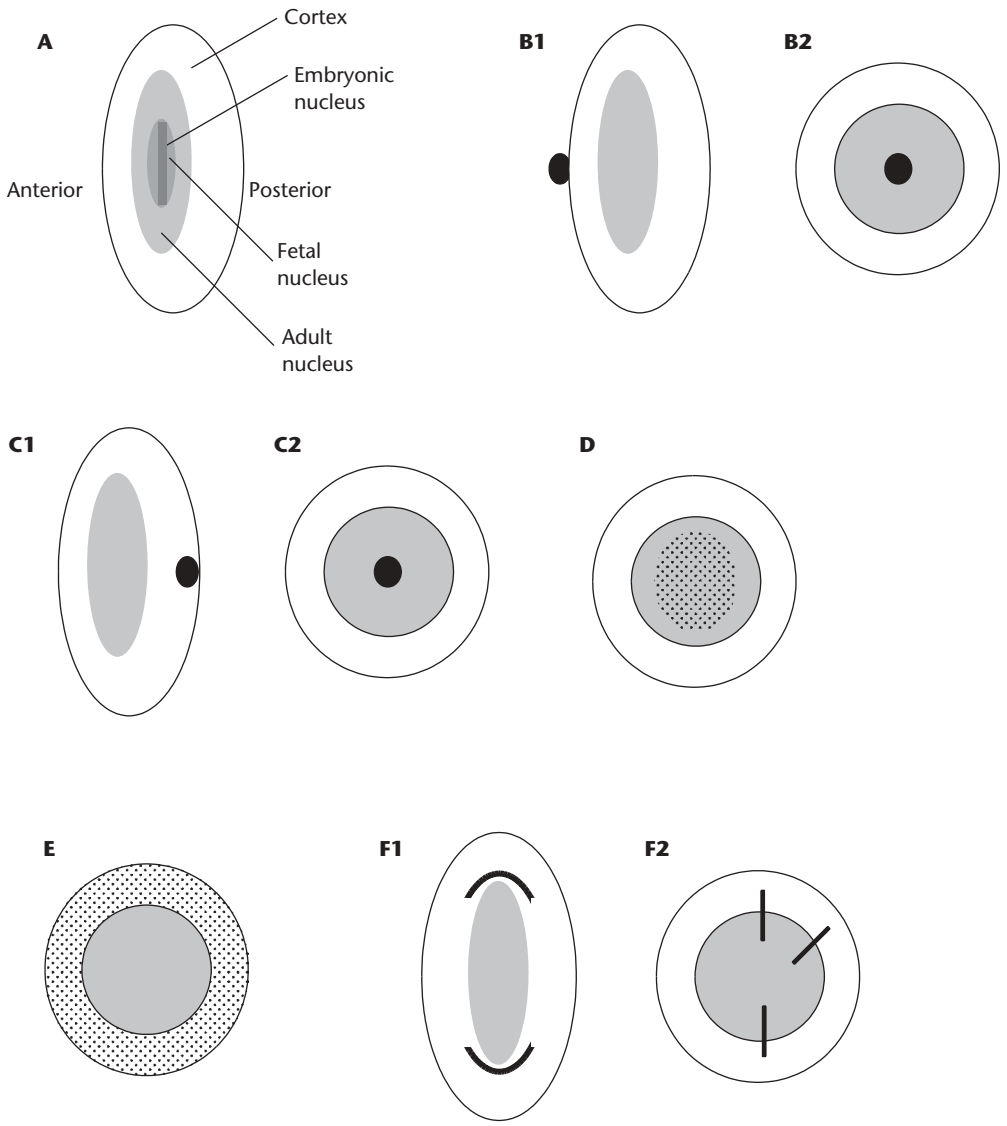
Figure 15.9 Organization of the human lens. New fiber cells are formed at the region of the epithelium that lies just anterior to the equator.

not known why these particular *PAX6* gene mutations have no demonstrable impact on the iris. However, in accordance with its role as a transcription factor for genes that contribute proteins to different parts of the eye, the *PAX6* gene is expressed in cells that give rise to the corneal epithelium, lens, posterior layers of the iris, ciliary body, retina, and central nervous system. On this basis, it is understandable that *PAX6* mutations would affect a range of ocular structures.

Cataracts

The lens of the eye is a biconvex transparent structure that refracts incoming light onto the retina (Figure 15.9). The central part of the lens is formed during embryonic development by elongating epithelial cells of the posterior lens vesicle and contains the lens primary nucleus. The front of the lens is covered with a thin layer of epithelial cells. Throughout life, newly formed fiber cells are produced from dividing epithelial cells just anterior to the central region (equator) of the lens. Over time, these cells differentiate into fiber cells by elongating and losing their nuclei and other intracellular organelles. And, as part of this continuous process, fiber cells migrate toward the center of the lens to form the lens cortex and outer layers of the nucleus.

The transparency and refractive properties of the lens are the result of the physiochemical properties and molecular packing of the major lens proteins called crystallins, which make up about 90% of total lens proteins. In humans, there are three major classes of lens crystallins, designated as the α-, β-, and γ-crystallins. A cataract is defined as any loss of transparency of the lens (lens opacity). Lens opacities are often regionalized. They can be confined to the anterior or posterior lens surface (capsular cataract) or below the surface (subcapsular cataract), to the lens nucleus (nuclear cataract), or to the lens cortex (cortical cataract). In addition, some cataracts have distinctive physical features within particular locations in the lens. For example, zonular (lamellar) cataract is the designation for a lens opacity that may be uniform or dotted and is limited to a discrete layer of fiber cells adjacent to the nucleus. When the opacity is made up of fine particles, the term "pulverulent" is often applied. A Y-shaped opacity occurs in some cataracts at the boundaries (sutures) where the major zones of fiber cell growth meet. Sutural cataracts usually do not impair vision. In addition, lens opacities can be needlelike, diffuse, or radiating. The classification of cataracts is complicated and controversial, with no



standardized nomenclature for the different types of cataracts. As a reference guide, schematic representations of some of the commonly encountered inherited cataracts are depicted in Figure 15.10.

Figure 15.10 Schematic representations of the location and appearance of inherited cataracts. (A) Cross section of a mature lens. (B) Anterior polar cataract. (C) Posterior polar cataract. (D) Pulverulent cataract. (E) Cerulean cataract. (F) Lamellar cataract. (G) Nuclear cataract. (H) Cortical cataract. (I) Sutural cataract. (J) Aculeiform cataract. (K) Total cataract. The lens is presented in cross section (ellipse) and frontal view (circles). The lens nucleus (gray), cortex (white), and both the location and the appearance of the cataract (black) are shown.

Throughout the world, cataracts affect about 16 million individuals and account for approximately 46% of all cases of blindness. Currently, it appears that a large number of different circumstances, both genetic and nongenetic, are responsible for cataract formation. Congenital cataracts, which are present in newborns, may be the consequence of infection, developmental defects, and gene mutations that affect lens proteins. Adult-onset (age related) cataracts are often caused by ultraviolet radiation, exposure to smoke, or other environmental agents. Although it has not been fully established, the formation of reactive compounds, such as hydrogen peroxide (H_2O_2), probably underlies the development of age-related cataracts. In these cases, a change in the oxidative state in the lens damages proteins and lipids including, possibly, the protein αA-

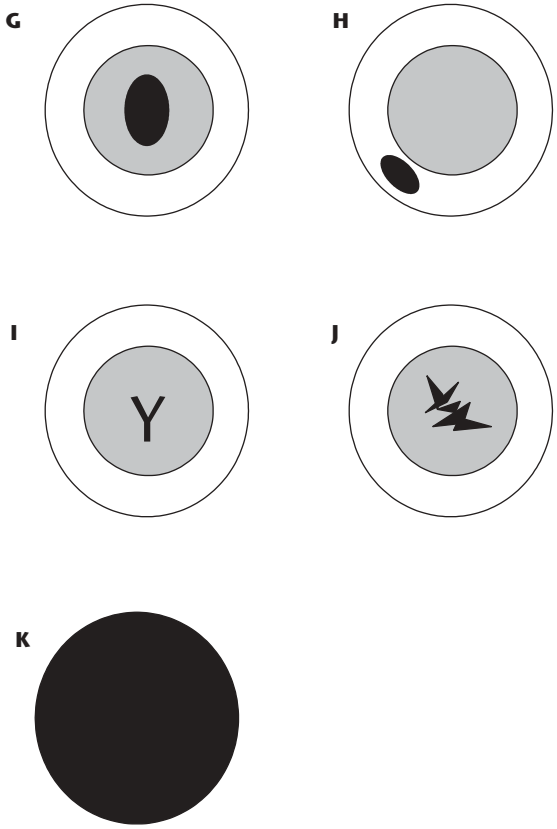


Figure 15.10 *continued*

crystallin, which maintains the structural integrity of other proteins. About one-third of all cataracts in infants are familial, whereas only a small proportion of age-related cataracts appear to be inherited.

Linkage studies, usually with large single kindreds, have identified the chromosome locations for a number of different autosomal dominant cataracts, some autosomal recessive loci, and at least one X-linked site. Fourteen genes that are responsible for primary cataract formation have been characterized (Table 15.2). Not surprisingly, seven of these genes encode lens crystallins. Overall, different gene mutations and alleles of the same gene produce an array of cataract phenotypes. Generally, the crystallin gene mutations may alter protein conformations that make them more susceptible to misfolding and aggregation, prevent essential protein-protein interactions that maintain lens transparency, and/or decrease solubility that results in the formation of protein agglomerates. The type of cataract that forms may depend on the timing of expression of a crystallin gene during lens development.

Mutant connexins 50 and 46 in the lens might hinder the distribution of sufficient small molecular compounds to some fiber cells and, as a result, induce localized protein aggregations. Alternatively, aberrant gap junction proteins may interfere with the proper production and/or formation of fiber cells. A defective water transport system may change the solubility conditions within lens cells that, as with *MIP* gene mutations, bring about cataract formation (caractogenesis). Disruption of the interaction between the cytoskeleton

Table 15.2 Genes that are responsible for inherited cataracts.

Gene	Site	Protein	Cataract
CRYAA	21q22.3	αA-crystallin	Nuclear
CRYAB	11q23.1	αB-crystallin	Posterior polar
CRYBB1	22q12.1	βB1-crystallin	Pulverulent
CRYBB2	22q11.2	βB2-crystallin	Cerulean; nuclear; pulverulent
CRYBA1	17q11.2	βA1/A3-crystallin	Nuclear; pulverulent; lamellar; sutural
CRYGC	2q33.3	γC-crystallin	Pulverulent; lamellar;
CRYGD	2q33.3	γD-crystallin	Nuclear; lamellar; cerulean; aculeiform; pulverulent
MIP	12q13.3	Lens fiber major intrinsic protein (aquaporin 0); water transport channel protein	Lamellar; variable morphology (polymorphic)
GJA8	1q21.1	Connexin 50; gap junction protein	Nuclear; pulverulent
GJA3	13q12.11	Connexin 46; gap junction protein	Nuclear; pulverulent
BFSP2	3q22.1	Beaded filament structural protein 2 (phakinin); lens-specific cytoskeleton protein	Lamellar; pulverulent
PITX3	10q24.32	Paired-like homeodomain transcription factor 3	Total; pulverulent
MAF	16q23.1	Leucine zipper transcription (bZIP) factor	Pulverulent
HSF4	16q22.1	Heat shock transcription factor 4	Lamellar

protein BSFP2 and αA-crystallin may be caractogenic. Finally, the roles of the three transcription factors (PITX3, MAF, HSF4) in the development and maintenance of the lens are not known, nor is there any indication how mutations of the *PITX3*, *MAF*, and *HSF4* genes produce cataracts.

Retinitis Pigmentosa

Over the past 150 years, researchers and clinicians have been particularly adroit at identifying a vast array of disorders of the retina. In many cases, these syndromes include degeneration (dystrophy) of part of or the entire retina. There are four major criteria shared among the retinal dystrophies. First, they tend to be familial. Second, the condition often becomes worse after its initial appearance (progressive), although in certain syndromes it remains the same (stationary) throughout life. Third, both eyes are affected. Fourth, the pattern of degeneration is symmetrical.

Understanding the molecular basis of retinal dystrophies is no easy task, because of the extensive genetic and allelic heterogeneity. Although much of the research has focused on retinitis pigmentosa (RP), the genetics of other retinopathies have also been studied, including vitreoretinal dystrophies, where both the vitreous chamber and the retina are affected; congenital stationary night blindness (CSNB), which is a nonprogressive lifelong disturbance of

night vision; and both cone and cone-rod dystrophies, where photoreceptor cell loss is initially confined to either the macula or fovea.

Retinitis pígmentosa, the most common of the progressive disorders of the retina, affects about 1 in 4000 individuals. The symptoms in an early stage include both reduced night vision (nyctalopia) and loss of peripheral sight. Later, as RP proceeds, the field of vision becomes extremely narrow, and blindness often occurs. Initially, rod cells undergo degeneration, and, in the later stages of the disease, there is a loss of cone cells. As the rod photoreceptor cells degenerate, the cells of the retinal pigment epithelium (RPE) extend into the nerve layer of the retina. The presence of the RPE produces a distinctive mottling of the retina, as the result of regions of underpigmentation adjacent to sectors that are excessively pigmented. Blood vessels in the choroid constrict, and the optic disk develops a yellow, waxy appearance. These changes are the hallmarks of RP. Although there are childhood cases, RP is usually an adult-onset disorder. Regardless of the age of onset, RP follows a similar course and takes a number of years before vision is lost completely.

Approximately 20% of RP cases are autosomal dominant (ADRP, adRP), 15% are autosomal recessive (ARRP, arRP), 10% are X-linked (XLRP, xlRP), and the rest are sporadic (nonfamilial, isolated, simplex). Generally, RP develops more rapidly in XLRP and ARRP than ADRP. More than 35 loci have been identified solely with RP (nonsyndromic RP, primary RP). Another 100 or more loci feature RP as part of multisystem conditions (syndromic RP, secondary RP). Linkage studies helped map many of the nonsyndromic RP loci to chromosome locations. Also, homologous retina-specific genes from mice, positional and candidate gene cloning, large mutational screening programs with retina-specific genes, and various gene-based strategies have been used to identify RP-causing genes (Table 15.3). The current collection of nonsyndromic RP genes is diverse. Some of these genes, such as *RHO*, *PDE6A*, *PDE6B*, and *CNGA1*, encode proteins that directly participate in the phototransduction cascade in rod cells. It is also common for RP genes that affect the integrity of the disks of photoreceptors, the relationship between the inner and outer segments of photoreceptor cells, and RPE-specific proteins involved in the synthesis of 11-*cis*-retinal to give rise to other retinopathies in addition to RP. Although it is difficult to determine exactly, about 20% and 8% of all cases of RP appear to be the result of mutations of the *RPGR* and *RHO* genes, respectively, and the rest of the known nonsyndromic RP loci contribute another 5% to 10% of occurrences of RP. In other words, a large number of RP genes remain to be identified.

More than 100 distinct mutations of the rhodopsin (*RHO*) gene have been reported from both familial and sporadic cases of RP. Many of these mutations are observed in kindreds with ADRP and account for approximately 30% of all cases of ADRP. The majority of these genetic changes are missense mutations, with the amino acid alterations occurring in most domains of the rhodopsin molecule (Figure 15.11). Some deletions and termination mutations have also been recorded. The P23H mutation accounts for about 10% of the cases of ADRP in the US. In addition to rhodopsin mutations that cause ADRP, two homozygous mutations (E150K, E249X) have been found in families with ARRP. And, surprisingly, two missense *RHO* mutations (G90D,

Table 15.3 Some of the genes that cause nonsyndromic retinitis pigmentosa.

Gene	Site	Protein	Function
RHO	3q22.1	Rhodopsin	Phototransduction cascade
RDS	6p21.2	Peripherin/RDS	Rim protein of photoreceptor disks
PDE6A	5q33.1	Rod α subunit cGMP phosphodiesterase	Phototransduction cascade
PDE6B	4p16.3	Rod β subunit cGMP phosphodiesterase	Phototransduction cascade
CNGA1	4p12	Rod α subunit cGMP-gated channel	Phototransduction cascade
CNGB1	16q13	Rod β subunit cGMP-gated channel	Phototransduction cascade
SAG	2q37.1	Arrestin	Deactivates activated rhodopsin
RP2	Xp11.23	Tubulin-specific chaperone cofactor C	Link from plasma membrane to cytoskeleton; triggers tubulin GTPase activity
RPGR	Xp11.4	RP GTPase regulator	Protein transport
ABCA4	1p22.1	Retina ATP-binding cassette transporter	Retinoid cycle
RLBP1	15q26.1	Retinal pigment epithelium (RPE) retinaldehyde-binding protein	Retinoid cycle
PRPF8	17p13.3	Pre-mRNA splicing factor C8	Intron removal
PRPF31	19q13.42	Pre-mRNA splicing factor C31	Intron removal
RPE65	1p31.2	Retinal pigment epithelium-specific protein	Retinoid cycle
NRL	14q11.2	Neural retina leucine zipper	Transcription factor
IMPDH1	7q32.1	Inosine monophosphate dehydrogenase	Guanine synthesis
RGR	10q23.1	RPE-retinal G protein-coupled receptor	Retinoid cycle

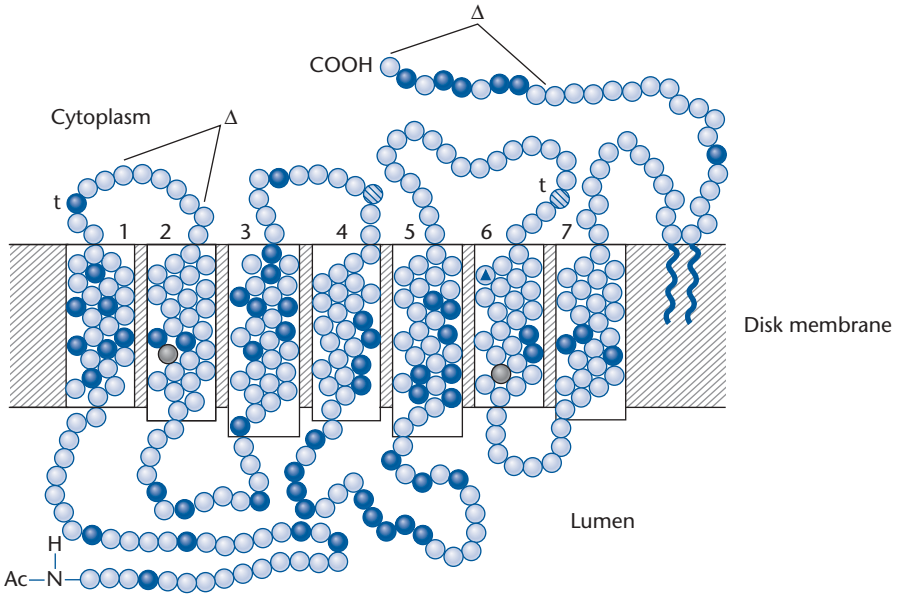


Figure 15.11 Schematic representation of rhodopsin within the disk membrane of the rod outer segment. The circles represent amino acid residues. Dark blue circles mark some of the sites of amino acid substitution associated with autosomal dominant and sporadic cases of retinitis pigmentosa. The circles with hatching represent amino acid sites that are changed in cases of autosomal recessive retinitis pigmentosa. The gray circles in transmembrane domains 2 and 6 indicate amino acid sites that are altered in some cases of congenital stationary night blindness. A t denotes a site where a termination codon has been observed. Deletions of consecutive amino acids that occur with ADRP are noted by the symbol delta (Δ), and the site of a single amino acid deletion is marked by a solid triangle. The numbers (1–7) indicate transmembrane domains. The squiggly lines within the disk membrane represent two palmitoyl groups that are added to two adjacent cysteine residues. The outer and inner sides of the disk membrane are marked as cytoplasm and lumen, respectively.

A292E) were observed in individuals with congenital stationary night blindness (CSNB). Congenital stationary night blindness is rare and is characterized by either poor or no night vision (nyctalopia), which persists to the same extent throughout the life of the patient, with no obvious signs of rod cell degeneration.

It is of considerable interest to determine how *RHO* mutations bring about RP. Currently, there is no simple answer to this question. At the molecular level, rhodopsin is a major disk membrane protein that makes up about 80% of the protein of a normal rod outer segment. The dominant behavior of the rhodopsin mutations suggests that the presence of a mutated rhodopsin interferes with the activity of the normal form or damages the photoreceptor in some way. This interpretation is supported by studies of individuals who have only one rhodopsin gene and no symptoms of impaired photoreceptor cell function. In these cases, the rhodopsin produced by one gene is sufficient for normal retinal activity. Conversely, it can be deduced that a mutated rhodopsin is the principal cause of retinal degeneration.

A number of RP *RHO* mutations cause protein misfolding. A misfolded rhodopsin probably interferes with the packing of normal rhodopsin molecules into the disk membranes, disrupting disk formation and stability. In turn, defective disks cause a shortening of the rod outer segment, eventually initiating a series of biochemical events that result in programmed cell death (apoptosis) and destruction of an entire photoreceptor. Over time, as more and more rod cells degenerate, the clinical features of RP become evident.

The human retinal degeneration slow (*RDS*) gene is a homolog of the *rds* gene, which causes slow retinal degeneration in an inbred strain of mice. The *RDS* gene encodes a 346-amino acid transmembrane glycoprotein originally named peripherin because it is confined to the rims of the disks of the outer segments of rod and cone cells, where presumably it stabilizes the structure of the membrane. The designation peripherin/RDS often is used for this protein to distinguish it from a neurofilament protein also called peripherin. Mapping studies localized the *RDS* gene close to the 6p12 region. With PCR-SSCP and other mutation screening techniques, a number of *RDS* mutations were found in ADRP families and in unrelated individuals with RP. About 3% of all cases of ADRP are the result of *RDS* mutations. In addition and somewhat unexpectedly, mutations of the *RDS* gene were also found to be responsible for a wide variety of retinal dystrophies including macular dystrophies, some dystrophies that form distinctive patterns of degeneration (pattern dystrophies), dystrophies that originate in the retina pigment epithelium, and both cone and cone-rod dystrophies.

Allelic heterogeneity is not an uncommon phenomenon, but rarely do different missense mutations at the same site produce contrasting phenotypes. Mutations at the N244 site of the *RDS* gene provide an example of such an unusual genetic phenomenon. An N244K mutation was found in individuals with ADRP, where, as expected, rod cell degeneration preceded the loss of cone cells. However, an N244H mutation was associated in a family with autosomal dominant cone-rod degeneration where cone cells degenerated before rod cells. Phenotypic heterogeneity also can arise among individuals who carry the same mutation. For example, the deletion of one of the two consecutive lysine (K)

codons at sites 153 and 154 of the *RDS* gene engendered cone dystrophies (fundus flavimaculatus, macular dystrophy) and adult-onset RP in different members of the same family.

There are no simple explanations for the various phenotypes that result from *RDS* gene mutations. Perhaps environmental factors or genes at other sites modify phenotypic outcomes. For the moment, however, we have to be satisfied and somewhat fascinated with the knowledge that mutations within a single gene and at a single site can produce an array of conditions. In this context, it seems likely that the diagnostic criteria for different retinopathies are too narrow and that many of the clinical differences may be merely part of a continuum.

Congenital Stationary Night Blindness

Congenital stationary night blindness (CSNB) is characterized by nonprogressive impaired vision in dim light as the result of defective rod receptor response. In addition, some individuals with CSNB have a rolling or jerky oscillation of the eyeballs (nystagmus). CSNB has been divided into two groups based on phenotypic differences. In complete CSNB, patients have no observable rod cell activity, moderate to severe shortsightedness (myopia), and unimpaired cone vision. In incomplete CSNB, rod and cone functions are reduced, with either severe myopia or farsightedness (hyperopia). Oguchi disease is a variant type of CSNB that features a golden-brown or gray-white tint at the back of the eye (fundus). This distinctive coloration disappears after 45 min in the dark and reappears when the eyes are reexposed to light.

Parenthetically, "sightedness" is defined by the location at which the lens focuses incoming light from distant and near objects with respect to the retina. If the light from a distant object tends to converge in front of the retina and the light from a near object focuses on the retina, then the condition is shortsightedness. If the point of focus is behind the retina from a near object and on the retina from a far object, then a person is said to be farsighted.

Congenital stationary night blindness is genetically heterogeneous, with autosomal dominant, autosomal recessive, and X-linked forms. The genes for at least eight CSNB loci have been identified. Mutations of the *RHO* and *PDE6B* genes occasionally produce CSNB. Large-scale mutational screening of an autosomal dominant CSNB, traceable back to the original founder, Jean Nougaret (1637–1719), established that the disorder in this family was caused by mutations of the *GNAT1* gene at chromosome 3p21.31, which encodes the α subunit of rod cell transducin. The *CACNA1F* gene at chromosome Xp11.23 encodes the $\alpha 1$ subunit of the photoreceptor Ca^{2+} voltage-gated channel and is responsible for incomplete CSNB. The CSNB *CACNA1F* mutations probably do not allow the Ca^{2+} channel to open during the phototransduction cascade. As a consequence, the release of neurotransmitter molecules does not occur, and a nerve impulse is not generated in postsynaptic retinal neurons. The Oguchi form of CSNB is an autosomal recessive trait and the result of mutations in either the rhodopsin kinase (*RHOK*, chromosome 13q34, Oguchi disease-1) or arrestin [*SAG* (S-antigen), chromosome 2q37.1, Oguchi disease-2] genes. Both rhodopsin kinase and arrestin are involved in the deactivation of activated rhodopsin. Briefly, under normal conditions, activated rhodopsin

is phosphorylated by rhodopsin kinase. Arrestin binds to this form of rhodopsin and allows the completion of the deactivation process, which regenerates rhodopsin molecules that can respond to light.

Choroideremia

Choroideremia (CHM) is a rare X-linked disorder, affecting about 1 in 100,000 individuals and characterized by progressive dystrophy of the choroid, retinal pigment epithelium, and retina. Males with CHM have progressive night blindness and loss of visual field from early childhood. By the fifth decade of life, the choroid often is severely atrophied, and complete blindness occurs. Heterozygous females (carriers) usually show patchy pigmentation in the retinal pigment epithelium but are not visually impaired.

Translocations and deletions of the X chromosomes of CHM individuals provided the starting point for the cloning of the choroideremia gene, which was localized to chromosome Xq21.2. After clones of various putative coding regions were obtained, mutation studies established which one represented the choroideremia gene (*CHM*). Molecular characterization revealed that the *CHM* gene has 15 exons and encodes a protein of 653 amino acids. In addition to translocations and deletions, the *CHM* genes of affected individuals often have frameshift or nonsense mutations that produce nonfunctional truncated proteins. Missense mutations in the *CHM* gene are very rare, and the few known instances actually may be splice site mutations that cause the removal of exons during mRNA processing.

The *CHM* gene is expressed in the retina, retinal pigment epithelium, choroid, and other cell types, including lymphoblasts. A portion of the *CHM* coding region is significantly similar to a gene that encodes a component of a multisubunit complex that enables protein trafficking between vesicles. The enzymic component of this complex adds chains with 20 carbon units (isoprenyl chains) to low-molecular-weight GTP-binding proteins called Rab proteins. The isoprenyl chains secure Rab proteins to the cytoplasmic surface of the membranes of various organelles. In general, Rab proteins facilitate membrane fusion during both endocytosis and exocytosis. They also control the fluidity of membranes. Some Rab proteins are modified by the addition of a 20-carbon isoprene chain, called geranylgeranyl (GG), to specific cysteine residues by the enzyme RabGG transferase (RGGT; RabGGTase).

Molecular and in vitro enzymatic studies have shown that the X-linked *CHM* gene encodes the A component (Rab escort protein-1, REP-1) of one of the RabGG transferases. This subunit enables the RGGT complex to bind to a particular Rab protein(s), followed by the B subunit of the RGGT that catalyzes the attachment of a GG group to a cysteine residue of the Rab protein. In addition, REP-1 guides the GG-Rab to specific membrane sites. How a defect in RGGT causes choroideremia is not understood. Clearly, when a specific Rab protein of the choroid, retinal pigment epithelium, or retina is not geranylgeranylated, degeneration of these ocular layers ensues. The loss of activity of a ubiquitous Rab protein would probably be lethal in humans, as it is in mice. When a *CHM* mutation is present, another Rab escort protein may be synthesized to offset the REP-1 deficiency in all nonocular tissues. For example, under these circumstances, the *CHML* gene at Xq21.2 that encodes

REP-2 may be switched on and restore RGGT activity to nonocular cells. Alternatively, a truncated REP-1 may not interact with an ocular-specific protein that, under normal conditions, also requires geranylgeranylation to maintain the integrity of the membranes of retinal tissues.

Color Vision Defects

Human color vision is a complex phenomenon that depends on three types of cone photoreceptors, designated as red, green, and blue cones. In a normal retina, there are about 1.0×10^6 blue, 0.6×10^6 green, and 4.4×10^6 red cones. Each type of cone has a visual pigment (opsin, color pigment) that responds to light within a specific spectral range. The blue (short wavelength sensitive) opsin absorbs light maximally at about 426 nm, the green (middle wavelength sensitive) opsin at about 530 nm, and the red (long wavelength sensitive) opsin at about 555 nm.

Changes within the genes that encode the opsins cause color vision defects. Some alterations decrease sensitivity to a specific wavelength, whereas others shift the absorption spectrum of an opsin to a longer or shorter wavelength. There is no absolute measuring procedure to define precisely the nature and extent of defective color vision. However, color matching and other kinds of tests can detect many of these deficiencies. A specific terminology has been developed to classify, in a broad way, the more commonly encountered kinds of color vision defects. This system uses slightly different terms for naming the actual color vision defect (condition) and describing an individual with a particular color vision condition (Table 15.4). Briefly, the total loss of red, green, or blue vision is called protanopia, deuteranopia, or tritanopia, respectively. Individuals with these conditions are protanopes, deuteranopes, or tritanopes, respectively. A lowered sensitivity to red, green, or blue light is referred to as protanomaly, deuteranomaly, or tritanomaly, respectively, and individuals with these conditions are called protans, deutans, or tritans.

Defects in red and green color vision are X-linked and affect about 8% of males and about 0.5% of females in most populations worldwide. Tritanomaly and tritanopia are autosomal disorders. The inability to perceive colors is a congenital, nonprogressive condition that, for the most part, has no serious biological consequences, although the world has a different look to a so-called colorblind person compared to someone with normal color vision. For example, for protanopes, all colors except blue are washed out (desaturated), greens and oranges have a yellowish appearance, and, in some cases, red and blue-green

Table 15.4 Nomenclature for red and green color vision defects.

General description	Condition	Individual
Red deficiency	Protanomaly	Protan
Extreme red deficiency	Extreme protanomaly	Extreme protan
No red sensitivity	Protanopia	Protanope
Green deficiency	Deuteranomaly	Deutan
Extreme green deficiency	Extreme deuteranomaly	Extreme deutan
No green sensitivity	Deuteranopia	Deuteranope

from the HUMAN GENETICS files

Searching for the Genetic Basis of Myopia

The inability to see distant objects clearly (myopia, nearsightedness) is the most common ocular disability worldwide. Approximately 25% of adults in the United States are myopic to some extent, and about 6% of blindness in United States school children is attributed to myopia. There are varying grades of myopia, which have been classified as low, moderate, or high, depending on the extent of the defect. Myopia is artificially corrected by prescription lenses and treated permanently with refractive surgery. If myopia is not treated, both headaches and fatigue are common symptoms, and, in children, learning may be impaired. Approximately 30% of those with myopia (2% of the United States population at large) have the high form of the disorder. In Asia, for reasons that are not understood, myopia is quite common. For example, as many as 70% of Taiwanese school children are nearsighted.

Myopia can occur as part of a constellation of other abnormalities or as the sole phenotypic defect. The basis of myopia is puzzling. It may involve malfunction of the refractive components of the eye and/or factors that regulate the axial length of the eye, that is, the distance from the front to the back of the eye. Both genes and environmental agents have been implicated as causes of myopia. One persistent and controversial viewpoint is that continuous close-up vision (near-work activity), such as reading or, more recently, working at the computer screen, may evoke elongation of the eyeball and/or affect the ability of the refractive system to accommodate to far distances. However, twin studies, the increased risk of myopia among close relatives, and the patterns of inheritance in some families indicate that myopia has a genetic component.

A linkage study using multigeneration families with myopia is a reasonable way of determining which loci, if any, are associated with the condition. One study of a five-generation family with myopia and other eye defects found linkage to Xq28. This locus was designated *MYP1*. A detailed study indicated that the *OPN1LW* (red opsin) and *OPN1MW* (green opsin) genes are probably not responsible for the myopia in this case.

Genome scanning of eight multigenerational families with high myopia, using 62 polymorphic microsatellite probes, indicated linkage to 18p, and, after haplotype analysis, the site of the locus was localized to 18p11.31. This form of autosomal dominant myopia was called *MYP2*. At least, one polymorphic site in the transforming growth factor-β-induced factor (*TGIF*) gene at 18p11.31, which may be the *MYP2* gene, is significantly associated with high myopia. The TGIF protein is a transcription repressor, and *TGIF* gene mutations are responsible for a disorder that entails forebrain, skull, and facial malformations (holoprosencephaly). A third locus, *MYP3*, was mapped to 12q21–23 in one four-generation family with high myopia. Similar analyses have identified loci for *MYP4* and *MYP5* at 7q36 and 17q21–q22, respectively. Finally, a haplotype-linkage disequilibrium study with dizygotic twin pairs localized a putative myopia-causing mutation to a nucleotide change within the promoter and/or regulatory region of the *PAX6* gene at 11p13. Obviously, once the potential genes for myopia have been definitively identified and fully characterized, researchers will be closer to understanding the regulation of the growth of the eye and how the focusing mechanism is altered.

are confused with white. Notwithstanding the lack of serious vision impairment, there has been a longstanding scientific curiosity about the molecular basis of red-green color vision defects.

Isolation of Human Rhodopsin and Color Opsin Genes

A bovine rhodopsin cDNA probe was used to isolate DNA clones of the four human visual pigments (photopigments). Because photoreceptor proteins probably evolved from a common ancestor, it was reasoned that each human gene would share some sequence similarity with the bovine rhodopsin sequence. The human rhodopsin gene was expected to be quite similar to the bovine rhodopsin sequence, whereas, because of evolutionary divergence, the genes for red, green, and blue opsins were thought to be less similar. To distinguish among these various degrees of similarity, a human genomic DNA clone bank was screened with the bovine rhodopsin DNA probe at different levels of hybridization stringency. With high stringency, a hybridizing DNA strand will form a stable complex with a DNA strand of the clone bank only if the two strands share long sequential stretches of complementary nucleotides.

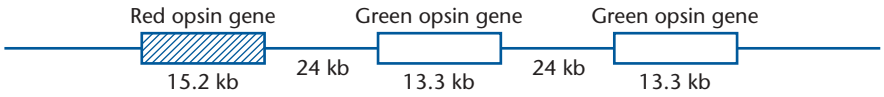


Figure 15.12 Tandem array of red and green opsin genes. The single red opsin gene is shown as a hatched rectangle with two successive green opsin genes as open rectangles. The lengths of the genes and intervening DNA regions are shown as kilobase pairs (kb). By convention, the left end of a segment of DNA is designated as the 5′ end and the right as the 3′ end.

Under low (relaxed)-stringency conditions, short stretches of complementary nucleotide sequences containing mismatched base pairs are stable. The sequence that was isolated under high-stringency conditions was the human rhodopsin gene (*RHO*, 3q22.1). The blue opsin gene (*OPN1SW*, opsin 1, short-wave-sensitive, 7q32.1) was isolated with moderate stringency, and the red opsin (*OPN1LW*, opsin 1, long-wave-sensitive, Xq28) and green opsin (*OPN1MW*, opsin 1, medium-wave-sensitive, Xq28) genes were isolated conjointly with low stringency. Once the clones for these genes were available, the chromosome locations were determined, and the genes were characterized. Amino acid sequence analysis of the human cone pigments, based on the gene sequences, showed that the red and green opsins are 96% identical to each other and about 40% identical with rhodopsin and the blue cone pigment. Interestingly, the red and green opsin genes are closely aligned in a tandem head-to-tail array. To date, all studies have found that a red opsin gene is at the 5′ end of the array and, in men with normal color vision, it is usually followed by two green opsin genes (Figure 15.12). A red-green cone pigment gene array will infrequently have only one green opsin gene and only very rarely will there be more than five green opsin genes following the red opsin gene. Moreover, despite some disputed evidence to the contrary, an array seldom has two or more red opsin genes in tandem.

The mechanisms that control the transcription of the red and green opsin genes in respective red and green cones are not well known. A DNA sequence (locus control region) that lies upstream from the 5′ end of the first gene of the array probably initiates transcription by making contact near the 5′ ends of the genes in either the first or second positions of the array. Much of the data indicate that the opsin gene in the first position is transcribed in red cones and the opsin gene in the second position is transcribed in green cones. Any opsin gene in the third, fourth, or a higher position has a very low probability of ever being transcribed.

Molecular Genetics of Red and Green Color Vision Defects

The genomic arrangements of the red and green opsin genes in men with color vision defects have been examined with Southern blot analysis and gene-specific PCR amplification. In general, these studies show that color vision abnormalities are most often the result of the formation of novel genes (hybrid genes) that contain sequences coding parts of the red and green opsin genes. Missense and nonsense mutations have rarely been associated with red or green color vision defects. Because of the high sequence similarity between the coding regions of the red and green opsin genes, mispairing can occur between these genes on homologous chromosomes during meiosis in females. If a recombination event (nonhomologous recombination, unequal crossing over) occurs within these misaligned DNA regions, a red-green opsin hybrid gene can be formed (Figure 15.13). To produce a hybrid opsin, a red-green opsin

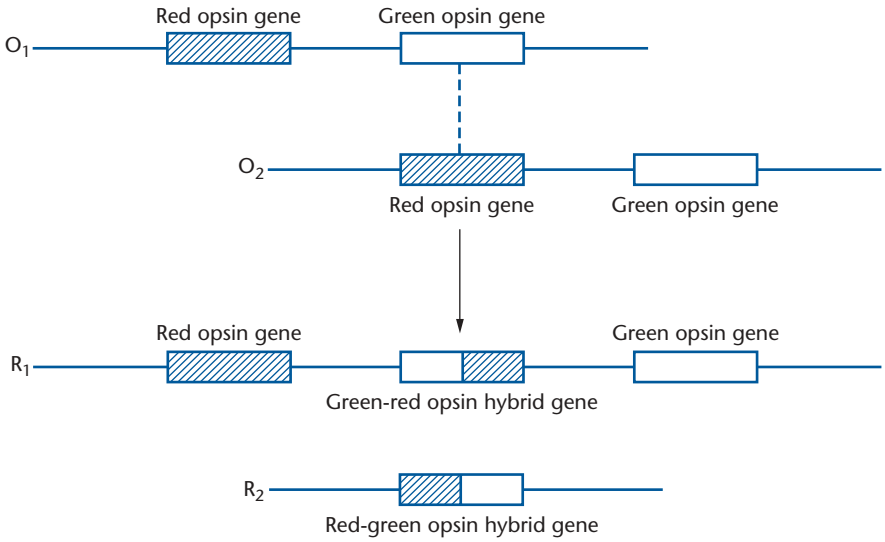


Figure 15.13 Schematic representation of nonhomologous recombination between red and green opsin genes. The vertical dashed line denotes the site of a reciprocal recombination between two of the four of the original chromatids (O_1, O_2). After recombination, one of the recombined chromatids (R_1) has a red opsin gene, a 5′ green-red opsin hybrid gene, and a green opsin gene. The other chromatid (R_2) has a single 5′ red-green opsin hybrid gene.

hybrid gene must have an intact reading frame and a complete set of exons. Depending on the site of the DNA exchange, a red-green opsin hybrid gene can contain more red opsin exons than green opsin exons, or vice versa. When the coding sequences for the red and green opsins are at the 5′ and 3′ ends, respectively, of a hybrid gene, the gene is designated as a 5′ red-green hybrid. Nonhomologous recombination can also create a 5′ green-red hybrid gene (Figure 15.13).

Various alterations of the red-green opsin gene array have been associated with specific color vision defects (Table 15.5). For example, in many protanopes, the first gene in the red-green opsin gene array is a 5′ red-green hybrid gene, and the rest of the genes encode green opsin. In the red cones of these individuals, the hybrid gene encodes an opsin that has lost all sensitivity to red light, whereas the green opsin in their green cones is probably produced from the second gene in the array. Other protanopes have a single 5′ red-green hybrid gene. In these cases, the hybrid gene probably encodes a hybrid green opsin with an absorption spectrum very similar to a normal green opsin. For most protanomolous subjects, the first gene in the array is usually a 5′ red-green hybrid gene, and the second gene encodes green opsin. In these individuals, the hybrid opsin retains either a moderate or marginal sensitivity to red light and is produced in red cones, whereas in green cones a normal green opsin is expressed from the second gene in the array. Often only one normal red opsin gene and no green opsin genes are found in the DNA of deuteranopes. In other deuteranopes, in addition to a red opsin gene in the first position, the second gene is a 5′ green-red hybrid gene, which presumably encodes an opsin that is insensitive to green light. Deuteranomaly can occur when there is a normal red opsin gene in the first position and a 5′ red-green hybrid gene that encodes an opsin with reduced sensitivity to green light in the second position.

Because there is no loss of visual acuity in individuals with color vision defects, cone cell degeneration is minimal, if it occurs at all, although some selective cone cell depletion cannot be precluded. When a single red opsin,

Table 15.5 The relationships among some genomic alterations of the red-green opsin gene array, opsin production by red and green cones, and color vision phenotypes.

Genomic alteration					
Gene in the first position of the array	Gene in the second position of the array	Red cone opsin	Green cone opsin	Phenotype	Comment
5′ Red-green hybrid gene	No green opsin gene	Hybrid green opsin	Hybrid green opsin	Protanopia	Hybrid opsin is sensitive to green light and is synthesized in both types of cones
5′ Red-green hybrid gene	Normal green opsin gene	Hybrid green opsin	Green opsin	Protanopia	Hybrid opsin is sensitive to green light
5′ Red-green hybrid gene	Normal green opsin gene	Hybrid red opsin	Green opsin	Protanomaly	Hybrid opsin has reduced sensitivity to red light
Normal red opsin gene	No green opsin gene	Red opsin	Red opsin	Deuteranopia	No green opsin is produced and red opsin is synthesized in green cones
Normal red opsin gene	5′ Red-green hybrid gene	Red opsin	Hybrid red opsin	Deuteranopia	Hybrid opsin is insensitive to green light
Normal red opsin gene	5′ Red-green hybrid gene	Red opsin	Hybrid green opsin	Deuteranomaly	Hybrid opsin has reduced sensitivity to green light

green opsin, or hybrid gene is present, it is likely to be expressed in both red and green cone cells. For example, deuteranopes with a single red opsin gene probably produce red opsin in both of their red and so-called green cones. Among individuals with red-green color vision defects, approximately 17%, 17.5%, 50.5%, and 15% have protanopia, deuteranopia, deuteranomaly, and protanomaly, respectively. The lack of equality among these groups suggests that nonhomologous recombination is not a random process and that hybrid opsins are more likely to have reduced sensitivity to green than to red light.

Sequence comparisons of human and primate red and green opsins and spectral analyses of hybrid opsins have identified some amino acid sites that may contribute to the difference between red and green sensitivity and account for the light responses of various hybrid opsins. The presence and location of hydroxyl-containing amino acids, such as tyrosine (Y), threonine (T), and serine (S), probably determine the spectral response of the red and green opsins. For example, amino acids at sites 277, 285, and 309 in exon 5, and site 65 in exon 1 are hydroxylated in human red opsin, whereas these sites are occupied by nonhydroxylated amino acids in the green opsin (Figure 15.14). On the other hand, the green opsin has hydroxylated amino acids at sites 230 and 233 in exon 3, and the red opsin has nonhydroxylated amino acids at these sites. Sites 277, 285, and 309 in exon 5 are important in determining the spectral properties of a hybrid opsin. If, for example, the first four exons of a 5′ red-green hybrid gene originate from a red opsin gene and the last two exons from a green opsin gene, then the hybrid opsin has more green sensitivity and reduced red sensitivity. Conversely, a 5′ green-red hybrid gene with exons 5 and

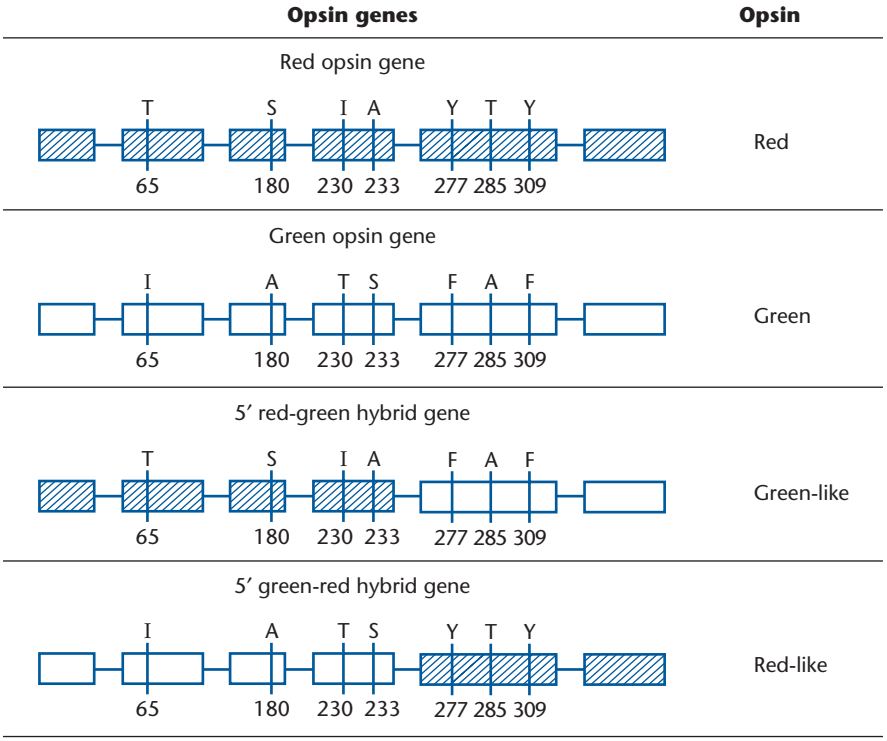


Figure 15.14 Schematic representation of amino acid sites of the red and green opsin genes that contribute to the spectral features of the red and green opsins. The hatched and open rectangles denote exons of the red and green opsin genes, respectively. The key amino acids are numbered, and the single-letter amino acid codes are used for the amino acids. The so-called greenlike and redlike opsins are thought to have spectral sensitivities similar to normal green and red opsins, respectively.

6 from a red opsin gene would encode a hybrid opsin that is shifted to greater sensitivity to red light (Figure 15.14).

Although not involved in color vision defects, site 180 in exon 3 of the red and green opsins is of interest. Alanine (A), a nonhydroxylated amino acid, is at site 180 of all normal green opsin molecules. By contrast, codon 180 is polymorphic in the red opsin gene. About 62% of males have a red opsin with a hydroxylated amino acid (serine) at site 180, and the remaining males have alanine at this site. With a serine residue at site 180, the maximal absorption of the red opsin is 552 nm and is shifted 5 nm to a longer wavelength (red shift) than when the site is occupied by alanine. In other words, the world may be marginally rosier for men with serine180 red opsin in comparison to those with alanine180 red opsin, at least as far as color perception is concerned.

Blue Cone Monochromacy

The loss of all red and green color vision [blue cone monochromacy (BCM); X-linked incomplete achromatopsia; blue cone monochromatism] is a rare X-linked disorder affecting about one in 100,000 worldwide. A hallmark of BCM is the absence of both red and green cones, which, despite the presence of blue cones, abolishes all color discrimination and produces a colorless world. In addition, decreased visual acuity, nystagmus, and, in some individuals, progressive degeneration of the central retina are associated with BCM. The molecular status of the red and green opsin genes in individuals with BCM has been examined, and two classes of genetic change were observed. First, some individuals with BCM have either one red opsin gene or a single 5′ red-green hybrid gene. Neither of these changes by itself necessarily accounts for the

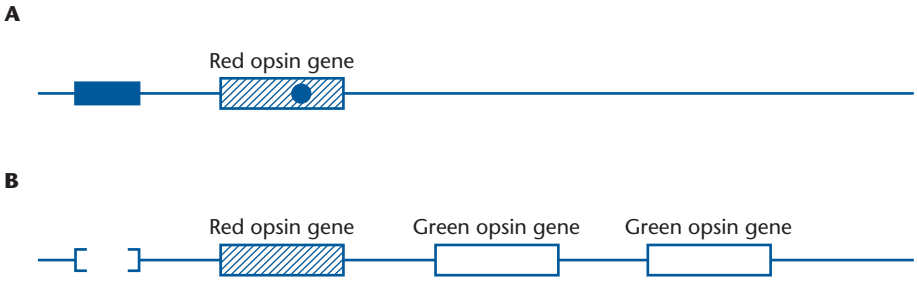


Figure 15.15 Genetic changes associated with blue cone monochromacy. (A) Some individuals with blue cone monochromacy (BCM) have a single red opsin gene with a point mutation (•). The upstream controlling sequence that plays a role in the transcription of the red and green opsin genes is depicted by a solid rectangle. (B) In some individuals with BCM, a common chromosomal region, probably containing the upstream controlling sequence that promotes the transcription of red and green opsin genes, is deleted (brackets).

BCM phenotype. However, after DNA sequencing, a number of BCM individuals with a single opsin gene were found to have an opsin-inactivating mutation (Figure 15.15). For example, in many cases, a single red opsin gene has a C203R mutation that produces an unstable and nonfunctional opsin. Other inactivating mutations of a single opsin gene have been observed in BCM individuals. In rare instances, BCM occurs when there are only two opsin genes in an array and both genes have a C203R mutation.

In the second class of BCM, the red-green opsin gene array is intact. However, there are deletions upstream from the 5′ end of the red opsin gene. Analysis of the overlaps of these deletions revealed that a common 579-base pair region located about 4 kilobase pairs upstream from the red opsin gene was always missing (Figure 15.15). This common region must contain a sequence (locus control region, LCR) that is essential for the transcription of red and green opsin genes, and, in its absence, neither a red nor green opsin is synthesized.

Blue Color Vision Defect

Tritanopia is an autosomal disorder characterized by virtually no sensitivity to blue light and may affect as many as one person in 500. Tritanopia, as in red and green color vision defects, is not associated with any obvious cone cell degeneration. By PCR-DGGE with specific pairs of primers for the exons of the blue opsin gene, unrelated tritanopes were analyzed for the presence of mutations. Missense mutations (G79R, S214P, P264S), not found in unaffected individuals, were observed. Each of these mutations is in a transmembrane domain of the blue opsin and may affect its spectral properties.

Rod Monochromacy

The total loss of color vision [rod monochromacy (RM); rod monochromatism; complete achromatopsia; total color blindness] is a rare autosomal recessive condition that affects about one person in 30,000. The retinal cones are either absent or defective. Accompanying features include nystagmus, impaired visual acuity, and extreme sensitivity to light (photophobia). At least three RM genes have been characterized. Briefly, *CNGA3* at 2q11.2 and *CNGB3* at 8q21.3 encode the α and β subunits of the cone-specific cGMP-gated cation channel (cyclic nucleotide-gated channel) and *GNAT2* at 1p13.3 the α subunit of cone-specific transducin. These proteins are components of the cone phototransduction cascade, and, as a result of mutations, signals are not generated. Consequently, there is no color processing by the brain.

key terms

absorption spectrum
allelic heterogeneity
aniridia
blue cone monochromacy
cataract
cGMP
cGMP-gated cation channel
choroid
choroideremia
cones
congenital stationary night blindness
cornea
corneal dystrophies
crystallins
deuteranopia
deuteranomaly
genetic heterogeneity
glaucoma
hybrid gene
intraocular pressure
iris
keratoepithelin
lamellar cataract
lens
locus control region
myocilin
myopia
opsins (red, green, and blue)
PAX6
peripherin/RDS
phenotypic heterogeneity
photopigment
phototransduction cascade
protanomaly
protanopia
pulverulent cataract
retina
retinal neurons
retinitis pigmentosa (RP)
retinoid cycle
rhodopsin
rod monochromacy
rod outer segment
rods
trabecular meshwork
transducin
tritanomaly
tritanope

summary

Human vision is a complex process. The structural components of the eye, such as the cornea, iris, and lens, have distinctive properties that direct light onto the retina. The photoreceptors (rods and cones) of the retina transduce this radiant energy into nerve signals by a series of activation steps called the phototransduction cascade. Light activates rhodopsin, a protein photopigment localized in the disk membranes of the rod outer segments. The activated form of rhodopsin, metarhodopsin II, activates transducin, which in turn activates a cGMP phosphodiesterase that hydrolyzes cGMP to 5′-GMP. The elimination of cGMP from a cation channel protein of the plasma membrane of the rod outer segment closes the channel and induces hyperpolarization of the plasma membrane, which, in turn, generates a nerve impulse. Color perception is created from the signals generated by the red-, green-, and blue-opsin photoreceptor (cone) cells. The neural signals are transmitted from photoreceptor cells to retinal neurons and then by nerve fibers to the brain, where visual images are formulated.

Hundreds of inherited conditions affect the eye. The chromosome locations of many of these genes have been determined by linkage studies and genomic scans. Moreover, positional cloning, candidate gene studies, large-scale mutational screening programs of known ocular-specific genes, and other approaches have been used to identify genes that affect different structures of the eye.

Various genes cause corneal dystrophy. Interestingly, mutations at one site of the *TGFB1* gene, formerly *BIGH3*, at chromosome 5q31 that encodes keratoepithelin are responsible for four clinically diverse corneal dystrophies.

At least 10 loci contribute to primary glaucoma. Of the known genes, *MYOC* encodes the protein myocilin, which probably affects water flow within the trbecular meshwork cells and increases intraocular pressure, leading to glaucoma.

The loss of activity of the *PAX6* gene is responsible for the incomplete development of the iris (aniridia) and other eye defects. The PAX6 protein is a transcription factor that activates many of the genes that participate in the development of the anterior portion of the eye.

Cataracts are the result of protein aggregations or disruption of cell morphology in parts of the lens that scatter incoming light instead of focusing it on the retina. As crystallins are the major lens proteins, it was no surprise that mutations of crystallin genes that probably cause misfolding produce cataracts. Mutations of some gap junction and transcription factor genes are also caractogenic.

Retinitis pigmentosa (RP) is a progressive retinal dystrophy that affects the rod cells. RP is extremely heterogeneous genetically, with about 35 different loci contributing to nonsyndromic forms of the disorder. Mutations of the rhodopsin gene are responsible for about 8% of the cases of RP. How mutations in many different genes cause the same pattern of retinal degeneration remains, for the most part, unresolved. A number of the mutated rhodopsin molecules have altered conformations that probably disrupt the integrity of the disks of the rod outer segment, which, in turn, induces programmed cell death and the loss of photoreceptors.

Mutations of the gene that encodes RabGG transferase are responsible for the degeneration of the choroid (choroideremia).

Color vision depends on the visual pigments (opsins) of the red, green, and blue photoreceptor cells (cones). The gene for the blue opsin (blue cone pigment) is on chromosome 7q32.1. The genes for the red and green opsins (red and green cone pigments) are on the X chromosome at Xq28 and form a head-to-tail tandem array with a red opsin gene at the 5′ end. Usually, an array consists of a red opsin gene followed by two green opsin genes. Generally, only opsin genes in the first two positions of the array are transcribed. Because of the high degree of sequence similarity between red and green opsin genes, nonhomologous recombination can occur during meiosis and may give rise to a chromosome with a solitary red opsin gene, a hybrid red-green gene, a hybrid green-red gene, or some other gene rearrangement. Proteins produced by hybrid opsin genes often have altered spectral properties that lead to color vision defects.

The absence of sensitivity to both red and green light is an inherited condition called blue cone monochromacy. At the genomic level, this disorder can occur in different ways. First, either a solitary red opsin or red-green hybrid gene has a point mutation that abolishes all sensitivity to red light. Alternatively, in rare instances, mutations of both red and green opsin genes produce red- and green-insensitive visual pigments. Finally, the loss of a common region of the DNA upstream from the tandem array prevents the transcription of both the red and green opsin genes, hence, these individuals do not synthesize either a red or green opsin. Mutations of genes that encode members of the phototransduction cascade of cone cells fail to produce neural signals. As a consequence, the brain does not receive any information from cone cells and there is a total loss of color vision, that is, rod monochromacy.

References

Bloome, M. A. and C. A. Garcia. 1982. *Manual of Retinal and Choroidal Dystrophies*. Appleton-Century-Crofts, New York.

Cvekl, A. and E. R. Tamm. 2004. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. *Bioessays* **26**:374–386.

El-Ashry, M. F. et al. 2002. Identification of novel mutations in the carbohydrate sulfotransferase gene (*CHST6*) causing macular corneal dystrophy. *Invest Ophthalmol Vis Sci* **43**:377–382.

Farrar, G. J., P. F. Kenna, and P. Humphries. 2002. On the genetics of retinitis pigmentosa and on mutation-independent approaches to therapeutic intervention. *EMBO J* **21**:857–864.

Fingert, J. H. et al. 2002. Myocilin glaucoma. *Surv Ophthalmol* **47**:547–561.

Gong, G. et al. 2004. Genetic dissection of myocilin glaucoma. *Hum Mol Genet* **13**:R91–102.

Hammond, C. J. et al. 2004. A susceptibility locus for myopia in the normal population is linked to the *PAX6* gene region on chromosome 11: A genomewide scan of dizygotic twins. *Am J Hum Genet* **75**:294–304.

Hanson, I. M. et al. 1994. Mutations at the *PAX6* locus are found in heterogeneous anterior segment malformations including Peters' anomaly. *Nat Genet* **6**:168–173.

Jagla, W. M. et al. 2002. The molecular basis of dichromatic color vision in males with multiple red and green visual pigment genes. *Hum Mol Genet* **11**:23–32.

Khaw, P. T., P. Shah, and A. R. Elkington. 2004. Glaucoma—1: diagnosis. *BMJ* **328**: 97–99.

Khorana, H. G. 1992. Rhodopsin, photoreceptor of the rod cell. An emerging pattern for structure and function. *J Biol Chem* **267**:1–4.

Klintworth, G. K. 1999. Advances in the molecular genetics of corneal dystrophies. *Am J Ophthalmol* **128**:747–754.

Lam, D. S. et al. 2003. TGFβ-induced factor: A candidate gene for high myopia. *Invest Ophthalmol Vis Sci* **44**:1012–1015.

Lines, M. A., K. Kozlowski, and W. A. Walter. 2002. Molecular genetics of Axenfeld-Rieger malformations. *Hum Mol Genet* **11**:1177–1184.

Liu, Y. and D. Vollrath. 2004. Reversal of mutant myocilin non-secretion and cell killing: implications for glaucoma. *Hum Mol Genet* **13**:1193–1204.

Michaelides, M., D. M. Hunt, and A. T. Moore. 2004. The cone dysfunction syndromes. *Br J Ophthalmol* **88**:291–297.

Milam, A. H., Z. Y. Li, and R. N. Fariss. 1998. Histopathology of the human retina in retinitis pigmentosa. *Prog Retin Eye Res* **17**:175–205.

Nathans, J. et al. 1989. Molecular genetics of human blue cone monochromacy. *Science* **245**:831–838.

Nathans, J. et al. 1992. Molecular genetics of human visual pigments. *Annu Rev Genet* **26**:403–424.

Nathans, J. et al. 1986. Molecular genetics of inherited variation in human color vision. *Science* **232**:203–210.

Nathans, J., D. Thomas, and D. S. Hogness. 1986. Molecular genetics of human color vision: the genes encoding blue, green, and red pigments. *Science* **232**:193–202.

Neitz, M. and J. Neitz. 1995. Numbers and ratios of visual pigment genes for normal red-green color vision. *Science* **267**: 1013–1016.

Newell, F. W. 1986. *Ophthalmology: Principles and Concepts*. 6th ed. C. V. Mosby, St. Louis, MO.

Paluru, P. et al. 2003. New locus for autosomal dominant high myopia maps to the long arm of chromosome 17. *Invest Ophthalmol Vis Sci* **44**:1830–1836.

Rattner, A., H. Sun, and J. Nathans. 1999. Molecular genetics of human retinal disease. *Annu Rev Genet* **33**:89–131.

Ray, K., A. Mukhopadhyay, and M. Acharya. 2003. Recent advances in mole-

cular genetics of glaucoma. *Mol Cell Biochem* **253**:223–231.

Reddy, M. A. et al. 2004. Characterization of the G91del CRYBA1/3-crystallin protein: a cause of human inherited cataract. *Hum Mol Genet* **13**:945–953.

Reddy, M. A. et al. 2004. Molecular genetic basis of inherited cataract and associated phenotypes. *Surv Ophthalmol* **49**: 300–315.

Rezaie, T. et al. 2002. Adult-onset primary open-angle glaucoma caused by mutations in optineurin. *Science* **295**:1077–1079.

Rivolta, C. et al. 2002. Retinitis pigmentosa and allied diseases: numerous diseases, genes, and inheritance patterns. *Hum Mol Genet* **11**:1219–1227.

Saliba, R. S. et al. 2002. The cellular fate of mutant rhodopsin: quality control, degradation and aggresome formation. *J Cell Sci* **115**:2907–2918.

Seabra, M. C., E. H. Mules, and A. N. Hume. 2002. Rab GTPases, intracellular traffic and disease. *Trends Mol Med* **8**:23–30.

Stojanovic, A. et al. 2003. Retinitis pigmentosa rhodopsin mutations L125R and A164V perturb critical interhelical interactions: new insights through compensatory mutations and crystal structure analysis. *J Biol Chem* **278**:39020–39028.

Stryer, L. 1991. Visual excitation and recovery. *J Biol Chem* **266**:10711–10714.

Thompson, D. A. and A. Gal. 2003. Vitamin A metabolism in the retinal pigment epithelium: genes, mutations, and diseases. *Prog Retin Eye Res* **22**:683–703.

Tsujikawa, M. et al. 1999. Identification of the gene responsible for gelatinous drop-like corneal dystrophy. *Nat Genet* **21**:420–423.

van den Hurk, J. A. et al. 2003. Novel types of mutation in the choroideremia (CHM) gene: a full-length L1 insertion and an intronic mutation activating a cryptic exon. *Hum Genet* **113**:268–275.

van Heyningen, V. and K. A. Williamson. 2002. *PAX6* in sensory development. *Hum Mol Genet* **11**:1161–1167.

Weinreb, R. N. and P. T. Khaw. 2004. Primary open-angle glaucoma. *Lancet* **363**:1711–1720.

Wissinger, B. et al. 1998. Human rod monochromacy: linkage analysis and mapping of a cone photoreceptor expressed candidate gene on chromosome 2q11. *Genomics* **51**:325–331.

Yamaguchi, T., A. G. Motulsky, and S. S. Deeb. 1997. Visual pigment gene structure and expression in human retinae. *Hum Mol Genet* **6**:981–990.

Young, T. L. et al. 2004. X-linked high myopia associated with cone dysfunction. *Arch Ophthalmol* **122**:897–908.

review questions

1. Draw a comprehensive representation of the human eye without referring to an existing illustration.
2. Outline the events of the phototransduction cascade in rod cells.
3. What is corneal dystrophy?
4. Describe the relationship between mutations of the *TGFB1* gene and corneal dystrophy.
5. What is glaucoma?
6. Describe the relationship between mutations of at least three genes and glaucoma.
7. Describe how the gene that causes aniridia was discovered.
8. What kind of gene determines the formation of an iris?
9. Comment on the functions of the domains of the PAX6 protein.
10. What is a cataract?
11. Describe the relationship between mutations of at least three genes and cataract formation.
12. What is genetic heterogeneity? Explain this term using specific examples from studies of the molecular genetics of components of the eye.
13. What is allelic heterogeneity? Explain this term using specific examples from studies of the molecular genetics of components of the eye.
14. What is the retinoid cycle? Why would mutations of this cycle cause retinitis pigmentosa?
15. What is RabGG transferase?
16. Describe the consequences of mutations of the gene that encodes REP-1.
17. What genetic changes cause deuteranopia and protanomaly in males?
18. Describe the genetic changes that lead to BCM and how they can be detected.
19. What is the genetic basis of rod monochromacy?
20. Describe the clinical features and molecular genetics of Stargardt disease, cone-rod dystrophy, and age-related maculopathy. Use OMIM, PubMed, and other relevant Internet resources for information.

chapter 16

Molecular Genetics of Cancer Syndromes

. . . some combinations of genes yield bodies that are much more prone than others to break out into that unregulated growth that is called cancer.

H. S. JENNINGS (1868–1947)

Cancer is a disease of growth (sometimes visible; more characteristically, inside), of abnormal, ultimately lethal growth that is measured, incessant, steady.

SUSAN SONTAG (1933–2004)

. . . Cancer's a funny thing.
Nobody knows what the cause is,
Though some pretend they do;
It's like some hidden assassin
Waiting to strike at you.

W. H. AUDEN (1907–1973)

FEW DISEASES CAUSE AS MUCH DREAD and grief as cancer. Everyone knows someone who has died of cancer. A university professor at the prime of his career has blurred vision and headaches, goes to the doctor, is diagnosed with a brain cancer, glioblastoma, and despite therapy dies within two years. A tennis player is unusually fatigued, goes to the doctor, is diagnosed with chronic myeloid leukemia (CML), and although therapy prolongs his life, he dies nine years after onset of the cancer. A mother feels a continual soreness in the abdominal region, goes to the doctor, is diagnosed with liver cancer, which probably arose from a previous bout with breast cancer, and dies within a month. The most frightening aspect of cancer is that it begins suddenly and by chance in outwardly healthy individuals. Despite the pall that cancer spreads, many patients respond to treatment and have lifelong remissions.

Explanations for the apparent upsurge in cases of cancer in the twentieth century are controversial. Cancer certainly is not a new disease; therefore, increased awareness may be the result of better and more definitive diagnoses. And, as infectious diseases have decreased and life spans lengthened, it has become increasingly likely that more people will live to develop some sort of cancer. Environmental pollutants and lifestyle choices, such as cigarette smoking, contribute significantly to the occurrence of cancer. However, many cancers arise without any obvious contributory determinants. In some of these

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cases, a genetic predisposition, in the form of a preexisting mutation, may facilitate cancer formation. Understanding how cancer is initiated and devising therapies would be much easier if cancer were a single disease, rather than a number of different disorders that result in many instances from similar biological malfunctions. Basically, cancers develop when there is a failure in the processes that induce cells to divide, control the cell division cycle, repair DNA damage, and/or implement programmed cell death (apoptosis).

Cell Proliferation and Cancer

The formation of many cells (multicellularity) from a single fertilized egg involves a complex, highly coordinated series of processes that include cell proliferation, cell quiescence, apoptosis, and activation of dormant cells by extracellular factors. After embryogenesis, under normal conditions, most of the cells are in a nondividing, quiescent state called G_0. Some continue to divide as needed, and apoptosis ensures that there is not an excessive accumulation of cells in any tissue or organ (Figure 16.1). Dozens of different proteins are required to induce or constrain cell proliferation, maintain or prevent cell division cycles, and initiate or inhibit apoptosis. Cell proliferation is triggered by a cascade of activation and inactivation steps, which, for the most part, are mediated by protein kinases that phosphorylate tyrosine, threonine, or serine residues of cytoplasmic and nuclear proteins. The nuclear proteins usually are transcription factors that collectively regulate the expression of scores of genes. In addition to the proteins required for activation of cell proliferation, cell division cycle, and apoptosis, others that help stave off cancer are used to repair damage to genomic DNA.

Cancer is the all-inclusive term for unrestrained cell growth. Generally, a cancer cell is a somatic cell with accumulated mutations in different genes that collectively cause a loss of control of cell proliferation. As a consequence, a mass of cells (tumor) forms. Some tumors continue to grow at the original site, and others stop and recede. In the worst case, tumor cells can become migratory (metastasize) and move into other sites, where, quite often, cell proliferation occurs unabated. A tumor that invades other tissues is malignant, and one that remains at its original site is benign. The continuous growth of cancer cells leads to severe tissue damage, organ failure, and, eventually, death. To date, more than 200 types of cancer syndromes have been identified affecting almost

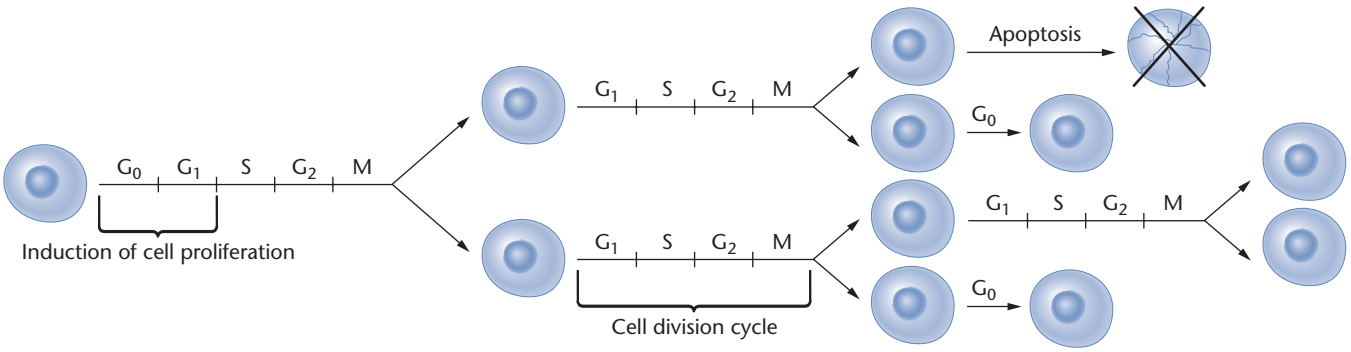


Figure 16.1 Induction of cell proliferation, cell division cycle, cell quiescence, and apoptosis. A cell proliferation signal transduction pathway induces the expression of proteins that enable a quiescent cell to leave the G_0 phase and enter the cell division cycle. The phases of the cell division cycle are G_1, S (DNA synthesis), G_2, and M (mitosis). After a number of embryonic cell divisions, nearly all the cells of most tissues enter the G_0 state. Some continue to divide, and others die by apoptosis.

every type of cell, tissue, and organ system. A cancer often is classified according to its initial (primary) site of occurrence. About 85% of all cancers are carcinomas and arise from cells that line organs and form part of the skin; about 2% are sarcomas from cells of connective, bone, or muscle tissue; about 5% are lymphomas from white blood cells of the spleen and lymph nodes; and about 3% are leukemias from white blood cells (leukocytes) of the blood system. Cancers of glandular tissues are called adenocarcinomas; cancers of the nonneuronal cells of the brain are called gliomas and astrocytomas; and cancers of plasma cells are called myelomas.

Mutations of about 300 genes have been implicated in the formation and development of the vast array of different human cancers. About 20% and 90% of all cancers have germline and somatic mutations, respectively. Most germline cancer mutations have no effect unless a somatic mutation occurs in the other allele. Parenthetically, for cancer clinicians, the term "familial cancer" usually denotes a cancer that affects a number of members of a family and is not necessarily inherited, whereas "hereditary cancer" describes cancers that are inherited, and, in some instances, are familial. About 5% of all cancers are hereditary, with a germline mutation playing a major role (Table 16.1). In most hereditary cancer syndromes and in nearly all sporadic forms, a number of mutations in a somatic cell are required for the initiation and development of a cancer. In this context, cancer resembles an oligogenic trait.

The genetic basis of cancer is confounding, because different combinations of different mutant genes can give rise to the same type of cancer. Some gene mutations are common among different cancers, whereas others are confined to a specific cell type. Many of the mutations that lead to cancer occur spontaneously. Others are caused by chemical agents (carcinogens) in tobacco smoke, foods, and the workplace; by ultraviolet light and ionizing radiation; and by viruses that either commandeer the cell division process of a tissue or insert a viral genome into a locus responsible for cell proliferation.

In the United States, approximately 500,000 people die from cancer-based disorders annually, and more than 1 million new cases are diagnosed each year. Although only a small percentage of all cancers are hereditary, these examples have contributed significantly to current insights into the molecular aspects of these disorders. An understanding of how cancers arise depends on knowledge of the signal transduction pathways that induce cell proliferation, the proteins that control and constrain the cell division cycle, and the ways in which apoptosis is kept in check.

Signal Transduction Pathway for Cell Proliferation

A quiescent cell can be stimulated to initiate the cell division cycle by an extracellular factor (signal) that binds a cell-specific receptor. The binding triggers a sequential series of catalytic events that ends with the transcription of a large number of genes that specify proteins for cell proliferation. When an eliciting factor does not enter the target cell and the response to the signal is relayed by cellular processes, the signal is said to be transduced, and the overall process is called a signal transduction pathway (Figure 16.2). Briefly, for many cell proliferation signal transduction pathways, a growth factor binds to two receptor molecules. This dimerization changes the configuration of each receptor, which

Table 16.1 Some inherited cancer syndromes.

Cancer syndrome	Primary cancer	Site	Gene	Normal function
Retinoblastoma	Retinoblastoma	13q14.2	*RB1*	Transcription factor; cell cycle regulation
Li–Fraumeni syndrome	Sarcomas, breast cancer	17p13.1	*TP53*	Transcription factor; responds to DNA damage; regulates cell cycle and apoptosis
Familial adenomatous polyposis (FAP)	Colorectal cancer	5q22.2	*APC*	Regulation of β-catenin; adhesion signaling; microtubule stability
Hereditary nonpolyposis colorectal cancer (HNPCC)	Colorectal cancer	2p21 3p21.3 2q31–q33 7p22 2p16	*MSH2* *MLH1* *PMS1* *PMS2* *MSH6*	DNA mismatch repair
Neurofibromatosis type 1 (NF1)	Neurofibromas	17q11.2	*NF1*	Negative regulator of Ras signal transduction pathway
Neurofibromatosis type 2 (NF2)	Acoustic neuromas	22q12.2	*NF2*	Link between cytoskeleton and plasma membrane proteins
Wilms tumor	Kidney tumor	11p13	*WT1*	Transcription regulator
Nevoid basal cell carcinoma syndrome (NBCCS)	Basal cell skin cancer	9q22.32	*PTCH*	Transmembrane receptor
Familial breast cancer 1	Breast cancer	17q21.31	*BRCA1*	Transcription factor; DNA repair; cell cycle regulation; chromatin remodeling; marking proteins for degradation (ubiquitination)
Familial breast cancer 2	Breast cancer	13q13.1	*BRCA2* (*FANCD1*)	DNA repair
von Hippel–Lindau syndrome (VHL)	Kidney cancer	3p25.3	*VHL*	Regulation of transcription elongation
Hereditary papillary renal cancer (HPRC)	Kidney cancer	7q31.2	*MET*	Hepatocyte growth factor, tyrosine protein kinase
Familial melanoma	Melanoma (pigment cell cancer)	9p21	*CDKN2A*	Cyclin-dependent kinase inhibitor 2A
Multiple endocrine neoplasia type 1 (MEN1)	Pancreatic islet cell cancer	11q13.1	*MEN1*	Repressor
Multiple endocrine neoplasia type 2 (MEN2)	Medullary thyroid cancer	10q11.21	*RET*	Receptor tyrosine protein kinase
Cowden disease	Breast cancer, thyroid cancer, multiple cancers	10q23.31	*PTEN*	Phosphatidylinositol-3,4,5-triphosphate 3-phosphatase
Ataxia telangiectasia	Lymphoma	11q22.3	*ATM*	PI3/PI4 kinase, cell cycle checkpoint kinase, regulates tumor suppressor genes
Bloom syndrome (BLM, BS)	Solid tumors at a variety of sites	15q26.1	*BLM*	RecQ DNA helicase
Xeroderma pigmentosum (XP)	Skin cancers	9q22.3 2q14.3 3p25.1 19q13.32 11p11.2 16p13,12 13q33.1	*XPA* *ERCC3* *XPC* *ERCC2* *DDB2* *ERCC4* *ERCC5*	Nucleotide excision repair
Fanconi anemia	Acute myeloid leukemia and other cancers	16q24.3 9q22.3 3p25.3 6p21.31 11p15 9p13	*FANCA* *FANCC* *FANCD2* *FANCE* *FANCF* *FANCG*	DNA repair

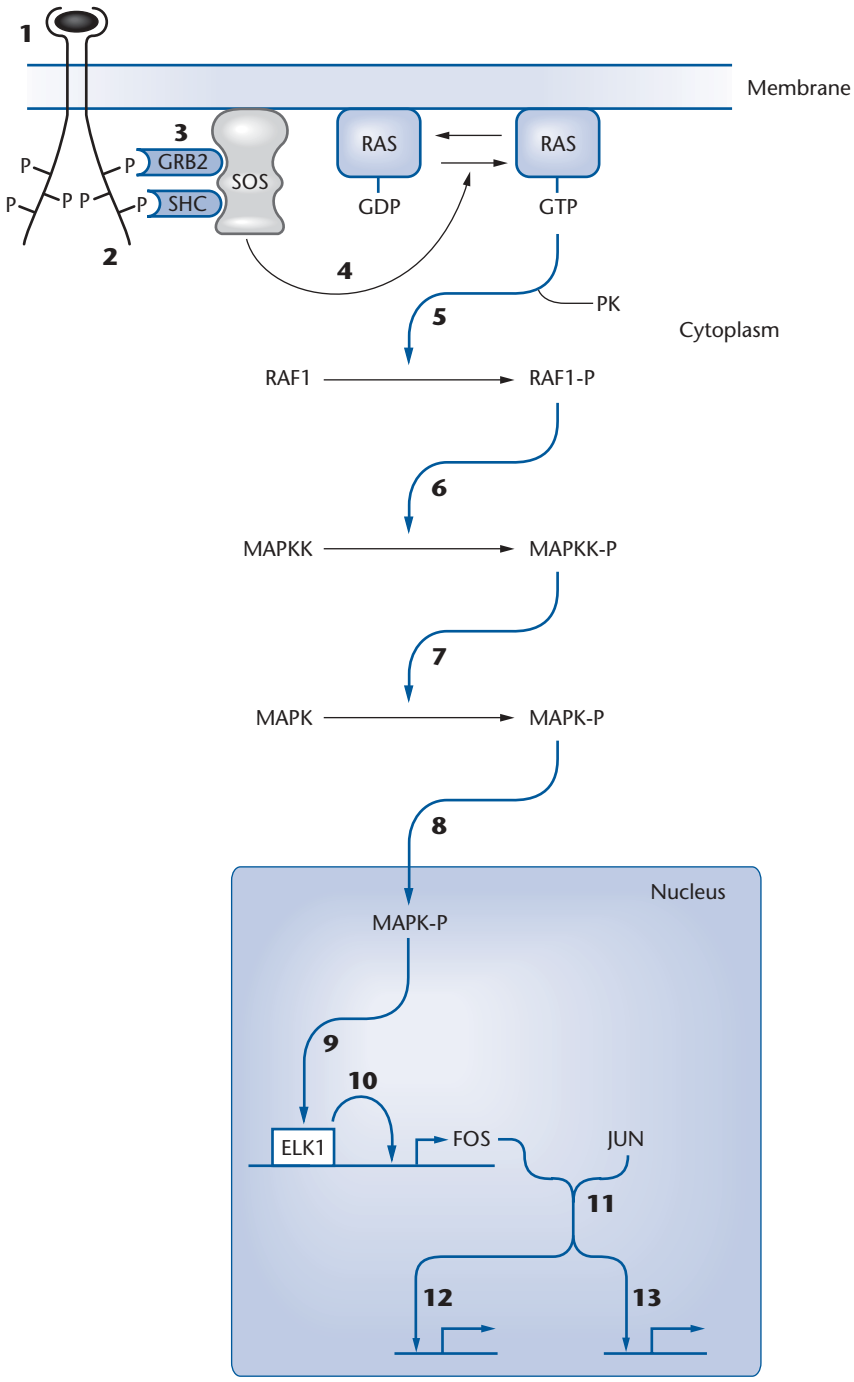


Figure 16.2 A cell proliferation signal transduction pathway. A growth factor ( ) binds two tyrosine protein kinase receptors to form a dimer (1) and triggers reciprocal phosphorylation of tyrosine residues of the cytoplasmic portion of each receptor protein (2). The GRB2 and SHC proteins bind to the phosphorylated tyrosine residues of the tyrosine protein kinase receptor, and the SOS protein binds to the complexed GRB2-SHC proteins (3). The complex-bound SOS protein activates the RAS protein (4). The activated RAS protein (RAS-GTP) recruits RAF1, which is activated by a protein kinase (PK) (5). Phosphorylated RAF1 activates MAPKK by phosphorylation (6), and activated MAPKK-P activates MAPK (7). Phosphorylated MAPK (MAPK-P) enters the nucleus (8) and phosphorylates ELK1 (9), which in turn induces the transcription of the *FOS* gene (10). The FOS and JUN proteins combine (11) and induce transcription of a number of other genes (12, 13).

induces the intracellular kinase domain of one receptor to phosphorylate tyrosine residues in the domain of the other molecule and vice versa. As a consequence of this phosphorylation, cytoplasmic proteins bind to the phosphorylated tyrosine residues. Then, after another protein attaches to these proteins, the conversion of a membrane-associated inactive protein (RAS-GDP) to its active state (RAS-GTP) is facilitated. For the RAS activation

reaction, the guanosine diphosphate (GDP) bound to the RAS protein is exchanged for guanosine triphosphate (GTP). Next in this particular pathway, the inactive form of the RAF1 serine/threonine kinase binds to the active RAS-GTP protein and is phosphorylated, presumably by a membrane-associated protein kinase. The phosphorylation of RAF1 activates its serine/threonine kinase activity. One of the targets of active RAF1 is inactive MAPKK (mitogen-activated protein kinase kinase). RAF1 phosphorylates threonine residues of MAPKK. The phosphorylated form of MAPKK activates MAPK (mitogen-activated protein kinase) by phosphorylation. Phosphorylated MAPK (MAPK-P) enters the nucleus, where it phosphorylates various transcription factors, including ELK1. Phosphorylated ELK1 activates the transcription of the FOS gene. Then, the FOS protein joins with the JUN protein, and this combination activates the expression of a number of genes that drive cell proliferation.

The RAS protein plays a pivotal role as an on-off switch to regulate intracellular events leading to the induction of cell proliferation. The active RAS form (RAS-GTP) is converted to the inactive form (RAS-GDP) by hydrolysis of GTP mediated by GTPase-activating proteins (GAPs). The short life of a growth factor also helps to turn off a signal transduction pathway. When the growth factor is cleared, the tyrosine protein kinase domain of the receptor molecule reverts to a conformation that prevents phosphorylation, and the subsequent cascade of phosphorylations does not occur. Also, phosphatases dephosphorylate activated kinases and restore them to an inactive state.

Because most of the proteins of a cell proliferation signal transduction pathway are members of gene families, different combinations of proteins occur in diverse cell types. Moreover, a single cell may have a number of different receptors that respond to different growth factors. In some cells signal transduction pathways interact (cross talk) with each other, whereas in other cells the pathways are independent of one another. Despite the intricate and labyrinthine aspects of signal transduction pathways, it is clear that mutations in any number of the genes that encode pathway proteins could lead to uncontrolled cell growth and, hence, cancer. For example, a tyrosine protein kinase receptor mutation that causes continual phosphorylation in the absence of a growth factor would keep a cell proliferation signal transduction pathway permanently turned on. Similarly, *RAS* gene mutations that prevent the conversion of RAS-GTP to RAS-GDP would stimulate continuous cell growth. For the most part, the genetic bases of many cancers involve genes that encode components of cell proliferation signal transduction pathways in specific cell types (Table 16.2). Genes with the potential to cause cancer are called proto-oncogenes. Proto-oncogenes become oncogenes when point mutations or chromosome rearrangements turn off gene expression, overproduce certain proteins, or cause other alterations that lead to the development of cancer.

Parenthetically, the names of the genes and proteins of cell proliferation signal transduction pathways are often idiosyncratic. Many of the human genes involved in these processes were identified because they are related evolutionarily to gene sequences that cause cancers in other organisms. For example, the human *RASA1* gene has a sequence similar to one that induces sarcomas in rats and was originally dubbed the rat sarcoma-inducing gene or *Ras* gene. On

Table 16.2 Association of cell proliferation signal transduction pathway oncogenes with human cancers.

Oncogene	Function	Cancer
EGFR	Epidermal growth factor receptor	Glioma
KIT	Tyrosine protein kinase receptor	Gastrointestinal stromal tumor, acute myelogenous leukemia, testicular germ cell tumor
RET	Tyrosine protein kinase receptor	Papillary thyroid carcinomas, multiple endocrine neoplasia type 2
JAK2	Tyrosine protein kinase receptor	Acute lymphocytic leukemia, acute lymphocytic leukemia
LCK	Tyrosine protein kinase receptor	T cell acute lymphoblastic leukemia
NRAS	GTP-binding protein	Acute myelogenous leukemia, thyroid cancer, melanoma, multiple myeloma
HRAS	GTP-binding protein	Sarcoma
KRAS2	GTP-binding protein	Colon, lung, pancreatic, and thyroid cancers, acute myelogenous leukemia
ABL1	Nonreceptor tyrosine kinase	Chronic myeloid leukemia, acute lymphocytic leukemia
MYC	Transcription factor	Small cell lung cancer, breast cancer, cervical carcinoma, Burkitt lymphoma
MYCN	Transcription factor	Neuroblastoma, lung cancer
MYCL1	Transcription factor	Small cell lung cancer

the basis of the nomenclature for human genes, the human homologs of the rat *Ras* genes were designated *RAS*. The *RASA1* gene is one of the members of the family of human *RAS* genes. Although the derivation of most of these designations are known, some proteins, for example, RAF, and their corresponding genes, remain undefined. In addition, some of the names of proteins are acronyms for descriptive designations assigned when these proteins were initially discovered, such as MAPKK for mitogen-activated protein kinase kinase. Unavoidably, any discussion of the molecular basis of cancer is liberally sprinkled with these kinds of obscure labels.

Regulation of the Cell Division Cycle

The cell division cycle requires a fine-tuned, temporal coordination of many sequential, diverse, and simultaneous events to ensure that, among other things, genomic DNA is precisely duplicated within a specified time period, DNA errors are repaired, chromosome compaction and the chromosome distribution process are precisely synchronized, and the splitting of a cell is not premature. Although much has been learned in the last 10 years about how the cell division cycle is controlled, there are still significant gaps in our understanding of the way the system functions at the molecular level.

Phosphorylations of target proteins by kinases drive the passage from the G_1 phase to the S phase (G_1/S transition) and from the G_2 phase to mitosis (G_2/M transition). As a cell enters G_1 from either G_0 or M, proteins called D- and E-cyclins and cyclin-dependent kinases (CDK2, CDK3, CDK4, CDK6) are synthesized. The joining of a cyclin to a CDK forms a complex that activates the kinase activity of the CDK component. Often a cyclin is a member

of a family of related cyclins. In addition, different cyclins, one at a time, can bind to the same CDK. As part of the regulatory system, various inhibitor proteins can block either the formation or the phosphorylation capability of various cyclin-CDK complexes.

During G_1, members of the set of D cyclins [cyclin D1 (CLND1), cyclin D2 (CLND2), and cyclin D3 (CLND3)] independently combine with CDK4 or CDK6 to form complexes that phosphorylate a binary complex consisting of retinoblastoma protein 1 (RB1) and the E2F protein (RB1-E2F). The phosphorylation of RB1 releases E2F. The transcription factor E2F enters the nucleus and promotes the expression of a number of genes, including those that specify proteins for DNA synthesis. As part of the regulatory process, the phosphorylation function of CLND-CDK4,6 complexes is blocked by various CDK inhibitors including CDKN2B (p15 INK4B, inhibitor of CDK4, protein B), CDKN2A (p16, INK4A), CDKN2C (p18, INK4C), and CDKN2B (p21, WAF1, wild-type p53 activating fragment 1). After the formation of the CLND-CDK4,6 complexes, the ternary complex CLNE-proliferating cell nuclear antigen (PCNA)-CDK2 is assembled. The CLNE-PCNA-CDK2 complex also phosphorylates RB1-E2F and releases E2F (Figure 16.3). The activations, the expression of various genes, and the production of specific proteins enable a cell to exit the G_1 phase. In addition, there are checkpoints that prevent cells from entering the next mitotic cycle phase before all the requirements of a preceding phase are met.

During S phase, CLNE-CDK2 contributes CDK2 to CLNA to form a CLNA-CDK2 complex that is phosphorylated by the CLNH-CDK7 complex and a cell division cycle phosphatase called CDC25A. The CLNE-CDK2 complex also activates CDC25A phosphatase. After it is activated, CLNA-CDK2 phosphorylates RB1 of the RB1-E2F complex, releasing E2F. In combination with other proteins, activated CLNA-CDK2 promotes the initiation

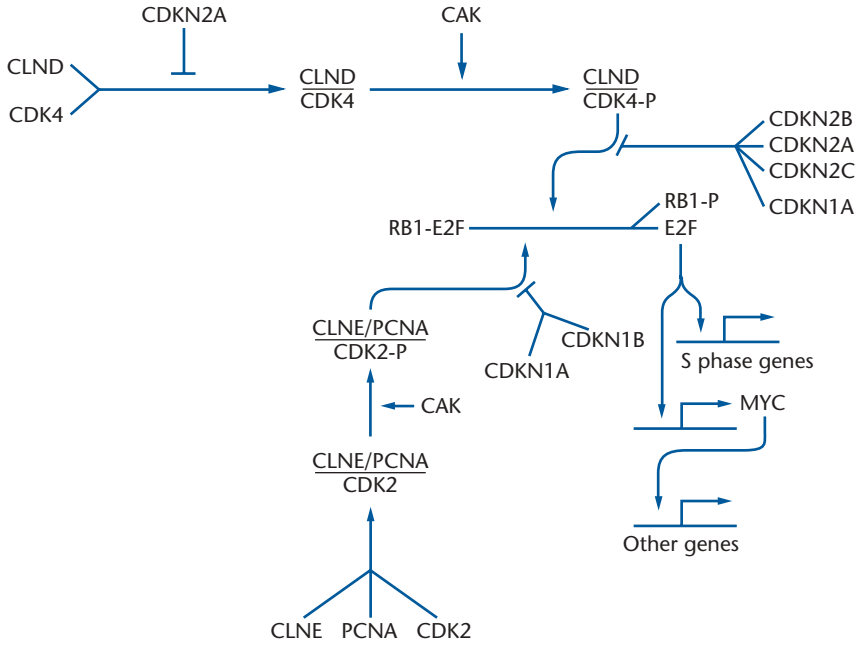


Figure 16.3 Molecular interactions during the transition from G_1 to S phase of the cell division cycle. Activation and inhibition of cyclin/cyclin-dependent kinases and dissociation of retinoblastoma protein 1 (RB1) and transcription factor E2F during the transition from G_1 to S phase. Cyclin D (CLND) combines with cyclin-dependent kinase 4 (CDK4). The individual members of the cyclin D family (D1, D2, D3) and the CLND-CDK6 complex are not shown. Cyclin E (CLNE), proliferating cell nuclear antigen (PCNA), and CDK2 form a complex later in the G_1 phase than the CLND-CDK4 complex. Both sets of complexes are phosphorylated (P) by cyclin-dependent kinase-activating kinase (CAK). Phosphorylation of the RB1-E2F complex by either of the CLN-CDK complexes promotes the release of E2F. Transcription factor E2F induces the expression of S phase-specific genes and the *MYC* gene. The MYC transcription factor acts on other genes. Various activation steps in this process are inhibited by cyclin-dependent kinase inhibitors CDKN1A, 1B, 2A, 2B, and 2C. A perpendicular sign (⊥) indicates the inhibition of a reaction. A line with a right-angled rightward arrow (⌐→) represents transcription of a gene. A more comprehensive view of the protein interactions and posttranslational modifications during the human cell division cycle is available at http://www.genome.ad.jp/kegg/pathway/hsa/hsa04110.html

of DNA replication. Inhibitors CDKN1A and CDKN1B block the activity of both CLNE-PCNA-CDK2 in G_1 and CLNA-CDK2 in S phase. In G_2, the CDC25A and CDC25B phosphatases activate the CLNA-CDK1 and CLNB-CDK1 complexes, respectively, and E2F is released from an RB1-E2F complex by CLNA-CDK1 phosphorylation of RB1. An activated CLNB-CDK1 complex facilitates both entry into mitosis and transit from mitosis into the G_1 phase.

Additional cyclins, such as CLNI and CLNF, and other proteins are involved in the cell division cycle. In addition to proteins that inhibit CLN-CDK complexes, proteolytic enzymes specifically degrade cyclins, so that the phase-specific cyclins are not present at the wrong times during the cell cycle. Some CLN-CDK complexes have more than one task during a phase of the cell division cycle. Moreover, certain cell division cycle components are used for other cellular activities. For example, CDK-activating kinase (CAK), which may be equivalent to CLNH-CDK7, plays a role in mRNA transcription. In other instances, the inhibitor CDKN1A participates in DNA replication and may also block the differentiation of keratin-producing cells. The RB1-E2F complex may be involved in the formation of muscle cells.

Unless damage to chromosome DNA is repaired, both chromosome rearrangements and mutations can accumulate, which can lead in some cases to the onset of cancer or, in other instances, to cell death. Fortunately, cells have elaborate systems for recognizing and repairing DNA damage. However, the cell division cycle must be halted to implement these corrective processes. A significant mediator of the cellular responses to DNA damage is the protein p53. When DNA damage occurs, the amount of p53 increases, and, among its many functions, p53 stimulates the expression of the CDK inhibitor CDKN1A. As the level of CDKN1A rises, the activations carried out by CLND-CDK4,6, CLNE-PCNA-CDK2, and CLNA-CDK2 are blocked, and the cell division cycle stops in G_1 or S phase.

Point mutations and other genetic alterations of genes that encode cell division cycle proteins are oncogenic, that is, cancer-causing. For example, inactivation of both *RB1* genes leads to the formation of tumors of the retina (retinoblastoma) in children and to lung, bladder, pancreatic, and breast cancer in adults. Mutations of the *CDKN2* gene family that encodes the cyclin-dependent kinase inhibitors (2A, 2B, 2C) have been found in many cancers, including acute lymphoblastic leukemia, pancreatic adenocarcinoma, glioblastoma, astrocytoma, esophageal squamous cell cancer, lung cancer, bladder carcinoma, and pituitary tumors. Oncogenic mutations of the *CDKN1A* and *CDKN1B* genes are less common but have been found in some gliomas, leukemias, and cancers of the prostate, breast, lungs, and liver. Both mutations and overexpression of the *CDK4* gene have been found in some melanomas and glioblastomas. Also, overexpression of the cyclin D1 gene (*CCND1*) occurs in some breast, gastric, and esophageal cancers. A translocation between the *CCND1* gene at chromosome 11q13 and the immunoglobulin heavy chain gene cluster (*IGH@*) at chromosome 14q32 is found in several lymphomas, leukemias, and myelomas. To a lesser extent, cyclin D2 (*CCND2*) and D3 (*CCDN3*) gene mutations occur in some colorectal, breast, and lymphatic system cancers.

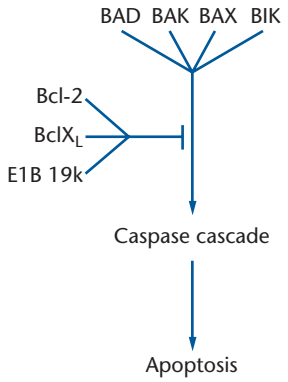


Figure 16.4 Inhibition and activation of apoptosis. The proteins Bcl-2 (B cell leukemia/lymphoma-2 protein), BclX$_L$ [long (L) form Bcl-like (X) protein], and E1B 19K (19 kDa homolog of adenovirus gene E1B) prevent the proteins BAD (Bcl-2-associated death protein), BAK (Bcl-2-antagonist/killer protein 1), BAX (Bcl-2-associated X protein), and BIK (Bcl-2-interacting killer protein) from activating a family of procaspases. After the conversion of the procaspases, caspases initiate the events (caspase cascade) that culminate in apoptotic cell death. The signal pathways that are triggered by specific apoptosis receptors and that initiate apoptosis are not shown. A more complete overview of apoptosis is available at http://www.genome.ad.jp/kegg/pathway/hsa/hsa04210.html

Apoptosis: Programmed Cell Death

Apoptosis is cell-initiated cell death. It performs tissue sculpturing during the development of the nervous system, digit formation, and other developmental programs by selective cell destruction. Apoptosis also removes cells that have sustained irreparable DNA damage, acts as a noninflammatory defense against viral infection, and prevents cell masses from becoming too large. The process of apoptotic cellular breakdown includes DNA fragmentation by a Mg^{2+}-dependent endonuclease, chromatin condensation, compaction of cell organelles, blebbing of the cell membrane, cell shrinkage, and, in the end, partitioning of the cell into small membrane-bound packages that are absorbed by other cells. Members of the Bcl-2 family of transmembrane proteins inhibit other members of the Bcl-2 family of proteins, such as BAD (Bcl-2-associated death protein), BAX (Bcl-2-associated X protein), BIK (Bcl-2-interacting killer protein), and BAK (Bcl-2-antagonist/killer protein 1), from initiating apoptosis (Figure 16.4). The key feature of apoptosis is the activation of several cell death proteases (caspases) by a proteolytic cascade. Under normal conditions, the caspases are almost totally inactive proenzymes. However, the extracellular and intracellular signals that trigger the cell death processes cause inactive caspases (procaspases) to aggregate. This clustering increases the likelihood that the low level of intrinsic proteolytic activity of inactive caspases will activate some caspase molecules, which, in turn, initiate a full-blown caspase proteolytic cascade. Caspases, which cleave after an aspartic acid residue in target proteins, are activated by the removal of a portion of the proenzyme. Once activated, caspases attack specific cellular proteins and structures, which releases factors that activate other proteases, nucleases, and additional destructive enzymes.

When mutations or genomic alterations cause increased levels of apoptosis-inhibiting Bcl-2 proteins, apoptosis is blocked, and cell survival is prolonged. When *BCL2* gene mutations of this type occur in combination with oncogenic mutations, the likelihood of cancer is increased, because potential cancer cells are not destroyed by apoptosis. Overproduction of Bcl-2 proteins has been associated with chronic lymphocytic leukemia (CLL) and cancers of the lymph nodes, prostate, lungs, breast, nose, and throat.

The protein p53 plays an important role in the induction of apoptosis (Figure 16.5). When DNA damage is extensive, wild-type p53, among other functions, represses the transcription of the *BCL2* gene and increases the expression of the *BAX* gene. Both of these actions initiate apoptosis. On the other hand, mutations of the *TP53* gene that do not invoke apoptosis in response to DNA damage allow cells to proliferate, accumulate oncogene mutations, and become cancerous. In addition to its role as an inducer of apoptosis, p53 is involved in the control of G_1/S and G_2/M transitions. In these instances, p53 halts the cell division cycle to allow damaged DNA to be repaired. Approximately 50% of all cancers have a mutated *TP53* gene, which makes it the most commonly observed mutated oncogene. Metaphorically, p53 is the cellular equivalent of the Roman hero Horatius, who single-handedly held a small bridge against the onslaught of a large number of enemy soldiers!

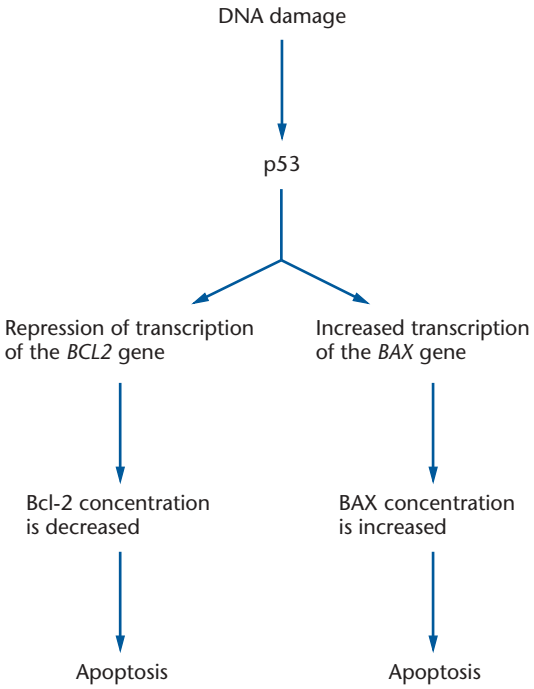


Figure 16.5 Association of DNA damage, p53, and apoptosis. When DNA is damaged, a signal is sent to p53, which, among other actions, represses and stimulates transcription of the *BCL2* and *BAX* genes, respectively. Each of these activities facilitates apoptosis.

Tumor Metastasis

The generation of a cancer requires a number of mutations, which, in succession, establish a predisposition for cancer formation, initiate and promote cancer development, and, finally, cause immortalized cells to become migratory and invasive (metastatic). Although much is known about the genetic basis of the early stages of cancer formation, there is little information about the genes that suppress metastasis in normal cells and those mutated genes responsible for metastasis in cancer cells.

Genes that specify cell adhesion proteins and those for proteases that specialize in the degradation of extracellular matrices are likely candidates for tumor metastasis. However, the search for such mutant genes will not be easy, because there are more than 40 different proteins that act as cell-to-cell adhesion molecules and more than 20 proteases that break down components of extracellular matrices.

The cadherin protein family, with about 20 different members, consists of transmembrane glycoproteins that attach to each other and anchor one cell to another. Mutations of the E-cadherin *CDH1* gene at chromosome 16q22.1 have been detected in a number of cancers, including endometrial and ovarian carcinomas. The loss of E-cadherin in cancer cells has also been associated with cell invasiveness.

Cancer cells with metastatic competence often have high levels of one or more matrix metalloproteinases (MMPs). Members of this enzyme family degrade extracellular matrices. Uncontrolled production of MMPs would remove the extracellular matrix and allow migratory cells to escape from a primary site. In addition, MMPs could create openings in other extracellular matrices, allowing cancer cells to invade other tissues. In this context, muta-

tions of genes that encode tissue inhibitors of metalloproteinases (TIMPs) may also be important for metastasis. Both in vitro and in vivo studies have indicated that wild-type TIMPs prevent metastasis and invasion of cancer cells, whereas cells become metastatic when the synthesis of a TIMP is blocked.

It would be surprising if there were only a few common gene mutations that controlled metastasis in all cancers. It is more likely that different combinations of these mutations occur in various kinds of tumors. The possibility of glioma-specific metastatic changes has been noted. Gliomas are extremely invasive cancers. About 20,000 new cases are diagnosed each year in the United States, and, despite various therapeutic regimens, life expectancy is at best two years. The basis for glioma invasiveness is not known. However, some gliomas produce a protein that binds to the major component of the extracellular matrix in the brain and, presumably, facilitates the movement of glioma cells through this structure. In other words, this protein lays down a path for glioma cells. Other glioma cells have a chloride ion channel not found in any other cell type, including normal glia cells. This glioma-specific ion channel may pump water out of the glioma cells, making them slender and able to pass readily through gaps in the extracellular matrix. It is not known whether these two changes occur together or whether each is sufficient for glioma invasiveness. In addition, there is no information about the genetic basis for these alterations.

Oncogenes

The convergence of cytogenetics of human leukemias, studies of retrovirus-induced cancers in rodents and other animals, and recombinant DNA technology created a foundation for understanding the molecular basis of cancer. From the beginning of the twentieth century, researchers were aware that chromosome abnormalities were common in cancer cells. However, the cause-or-effect issue concerning these changes was controversial. Some argued that both loss of chromosomes and structural alterations were the consequences of cancer. Others suggested that the changes were responsible for the cancer. The question was not resolved until the 1970s, when chromosome banding techniques were developed and chromosome preparations of human cancer cells could be examined routinely. Karyotype studies revealed that some cancers had the same chromosome aberrations. The most striking example was chronic myeloid leukemia (CML), where more than 90% of patients had a translocation between chromosomes 9 and 22 [t(9;22)(q34.1; q11.21)]. Before it was known that chromosome 22 was actually part of a translocation and not merely a truncated chromosome, it was called the Philadelphia (Ph′) chromosome. In other leukemias, specific translocations were also observed (Table 16.3), but not nearly to the extent of the Ph′ chromosome in CML patients. The association of specific chromosome abnormalities with specific cancers indicated a causal relationship. Otherwise, it would be expected that chromosome abnormalities would be distributed randomly among all types of cancers. However, at the time, no one knew which genes were disrupted or unregulated by the various translocations. In addition to translocations, cancer cells often have a large number of chromosome abnormalities with gains and losses of entire chromosomes or parts of chromosomes. Although genetic and chromosome modifications are prevalent in all tumors, it is not always known which changes are

Table 16.3 Chromosome translocations associated with human leukemias.

Leukemia	Chromosome translocation
Chronic myeloid leukemia	t(9;22)(q34.1;q11.21)
Chronic lymphocytic leukemia	t(11;14)(q13;q32)
Burkitt lymphoma	t(8;14)(q24.13;q32)
Acute nonlymphocytic leukemia	t(15;17)(q22;q11.2)
T-cell acute lymphocytic leukemia	t(7;19)(q35;p13)
Acute myeloid leukemia	t(8;21)(q22;q22)
Acute lymphocytic leukemia	t(9;11)(q21;q23)
Pre-B cell acute lymphocytic leukemia	t(1;19)(q23;p13.3)
Acute lymphoblastic leukemia	t(12;21)(p13;q22)
Acute promyelocytic leukemia	t(11;17)(q23;q21)

specific for certain cancers and which are merely the consequence of genomic instability. In this context, a group of tumor cells resembles a microevolutionary population, because, as genetic and chromosome mutations accumulate, the individual cells become different from one another. And, by chance, subsequent changes might occur that allow a tumor to pass from one stage to another.

For a number of years, researchers had known that a very large number of species-specific RNA viruses (retroviruses) cause cancers in rodents, cats, chickens, and other animals. By the 1970s, samples containing cancer-causing retroviruses were shown to comprise two different forms of the same virus. One viral type was able to proliferate and rarely caused cancer. The other type could not proliferate on its own (i.e., it was defective), and it frequently induced cancers. Molecular analysis indicated that a virus that could proliferate contained a complete set of retroviral genes. The genome of the defective form usually retained the ends of the retroviral genome but had an internal portion that was not equivalent to the viral genomic sequence. The sources and functions of these replacement sequences (inserts) that were different in different cancer-causing retroviruses became the focus of interest. Hybridization studies showed that the inserts were originally derived from the genomes of the host organisms and not from retroviral sequences. In addition, the inserts represented highly conserved sequences that were also present in humans. Additional work established that each insert originates from a messenger RNA molecule of the host cell. As part of the virus, these sequences, after infecting a host cell, either stimulate cell proliferation or prolong the life of the host cell. The presence of wild-type retrovirus is necessary to ensure that the defective virus genomes are perpetuated. Moreover, during many replication cycles through repeated infections, an insert in a retroviral genome accumulates mutations and diverges from the original sequence. These mutations make the protein encoded by an insert an exceptionally efficient inducer of cancer in a host cell.

As part of the normal life cycle of a retrovirus, the viral genome acts as a template for the production of a double-strand DNA version, which is incorporated into the genome of the host cell. When the DNA version of a defective retrovirus genome is integrated into a chromosome site of a cell, the protein encoded by the insert disrupts the constraints on cell proliferation and cancer develops. Parenthetically, the rare instances of induction of cancer by a wild-

type retrovirus result from the insertion of the viral genome into a site in the host cell genome that controls cell proliferation. Clearly, it was imperative to learn as much as possible about each human gene homolog of each retroviral insert sequence.

The highly diverged cancer-causing sequences in retroviral genomes were called v-oncogenes, and the host sequences that gave rise to v-oncogenes were designated proto-oncogenes. The specific names for v-oncogenes and, subsequently, proto-oncogenes are often three-letter abbreviations derived, in part, from the name of the retrovirus that carries a particular v-oncogene (Table 16.4). Later, when human cancers were observed with mutated proto-oncogenes, these alleles were called oncogenes.

In situ hybridizations were carried out with cloned v-oncogenes from various sources to pinpoint the chromosome sites of the corresponding human genes. It was of more than considerable interest when the locations of some of the human homologous genes of the v-oncogenes coincided with breakpoints of translocations found in various leukemias. For example, the v-*abl* oncogene identified the homologous human *ABL* gene at chromosome 9q34.1, which is at the site involved in the Ph′ chromosome. Thus it was likely that an alteration of the *ABL* gene contributed to CML. In this way, a number of v-oncogenes were used to determine which human genes might be involved in the formation of cancers. The focus of cancer research shifted from v-oncogenes to human oncogenes. As the research progressed, in addition to the v-oncogene human homologs, oncogenes were discovered that did not exist as retroviral v-oncogenes.

One strategy for identifying human oncogenes involves first testing whether DNA from a human cancer alters the growth characteristics of a mouse cell line in culture and, if so, then getting rid of extraneous human DNA before cloning the human DNA fragment with the oncogene. One of a number of differences between normal and cancer cells in culture is that normal cells stop

Table 16.4 Cancers caused by retroviral v-oncogenes.

Retrovirus	v-Oncogene	Origin	Cancer
Rous sarcoma virus	v-*src*	Chicken	Sarcoma
Feline sarcoma virus	v-*fes*	Cat	Sarcoma
Avian myeloblastosis virus	v-*myb*	Chicken	Myeloblastosis
Avian myeloblastosis virus MC29	v-*myc*	Chicken	Leukemia
Avian sarcoma virus Y73	v-*yes*	Chicken	Sarcoma
Gardner–Rasheed feline sarcoma virus	v-*fgr*	Cat	Sarcoma
Simian sarcoma virus	v-*sis*	Monkey	Sarcoma
Finkel, Biskis, and Jinkins murine osteosarcoma virus	v-*fos*	Mouse	Osteosarcoma
Avian erythroblastosis virus	v-*erbA*	Chicken	Erythroblastosis
Sloan–Kettering virus	v-*ski*	Chicken	Carcinoma
Abelson murine leukemia virus	v-*abl*	Mouse	Leukemia
Avian sarcoma virus UR2	v-*ros*	Chicken	Sarcoma
Moloney sarcoma virus	v-*mos*	Mouse	Sarcoma
Avian reticuloendotheliosis virus	v-*rel*	Turkey	Lymphoid leucosis
Harvey murine sarcoma virus	v-*Hras*	Rat	Sarcoma
Kirsten murine sarcoma virus	v-*Kras*	Rat	Sarcoma

growing after they form a monolayer on a petri plate, whereas cancer cells continue to grow on top of each other. Normal cells in culture that acquire cancer cell attributes are said to be transformed. If a mouse cell line is transformed by DNA from a human cancer, there is a strong likelihood that a cancer-causing version of a human oncogene was integrated into the mouse genome. In the next step, DNA is isolated from the transformed mouse cells and transfected into an untreated mouse cell line and a second round of transformed cells are selected. By repeating this process four or five times, the DNA segment carrying the oncogene is reduced to the point where it can be cloned into a vector. A library is constructed with DNA from the last transformed cell line. The clones with human DNA, which can be distinguished from clones with mouse DNA inserts by the presence of *Alu* sequences, are isolated and characterized. In this way, the oncogene from the original human cancer can be identified (Figure 16.6). Currently, more than 70 oncogenes have been identified as participating in cellular proliferation, cell division cycle, apoptosis, and other related cellular processes.

Alteration of Oncogenes in Cancer Cells

Different kinds of genomic changes can produce oncogenic effects. Some oncogenes have point mutations that alter the function of the encoded protein. In other cases, the activity of an oncogene can be affected by a translocation. In some instances, the rearrangement places an active promoter region or transcription regulatory element next to an oncogene. Consequently, the normal control mechanisms that constrain the oncogene are undermined, the oncogene is continually expressed, and the cell proliferation mode is established. For example, the t(8;14)(q24;32) translocation for Burkitt lymphoma places the *MYC* gene under the control of the very active promoter for the heavy immunoglobulin chain gene cluster. Thus the MYC protein is overproduced, and cell proliferation genes are expressed. In other cases, the breakpoints of a translocation occur within introns of two genes, and, after joining, a single reading frame is established with genetic information from both genes and a fusion (hybrid, chimeric) protein is synthesized. If such a fusion protein is under the control of an active promoter, then the oncogenic protein that is part of the fusion protein is synthesized. For example, the translocation represented by the Ph′ chromosome creates a chimeric gene that specifies segments from the *BCR* and *ABL* genes and is under the control of the *BCR* promoter. The *ABL* portion of the chimeric gene encodes tyrosine protein kinase. Thus, as part of the fusion protein, tyrosine protein kinase activity is continuously present, the signal transduction pathway is turned on, and cell proliferation persists.

Overproduction of oncogenic proteins often occurs when there is repeated replication (DNA amplification) of a specific chromosome region. The mechanism that brings about localized chromosome DNA amplification is not fully understood. When the amplification process generates a set of repeated DNA segments that are confined to a region of a chromosome, a distinctive repetitive chromosome banding pattern, called a homogeneous staining region (HSR) is produced. Alternatively, multiple DNA elements formed by repeated replication of a chromosome region are released into the nucleoplasm. After

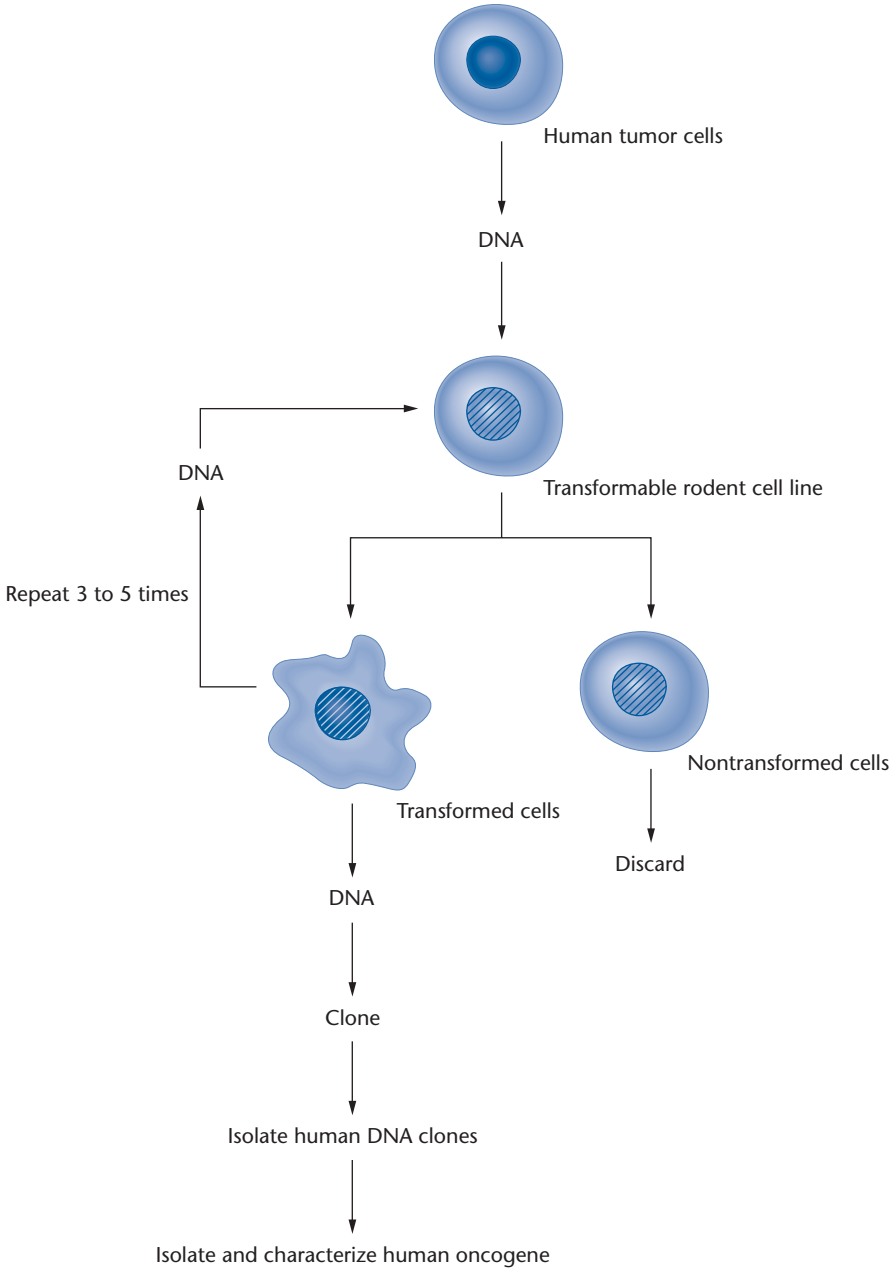


Figure 16.6 Strategy for isolating human oncogenes. DNA is extracted from a human tumor and introduced into cells of a rodent cell line that can be readily transformed in vitro. Transformed cells are identified and grown to ensure that the transformation is stable. Nontransformed cells are discarded. DNA is extracted from the transformed cells and introduced into nontransformed cells of the transformable cell line, and a second round of transformed cells is selected and grown. This selection process is repeated three or four more times, and then DNA is extracted from the last round of transformed cells and cloned into a vector. The clones containing human DNA are identified, and the inserts are characterized.

DNA staining, these extrachromosomal DNA components have a joined double-dot appearance (●●) and are called double minutes (DMs). A double minute is a pair of circular, 1-Mb DNA fragments. Because they do not have centromeres, DMs are randomly distributed to daughter cells. In human tumors, about 95% of cases of amplification are DMs and the rest are HSRs. In either instance, the end result is often an enormous increase in the number of copies of an oncogene and, as a result, the overproduction of an oncogenic protein. Amplification of an oncogene occurs in a variety of cancers (Table 16.5).

Table 16.5 Oncogene DNA amplification.

Cancer	Oncogene	Amplification*
Promyelocytic leukemia	*MYC*	20×
Small-cell lung carcinoma	*MYC*	5–30×
Neuroblastoma	*MYCN*	>100×
Acute myeloid leukemia	*MYB*	10×
Colon carcinoma	*MYB*	10×
Epidermal carcinoma	*EGFR*	30×
Lung cancer	*KRAS*	10×
Bladder carcinoma	*KRAS*	~10×
Mammary carcinoma	*NRAS*	~10×
Breast cancer	*ERRB2*	~10×

*Normal copy number is 1×.

Tumor Suppressor Genes

Genes specifying proteins that constrain uncontrolled cell proliferation and cell division cycles are called tumor suppressor genes (TSGs). Among the repertoire of oncogenic mutations in cancers, the inactivation of both alleles of a tumor suppressor gene is quite common. In many instances, an individual will inherit a TSG allele with an inactivating point mutation and then, in a somatic cell, the wild-type allele at the TSG locus is deleted. Such deletions are probably the consequence of faulty DNA replication or chromosome instability. The deletion of a wild-type allele in a heterozygous individual causes a loss of heterozygosity (LOH). Under these conditions, no functional TSG protein is produced, and the capability to prevent cancer formation is lost. In addition to LOH at the TSG locus, a chromosome deletion will remove some of the neighboring alleles. If any of these flanking alleles are polymorphic, then the likelihood is high that loss of heterozygosity will also occur at these loci. The detection of LOH of polymorphic alleles has led to the identification of more than 20 TSGs. These genes have diverse functions, and many are involved in hereditary forms of cancer, such as retinoblastoma, colorectal cancer, neurofibromatosis type 1, nevoid basal cell carcinoma, and renal cell carcinoma.

Generally, PCR-based short tandem repeat polymorphism (STRP) analysis of DNA from normal and cancerous cells of the same individual is used to detect LOH and localize it to a specific chromosome region. The rationale for this strategy is based on the likelihood that missing polymorphic alleles in cancer cells are closely linked to a TSG locus. Briefly, a large panel of STRP probes are used to screen DNA from normal and cancer cells of a large number of patients with the same cancer. The occurrences of losses of polymorphic marker loci are noted, and, based on STRP chromosome maps, the region with the smallest deletion is recognized (Figure 16.7). If this region does not contain a known TSG, then the STRP probes that delineate the smallest observed deletion are used to isolate clones from a cDNA or genomic DNA library. These clones then are analyzed for a TSG. Although the LOH phenomenon in cancer cells commonly entails a chromosome deletion, point mutations that inactivate the functional TSG allele have also been noted. And, in rare instances, deletions of both alleles of a TSG locus have been found in some cancer cells. Over all, cancer is a chance phenomenon that requires various mutations in a sus-

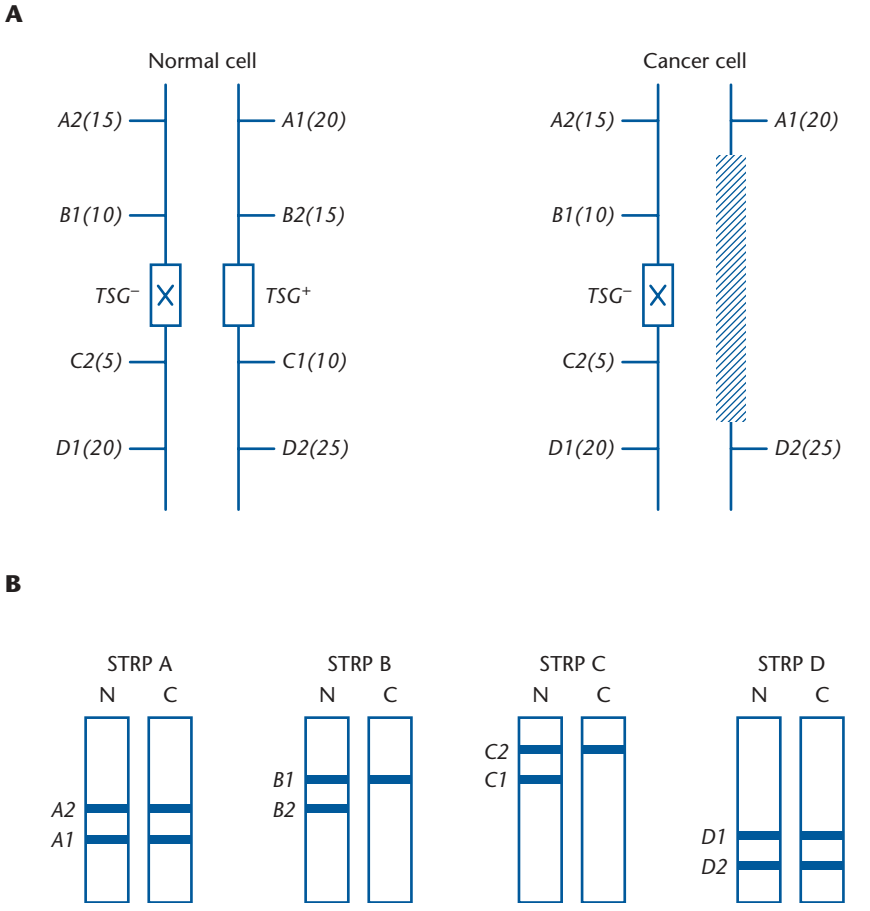


Figure 16.7 Localization of a tumor suppressor gene by detecting loss of heterozygosity. (A) Genotypes of a normal and a cancer cell from the same individual. The alleles of the short tandem repeat polymorphism loci are designated with an uppercase italicized letter and number, for example, *A1*, and the number in parenthesis, for example, (20), denotes the dinucleotide repeat length of a short tandem repeat polymorphic locus. The tumor suppressor gene (*TSG*) locus is heterozygous in the normal cell. The chromosome deletion in the cancer cell, which removes the TSG^+ allele and two flanking short tandem repeat polymorphism (STRP) alleles, is shown by the hatched region. (B) Schematic representation of the results of PCR-based STRP analysis of normal (N) and cancer (C) cell DNA with four separate sets of STRP primers (STRP A–D). The PCR banding patterns indicate loss of one of the alleles from each of the *B* and *C* loci and retention of both alleles of the *A* and *D* loci, which places the *TSG* locus between the *A* and *D* loci.

ceptible somatic cell. However, inheritance of a mutated oncogene increases the likelihood that a cancer will form and develop.

Neurofibromatoses

Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1, von Recklinghausen disease) is a very common autosomal dominant disorder with a high mutation rate of 1 in 10,000, affecting about 1 in 3500 people worldwide. Although penetrance is high, the phenotype is quite variable, even among members of the same family. More than 90% of NF1 patients have hundreds of lightly pigmented spots (café-au-lait spots), which are 15 mm or larger in diameter and scattered over the entire body; hundreds of benign skin tumors (neurofibromas) originating from peripheral nerve sheaths on the torso, legs, arms, and face; and tiny cell growths (hamartomas, Lisch nodules) on the surface of the iris. Curvature of the spine (scoliosis), enlarged head, short stature, inability to learn, disruption of brain stem functions, and weakening of the walls of blood vessels also occur to lesser extents. In about 30% of NF1 individuals, very large cell growths associated with interior nerves (plexiform neurofibromas) cause severe body deformations and facial disfigurement and often become malignant and frequently fatal.

Cancer of the adrenal medulla (pheochromocytoma) and malignant tumors of the CNS, spinal cord, and peripheral nerves occur in about 5% of those with NF1.

The cells chiefly affected by NF1 are derived from the neural crest. The neural crest, formed when CNS development is initiated during embryogenesis, is a series of paired clusters of cells that accumulate adjacent to the fused neural tube. Neural crest cells are migratory and, at their destinations, form sensory nerves, ganglia, the adrenal medulla, part of the adrenal glands, some Schwann and satellite cells of the PNS, cartilage cells of the face and skull, and all the pigment cells of the body, except those of the retina.

The age of onset of NF1 ranges from less than 10 years to 40 years or older. After onset, the severity of the clinical features increases with age. Generally, life expectancy is reduced as a result of complications caused by recurring benign tumors. If malignant tumors develop, the prognosis is poor.

Linkage studies with large numbers of NF1 families placed the *NF1* gene at or near 17q11.2. This conclusion was bolstered by cytogenetic observations. In two NF1 individuals with chromosome translocations, one of the breakpoints in each case was at 17q11.2. In addition, some NF1 patients had deletions that removed 17q11.2. Positional cloning, using the translocation breakpoints and a set of NF1-overlapping deletions, identified a conserved sequence in a nerve cDNA library. Mutation analysis, using primers from the putative NF1 cDNA, revealed mutations in some NF1 individuals. And, as confirmation, with the NF1 cDNA as a probe, altered NF1 transcripts were found in the NF1 patients who had chromosome 17 translocations.

The *NF1* gene is very large. It covers more than 350 kb of genomic DNA and has 60 exons. It encodes an mRNA of about 12 kb. Although probably not an important aspect of the NF1 phenotype, the *NF1* gene has a novel genetic feature. In intron 27b, which is about 50 kb in length, three functional genes are transcribed from the DNA strand that is not used as the template for the NF1 mRNA. The 2818-amino acid protein specified by the *NF1* gene was named neurofibromin and is found in the cytoplasm of most tissues, as well as being abundant in neurons, Schwann cells, and oligodendrocytes.

Because of its size, neurofibromin probably has multiple cellular functions. In one capacity, it acts as RAS-specific GTPase and converts active RAS (RAS-GTP) to the inactive form (RAS-GDP) (Figure 16.2). Most of the *NF1* gene mutations cause protein truncation or inactivate the protein. Therefore, if neurofibromin is either absent or at a reduced concentration in cells where it is the prevalent RAS-GTPase, then the amount of RAS-GTP increases, the cell proliferation signal transduction pathway is operative, and benign tumors form. In addition to negatively regulating a RAS pathway, neurofibromin mediates other signaling cascades in various cell types.

The *NF1* gene has all the features of a TSG. This conclusion is supported by various observations. First, both malignant and benign tumors in many NF1 individuals show a loss of heterozygosity at the 17q11.2 locus, and, in some instances, both *NF1* alleles are inactivated. Second, children with NF1 are 200 to 500 times more likely to develop malignant myeloid (bone marrow) disorders. Third, in some instances, astrocytoma and myelodysplasia cancer cells in non-NF1 patients have mutated *NF1* genes.

Neurofibromatosis Type 2

Neurofibromatosis Type 2 (NF2, central neurofibromatosis) is an autosomal dominant trait affecting about 1 in 40,000 people worldwide. The major clinical features are the formation of tumors of the CNS. The most common NF2 tumor is bilateral vestibular schwannoma (acoustic schwannoma, acoustic neuroma), which forms around both acoustic nerves (cranial nerve VIII, vestibulocochlear nerve) in the brain. A schwannoma is a tumor that originates from the sheath cells of neurons. The acoustic nerves emanate from the junction of the pons and medulla oblongata (pontomedullary junction) in both halves of the brain and proceed to each ear. In each of these nerves, one branch (vestibular division) innervates the balance control center of the ear and the other branch (cochlear division) innervates the part of the ear that receives and transmits auditory signals. In NF2 patients, schwannomas often compress each cochlear branch and cause deafness. In addition to bilateral vestibular schwannomas, tumors at other sites, such as meningiomas of the membrane layers (meninges) that encapsulate the brain and, occasionally, rare glia cell tumors (ependymomas) afflict NF2 patients. In about 50% of NF2 cases, the lenses become opaque, cataracts form, and vision is impaired. Generally, NF2 is either mild or severe. In the mild form (Gardner NF2), onset is after age 25, only slow-growing bilateral vestibular schwannomas develop, and survival is beyond 50 years of age. By contrast, with the severe form (Wishart NF2), onset is before the second decade, numerous aggressive cranial tumors including vestibular schwannomas form, and patients rarely live past 50 years of age.

Positional cloning was used to isolate the *NF2* gene, which had been localized to 22q12.2. This gene encodes a protein that links transmembrane proteins to the cytoskeleton. The type of mutation correlates with the extent of the NF2 phenotype. The phenotype is severe when mutations cause protein truncation. Alternatively, missense mutations are associated with the milder form. The NF2 protein was called schwannomin by one group of researchers and merlin by another. The designation merlin, an abbreviated form of moesin, ezrin, radixin-like protein, was chosen because the NF2 protein shares sequence similarity with members of the moesin, ezrin, radixin protein family, which links actin of the cytoskeleton to the plasma membrane. Schwannomin/merlin is found in cells of the CNS, lens, rod and cone cells, retinal pigment epithelium, and muscle cells.

Most of the *NF2* germline mutations produce a truncated protein, and the wild-type *NF2* allele is usually lost in tumors of NF2 patients. In other words, the *NF2* gene is a TSG. Loss of heterozygosity at the *NF2* locus is prevalent among schwannomas and meningiomas, although a double inactivation of *NF2* genes is occasionally observed in other kinds of cancers. The mode of action of schwannomin/merlin in NF2 is unresolved, although it may be part of a signaling pathway because it interacts with Rac1-GTPase and at least one receptor protein.

Breast Cancer

In developed countries, approximately 2% of the women who live to 50 years and approximately 10% of those who survive to 80 years will experience the

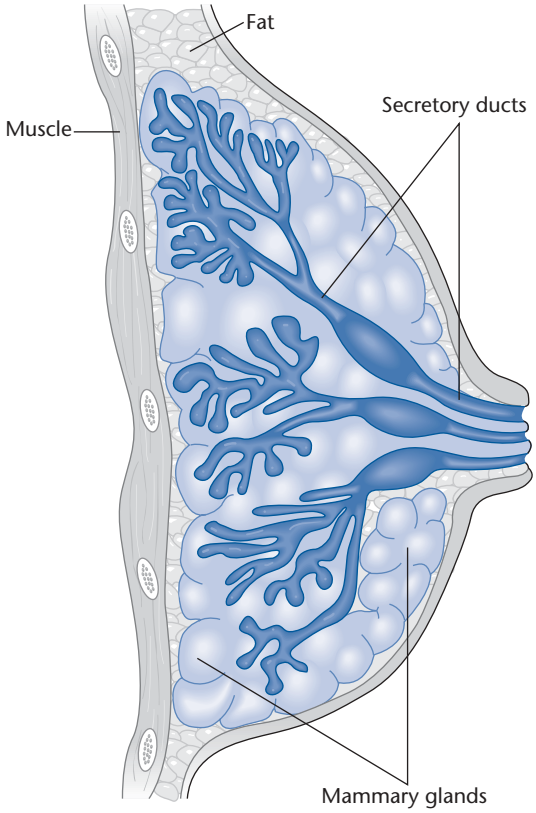


Figure 16.8 Sagittal section of the human female breast.

anguish of breast cancer. Despite early detection and improved treatments, many affected women will have breasts removed and about two-thirds will die within 10 years of diagnosis as a result of metastasis to the liver or other organs. Each year in the United States, about 180,000 new cases of breast cancer are diagnosed and 46,000 women die from the disease.

The human female breast is an enlarged secretory gland (Figure 16.8). During lactation, grapelike clusters of lobes (mammary glands) produce milk that fills and enlarges the multibranched duct system. About 90% of all breast cancers originate from the epithelial cells of the secretory ducts. Initially, breast tumors form within the ducts, although some cancer cells later may leave the inside of a duct and form cell masses on the outside of the duct epithelium. After metastasis of breast tumors, secondary sites may include the chest wall, lung, liver, brain, bone, and other locations. Half of all breast cancers are detected in the upward, lateral (outward) quadrant of the breast. Cancers of the lobes and the skin of the breast are rare.

Linkage studies using families with multiple cases (≥3) of early onset (≤40 years) autosomal dominant breast cancer indicated significant association with 17q21. The putative gene was called *BRCA1*, because it was the first (1) inherited breast cancer-predisposing gene to be localized to a chromosome region. After an intensive effort that included examining 65 potential candidate genes in the 17q21 region, a gene was identified as mutated in affected pedigree members but not in control samples. The *BRCA1* gene at 17q21.31 encodes a protein, BRCA1, of 1863 amino acids. A number of families with multiple

cases of early-onset breast cancer that did not show linkage with 17q21 were screened with other DNA probes, and linkage was discovered at chromosome 13q12–13. This second breast cancer gene at 13q13.1 was designated *BRCA2* and was isolated by positional cloning. Later, the *BRCA2* gene was found to be the same as one of the genes, *FANCD1*, that causes Fanconi anemia (FA). The major clinical feature of this rare, autosomal recessive disorder is the failure of development of all types of blood cells that originate from bone marrow cells (pancytopenia). The BRCA2/FANCD1 protein, which has 3418 amino acids, shares no significant sequence similarity with BRCA1 or any other known protein. This notwithstanding, the *BRCA1* and *BRCA2* genes are coregulated and BRCA1 and BRCA2 interact to facilitate the repair of DNA damage.

Extensive mutation screening studies have been conducted with PCR primers for the *BRCA1* and *BRCA2* genes. The majority of the mutations cause protein termination, suggesting that they are TSGs. Among families with early onset and multiple cases (≥4) of breast cancer, the *BRCA1* and *BRCA2* genes account for about 50% and 30%, respectively, of the gene mutations, which indicates that there are other genes for hereditary breast cancer. Mutations of the *BRCA1* and *BRCA2* genes are present in 80% and 14%, respectively, of families with individuals who have both breast and ovarian cancers. Male breast cancer occurs with *BRCA2* but not *BRCA1* mutations. When germline *BRCA2* mutations are associated with ovarian cancer, the mutated sites are confined to a region within exon 11 that has been named the ovarian cancer cluster region (OCCR). Comparable mutational clustering does not occur in the *BRCA1* gene. Interestingly, neither *BRCA1* nor *BRCA2* gene mutations are significantly represented in sporadic breast cancers. Overall, the *BRCA1* and *BRCA2* gene mutations account for less than 5% of all breast cancers.

Numerous studies have established that BRCA1 is multifunctional and interacts with many different proteins. Although specifics are lacking, in the main, BRCA1 responds to signals initiated by other proteins, and possibly itself, that detect DNA damage and, subsequently, up- and downregulates the transcription of a number of genes that block the cell cycle and implement DNA repair (Figure 16.9). In addition, BRCA1 directly interacts with DNA repair proteins as well as participating in different cellular pathways. By contrast, BRCA2 is principally involved with DNA repair. The absence of BRCA1 has at least a twofold impact. First, DNA damage including deletions accumulates with the increased probability that other TSGs will be affected. Second, control of the cell cycle is reduced. As a result, the development of cancer is highly likely. It is not known why cancer-causing *BRCA1* and *BRCA2* mutations predominantly affect breast and ovary cells.

Currently, there are no obvious oncogenes and TSGs that consistently contribute to sporadic breast cancers, although alterations of genes that encode growth factors, tyrosine protein receptors, nuclear factors, and cell division components have been reported. In addition, the possible oncogenes from the 20 or so different chromosome regions that are amplified in the genomes of sporadic breast cancer cells have not yet been identified.

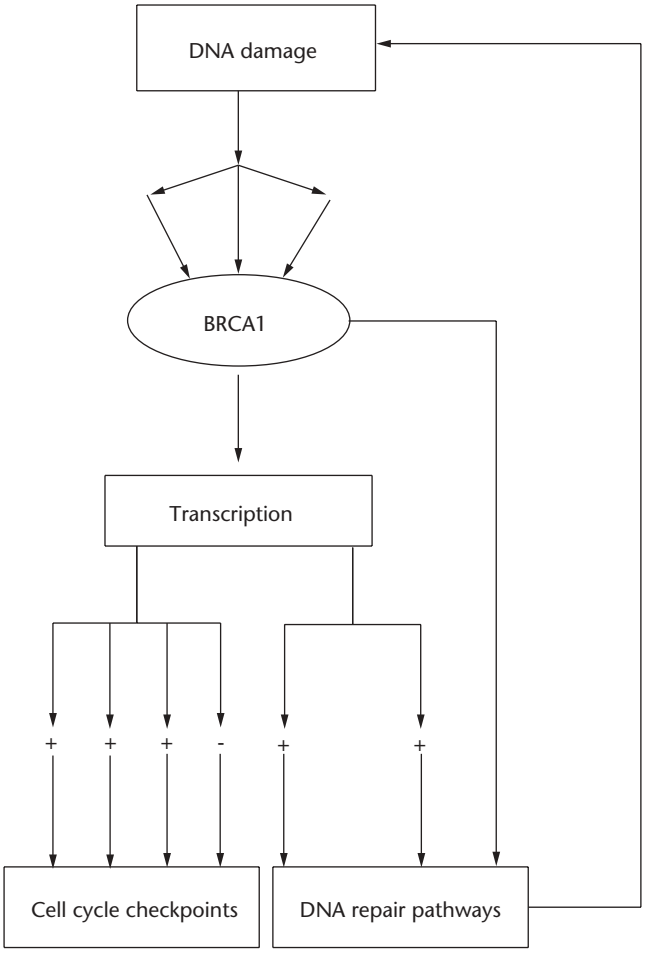


Figure 16.9 Schematic representation of the responses of BRCA1 to DNA damage. Proteins inform BRCA1 of DNA damage. BRCA1 responds by upregulating (+) and downregulating (–) the transcription of genes that encode cell cycle arrest and DNA repair proteins and by activating or combining with DNA repair proteins. The DNA repair proteins attend to the DNA damage.

Retinoblastoma

Inherited retinoblastoma is a rare malignant tumor of the retina of children who are younger than 7 years old. It is an autosomal dominant trait that affects about 1 in 20,000 children worldwide and accounts for about 0.35% of all childhood tumors. Both eyes develop tumors, and, without medical intervention, serious eye abnormalities develop. About 40% of all cases of retinoblastoma are hereditary. The genomic changes in retinoblastomas are the same for both hereditary and sporadic forms and invariably involve the loss of both alleles at the retinoblastoma (*RB1*) locus, which indicates that the *RB1* gene is a TSG. With hereditary retinoblastoma, individuals inherit one defective *RB1* gene, and, after mutation of the wild-type allele in retinal precursor cells, tumors develop at multiple sites in the retinas of both eyes. In sporadic cases of retinoblastoma, mutations that inactivate both *RB1* alleles occur independently when retinal precursor cells are proliferating or during retinal formation. As a consequence of the timing of the second *RB1* gene mutation in sporadic retinoblastoma, tumors often develop in only one eye. There is a loss of one of the *RB1* alleles in about 70% of all of the cases of retinoblastoma. Mutational

events at other loci such as 6p22, 1q31, and 16q22 are important for retinoblastoma formation. With the hereditary and sporadic forms of retinoblastoma, retinal tumors are, for the most part, the only cancers, although individuals with inherited retinoblastoma have an increased risk of osteosarcoma. Loss of activity of the *RB1* alleles has been observed in about 25% of the cell lines established from non-small-cell lung, bladder, pancreatic, and breast cancers in patients with no signs of retinoblastoma.

Linkage studies of families with retinoblastoma placed the *RB1* gene at chromosome 13q14.2. The RB1 protein is an important multifunctional nuclear protein that plays a key role in cell division, differentiation, apoptosis, and other cellular proesses. It interacts with more than 100 different proteins including members of the E2F family of cell proliferation-associated transcription factors. As noted in Figure 16.3, one of the activities of RB1 is regulating transcription factors that initiate the expression of genes required for cells to pass through the G_1 phase and into the S phase. Thus inactivation of RB1 dysregulates the cell division cycle. In addition, RB1 represses genes that encode proteins for DNA synthesis and apoptosis. On the other hand, RB1 enhances the transcription of some genes, while regulating its own transcription. It is not clear why retinal cells, more than any other cell type, are affected by the loss of activity of both *RB1* genes. Perhaps retinal cells depend solely on the RB1 protein to keep cell proliferation in check, whereas other cell types express additional genes that encode tumor suppressors.

Wilms Tumor

Wilms tumor (WT), which affects mainly the kidneys in 1 out of 10,000 children worldwide, accounts for about 8% of all childhood cancers. In addition to kidney tumors, developmental abnormalities of the gonads and some other organs are part of the WT phenotype. Hereditary WT is very rare and represents only 1% of all cases of WT. Linkage studies of WT families and LOH analyses of cell lines identified four possible WT chromosomal loci. Of these, the most prevalent WT gene, *WT1*, at 11p13 has been isolated, characterized, and extensively studied. In both hereditary and sporadic forms, mutations of *WT1* genes occur in kidney precursor cells during development and solid tumors usually form between 26 and 40 months after birth. Surgical removal of an early detected tumor mass is sufficient to cure most patients. However, metastasis occurs in 15% of children, and the chance of survival is greatly diminished.

The *WT1* gene, which covers about 50 kb and has 10 exons, is expressed at the onset and during the development of the kidneys and in a wide range of tissues and organs after birth, including the kidneys, muscles, testes, spleen, spinal cord, brain, ovaries, heart, and lungs. The only WT1 tumor of significance occurs in the kidneys, although the *WT1* gene may be involved in some ovarian cancers and leukemias. The WT1 protein (Wilms tumor suppressor 1) has four protein binding domains, that is, zinc finger (ZF) regions, at its C-terminus. Other sites are involved in the regulation of gene expression and protein-protein interactions. A large number of *WT1* gene mutations have been recorded throughout the gene. Of these, mutations that alter the second and

third zinc fingers (ZF2, ZF3) cause a severe form of WT called Denys–Drash syndrome. In these rare instances, children develop kidney tumors and often die as a result of the destruction of cells of the glomerulus.

In vitro, cell, and animal model studies indicate that the WT1 protein and its many isoforms either induce or repress the expression of 20 or more genes, many of which are involved in cell proliferation, differentiation, and apoptosis. For example, WT1 suppresses the transcription of the apoptosis gene *BCL2*, the oncogenes *MYB* and *MYC*, and the growth factor gene *IGF2*. The IGF2 (insulin-like growth factor II) protein stimulates the proliferation of the cells that synthesize and release IGF2, that is, autocrine growth stimulation. Because the *IGF2* gene is overexpressed in WT tumor cells, the continuous production of IGF2 likely causes these cells to divide continuously. In addition, WT1 is associated with a complex that removes introns during the processing of primary transcripts to messenger RNA. Among other roles, WT1 is required for the development of the kidney, retina, and early gonads. All in all, the WT1 protein is a multifaceted transcription regulator, and how it impacts specifically on kidney cells has not been elucidated.

Multiple Endocrine Neoplasia Type 1

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant cancer syndrome that features tumors of the parathyroid, pituitary, and the islet cells of the pancreas. These tissues are part of the endocrine system, which produces and secretes various hormones. In response to different hormones, target cells control homeostatic processes, growth, development, and stress. Specifically, with regard to the sites of the MEN1 tumors, the parathyroid glands control levels of calcium and phosphate; the pituitary glands regulate metabolic activities of other endocrine glands and growth; and the pancreatic islet cells maintain blood glucose concentration. Depending on the site and the extent of the MEN1 tumors, symptoms may include excess calcium in the bloodstream (hypercalcemia), which causes kidney stone formation; low blood sugar concentration (hypoglycemia), resulting in dizziness, confusion, weight loss, and glucose intolerance; and disruption of the pituitary gland, accompanied by amenorrhea, impotence, and infertility. In addition, tumors of the gastrointestinal tract (gastrinomas) secrete excess gastrin (hyperacidity), and, consequently, peptic ulcers are observed commonly as part of the MEN1 phenotype. Infrequently, surplus somatotropin from pituitary gland cancer cells causes continual enlarging of the head, face, hands, and feet (acromegaly). MEN1 tumors are usually benign and form glandlike structures (adenomas) that compress adjacent tissues. When malignancy occurs, there is infiltration of both neighboring and distant tissues by hormone-producing cancer cells. There are no effective therapies for MEN1 tumors.

The incidence of MEN1 is about 1 in 50,000. With the hereditary form, tumors occur at various sites by 50 years of age. The *MEN1* gene was identified by mutation analyses after linkage studies placed it at 11p13.1. The encoded 610-amino acid protein was designated menin. Most *MEN1* germline mutations are truncating, with loss of the second allele a common occurrence in MEN1 tumor cells. *MEN1* mutations, including loss of heterozygosity, are

often present in nonfamilial MEN1 tumors. Thus *MEN1* has the features of a TSG.

Menin is a nuclear protein that inhibits a number of transcription factors that are involved with the cell division cycle, maintaining genomic stability, differentiation, and apoptosis. In addition, when menin combines with another protein (nm23) the complex has GTPase activity. Despite the large number of its known protein interactions, menin's molecular activities have not been fully elucidated.

Multiple Endocrine Neoplasia Type 2

The bilobate thyroid gland is located on the anterior surface of the trachea at the junction of the larynx and secretes a number of hormones essential for growth and development. Each lobe has many hollow spherical clusters (follicles) that are circumscribed by a single layer of cells. Parafollicular cells (C cells) occupy the spaces between follicles. The follicular cells synthesize and secrete several hormones, including thyroxine and triiodothyronine, which regulate metabolic rate and maintain the responsiveness of the cardiovascular system to nerve impulses. The parafollicular cells manufacture and release calcitonin, which regulates the concentration of calcium ions in the blood and other body fluids. Calcitonin decreases calcium ion concentrations, stimulates the production of bone, reduces the absorption of Ca^{2+} by the intestine, and stimulates the excretion of Ca^{2+} by the kidneys.

The incidence of both benign and malignant thyroid gland cancers is about 1 in 20,000 individuals worldwide. Cancers of the follicular and parafollicular cells are distinct biological entities. However, much research has focused on cancer of the parafollicular cells. Specifically, medullary thyroid cancer (MTC) is a parafollicular cell cancer with both autosomal dominant and sporadic forms. MTC usually is benign and treatable if detected early. The hereditary form of MTC is part of a cancer syndrome designated as multiple endocrine neoplasia type 2 (MEN2). Based on combinations of tumors in various MEN2 families, three MEN2 subtypes have been identified. In the first MEN2 subtype, the only cancer is MTC, and the subtype is called familial medullary thyroid cancer (FMTC). The second MEN2 subtype has three prevalent cancers and has been designated MEN2A. The types of MEN2A cancers are MTC, parathyroid gland cancer (parathyroid hyperplasia), and adrenal medulla cancer (pheochromocytoma), which are present in about 95%, 50%, and 30%, respectively, of MEN2A patients. Parathyroid hyperplasia effects the parathyroid glands, located on the posterior surface of the thyroid gland, that produce parathormone. This hormone increases calcium ion concentration in the blood, decreases blood phosphate concentration, stimulates the breakdown of bone, and, if the level of Ca^{2+} in the blood is abnormal, reduces the excretion of Ca^{2+} by the kidneys. The third MEN2 subtype is MEN2B and is rarely encountered. MEN2B individuals usually have MTC, pheochromocytoma, abnormal proliferation of ganglion cells of the intestinal tract (ganglioneuromas), and tumors (neuromas) of the lips, tongue, and the mucous membrane of the anterior surface of the eye. Parathyroid hyperplasia is not a feature of MEN2B. Onset of MEN2B is soon after birth, and the development of the tumors is

very rapid. By contrast, FMTC is only evident 40 to 50 years after birth, with diarrhea as the most common symptom. With the MEN2A subtype, the different cancers appear when individuals are between 20 and 30 years old. Patients with the three MEN2A cancers suffer from diarrhea as a result of MTC, both irritability and hypertension caused by pheochromocytoma, and kidney stones from parathyroid hyperplasia.

Linkage studies with MEN2 families, regardless of subtype, localized the putative gene to chromosome 10q11.2. Thus the three MEN2 subtypes probably represented mutations of the same gene or three closely linked genes. Previously, the *RET* (rearrangement during transfection) oncogene that encodes a tyrosine protein kinase receptor had also been mapped to 10q11.2, actually 10q11.21, and provided a likely candidate gene for the MEN2 cancers. The *RET* gene is expressed in kidney cells and both the central and peripheral nervous systems. A protein complex that includes the glial cell line-derived neurotrophic factor (GDNF) interacts with the RET protein to initiate a signal transduction pathway.

In addition, at about the same time that the MEN2-associated locus was discovered, the gene for an autosomal dominant condition called Hirschsprung disease (aganglionic megacolon), which affects about 1 in 5000 individuals, was mapped to chromosome 10q11.2. The principal phenotype of this noncancerous disorder is the absence of nerve cells (intrinsic ganglion cells) from a section of the terminal portion of the colon. Because there is no peristaltic movement in an aganglionic segment of a colon, waste material accumulates. In mild forms of Hirschsprung disease, a short segment of the colon is aganglionic, and constipation occurs. In severe cases, where a long segment of the colon lacks nerve cells, there may be complete colonic obstruction and abdominal distension. Often, with extreme colonic blockage, many small fissures develop and become infected. If untreated, the severe form is fatal when the colon ruptures. However, surgical removal of the aganglionic portion of the colon readily alleviates the condition. Interestingly, the primary phenotypic feature of Hirschsprung disease appears to be the converse of one of the sentinel features of the MEN2B subtype. With MEN2B, intestinal ganglionic cells proliferate and form enteric ganglioneuromas, whereas with Hirschsprung disease, a portion of the colon has no ganglionic cells.

Mutation analyses established that the *RET* gene was mutated in families with Hirschsprung disease, as well as in families with MEN2, regardless of the subtype. Therefore, a single gene is responsible for four different phenotypes. In a large study, more than 92% of 477 MEN2 families had a missense mutation in one of eight sites in the *RET* gene. The MEN2A mutations changed cysteine codons at sites 609, 611, 618, 620, 630, and 634. The amino acids encoded by these sites are near the transmembrane domain in the extracellular portion of the RET protein (Figure 16.10). Of these MEN2A mutations, which probably keep the protein tyrosine kinase activity turned on by abolishing the need for the receptor-specific factor, more than 85% are at codon 634, and, of these, 52% are C634R. More than 95% of the MEN2B mutations occur at codon 918 (M918T) within the tyrosine kinase domain of the RET protein. The *RET* mutations among the FMTC families are similar to those found in MEN2A individuals and include missense mutations at codons 618, 620, and

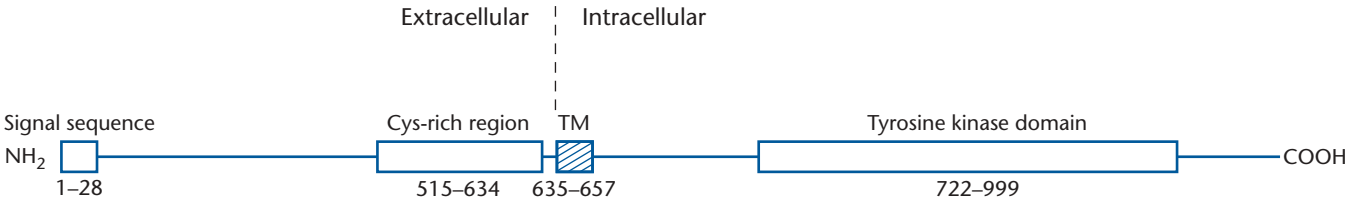


Figure 16.10 Schematic representation of the domains of the RET protein. Cys-rich and TM denote cysteine-rich and transmembrane domains, respectively. The numbers beneath the RET protein domains correspond amino acid sites. The first amino acid of the Cys-rich domain (515) is approximated. The domains are not drawn to scale.

634. However, the change at codon 634 is often C634Y and not C634R. Additional FMTC mutations have been observed at codons 768 (E768D) and 804 (V804L), which fall within the tyrosine kinase domain of the RET protein. Tumor cell genomes in about 45% of cases of sporadic MTC and 15% of cases of pheochromocytoma have *RET* missense mutations. Sporadic parathyroid adenomas do not have *RET* mutations. And loss of heterozygosity at the *RET* locus in thyroid cancers and other cancers is extremely rare. In contrast to the limited number of mutated sites in the *RET* gene among the different hereditary MEN2 subtypes, the *RET* mutations for Hirschsprung disease are found in various exons throughout the coding region and include both truncating and missense mutations.

The *RET* mutations of the MEN2 subtypes cause factor-independent protein tyrosine kinase activity that is not inactivated by normal cellular mechanisms. This type of change impacts on the cell types that express the *RET* gene by keeping signal transduction pathways turned on and thereby promoting cell proliferation. By contrast, the mutations of the *RET* gene that cause Hirschsprung disease are loss-of-function mutations that probably do not affect directly the signal transduction pathway capability of the RET protein but instead cause a deficiency in the amount of the RET protein needed for another purpose. Possibly, this haploinsufficiency prevents the neural crest cells that form enteric ganglia during embryogenesis from reaching their final destination, and, consequently, the terminal portion of the colon is not innervated properly. The association of one gene with four syndromes is quite unusual but not totally unexpected if a gene encodes a protein with diverse functions that are implemented at different times during the life cycle and in disparate cell types.

Li–Fraumeni Syndrome

After examining medical records and death certificates of a large number of children with malignant skeletal muscle cancer (rhabdomyosarcoma), Frederick P. Li (b. 1940) and Joseph F. Fraumeni, Jr. (b. 1933) identified five families with a variety of early-onset cancers in successive generations. Since the original report in 1969, many families with a pattern of early-onset cancers have been observed and studied (Figure 16.11). Subsequently, this rare autosomal dominant cancer disorder was called Li–Fraumeni syndrome (LFS). There are three main criteria for a diagnosis of LFS. The first requirement is an individual with a sarcoma before 45 years of age who was the initial contact for the assembly of his/her pedigree. The first member of a pedigree to be diagnosed with the inherited trait is designated as the proband (index case). The second feature is that the proband must have a first-degree relative (mother,

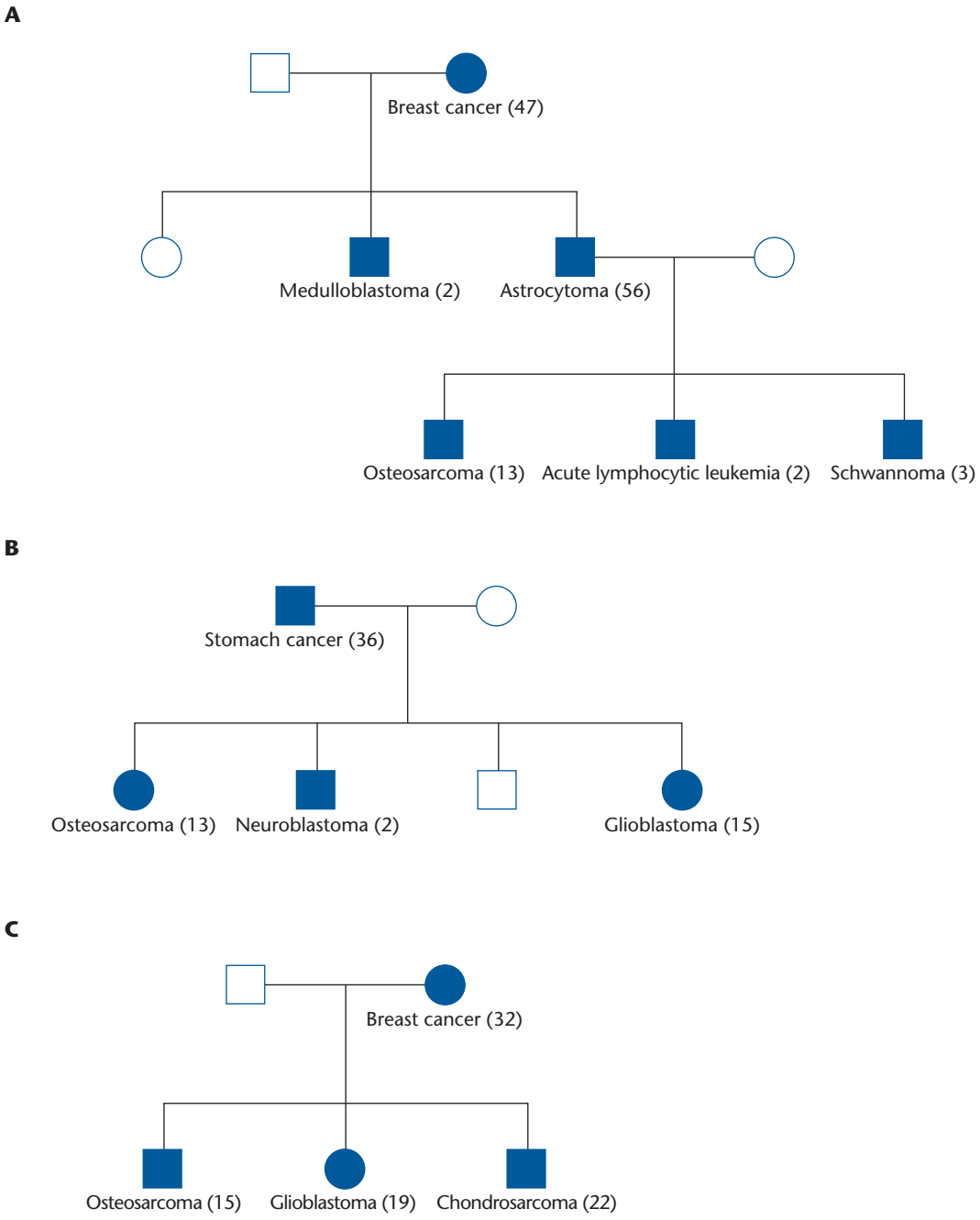


Figure 16.11 Pedigrees with Li–Fraumeni syndrome. Solid symbols represent affected individuals. The specific cancer is named below each solid symbol. The numbers in parentheses denote the age at which the cancer was diagnosed.

father, son, or daughter) with a cancer before 45 years of age. Finally, the proband must also have either another first-degree relative or a second-degree relative (grandmother, grandfather, sister, brother, granddaughter, or grandson) with either any type of cancer before 45 years of age or a sarcoma at any age. Breast carcinoma, soft tissue sarcoma, brain tumor, osteosarcoma, leukemia, and adrenocortical cancer often are associated with LFS.

Initially, the genetic basis of LFS was difficult to establish. Cytogenetic studies did not reveal any common chromosome abnormality. Linkage studies

were not feasible, because the condition is rare, families are small, and mortality is high. However, as oncogenes and TSGs were cloned, the candidate gene strategy could be implemented. In this context, because of the wide spectrum of cancers, it was postulated that the likely germline mutation for LFS would be a TSG. Of the possible candidates available for mutation analyses in 1990, both *RB1* and *WT1* were considered unlikely, because retinoblastomas and childhood kidney tumors do not occur in LFS families. The only other tumor suppressor gene that had been characterized at that time was *TP53*. This gene encodes the nuclear protein p53 and, as later research established, plays ubiquitous and important roles in regulating cell proliferation, growth arrest, apoptosis, and DNA repair.

Mutation analysis of the *TP53* gene revealed germline mutations in many, but not all, of the LFS families. Those LFS families without *TP53* mutations could have mutations in the regulatory regions preceding or following the *TP53* coding sequence, in other genes that encode proteins that interact with p53, or in other gene products that are independent of p53 but also behave as tumor suppressors. Consistent with its action as a TSG, loss of heterozygosity of the *TP53* locus at 17p13.1 is very common in the DNA of sporadic cancers. Similarly, the wild-type *TP53* gene is often missing in the DNA of cancers from LFS family members with *TP53* germline mutations. Moreover, germline *TP53* mutations have also been found in families with various cancers that do not meet the criteria for LFS. Clearly, the presence of a germline *TP53* mutation increases the risk of cancer in a number of different tissues. Overall, about 50% of all cancer cells do not have an active *TP53* gene.

The most common *TP53* germline mutations and many of those found in sporadic cancers occur within codons 175, 248, 273, and 282 of the p53 DNA binding region (Figure 16.12). Each of these mutations abolishes the p53 binding to target DNA sequences. Specifically. the mutations at codons 248 and 273 are unable to interact with the minor and major DNA grooves, respectively, whereas changes at the two other codons alter the conformation of the DNA binding region and prevent attachment to DNA.

Colorectal Cancer

Each year about 57,000 people in the United States die from complications caused by colorectal cancer (CRC) and approximately 147,000 new cases are diagnosed. Moreover, at least 1 in 2 people worldwide will develop a benign colorectal tumor by the age of 70. In 10% of these cases, malignancy will develop and the cancer will spread to other sites. Colorectal cancer need not be life threatening if it is detected early and the tumor(s) is removed surgically. The initial signs of CRC are blood in the stool, diarrhea or constipation, and a persistent dull abdominal pain.

The walls of the colon and rectum are multilayered. The highly folded cell layer lining the lumen is composed of different types of glandular epithelial

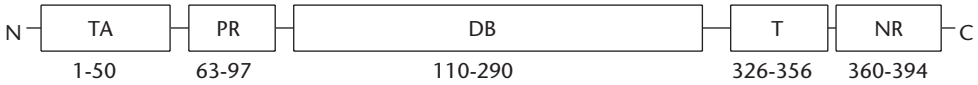


Figure 16.12 Schematic representation of p53. The functional domains are transcriptional activation (TA), proline-rich (PR), DNA-binding (DB), tetramerization (T), and negative regulation (NR). N and C mark the amino- and carboxyl-terminal ends, respectively. The amino acid sites for the domains are numbered.

cells with secretory, receptor, and absorptive functions. Cells at the tips of the folds are continually being replaced by cells produced in the deep parts (crypts) of the folds. The epithelial layer is supported by a muscle layer. The other layers beneath the muscle layer include circular muscles and regions containing nerves that relay signals to the gland cells of the epithelial layer. Most colorectal cancers are derived from cells of the glandular epithelium.

Although it is difficult to track the development of all colorectal cancers, many probably begin as localized outcroppings of epithelial cells (polyps). A polyp that originates from a glandular epithelial cell is called an adenomatous polyp (adenoma). Polyps may have different colors and shapes. For example, fingerlike, villous polyps extend from the colonic epithelium into the lumen and are attached by a fibrous stalk to the muscle layer that lies beneath the epithelial layer. About 40% of villous polyps proceed to malignancy. As mutations occur, a polyp (an early adenoma) grows by cell proliferation into an advanced adenoma. With additional mutational events, it forms a large mass of growing cells (high-grade dysplasia), which eventually becomes a carcinoma that spreads through the entire wall of the colon. In addition, at the carcinoma stage, some cells metastasize and either invade other nearby tissues directly or are carried to other sites by the lymphatic system. Generally, an adenomatous polyp has a 5% chance of forming a colorectal cancer. The transition from early adenoma to carcinoma takes about five years.

Much of the understanding of the molecular basis of CRC is grounded on studies of the action of the genes that contribute to two hereditary forms. Specifically, familial adenomatosis polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) have been studied in detail. Overall, FAP and HNPCC account for less than 1% and about 10% of all colorectal cancers, respectively.

Familial Adenomatous Polyposis

Familial adenomatous polyposis (FAP) is an almost completely penetrant autosomal dominant trait that affects about 1 in 8000 individuals. The major clinical feature is the development of hundreds to thousands of colonic polyps. By 40 years of age, most FAP patients usually have malignant colorectal carcinoma, although the onset of CRC can occur in adults more than 70 years of age or in young children. FAP cancers tend to be localized in the terminal portion of the colon (sigmoid colon) and the part of the rectum adjacent to the colon (Figure 16.13). Excessive polyposis causes obstruction of the lower bowel, bloody diarrhea, weight loss, and an inability to assimilate food. In the absence of treatment, there is about a 95% probability that CRC will develop in FAP individuals. In these cases, the disease is often fatal before individuals reach 50 years of age. In addition to polyps and CRC, the FAP phenotype may include localized proliferation of cells of the retinal pigment epithelium (congenital hypertrophy of the retinal pigment epithelium, CHRPE), formation of large abdominal tumors as a result of proliferation of fibroblasts (desmoid tumors), and cancers of the upper gastrointestinal tract, brain, jaw, liver, and bones. Often, notwithstanding treatment for CRC, cancers of the upper gastrointestinal tract or at other sites are fatal. There is a high degree of phenotypic variability among affected members of the same FAP family.

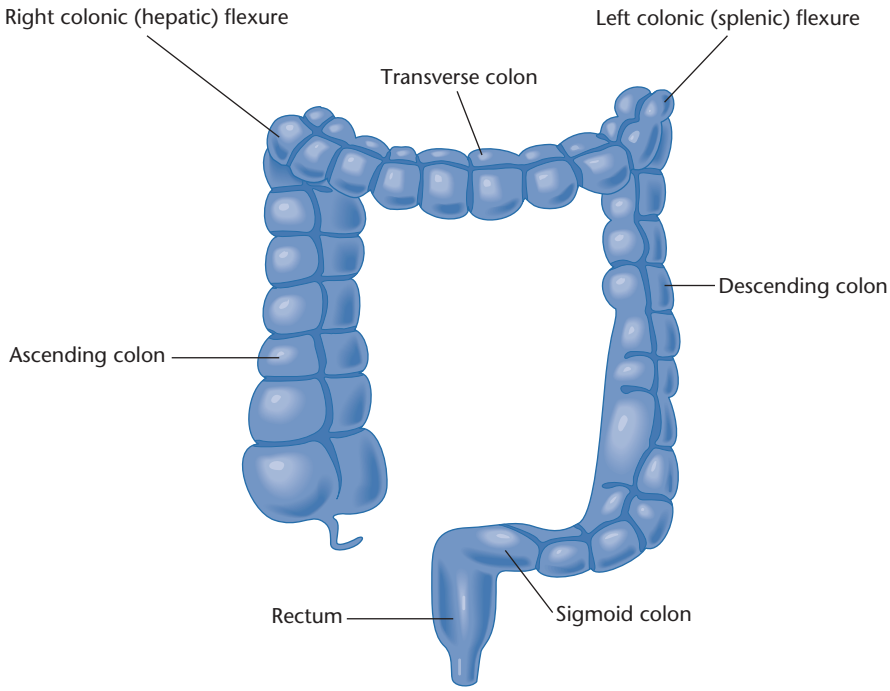


Figure 16.13 Schematic representation of the human colon. The small intestine is not shown.

After an FAP patient was observed with an interstitial deletion on chromosome 5q, the search for the gene focused on this chromosomal region. A series of linkage studies eventually localized the gene to chromosome 5q21, actually 5q22.2. Mutation analyses established the gene for FAP, which was designated *APC* for adenomatous polyposis coli, where "coli" means "of the colon." The APC protein has 2843 amino acids. Exon 15 of the *APC* gene is a bit of a curiosity because it has 6577 bp, which makes it the largest known exon. By comparison, most exons are from approximately 100 to 200 bp in length.

More than 96% of all *APC* mutations cause protein truncation, and more than 85% of FAP patients carry a germline *APC* mutation. Also, the *APC* gene is frequently mutated in sporadic cases of CRC. Mutation analysis of CRC DNA in FAP patients revealed that the wild-type *APC* allele was lost in almost all instances. Extensive mutation screening of the DNA of cells at different stages of the formation of CRC in FAP patients and in sporadic CRC cases indicated that the development of CRC requires mutations of a number of different genes. Although there is no unique sequential order of gene mutations leading to CRC, much of the evidence indicates that inactivation of APC activity is necessary for adenoma formation and that mutations of various genes, including *KRAS*, *SMAD4*, and *TP53* are important, but not exclusively required, for the transition from early to advanced adenoma. The switch to high-grade dysplasia requires the complete loss of p53 activity and mutations of other genes. Moreover, CRC may result from combinations of gene mutations that, eventually, produce the same cancer phenotype. Finally, additional gene mutations are necessary for the cancer to spread and occupy other sites.

The APC protein is found in both the cytoplasm and the nuclei of many cell types, including colon cells and neurons. It is a multifunctional protein that may be involved in cell adhesion, progression of cells through the cell cycle, cell migration, cytoskeletal functions, regulation of transcription, and neuronal activities. In addition, APC binds a number of different proteins, including β-catenin, which, among its different functions, forms part of the cell adhesion system. Although the relationship between APC and the formation of CRC is not fully understood, evidence is accumulating that the wild-type APC indirectly suppresses the transcription of the *MYC* oncogene. The downregulation of the *MYC* gene curtails the transcription of a number of cell proliferation genes activated by the MYC protein. As part of this regulatory system, the binding of APC and other proteins to β-catenin selectively targets β-catenin for degradation. As a result, there are few free β-catenin molecules in the cytoplasm available to bind to the transcription factor TCF4. Ordinarily, β-catenin-TCF4 complexes enter the nucleus and promote transcription of the *MYC* gene. Thus, under normal conditions, APC maintains the expression of the *MYC* gene at a low level. By contrast, when there is no APC activity, β-catenin molecules accumulate and there is an increased amount of the β-catenin-TCF4 complex. Consequently, the *MYC* gene is overexpressed, a number of genes involved in the cell division cycle are activated, colonic epithelial cells proliferate, and adenomas form. Interestingly, about 50% of the individuals with wild-type *APC* genes and colorectal adenomas have mutated β-catenin genes (*CTNNB1*, 3p22.1), In these cases, β-catenin is less susceptible to proteolysis, whereas the ability to form active transcription complexes with TCF4 is not affected. In other words, these *CTNNB1* mutations produce the same effect as FAP *APC* mutations.

In addition to forming a complex with β-catenin and other proteins (destruction complex), APC binds to kinetochores during mitosis. Spindle fibers do not connect efficiently to kinetochores without APC, which leads to chromosome loss (chromosome instability, CIN). In fact, aneuploidy is 10 to 100 times greater in APC-negative cells than in normal cells. Thus, with regard to CRC, *APC* mutations have a double impact. First, regulation of cell proliferation is impaired and cells divide continously. Second, there is a high probability that chromosomes with tumor and malignancy suppressor genes will be lost.

Mutations of the *MUTYH* gene at 1p34.1 in the presence of wild-type *APC* genes are responsible for a form of autosomal recessive colorectal adenomatosis polyposis. The MUTYH protein is a DNA glycosylase that removes adenine residues that are mispaired with reactive oxygen-modified 8-hydroxyguanosine in DNA. The specific relationship between diminished MUTYH activity that increases the frequency of GC→TA transversions and CRC has not been established.

Hereditary Nonpolyposis Colorectal Cancer

Hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome), which affects about 1 in 200 individuals, is the most common inherited cancer syndrome. It behaves as an autosomal dominant trait. The phenotype is characterized by few colonic polyps (<100) and early onset of multiple tumors in the

from the HUMAN GENETICS files

Philadelphia Chromosome, Two-Hit Hypothesis, and Comparative Genomic Hybridization

In the early 1970s, when chromosome banding was a newly developed technique, Janet Rowley (b.1925) demonstrated the first consistent association between a chromosome translocation [t(8;21)(q22;q22)] and a cancer (acute myeloblastic leukemia, AML). Shortly afterward, she solved the mystery of the Philadelphia chromosome (Ph′). Since 1960, the Philadelphia chromosome, named after the city where it was first discovered, had been observed consistently in cases of chronic myeloid leukemia (CML). On the basis of whole chromosome staining, the Philadelphia chromosome appeared to consist of half of either chromosome 21 or 22. At this level of resolution, there was no evidence that it was part of a translocation. Rowley found that the Philadelphia chromosome was actually a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)]. After finding a third example of a specific association of a translocation [t(15;17)(q22;q11.2)] with a cancer (acute promyelocytic leukemia, APL), Rowley was convinced that these chromosome abnormalities were not coincidental and that chromosomal changes in leukemias, and probably other cancers, did not arise after the cancer was formed but instead contributed to its onset. As she has said, ". . . Chromosome changes were an essential component of the leukemogenic process, and I soon became a 'missionary,' attending hematology meetings in the 1970s and early 1980s carrying the 'gospel' that chromosome abnormalities were an essential component of hematologic malignant diseases to which the clinical community should pay attention."

As more correlations between chromosome abnormalities and specific cancers accumulated, it became clear that Rowley was right. Her work focused the attention of cancer researchers on chromosomes and raised provocative questions about the genes that were disrupted by translocations in the various cancers. By 1982, with the development of recombinant DNA technology, the t(8;14)(q24;q32) breakpoint was cloned. This translocation is associated with both acute lymphocyte leukemia and Burkitt lymphoma. Within a few years, the genes involved in the t(9;22) translocation and other cancer-specific chromosome abnormalities were isolated. Once these genes were available for study, it became clear that the translocations in leukemias and other cancers activate genes by either putting them under the control of the expression elements of other genes or creating fusion proteins as a result of the joining of two reading frames from different genes. Not only did this work establish the molecular basis of cancer, but it facilitated the development of new diagnostic and management protocols.

As Rowley was painstakingly matching chromosome bands, Alfred Knudson (b. 1922) was delving into the relationship, if any, between hereditary and nonhereditary forms of the same cancer. Specifically, he examined the features, including onset, of bilateral (hereditary) and unilateral (nonhereditary) retinoblastoma (RB). He concluded that both forms of RB were related and postulated that both were caused by two independent, successive mutation events, that is, two hits. In cases of hereditary RB, one of the mutations preexists in the germline cells and is passed on to all somatic cells during embryogenesis. The second hit occurs, by chance, in a somatic cell. Under these circumstances, one would expect that cells in both retinas likely would have two hits and bilateral tumors would develop. By contrast, in nonhereditary RB, the two mutations occur independently in the same somatic cell. In this case, it is highly unlikely that cells in both retinas would accumulate two hits, and, therefore, unilateral tumor formation is expected. Although there were other possibilities, Knudson opted for the two-hit hypothesis, because it was the simplest interpretation of his observations. The loss of activity in two alleles of a gene raised the possibility that a wild-type gene product could prevent tumor formation. Knudson called such a gene an "anti-oncogene." This term has been replaced by "tumor suppressor gene."

The two-hit hypothesis was supported when chromosome G-banding analyses showed a deletion at 13q14 in the tumor cells of many patients with hereditary RB. After the retinoblastoma gene (*RB1*) was isolated and characterized, mutational screening established conclusively that both *RB1* alleles were mutated in retinoblastoma tumor DNA. Knudson's insights formed the conceptual framework for understanding tumor suppressor genes.

Cancer cytogenetic studies have shown that chromosome abnormalities are among the hallmarks of cancer cells. In addition to losses or gains of whole chromosomes (aneuploidy) and translocations, more subtle changes can lead to the loss and/or gain of chromosome subregions. A number of methods have been developed to detect gain and loss of interstitial chromosome regions. Of these, a two-color FISH procedure called comparative genomic hybridization (CGH) has been quite effective. Briefly, for CGH, total genomic DNA is extracted from a tumor and labeled with a green fluorescent dye. DNA from normal cells is labeled with a red fluorescent dye. The two DNA samples are combined and hybridized, along with an unlabeled sample of highly repetitive DNA, to normal human metaphase spreads from noncancerous cells. The unlabeled repetitive DNA is included in the hybridization mixture to prevent labeled repetitive sequences in the DNA samples from binding to chromosome DNA. Consequently, only labeled single-copy DNA sequences hybridize to chromosome DNA.

If a chromosome region has the same copy number in both of the labeled DNA samples, that is, in cancer and noncancer cells, then after hybridization the fluorescence will be a blend of green and red fluorescent dyes. If DNA is lost from a chromosome subregion in tumor cells, then the comparable regions of the hybridized chromosomes will fluoresce

with more red than green dye. Finally, if a chromosome region is amplified in tumor cells, then after hybridization the corresponding chromosome regions will fluoresce with more green than red dye. Comparative genomic hybridization also detects the loss or gain of whole chromosomes. Computer-assisted image analysis distinguishes differences in fluorescence and calculates the relative amount of each fluorescent dye in defined segments of each chromosome. As part of the analysis, a green-to-red intensity ratio profile is calculated for sections of each chromosome; significant deviations from 1.0 are localized to specific chromosome regions; and, depending on whether the green-to-red ratio is greater or less than 1.0, the gain or loss, respectively, of chromosome material is noted. For example, when pancreatic carcinomas were screened by CGH, gains of chromosome DNA were consistently found on 3q, 5p, 7q, 8q, 12p, and 20q and losses in 8p, 9p, 17p, 18q, 19p, and 21.

Comparative genomic hybridization has been used to track chromosome copy number changes during the progression of a cancer. Generally, CGH overcomes the problems of poor metaphase chromosome spreads from tightly bound cancer cells and the relatively low frequency of cells in metaphase in many tumors. As well, the level of resolution detects amplifications as small as 1 Mb. On the other hand, CGH is blind to translocations. Thus, for a complete cytogenetic picture of a cancer cell, a combination of techniques, including spectral karyotyping, G-banding, and FISH is often used. For the most part, cancer cytogeneticists are interested in identifying consistent chromosome abnormalities that can be used as diagnostic indicators and demarcating chromosome regions that carry cancer-causing genes.

transverse and ascending portions of the colon (Figure 16.13). In a number of HNPCC families, tumors of the ovary, kidney, brain, pancreas, stomach, and the innermost layer of the uterus (endometrium) also occur, but to a lesser extent than colon cancer. Generally, HNPCC tumors appear in patients before they are 45 years old.

Stringent criteria were established for the clinical designation of a family with HNPCC to minimize heterogeneity among data sets to be used for linkage and other studies. These requirements, which have been dubbed the Amsterdam criteria, are (a) at least three relatives must have colon cancer, with at least one of them being a first-degree relative to the other two, (b) at least two successive generations must have affected individuals, (c) at least one family member should have colorectal cancer before the age of 50, and (d) FAP should be excluded as the basis of the disorder. As knowledge about a disease accumulates, diagnostic guidelines are reworked. Currently, the revised Bethesda guidelines (2003) are often used to define cases of HNPCC (Table 16.6).

Linkage studies with large HNPCC kindreds indicated that the disorder was genetically heterogeneous. Sites for HNPCC were mapped to chromo-

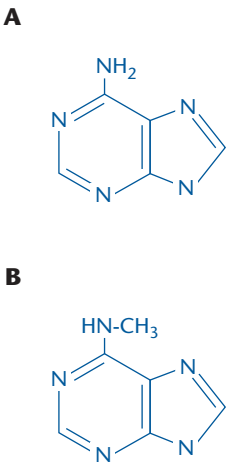
Table 16.6 Revised Bethesda (2003) guidelines for diagnosing HNPCC.

For a diagnosis of HNPCC, one of the following must be observed:
1. Diagnosis of colorectal cancer before the age of 50 yr
2. Synchronous (at the same time) or metachronous (at nearly the same time) instances of colorectal cancer or other HNPCC-related tumors including stomach, bladder, ureter, renal pelvis, biliary tract, glioblastoma, sebaceous gland adenomas, keratoacanthomas, and carcinoma of the small bowel, regardless of age
3. Colorectal cancer with a high-microsatellite-instability morphology that was diagnosed before the age of 60 yr
4. Colorectal cancer with one or more first-degree relatives with colorectal cancer or other HNPCC-related tumors. One cancer must be have been diagnosed before the age of 50 yr, including adenoma, which must have been diagnosed before the age of 40 yr
5. Colorectal cancer with two or more relatives with colorectal cancer or other HNPCC-related tumors, regardless of age

somes 2p and 3p in many, but not all, of the families. To determine whether the HNPCC phenotype was the consequence of loss of heterozygosity at 2p, 3p, or other sites, short tandem repeat (STR) polymorphic probes for all chromosomes were tested with tumor DNA from HNPCC individuals. No obvious loss of heterozygosity was observed for any chromosome region. However, surprisingly, almost all the dinucleotide sequences demarcated by the STR polymorphic probes were either expanded or contracted by one or a few nucleotides in comparison to the corresponding alleles in nontumor DNA. On this basis, it was deduced that the HNPCC genes at 2p and 3p encode proteins that help maintain the integrity of the genome. The appearance of changes in the lengths of microsatellites was called microsatellite instability (MSI). The occurrence of MSI, depending on the data set of HNPCC kindreds, varies from about 45% to more than 90% of the families. However, a more inclusive designation was devised when it was realized that nucleotide changes occur at sites other than microsatellite sequences. Because replication errors probably cause the observed genomic instability, the abbreviation RER was adopted. When replication errors are observed, the phenotype is designated as RER^+. About 20% of sporadic colorectal cancers are RER^+, and nucleotide errors probably occur throughout the genome.

Changes to chromosomal DNA, for the most part, occur as a consequence of environmental or internal mutagens, errors during DNA replication, or faulty recombination events. Generally, errors during replication, especially in stretches of repeat nucleotide sequences, are the result of strand slippage. When strand slippage occurs, there is a mismatch of nucleotides between a template and the newly synthesized strand. If a mismatch is not corrected, then, after the next round of DNA replication, one of the DNA molecules will have nucleotide alterations that will be perpetuated. Fortunately, both prokaryotes and eukaryotes have systems for detecting and repairing postreplication mismatched nucleotides. A mismatch repair (MMR) system consists of a set of proteins that recognizes mismatched nucleotide pairs, removes them from the newly synthesized strand, and fills in the correct nucleotide sequence using the original strand as the template. The mismatch repair system of *E. coli* has been studied in detail. In *E. coli*, a newly synthesized DNA strand is not methylated immediately after DNA replication, which distinguishes it from the template strand, which is methylated. The presence of methylated adenine residues (Figure 16.14) in the template strand enables the MMR system to recognize a newly synthesized nonmethylated strand in the vicinity of a nucleotide mismatch. The products of four *E. coli* genes called *MutS*, *MutL*, *MutH*, and *UvrD* (helicase II) play important roles in the repair of mismatched DNA. The MutS protein binds to a GATC sequence near the site of a DNA mismatch, and then MutL and MutH bind to the DNA-bound MutS. The MutH protein cleaves 5′ to the guanine (G) and 3′ to the adenine (A) in the GATC sequence in the nonmethylated DNA strand. The ensuing steps of the mismatch repair process require a number of other proteins, including helicase II, which unwinds the DNA from the MutH cleavage sites (nicks); 3′- and 5′-exonucleases, which remove about 1000 nucleotides in either direction from each MutH cleavage site; DNA polymerase III, which initiates DNA synthesis from the 3′-OH ends after removal of nucleotides by the exonucleases and uses the original template

Figure 16.14 Structures of adenine (A) and N6-methyladenine (B).



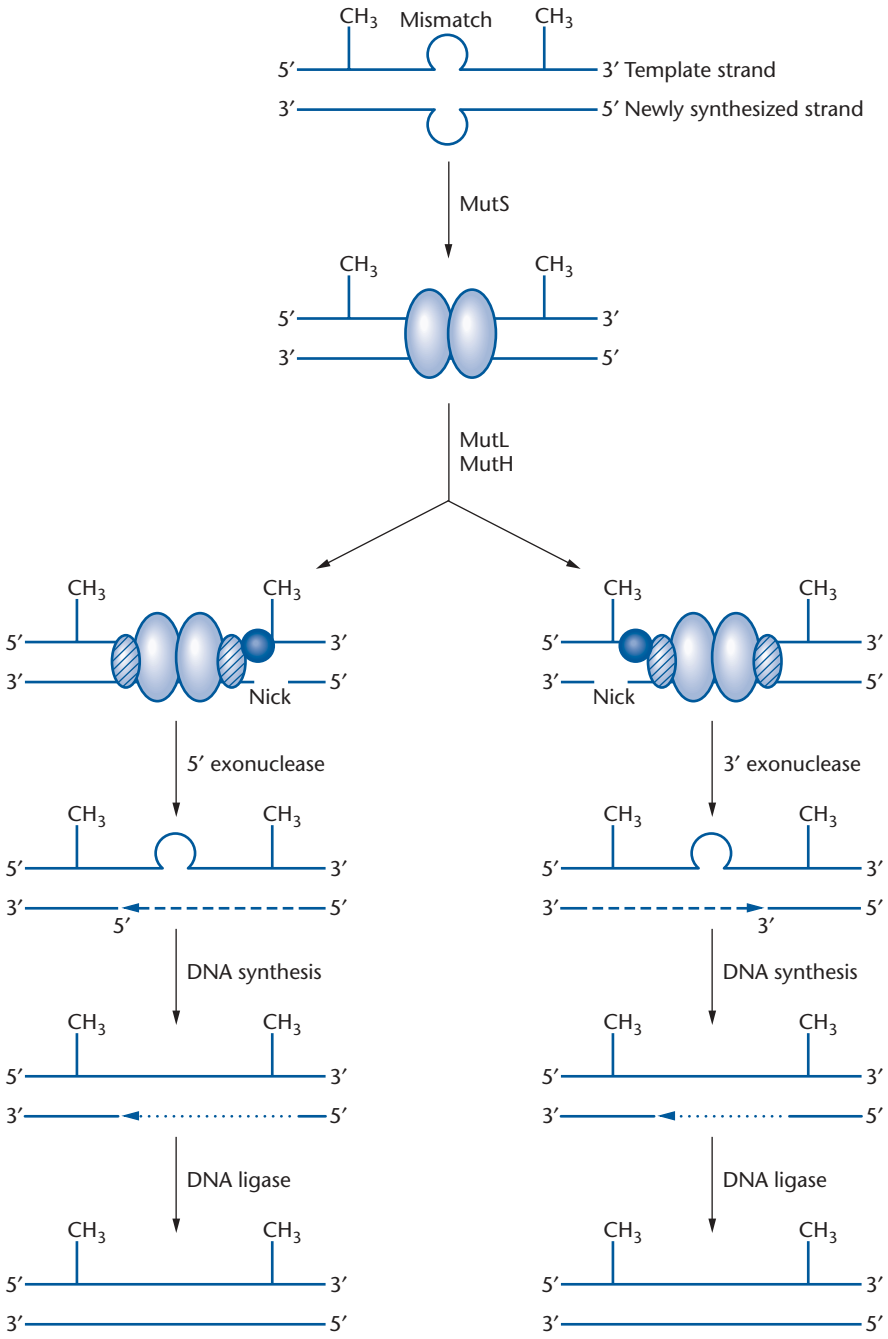


Figure 16.15 The *E. coli* mismatch repair system. Repair of a DNA mismatch is initiated by the binding of MutS (shaded ovals) to the mismatched nucleotides. MutL (hatched ovals) and MutH (solid circle) bind to bound MutS. MutH cuts the newly synthesized, non-methylated DNA strand after the 5′ position of the G residue in a GATC sequence. Excision of nucleotides (dashed arrows) from the newly synthesized strand occurs in the 5′ and 3′ directions driven by 3′ and 5′-exonucleases, starting from the MutH-induced nicks and continuing for about 1 to 2 kilobases. The excision process removes mismatched nucleotides from the newly synthesized strand. DNA synthesis (dotted arrows) directed by DNA polymerase III holoenzyme, which does not have intrinsic 5′-exonuclease activity, fills in the excised nucleotides using the original strand as the template. DNA ligase joins the 3′ end of the filled-in portion to a 5′ nucleotide of the newly synthesized strand. The end result of the mismatch repair process is a corrected DNA molecule.

strand as the template for the repair synthesis; single-strand binding protein, which keeps the strands apart during the repair DNA synthesis; and DNA ligase, which seals the 3′ to 5′ ends after repair DNA synthesis, forming an intact double-stranded DNA molecule (Figure 16.15). Mutations of the *MutS*, *MutL*, or *MutH* genes increase the mutation frequencies of other genes by 100- to 1000-fold. Also, in strains with mutations of both the *MutS* and *MutL* genes, the frequency of insertions and deletions in a cloned $(CA)_{10}$ dinucleotide repeat is increased about 500-fold.

Studies with the yeast *Saccharomyces cerevisiae* indicated that its mismatch repair system uses genes homologous to the *E. coli MutS* and *MutL* genes. Some of the yeast MMR genes were named after their *E. coli* homologs. For example, the yeast gene *MSH2* represents the second (2) MutS homolog in yeast, and *MLH1* stands for the first (1) yeast MutL homolog. Two other yeast genes, *PMS1* and *PMS2*, also were discovered to be homologs of the *E. coli MutL* gene. Mutations of the *PMS1* and *PMS2* genes alter the frequencies of genes after meiosis, that is, during postmeiotic segregation. Mutations of the yeast MMR genes increase the frequencies of mutations at a number of different loci. The features of mismatch repair in yeast and humans resemble those of the *E. coli*, although the process of strand selection is not known for either the yeast or human systems. In addition, the excision of 1000 to 2000 nucleotides, which occurs in *E. coli*, is not a part of the eukaryotic MMR systems.

Because the RER^+ phenotype in HNPCC families appeared to be the result of a defect in mismatch repair, the conserved sequences from yeast MMR genes were used to develop PCR primers for screening human cDNA libraries. Mutation analyses of genes linked to HNPCC sites were also carried out. In these ways, human versions of the yeast MMR genes were isolated and characterized (Table 16.1). The *MLH1* and *MSH2* genes account for more than 90% of mutations in HNPCC kindreds. Invariably, individuals with a germline mutation of an MMR gene are heterozygous. The wild-type allele is inactivated by point mutations or deletions in individuals with HNPCC.

Many HNPCCs and sporadic colon cancers with the RER^+ phenotype do not have mutated MMR genes. This paradox was partially resolved when it was shown that in some of these tumors both copies of the *MLH1* genes in the tumor DNA are not expressed because of methylation of nucleotides of both promoters. This form of transcription inactivation is called gene silencing. The mechanism that initiates the abnormal methylation of the *MLH1* genes is not known. But it is of interest to learn whether gene silencing is a common feature of colon carcinogenesis and whether it affects other oncogenes in other types of cancers. In other cases of RER^+ without detectable *MLH1* and *MSH2* mutations, the promoter region of the *MSH2* gene was rearranged.

The relationship between MMR gene mutations and HNPCC has not been elucidated fully. Clearly, a mutation of an MMR gene establishes a predisposition for the development of colon cancers of the HNPCC type and, to a lesser extent, for cancers at other sites, including endometrial cancers. Any plausible scenario that accounts for HNPCC-based tumorigenesis must include a series of sequentially contingent events. For example, if the amount of an MMR protein, say MSH2, is reduced in heterozygotes, then the probability of mutation of genes with repeat nucleotide sequences is increased in dividing cells, such as colon cells. If these mutated genes happen to be responsible for growth arrest or apoptosis, then cell proliferation is augmented, and there is a chance of mutation of the wild-type MMR allele in one of these somatic cells. If the wild-type MMR allele mutates and no active form of a particular MMR protein is produced, then there is a significant chance that mutations will occur in other genes that suppress tumor formation or increase cell proliferation. If the latter mutations take place, then tumor formation is the likely outcome.

There is evidence that supports aspects of this scheme. In HNPCC tumors with the RER^+ phenotype, frameshift mutations have been observed in the transforming growth factor β, type II receptor (*TGFBR2*) gene. The TGF-β receptor is a transmembrane serine/threonine protein kinase that is part of a signal transduction pathway inhibiting cell growth. A repeat sequence of $(A)_{10}$ within the *TGFBR2* gene is susceptible to mutation in RER^+ cells. Similarly, the $(G)_8$ tract in the *BAX* gene is mutated in about 50% of colon cancers with the RER^+ phenotype. Presumably, the *TGFBR2* gene mutations alleviate growth arrest and allow cells to proliferate. The *BAX* gene mutations prevent apoptosis, facilitating the development of colon cancers. A number of other oncogenes with coding microsatellites such as *PTEN*, *E2F4*, *TCF4*, *BLM*, and *IGF2R* are vulnerable to mutation in RER^+ HNPCC individuals. In addition, a defective MMR system might make proliferating cells more susceptible to the impact of carcinogens, increasing the likelihood of gene mutations and cancer formation.

Bloom Syndrome

Bloom syndrome (BS) is a rare autosomal recessive disorder characterized by short stature, immune deficiency, redness and often infections as a result of localized capillary expansions and lesions (telangiectasis), primarily of the cheeks and nose, creating the so-called butterfly pattern, sensitivity to sunlight, and increased occurrence of a wide range of cancers, including lymphomas, leukemias, and laryngeal, cervical, and gastrointestinal carcinomas. Life expectancy is not much longer than 50 years. In contrast to cultured normal cells, those from BS patients have large numbers of chromosome abnormalities, such as deletions, duplications, broken chromosomes, and unusual chromosome configurations. An interesting chromosome phenomenon called sister chromatid exchange (SCE), which entails recombination between chromatids of duplicated chromosomes, is commonly observed in cultured BS cells. In addition to chromosome abnormalities, the rate of gene mutation is about 10 times higher in BS cells than in normal cells. Thus it appears that BS represents a defect in DNA repair and/or replication.

Both homozygosity and linkage disequilibrium mapping placed the gene for BS, which was designated *BLM*, to chromosome 15q26.1 and about 2000 kb from the nearest marker site. A strategy called somatic crossover point (SCP) mapping was devised to narrow down the region containing the *BLM* gene. SCP mapping starts by establishing cell lines from BS patients of nonconsanguineous kindreds because they are likely to be compound heterozygotes. The high rate of chromosome abnormalities in these cell lines can be observed by examining metaphase chromosomes. An exchange between the sites of two *BLM* mutations on two different chromatids of duplicated homologous chromosomes produces a wild-type *BLM* gene on one of the chromatids (Figure 16.16). After the original cell lines have been cultured for a number of cell divisions, clones are established, and each is examined for the frequency of chromosome abnormalities. Cell lines with a low-frequency of chromosome abnormalities and little or no SCE probably have a wild-type *BLM* gene. Haplotype analysis of the low-frequency SCE cell lines, using ordered

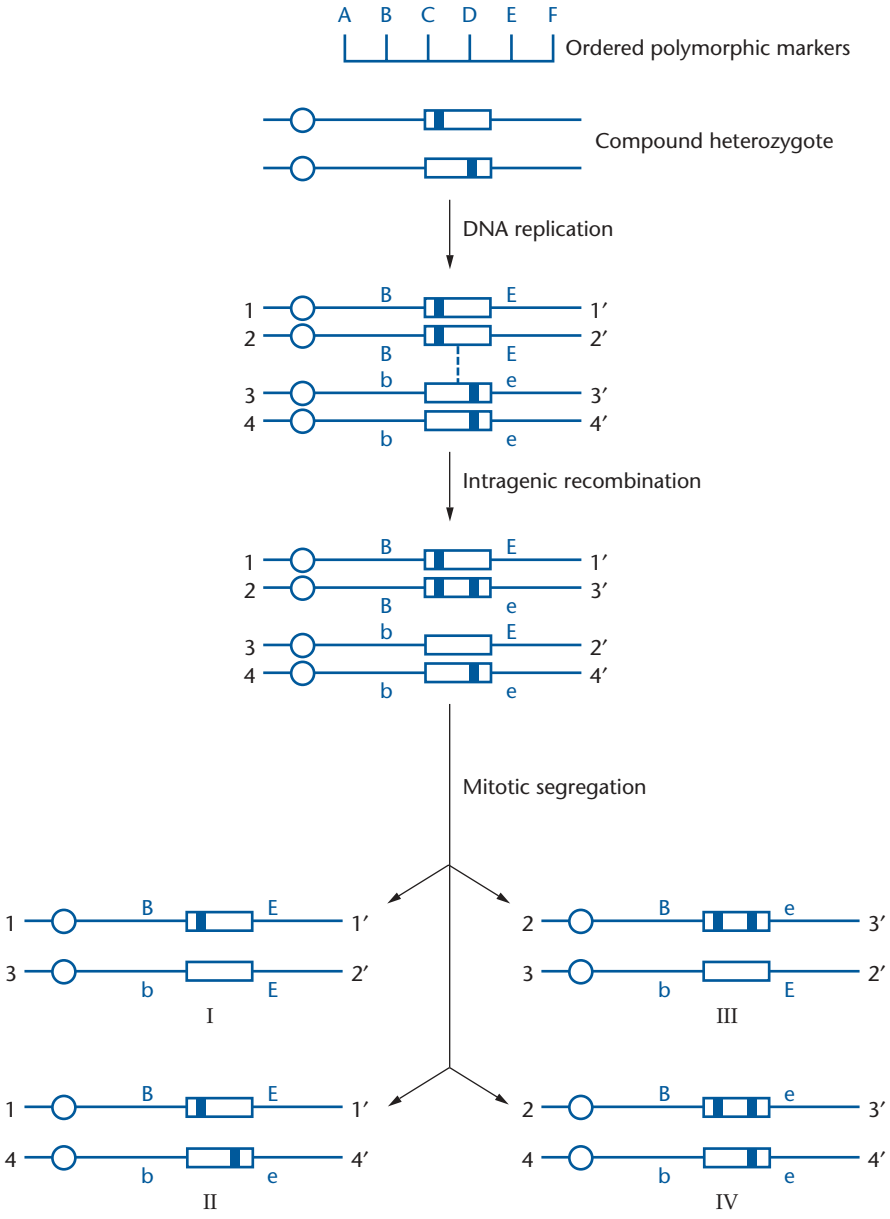


Figure 16.16 Somatic crossover point mapping. Cell lines are established from Bloom syndrome patients who are likely to be compound heterozygotes, cultured, and then subcultured from single cells. The extent of chromosome abnormalities are scored in these subcultures. The cell lines with a high frequency of chromosome abnormalities are the result of both *BLM* alleles carrying mutations (II, IV), and those with a low frequency have a *BLM* gene without any mutations that arose from intragenic recombination (I, III). A set of ordered polymorphic markers that identified heterozygous sites in each original cell line are used for haplotype analysis of each subcultured cell line with a low frequency of chromosome abnormalities to determine which loci remain heterozygous and which become homozygous. The target gene, or part of it, is bracketed by the two closest adjacent loci where one remains heterozygous and the other becomes homozygous. Nonrecombined chromatids are marked as 1-1′, 2-2′, 3-3′, and 4-4′. The recombined chromatids are 2-3′ and 3-2′. Intragenic recombination (dashed line) probably occurs during the G_2 phase of the cell division cycle. Open circles represent centromeres. The letters denote the alleles of the polymorphic markers. A solid bar indicates a mutated site within the *BLM* gene.

polymorphic markers shown to be heterozygous in the original cell line, indicate which sites become homozygous and which remain heterozygous. Marker sites that become homozygous lie distal to the region of recombination, and those that never become homozygous are proximal to the crossover region. Thus, based on the order of polymorphic markers and which loci remain heterozygous and which become homozygous, it is possible to bracket the target gene between two adjacent marker sites. In this way, the search for the *BLM* gene was narrowed down from 2000 kb to a chromosome segment of 250 kb.

Isolation and examination of cDNAs from the 250-kb region, followed by mutation screening of BS patients, revealed that the *BLM* gene encodes a 1417-amino acid protein. Sequence comparisons with known proteins indi-

Table 16.7 Human RecQ helicases and associated disorders.

Gene	Site	Disorder	Cancer	Noncancer features
BLM	15q26.1	Bloom syndrome	Lymphoma, acute lymphocytic leukemia, and many other types	Sensitivity to sun, cheek and nose lesions, immunodeficiency, subfertility, short stature
WRN	8p12	Werner syndrome	Soft tissue sarcoma, osteosarcoma, melanoma, meningioma	Premature development of age-associated disorders: atherosclerosis, arteriosclerosis, osteoporosis, diabetes, cataract formation, baldness, and subfertility
RECQL1	12p12.1	Unknown	Unknown	Unknown
RECQL4	8q24.3	Rothmund–Thomson syndrome	Soft tissue sarcoma, osteosarcoma, skin cancer	Growth deficiency, photosensitivity, cataracts, skeletal abnormalities, baldness
RECQL5	17q25.1	Unknown	Unknown	Unknown

cated that the BLM protein is homologous, in part, to a family of *E. coli* DNA helicases that are called RecQ helicases. DNA helicases move along one strand of duplex DNA, enzymatically cleaving hydrogen bonds that hold complementary nucleotides together and separating the two strands. Generally, these enzymes are found in both prokaryotes and eukaryotes and function in DNA repair and DNA replication. There are five RecQ helicases in humans (Table 16.7). Of these, BLM is multifunctional and interacts with proteins involved in DNA repair, homologous recombination, and sensing DNA damage. One key function of BLM is the restoration of DNA synthesis at blocked replication forks. Another is unwinding complex DNA structures such as hairpins and recombination junction points, which likely prevents aberrant recombination events. In sum, the chromosome abnormalities that characterize BS and lead to the formation of cancers result from alterations of DNA replication, recombination and/or repair.

Xeroderma Pigmentosum

Xeroderma pigmentosum (XP) is a rare, fatal autosomal recessive disorder that affects about 1 in 250,000 people worldwide. The major clinical feature of XP is the early onset of skin cancers that originate from the outermost (squamous) or innermost (basal) cells of the epithelium of the skin. Numerous pigment spots, which are often the sites of skin cancers, form in XP individuals. Other cancers associated with XP include malignant melanoma, keratoacanthomas, sarcomas, and adenocarcinomas. XP patients are extremely sensitive to light, develop lesions of the skin, eyes, and tongue, usually have neurological abnormalities, and demonstrate an impaired ability to learn. They seldom survive past 20 years of age.

The phenotype of cultured XP cells is characterized by extreme sensitivity to ultraviolet (UV) radiation, which includes both an increase in the frequency of chromosome abnormalities and a lowered ability to repair UV-damaged

DNA. The latter observation indicated that the XP defect is probably in the nucleotide excision repair (NER) system. This DNA repair system, which differs from MMR that is specific for mismatches, insertions and deletions, has been studied extensively in prokaryotes and eukaryotes.

Ultraviolet radiation induces the formation of stable linkages between adjacent pyrimidines; that is, T can be linked to T (T-T), C to T (C-T), and C to C (C-C). Such pairs of covalently linked nucleotides are called dimers. Generally, of these three types of dimers, thymine dimers (T-T) occur most frequently. Ultraviolet radiation is also responsible for other kinds of nucleotide crosslinks. In addition, some chemical compounds act as nucleotide crosslinking agents, and others add chemical groups to DNA bases. Dimers, nucleotide crosslinks, and nucleotide-specific side groups disrupt chromosomes and cause mutations. The NER system removes these damaged DNA nucleotides and reestablishes the correct nucleotide sequence. For example, after treatment with UV radiation, proteins of the NER system bind to a DNA thymidine dimer, DNA helicases of the NER complex unwind the DNA in both directions from the site of a dimer, endonucleases cut the damaged DNA strand a short distance on each side of the dimer, the oligonucleotide fragment containing the dimer is removed, the gap is filled in with DNA polymerase, and the strand is sealed by DNA ligase. Eukaryotic NER systems have more than 30 different proteins.

An operative NER system can be detected in UV-treated cultured cells by scoring with autoradiography the incorporation of radiolabeled thymidine (^{3}H-thymidine) into DNA. The UV-induced DNA synthesis, which is called unscheduled DNA synthesis (UDS), occurs in response to DNA damage and is not part of the normal S-phase DNA synthesis. Unscheduled DNA synthesis is usually not observed in cells derived from individuals with XP. Consequently, UDS assay can establish whether a person has XP. Moreover, the UDS response was also used to determine whether XP was genetically heterogeneous.

Briefly, cells from two individuals with XP are cultured separately and then fused. Only fused cells from individuals with different XP genes will show UDS after treatment with UV light. If the mutations are in the same locus in both XP individuals, there will be no UV-induced UDS in the fused cells. In other words, if the two original cell lines are homozygous recessive for different XP loci, that is, *aaBB* and *AAbb*, then the genotype of the fused cells is *AAbb*/*aaBB* and UDS occurs because all the components of the NER pathway are present and functional. Alternatively, if the original cells are homozygous recessive for the same gene, that is, both are *aaBB*, then the genotype of the fused cells is *aaBB*/*aaBB* and no UDS occurs because the NER pathway is incomplete and, therefore, defective. When two mutant cell types combine and produce a wild-type phenotype, they are said to belong to different complementation groups. It is assumed that each complementation group represents a single gene.

Cell complementation studies with XP patients established seven complementation groups (XPA to XPG). Expression cloning and homologous probes derived from *E. coli* and *S. cerevisiae* NER genes were used to identify, localize, and characterize most of the XP genes. Cell studies, amino acid homologies with NER proteins from other organisms, and biochemical reconstructions

Table 16.8 Xeroderma pigmentosum complementation groups.

Complementation group	Gene	Site	Function
XPA	*XPA*	9q22.3	DNA binding
XPB	*ERCC3**	2q14.3	DNA helicase, subunit of transcription complex
XPC	*XPC*	3p25.1	DNA binding
XPD	*ERCC2*	19q13.32	DNA helicase, subunit of transcription complex
XPE	*DDB2***	11p11.2	Damage-specific DNA-binding protein 2 subunit
XPF	*ERCC4*	16p13.12	Endonuclease
XPG	*ERCC5*	13q33.1	Endonuclease

*Excision repair cross-complementing rodent repair deficiency, complementation group 3.
**Damage-specific DNA-binding protein 2 subunit.

helped to determine the functions of the XP proteins (Table 16.8). The ERCC3 and ERCC2 proteins are members of the multiprotein complex TFIIH, which initiates transcription of structural genes. In this capacity, they participate in a NER subpathway called transcription-coupled repair (TCR) that corrects mistakes in actively transcribed genes. XP proteins are also involved in repairing damage to nontranscribed sites throughout the genome by the general genomic repair (GGR) subpathway. The phenotypic effects of the XP genes are not equivalent. For example, mutations of *XPA* and *XPC* cause severe, *ERCC2* and *ERCC4* mild, and *DDB2*, *ERCC3*, and *ERCC5* very mild impairment of NER. Furthermore, neurological abnormalities are often associated with mutations of *XPA*, *ERCC2*, and *ERCC5*.

About 20% of all cases of XP have an intact NER pathway, which has been designated as XP-variant (XPV). Mutations of the *POLH* gene that encodes DNA polymerase η (POLH) are responsible for XPV. POLH, unlike the primary DNA polymerases, efficiently corrects UV- and chemically induced DNA damage. For example, it inserts adenine residues opposite thymine dimers. This interesting property has been dubbed translesion synthesis (TSL). Mutant POLH molecules stop the replication process at the site of the lesion, and the damage is not corrected, which increases the overall mutation rate.

Mutations of the *ERCC3*, *ERCC2*, and *ERCC5* genes have been found in individuals with Cockayne syndrome (CS). The CS phenotype includes short stature and sensitivity to light but without the formation of skin cancers, deafness, ocular abnormalities, and cataracts. Death from CS occurs early in the second decade after birth. There are at least two other CS genes, neither of which is related to any of the XP genes. It is not clear why mutations of XP genes produce a CS phenotype. In a similar vein, trichothiodystrophy (TTD), which is a rare, noncancerous autosomal recessive disorder featuring dry, scaly skin (ichthyosis), short stature, intellectual impairment, and very brittle hair, may in some, but not all, cases be the result of either *ERCC2* or *ERCC3* gene mutations. Because these genes are part of the TFIIH complex, possibly, transcription activity and not DNA damage sensing is affected. The occurrence of gene mutations that determine different disorders underscores the inherent complexity of many inherited conditions.

key terms

adenoma
amplification
apoptosis
Bloom syndrome
cancer
carcinoma
cell proliferation signal transduction pathway
checkpoint
complementation group
complementation test
cyclin
cyclin-dependent kinase
DNA damage
DNA helicase
familial adenomatous polyposis
GTP-binding protein
hereditary nonpolyposis colorectal cancer
leukemia
Li–Fraumeni syndrome
loss of heterozygosity
lymphoma
metastasis
microsatellite instability
mismatch repair system
multiple endocrine neoplasia, types 1 and 2
neurofibromatosis, types 1 and 2
nucleotide excision repair
oncogene
Philadelphia chromosome
protein phosphorylation cascade
proto-oncogene
pyrimidine dimers
retinoblastoma
RER
retrovirus
sarcoma
somatic crossover point mapping
translocation
tumor suppressor gene
tyrosine protein kinase receptor
v-oncogene
Wilms tumor
xeroderma pigmentosum

summary

Cancers occur when cell proliferation goes awry. Many biological control systems have evolved to ensure that cell division does not become rampant. There are a number of different ways that cell proliferation can be initiated when it is required. Quiescent cells are activated by various signal transduction pathways entailing cascades of protein phosphorylations that activate successive intermediary proteins and, in the end, promote the expression of genes that encode cell division proteins. When necessary, apoptosis is triggered and cells are selectively destroyed. Usually cancer formation requires mutations in somatic cells that affect diverse aspects of the cell proliferation process, apoptosis, and the migratory behavior of cells.

Genes with alleles that cause cancer are called oncogenes. Tumor suppressor genes prevent uncontrolled cell proliferation under normal conditions. However, along with other mutations, the absence of a tumor suppressor is a common occurrence among different cancers. The genomic changes that affect cancer-causing genes are point mutations that either alter protein functions or inactivate tumor suppressor genes; deletions that remove wild-type tumor suppressor alleles; translocations that create new genomic arrangements leading either to overproduction of oncogenic proteins or formation of cell proliferation proteins that escape regulation; and localized DNA amplifications that increase the copy numbers of some oncogenes. Most cancer-causing genes are active in a range of cell types, and others are cell-specific.

There are many inherited forms of cancer. In most of these cases, inheritance of either a mutant oncogene or tumor suppressor gene predisposes an individual to a particular type of cancer. Despite the involvement of a number of genes in most cancers, in some instances, mutations of single genes are apparently sufficient to evoke specific cancers. For example, mutations of the *BRCA1* and *BRCA2* (*FANCD1*) genes seem to be the primary basis of early-onset breast cancer in many families. Interestingly, these genes are rarely mutated in sporadic breast cancers. The loss of a wild-type allele of a tumor suppressor gene in somatic cells of heterozygotes leads to the cancers associated with neurofibromatosis type 1, retinoblastoma, and Wilms tumor. Mutations of the *RET* oncogene that affect different domains of the RET protein, which is part of a signal transduction pathway, can produce different cancer phenotypes. Moreover, some mutations of the *RET* gene and other oncogenes give rise to noncancerous disorders. The latter circumstances highlight the fact that many proteins are multifunctional and act in different cells and at different times during development.

Some inherited cancer disorders, such as Li–Fraumeni syndrome (LFS), develop tumors in a broad range of cell types. In LFS families, mutations of the wild-type allele of the *TP53* gene that encodes p53 contribute to many kinds of early onset cancers. Wild-type p53 plays important roles in the cellular response to DNA damage, blocking the G_1/S and G_2/M transitions of the cell division cycle and inducing programmed cell death (apoptosis).

Colorectal cancer provides a notable paradigm for illustrating that sequential genetic changes in somatic cells are required for the formation of a cancer. In famil-

ial adenomatous polyposis (FAP) individuals, mutations of the *APC* gene cause localized cell proliferation and polyp formation of colon epithelial cells. Mutations of other genes convert benign polyp cells into cancer cells that proliferate in the wall of the colon. Finally, additional gene mutations bring about metastasis, and as a result, full-blown malignant colorectal cancer materializes and cancer cells invade other sites.

Gene mutations that decrease the ability of a cell to repair different types of DNA damage are associated with cancer syndromes such as hereditary nonpolyposis colorectal cancer, Bloom syndrome, and xeroderma pigmentosum. In these cases, although the relatively narrow range of cancers with each disorder is puzzling, an increased overall mutation rate almost inevitably generates cancer-causing gene mutations.

References

Adams, J. M. 2003. Ways of dying: multiple pathways to apoptosis. *Genes Dev* **17**:2481–2495.

Agarwal, S. K. et al. 2004. Molecular pathology of the *MEN1* gene. *Ann NY Acad Sci* **1014**:189–198.

Baker, S. J. and P. J. McKinnon. 2004. Tumour-suppressor function in the nervous system. *Nat Rev Cancer* **4**:184–196.

Balmain, A., J. Gray, and B. Ponder. 2003. The genetics and genomics of cancer. *Nat Genet* **33**:238–244.

Baser, M. E. et al. 2004. Genotype-phenotype correlations for nervous system tumors in neurofibromatosis 2: a population-based study. *Am J Hum Genet* 75.

Bordi, C. 2004. Multiple endocrine neoplasia (MEN)-associated tumours. *Dig Liver Dis* **36** Suppl 1:S31–S34.

Bright-Thomas, R. M. and R. Hargest. 2003. APC, β-Catenin and hTCF-4; an unholy trinity in the genesis of colorectal cancer. *Eur J Surg Oncol* **29**:107–117.

Chau, N. B. and J. Y. Wang. 2003. Coordinated regulation of life and death by RB. *Nat Rev Cancer* **3**:130–138.

Cichowski, K. et al. 2003. Dynamic regulation of the Ras pathway via proteolysis of the NF1 tumor suppressor. *Genes Dev* **17**:449–454.

Classon, M. and E. Harlow. 2002. The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* **2**:910–917.

Cory, S. and J. M. Adams. 2002. The BCL2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**:647–656.

Dasgupta, B. and D. H. Gutmann. 2003. Neurofibromatosis 1: closing the GAP between mice and men. *Curr Opin Genet Dev* **13**:20–27.

de Jong, M. M. et al. 2002. Genes other than *BRCA1* and *BRCA2* involved in breast cancer susceptibility. *J Med Genet* **39**: 225–242.

Deng, C.-X. and R. H. Wang. 2003. Roles of BRCA1 in DNA damage repair: a link between development and cancer. *Hum Mol Genet* **12**:R113–R123.

Fodde, R. 2002. The *APC* gene in colorectal cancer. *Eur J Cancer* **38**:867–871.

Fodde, R., R. Smits, and H. Clevers. 2001. *APC*, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer* **1**:55–67.

Frebourg, T. et al. 1995. Germ-line p53 mutations in 15 families with Li-Fraumeni syndrome. *Am J Hum Genet* **56**:608–615.

Friedberg, E. C. 2001. How nucleotide excision repair protects against cancer. *Nat Rev Cancer* **1**:22–33.

Friedberg, E. C. 2004. The discovery that xeroderma pigmentosum (XP) results from defective nucleotide excision repair. *DNA Repair* **3**:183–195.

Friedberg, E. R., G. C. Walker, and W. Siede. 1995. *DNA Repair and Mutagenesis*. ASM Press, Washington, DC.

Futreal, P. A. et al. 2004. A census of human cancer genes. *Nat Rev Cancer* **4**: 177–183.

Gismondi, V. et al. 2004. Prevalence of the Y165C, G382D and 1395delGGA germline mutations of the *MYH* gene in Italian patients with adenomatous polyposis coli and colorectal adenomas. *Int J Cancer* **109**:680–684.

Hahn, W. C. and R. A. Weinberg. 2002. Rules for making human tumor cells. *N Engl J Med* **347**:1593–1603.

Hesketh, R. 1997. *The Oncogene and Tumor Suppressor Gene Factsbook*. 2nd ed. Academic Press, San Diego, CA.

Hickson, I. D. 2003. RecQ helicases: caretakers of the genome. *Nat Rev Cancer* **3**:169–178.

Hoff, A. O., G. J. Cote, and R. F. Gagel. 2000. Multiple endocrine neoplasias. *Annu Rev Physiol* **62**:377–411.

Howlett, N. G. et al. 2002. Biallelic inactivation of *BRCA2* in Fanconi anemia. *Science* **297**:606–609.

Jacob, S. and F. Praz. 2002. DNA mismatch repair defects: role in colorectal carcinogenesis. *Biochimie* **84**:27–47.

Jones, S. et al. 2002. Biallelic germline mutations in *MYH* predispose to multiple colorectal adenoma and somatic G:C→T:A mutations. *Hum Mol Genet* **11**:2961–2967.

Kannouche, P. and A. Stary. 2003. Xeroderma pigmentosum variant and error-prone DNA polymerases. *Biochimie* **85**: 1123–1132.

Lehmann, A. R. 2003. DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. *Biochimie* **85**:1101–1111.

Lucci-Cordisco, E. et al. 2003. Hereditary nonpolyposis colorectal cancer and related conditions. *Am J Med Genet* 122A:325–334.

Lynch, H. T. and A. de la Chapelle. 2003. Hereditary colorectal cancer. *N Engl J Med* **348**:919–932.

Malumbres, M. and M. Barbacid. 2001. To cycle or not to cycle: a critical decision in cancer. *Nat Rev Cancer* **1**:222–231.

Mareel, M. and A. Leroy. 2002. Clinical, cellular, and molecular aspects of cancer invasion. *Physiol Rev* **83**:337–376.

Marsh, D. J. and R. T. Zori. 2002. Genetic insights into familial cancers—update and recent discoveries. *Cancer Lett* **181**:125–164.

McClatchey, A. I. 2003. Merlin and ERM proteins: unappreciated roles in cancer development? *Nat Rev Cancer* **3**:877–883.

Nichols, K. E. et al. 2001. Germ-line *p53* mutations predispose to a wide spectrum of early-onset cancers. *Cancer Epidemiol Biomarkers Prev* **10**:83–87.

Offit, K. 1998. *Clinical Cancer Genetics: Risk Counseling and Management*. Wiley-Liss, New York.

Olivier, M. et al. 2003. Li-Fraumeni and related syndromes: correlation between tumor type, family structure, and *TP53* genotype. *Cancer Res* **63**:6643–6650.

Pantel, K. and R. H. Brakenhoff. 2004. Dissecting the metastatic cascade. *Nat Rev Cancer* **4**:448–456.

Poisson. A., B. Zablewska, and P. Gaudray. 2003. Menin interacting proteins as clues toward the understanding of multiple endocrine neoplasia type 1. *Cancer Lett* **189**:1–10.

Rosen, E. M. et al. 2003. *BRCA1* gene in breast cancer. *J Cell Physiol* **196**:19–41.

Sancar, A. et al. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* **73**:39–85.

Sears, R. C. and J. R. Nevins. 2002. Signaling networks that link cell proliferation and cell fate. *J Biol Chem* **277**:11617–11620.

Sontag, S. 1979. *Illness as Metaphor*. Vintage Books, New York.

Stary, A. and A. Sarasin. 2002. The genetics of the hereditary xeroderma pigmentosum syndrome. *Biochimie* **84**:49–60.

Thoma, B. S. and K. M. Vasquez. 2003. Critical DNA damage recognition functions of XPC-hHR23B and XPA-RPA in nucleotide excision repair. *Mol Carcinog* **38**:1–13.

Thornberry, N. A. and Y. Lazebnik. 1998. Caspases: enemies within. *Science* **281**: 1312–1316.

Umar, A. et al. 2004. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* **96**:261–268.

van Brabant, A. J., R. Stan, and N. A. Ellis. 2000. DNA helicases, genomic instability, and human genetic disease. *Annu Rev Genomics Hum Genet* **1**:409–459.

Varley, J. M. 2003. Germline *TP53* mutations and Li-Fraumeni syndrome. *Hum Mutat* **21**:313–320.

review questions

1. What is a cell proliferation signal transduction pathway? Describe in detail its essential features.
2. What is an oncogene?
3. How were oncogenes initially discovered?
4. What is apoptosis?
5. What is the relationship between apoptosis and cancer?
6. What are cyclins? Describe the role of cyclins during the G_1 to S phase transition.
7. What is metastasis? Describe which kinds of genes might be involved in metastasis.
8. What is a tumor suppressor gene (TSG)? What are the criteria for a TSG? Discuss two examples of inherited cancer syndromes that result from the loss of TSGs.
9. What is the significance of LOH? How is it determined?
10. Why are certain chromosome translocations found in specific types of cancers?
11. Discuss the mode(s) of action of BRCA1. Why do *BRCA1* gene mutations cause cancer?
12. What are the key consequences of mutations of the *RET* gene?
13. Why is LFS phenotypically heterogeneous?
14. What is the MMR system?
15. Describe the genetic basis of HNPCC.
16. What is the NER system?
17. What is a complementation test? Discuss complementation testing to determine genetic heterogeneity.
18. What is comparative genomic hybridization? How does it work?
19. Discuss, with examples, the topic: Cancer is a multi-hit phenomenon.
20. Discuss the roles of germline and somatic mutations in hereditary and sporadic cancers.

chapter 17

Counseling, Diagnostic Testing, and Management of Genetic Disorders

Declare the past, diagnose the present, foretell the future.
HIPPOCRATES (~460–357 BCE)

Friendly counsel cuts off many woes.
ADAPTED FROM WILLIAM SHAKESPEARE (1564–1616)

We may give advice, but we never can compel behavior.
DUC DE LA ROCHEFOUCAULD (1613–1680)

There is nobody lonelier than a parent of a child just diagnosed with a genetic disorder.
ANONYMOUS

THE NEED FOR MEDICAL ATTENTION can arise a number of times during a lifetime. Accidents, infections, lifestyle excesses, extreme mood changes, sudden or chronic organ failure, environmental agents (toxins), or the onset of a genetic disorder can cause a serious complaint. Generally, when faced with an ill patient, a physician has to answer four primary questions. What is wrong? What is going to happen? What can be done about the ailment? Can the condition be avoided? In other words, these queries encompass diagnosis, prognosis, treatment, and prevention, respectively.

Ordinarily, effective treatment is not possible unless there is an accurate diagnosis. According to Peter Mere Latham (1789–1875), an influential British physician, diagnosis of a disorder "is often easy, often difficult, and often impossible." The perplexity of a difficult case is that the same set of symptoms may result from very different causes. For example, the clinical features of clinical hepatitis, which is a liver inflammation that has lasted for more than six months, are fatigue, malaise, and loss of appetite. If untreated, hepatitis can be fatal. However, a severe liver ailment can result from an infection with hepatitis virus B or C; excessive alcohol or drug use; autoimmunity; or genetic disorders such as hemochromatosis, Wilson disease, or α1-antitrypsin deficiency. Each of these possibilities has an explicit treatment. The diagnostic riddle is initially tackled by assembling a complete medical history including informa-

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tion about current medications, recent blood transfusions, lifestyle, and other family members with similar symptoms. Next, a thorough physical examination is conducted to determine the extent of the disorder. Sometimes, a probable diagnosis emerges on the basis of these data. For example, in the absence of other contributing factors, bronze or gray skin color in a patient who is 40 years or older with hepatitis is often indicative of hemochromatosis. In addition, biochemical and immunological tests help establish the cause of a disorder. Once a definitive diagnosis is reached, the appropriate treatment is initiated. For example, periodic removal of blood relieves the liver damage associated with hemochromatosis, whereas preventing the accumulation of dietary of copper will alleviate hepatitis caused by Wilson disease. If there is no specific therapeutic regimen for a particular disorder, supportive (palliative) care is administered to ease pain and discomfort.

A genetic condition is likely when, among other factors, a number of close relatives have identical symptoms, there is a delay in development after birth, a patient has two or more medical anomalies, and/or the condition is progressive. A primary physician will often refer a patient with a suspected genetic disorder to genetic counseling with either another physician (clinical geneticist) or a certified health professional (genetic counselor) who is trained to determine whether the features of a preliminary diagnosis represent an inherited condition.

Genetic Counseling

Formally, genetic counseling is defined by the American Society of Human Genetics as

> *a communication process which deals with human problems associated with the occurrence or the risk of occurrence of a genetic disorder in a family. The process of genetic counseling involves an attempt by one or more appropriately trained individuals to help the affected individual or family to: (1) comprehend the medical facts, including diagnosis, probable cause of the disorder, and the available management; (2) appreciate the way heredity contributes to the disorder, and the risk of recurrence in specified relatives; (3) understand the alternatives for dealing with the risk of recurrence; (4) choose the course of action which seems to be appropriate in view of its risk, their family goals, and their ethical and religious standards, and then to act in accordance with that decision; (5) make the best possible adjustment to the disorder in an affected family member and/or to the risk of recurrence of that disorder.*

The task of assigning the actual genetic basis of a disorder can be daunting. Frequently, the same apparent phenotype is associated with mutations of many different genes. For example, there are more than 400 inherited syndromes that have retinal degeneration as a major clinical feature. The first step in the genetic counseling consultation process is the construction of a complete pedigree to define the mode of inheritance of the disorder. Next, a comprehensive medical history of the patient is assembled. With this information, appropriate diagnostic tests are recommended for a definitive identification of the disorder.

Diagnostic Testing

Principles of Genetic Testing

The purpose of a genetic diagnostic test is to identify a specific component (indicator, target) that signifies whether an individual has or will develop a particular disorder. A positive result denotes the presence of the target and a negative outcome, its absence. A perfect test would always identify the target when it is present, that is, only a true positive (TP) is observed and every negative result would be a true negative (TN). Unfortunately, very few tests are absolutely accurate. There will be instances where a person with a disorder will test negative, that is, the test result is a false negative (FN), and normal individuals will have a positive test score, that is, a false positive (FP) result.

Clearly, false results, regardless of the cause, have important consequences. With a false positive result, a normal person will suffer extreme anxiety and possibly undergo unnecessary treatments. Routinely, when a positive result is recorded, the person is either retested or given more sophisticated tests to confirm the initial diagnosis. A false negative result can have very serious consequences because a treatable disease might be missed. Consequently, clinical tests are adjusted to minimize the occurrence of false negatives, often at the expense of increasing the frequency of false positives. The latter circumstance is preferred despite some short-term psychological consequences because the true negatives will be discovered among the positives after retesting.

Various indices, such as sensitivity and specificity, have been devised to judge the clinical validity of a diagnostic test. The relationships among positive and negative results and the presence or absence of the target can be represented by a two-by-two matrix (Table 17.1). Sensitivity is defined as the fraction of correctly identified people who truly have the target, that is, $\frac{TP}{TP+FN}$. Specificity represents the fraction of people who do not have the target out of all of those who are truly negative, that is, $\frac{TN}{TN+FP}$. Generally, a condition is likely to be present when a test result is positive and the specificity is high (>95%). On the other hand, the condition is probably absent when the results are negative and the sensitivity of the test is also high (>95%). Both high sensitivity and specificity are required before any diagnostic test can be used routinely for a clinical assessment.

Testing for Genetic Disorders

A genetic test is any diagnostic procedure that is used to determine an inherited disorder. The target may be a chromosome, protein, metabolite, or nucleic

Table 17.1 Outcomes of a diagnostic test.

	Test result	
Condition	**Positive**	**Negative**
Present (affected)	True positive (TP)	False negative (FN)
Absent (unaffected)	False positive (FP)	True negative (TN)

acid (RNA, DNA) sequence. Chromosome aberrations are usually identified by karyotype staining or fluorescent in situ hybridization (FISH). For example, multiple miscarriages often indicate an abnormal chromosome constitution in the fertilized egg. In these cases, the parents might be advised to have their karyotypes analyzed to check for a balanced chromosome translocation. Also, submicroscopic deletions that are characteristic of certain disorders such as Prader–Willi and DiGeorge syndromes are diagnosed by FISH.

The vast majority of the available genetic tests are designed for monogenic conditions. Biochemical and immunological assays of the blood and urine detect amino acids, organic acids, metabolites, or proteins that are specifically associated with genetic disorders. Currently, with a variety of formats, analytic procedures are able to detect more than 40 genetic diseases.

The Guthrie test, a microbial assay that was developed by the US scientist Robert Guthrie (1910–1995), has been used for over 30 years for the diagnosis of phenylketonuria (PKU) in newborns. For this screening procedure, drops of blood, usually from a newborn infant, are spotted as circles on a paper card. The spots are air-dried, punched out in the form of disks, and placed on an agar surface covered with the bacterium *Bacillus subtilis*. This bacterial strain requires phenylalanine for growth. The agar contains β-2-thienylalanine that inhibits the uptake of phenylalanine by the bacteria. If the blood on a disk contains a high level of phenylalanine, the phenylalanine diffuses into the agar and blocks the inhibitor. Under these conditions, a halo of bacterial cells forms around the periphery of the disk. A disk with a normal blood level of phenylalanine does not have a surrounding zone of bacterial growth because there is not enough phenylalanine to block the growth inhibitor. Disks with increasing concentrations of phenylalanine are used to calibrate the blood phenylalanine in the samples. The extent of a halo that is equivalent to 20 mg/dl phenylalanine or greater indicates PKU. Atypical or mild PKU falls between 7 and 20 mg/dl and normal blood phenylalanine levels are less than 7 mg/dl. Those with positive results, that is, 7 mg/dl or greater, are retested with a more sensitive assay for phenylalanine to confirm positive results. False negative results with the Guthrie test are rare (0.3%) when the blood sample is taken 72 h after birth. The basic principle of this simple test has been adapted for some other genetic disorders.

A large number of tests have been devised to detect genetic disorders that are caused by either the accumulation or deficiency of a metabolite. Because metabolic disorders have distinctive biochemical signatures, the diagnostic tests often require completely different laboratory protocols. However, with the development of tandem mass spectrometry (MS/MS), it may be possible to use a common analytical device to determine a number of different inherited metabolic disorders. Briefly, for MS/MS, a sample is subjected to fast atom bombardment, electrospray, or liquid secondary ion treatment to produce quasimolecular ions that are separated from one another in the first mass spectrometer on the basis of mass-to-charge (m/z) ratios. The separated ions are passed through a reaction chamber where they are fragmented, and the m/z values of the fragments are analyzed in a second mass spectrometer. The final m/z profile is compared to known standards to identify and quantify the target molecule. One complete MS/MS analysis takes less than 2 min, whereas con-

2,4-Dienoyl-CoA reductase deficiency
2-Methylbutryl-CoA dehydrogenase deficiency
3-Hydroxy long chain acyl-CoA dehydrogenase deficiency
3-Hydroxy-3-methylglutaryl-CoA lyase deficiency (HMG)
3-Ketothiolase deficiency
3-Methylcrotonyl-CoA carboxylase deficiency
3-Methylglutaconyl-CoA hydratase deficiency
5-Oxoprolinuria
Adenosylcobalamin synthesis defects
Argininemia
Argininosuccinate aciduria (ASA)
Carnitine palmitoyl transferase deficiency Type II (CPT II)
Carnitine/acylcarnitine translocase deficiency
Citrullinemia
Glutaric acidemia Type I (GA I)
Homocystinuria
Hyperammonemia, hyperornithinemia, homocitrullinuria syndrome (HHH)
Hypermethioninemia
Isobutryl-CoA dehydrogenase deficiency
Isovaleric acidemia (IVA)
Long chain hydroxyacyl-CoA dehydrogenase deficiency (LCHAD)
Malonic aciduria
Maple syrup urine disease (MSUD)
Medium chain acyl-CoA dehydrogenase deficiency (MCAD)
Methylmalonyl-CoA mutase deficiencies
Mitochondrial acetoacetyl-CoA thiolase deficiency
Multiple acyl-CoA dehydrogenase deficiency (MADD; Glutaric acidemia Type II, GA II)
Multiple CoA carboxylase deficiency
Nonketotic hyperglycinemia (NKH)
Phenylketonuria (PKU)
Proprionic acidemia (PPA)
Short chain acyl-CoA dehydrogenase deficiency (SCAD)
Trifunctional protein deficiency tyrosinemia Type I (TYR I)
Tyrosinemia Type II (TYR II)
Very long chain acyl-CoA dehydrogenase deficiency (VLCAD)

Figure 17.1 Genetic disorders that are detected with tandem mass spectrometry.

ventional diagnostic assays require hours and, in some cases, days. Tandem MS accurately detects and measures the amounts of many of the different metabolites that are indicators of various genetic disorders (Figure 17.1).

In the past, it was not possible to develop a reliable diagnostic test for a genetic disorder that did not have an unique chemical feature. Now, theoretically, because the sequence of every human gene is known, it is feasible to construct an assay that will identify mutations in any disease-causing gene. There are both indirect and direct mutation detection systems. If allelic specificity is not relevant and determination of a mutation anywhere in a gene is sufficient for diagnosis, a number of indirect methods are available such as single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and denaturing high-performance liquid chromatography (DHPLC). Alternatively, considerable attention has been directed to developing direct DNA-based tests for known mutations at specific sites within a gene.

Direct Mutation Detection Methods

A staggering number of PCR-based protocols have been devised to detect known gene mutations. Generally, these systems are assigned to various categories (Figure 17.2). The choice of a particular detection assay depends on

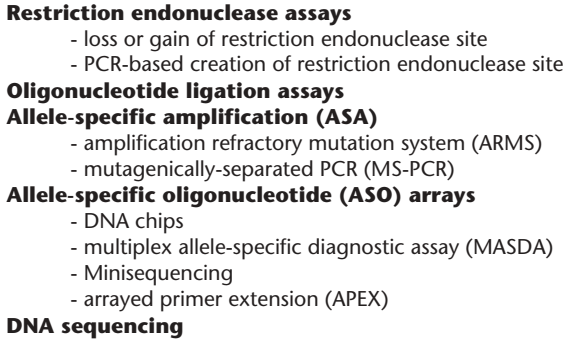


Figure 17.2 Mutation detection assays.

whether it is accurate, simple, rapid, and inexpensive. Because of cost and time, DNA sequencing is not a preferred approach unless there is no alternative. It is sufficient for some tests to target a single nucleotide. For others, a number of different mutations in a single gene are detected simultaneously. Such a test is called a multiplex assay.

A PCR-RFLP assay is feasible when a mutation causes either the loss or the gain of a restriction endonuclease site. The procedure entails amplifying the region containing the target, treating the amplified DNA with a restriction endonuclease, and determining the sizes of the fragments after separation by gel electrophoresis. If a restriction endonuclease site is lost because of mutation, then a larger than normal fragment is observed after gel electrophoresis (Figure 17.3A). Conversely, if a mutation creates a novel restriction endonuclease site, then treatment of the amplified DNA with the restriction endonuclease for this site produces fragments that are smaller than DNA with the normal sequence (Figure 17.3B). Genotypes can be readily determined with a PCR-RFLP assay (Figure 17.3).

The amplification refractory mutation system (ARMS) is a PCR assay that depends on the specificity of a primer for the normal sequence (normal primer) and another primer for the mutation (mutant primer). The normal primer is matched (complementary) at its 3′ end to the normal nucleotide, and, consequently, it is mismatched at its 3′ end with the mutant nucleotide. The other primer, the mutant primer, is matched and mismatched at its 3′ end to the mutant and the normal nucleotide, respectively. A mismatched 3′ end of a primer prevents nucleotide extension during the DNA synthesis phase of the polymerase chain reaction, and, as a result, no amplification occurs. A common primer that is perfectly matched to both normal and mutant DNA at a distance from the site of the mutation provides the second member of each pair of primers for PCR amplification. If required, a DNA sample can be amplified by primers that lie beyond the regions that are complementary to the normal, mutant, and common primers and then assayed for a specific mutation.

For an ARMS assay, an original or preamplified DNA sample (test sample) is divided into two aliquots. One portion is subjected to PCR with the normal and common primers and the other with the mutant and common primers. The formation of an amplification product is scored by gel electrophoresis. If a PCR product is formed when the normal primer is used, then the template

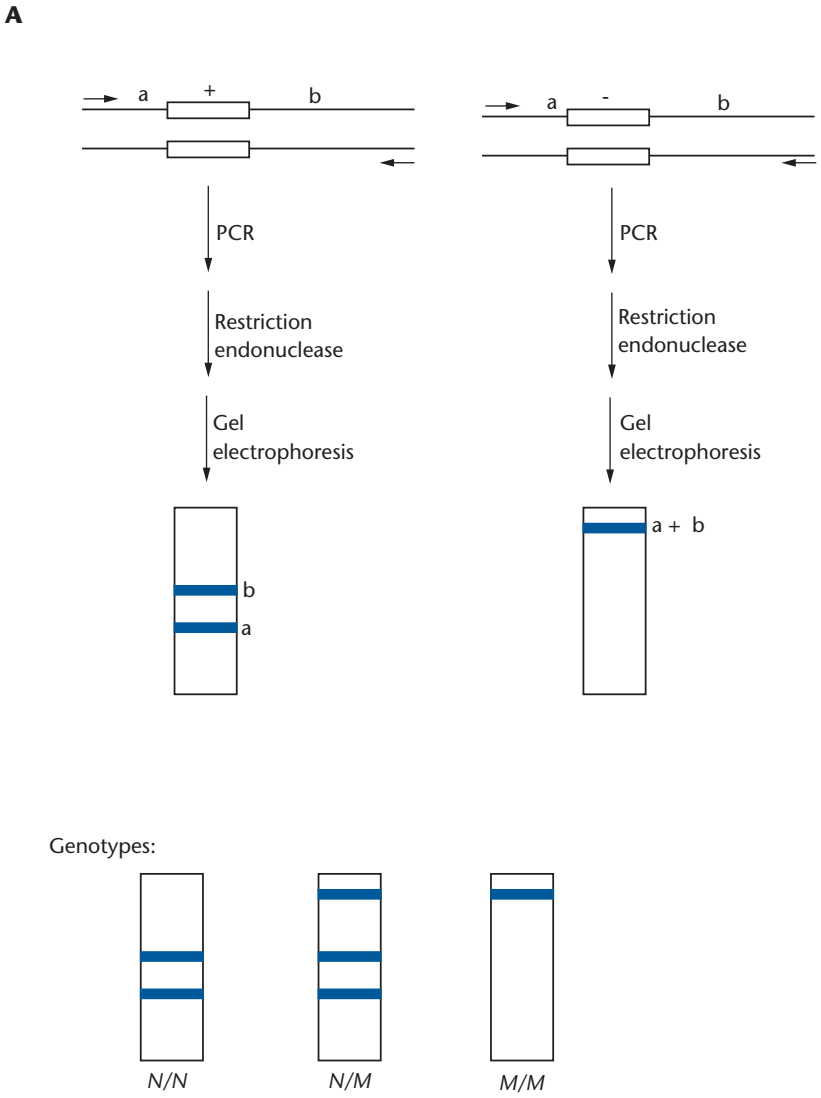


Figure 17.3 PCR-RFLP mutation detection system. (A) A mutation has caused the loss of a restriction endonuclease site (–). The normal restriction endonuclease site is designated with a plus sign (+). The horizontal arrows represent primers used for PCR amplification. A restriction endonuclease cuts amplified DNA with the corresponding restriction endonuclease site into two fragments (a, b). Gel profiles for homozygous normal (*N*/*N*), heterozygous (*N*/*M*), and homozygous (*M*/*M*) genotypes are shown, with *N* and *M* representing the normal and mutated sequences, respectively. (B) A mutation has caused the gain of a restriction endonuclease site (+). The horizontal arrows represent primers. The absence of the restriction endonuclease site is designated with a minus sign (–). The restriction endonuclease cuts the amplified DNA that acquired a restriction endonuclease site by mutation into two fragments (c, d). Gel profiles for homozygous normal (*N*/*N*), heterozygous (*N*/*M*), and homozygous (*M*/*M*) genotypes are shown, with *N* and *M* representing the normal and mutated sequences, respectively.

DNA came from an individual who is homozygous for the normal nucleotide. If there are PCR products in both reactions, then the person is a heterozygote. And if there is only a PCR product when the mutant and common primers are used, then the template DNA is from an individual who is homozygous for the mutant nucleotide (Figure 17.4).

There are many variants of the oligonucleotide ligation reaction [OLR; ligase chain reaction (LCR)] system for detecting known mutations. The basic principle of an OLR assay is that after two oligonucleotides are hybridized to a template DNA, they can only be ligated if the 5′ end of one of them is adjacent to the 3′ end of the other. If there is a mismatch at the 5′ end of the oligonucleotide that faces the 3′ end of the other oligonucleotide, then ligation does not occur. Thus, depending on the match or mismatch at the end of a particular oligonucleotide, the formation of a ligation product denotes the pres-

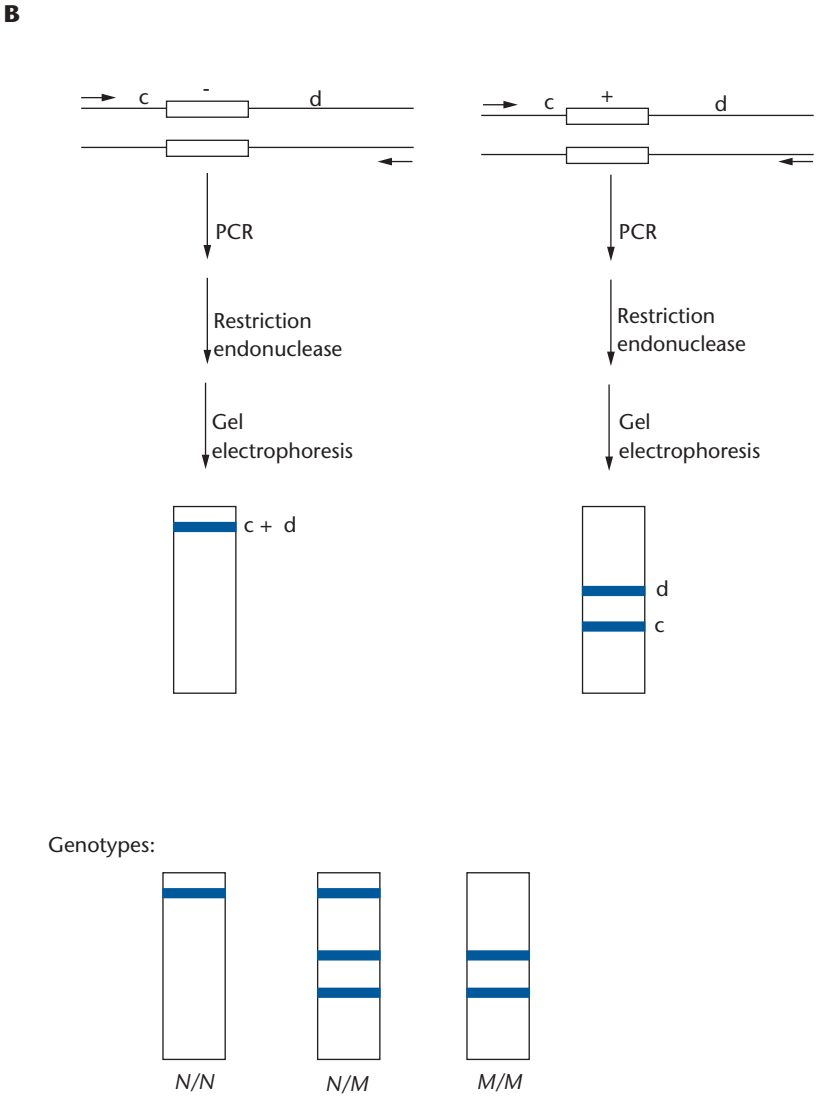
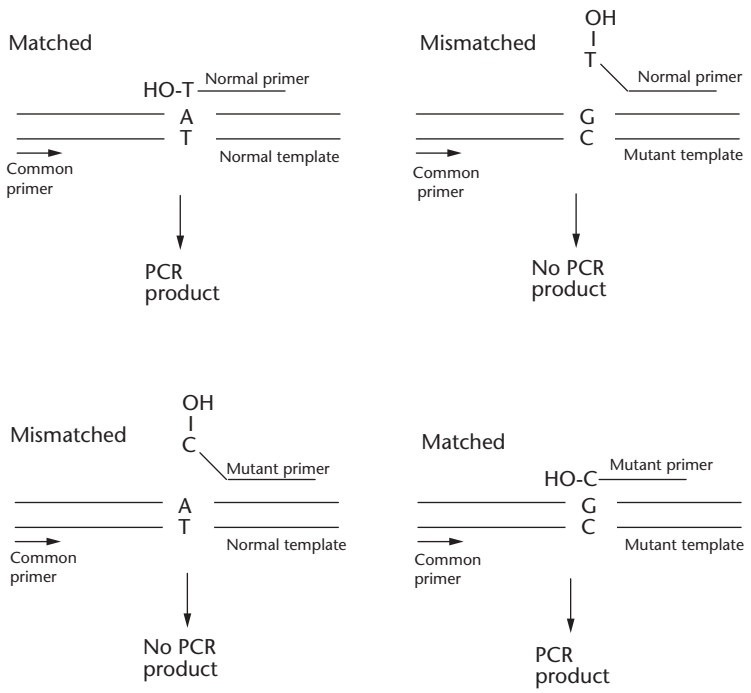


Figure 17.3 *continued*

ence or absence of a mutation (Figure 17.5A). In practice, the 5′ end of one oligonucleotide is matched to the normal (wild type) nucleotide, and the 5′ end of another oligonucleotide is matched to the mutant nucleotide. Another oligonucleotide that is common to both normal and mutant DNA has a 3′ end that is complementary to the nucleotide adjacent to the nucleotide site where the known mutation occurs.

Usually for OLR mutation assays, a DNA sample is amplified by PCR, denatured, and divided into two aliquots. The normal and common oligonucleotides are added to one aliquot and the mutant and common oligonucleotides to the other. Then DNA ligase is added to each tube and the presence or absence of a ligation product is scored. Parenthetically, for some OLR formats, the complete analysis can be carried out in a single reaction tube. The presence or absence of a ligation product, which identifies whether there is a mutation in the sample DNA, can be detected by gel electrophoresis if the normal and mutant oligonucleotides have different lengths. Or, to increase

A



B

	PCR product	
DNA sample	Normal/Common primer	Mutant/Common primer
Homozygous normal	+	-
Heterozygous	+	+
Homozygous mutant	-	+

Figure 17.4 Amplification refractory mutation system (ARMS) for detecting mutations. (A) The presence or absence of a PCR product is shown for combinations of normal and mutant primers with normal and mutant templates. (B) The relationship between each genotype and the presence (+) or absence (–) of a PCR amplification product with different pairs of primers are indicated.

resolution, fluorescent dyes can be used to mark the different oligonucleotides, so that each ligation product emits a distinctive signal that can be scored spectrophotometrically. Regardless of the ORL procedure, each genotype can be identified (Figure 17.5B).

For some mutation detection assays, either the sample (target) DNA or an allele-specific oligonucleotide (probe) is fixed to a solid support. The former format is called a dot blot (forward dot blot), and the latter is a reverse dot blot. High-density arrays (DNA chips) for all possible sequences have been created to detect any mutation in some genes such as *BRCA1*, *ATM*, and *CFTR*. Other diagnostic assays have been developed to analyze large numbers of samples for many different gene mutations at the same time. For example, the multiplex allele-specific diagnostic assay (MASDA) uses a dot blot protocol to detect over 100 different mutations in more than 500 samples. Briefly, each region in a DNA sample that may contain one of the targeted mutations is amplified by PCR. This DNA is denatured and bound as a spot to a membrane. Radiolabeled oligonucleotides (probes) that are specific for each mutation are pooled and hybridized to the membrane with the spots of immobilized PCR-amplified genomic DNA. Unhybridized (mismatched) oligonucleotides are washed away, and the spots on the membrane with hybridized oligonucleotides are detected by autoradiography. The hybridized probe from each spot

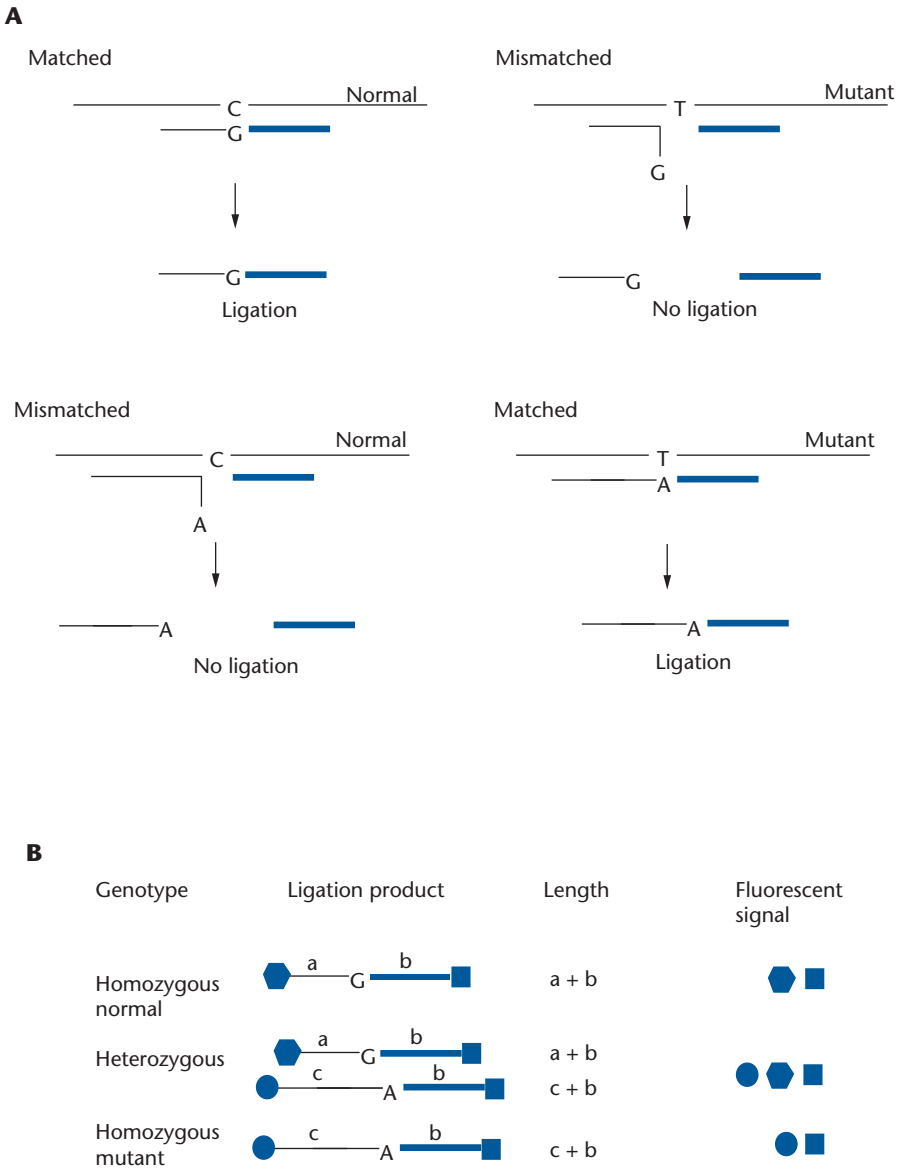


Figure 17.5 Oligonucleotide ligation reaction (OLR) system for detecting mutations. (A) The presence or absence of a ligation product is shown for combinations of matched and mismatched oligonucleotides with normal and mutant target DNA. The thick line denotes the common oligonucleotide. (B) The relationship between each genotype and the formation of a ligation product. Ligation is determined either electrophoretically on the basis of length of the ligation products or spectrophotometrically with combinations of fluorescent dyes after removal of the nonligated oligonucleotides. The lowercase letters denote the lengths of the oligonucleotides, and the solid symbols, indicate fluorescent dyes. Each combination of fluorescent dyes produces a distinct signal.

is individually released and identified by chemical cleavage or DNA sequencing. A mutation is immediately apparent because the sequence of each probe is unique and specifically corresponds to one particular mutation.

Clinical Applications of Genetic Tests

Broadly speaking, genetic tests are used to establish the basis of an existing disorder (diagnostic testing), determine the presence of genetic condition when there are no obvious symptoms (predictive testing), identify heterozygotes (carrier testing), and assess a fetus for abnormalities (prenatal testing). A positive diagnostic test result can lead to improved short- and long-term care. Predictive testing reveals whether an asymptomatic individual has a genotype that will inevitably lead to a disorder, that is, whether a person is presymptomatic.

A positive test result in the absence of any symptoms can be traumatic. Consequently, some potentially presymptomatic individuals favor the "right not to know" about their future health and refuse testing. Others use a positive result to prepare for the onset of the disorder. A negative predictive test result is usually good news, although, occasionally, it induces severe feelings of guilt because of the concern that other family members may not be as fortunate. Predictive testing can also detect the presence of a gene that increases the risk of a disorder. In this case, a positive result indicates a predisposition (susceptibility) for a particular condition with no certainty that the disease will ever arise. Genetic counseling provides the counselee with an understanding of the significance of this type of result.

Carrier testing identifies heterozygotes (carriers) for X-linked and autosomal recessive disorders that occur with high frequencies in certain populations. Theoretically, half the sons of a female carrier for an X-linked recessive allele will inherit the disorder. And, if both parents are carriers for an autosomal recessive disorder, there is a 25% chance that each offspring will have the genetic condition. Knowledge of carrier status gives couples the opportunity to make reproductive choices. If termination of pregnancy is not acceptable, couples at risk for an affected child may decide not marry, opt not to have children, or accept the possibility of having an affected child. Other couples may have the fetus tested for the genetic abnormality and terminate the pregnancy if the test result is positive. Couples who are either at risk of having a child with a serious abnormality or have experienced repeated fetal loss due to a chromosomal abnormality may seek in vitro fertilization with genetic testing of either second meiotic division polar bodies or cells taken from an 8-cell stage embryo before implantation (preimplantation diagnosis). Under these circumstances, as best as can be determined, only normal embryos are implanted. Therefore, the chance of either giving birth to an affected child or having spontaneous fetal loss early in a pregnancy is dramatically reduced. However, preimplantation diagnosis is experimental, expensive, and not easily accomplished.

Prenatal testing is performed during pregnancy to determine whether there is a significant risk of having a child with a serious disorder. A genetic abnormality is likely when either one of the parents or a previous child has had an inherited disorder, both parents are carriers or there is a high probability that both of them are carriers, or the woman has a family history of an X-linked recessive disorder. Fetal chromosome abnormalities that increase in frequency with maternal age such as Down syndrome (trisomy 21) (Table 17.2) are also detected by prenatal testing.

The growth and development of a fetus is generally monitored throughout a pregnancy. There are various indicators that a fetus may have an increased risk for either a chromosome abnormality or open neural tube defects such as spina bifida and/or anencephaly. First, an increased depth of the pocket of fluid at the back of the neck of a fetus (nuchal translucency) that is determined by an ultrasonogram is frequently associated with an increased probability of fetal chromosome abnormality. Second, determination of the amounts of α-fetoprotein (AFP), human chronic gonadotropin (hCG), and unconjugated estriol (UE3) in a mother's blood indicates whether there is an increased risk

Table 17.2 Risk of having a child with trisomy 21 and any chromosome aberration as a function of maternal age.

Maternal age, yr	Risk of trisomy 21	Risk of any chromosome aberration
20	1/1667	1/526
24	1/1250	1/476
28	1/1053	1/435
32	1/769	1/323
36	1/294	1/156
40	1/106	1/66
44	1/38	1/26
48	1/14	1/10

Adapted from Hook, E. B. 1981. Rates of chromosome abnormalities at different maternal ages. *Obstet Gynecol* **58**:282–285 and Hook, E. B., P. K. Cross, and D. M. Schreinemachers. 1983. Chromosomal abnormality rates at amniocentesis and in live-born infants. *JAMA* **249**:2034–2038.

of Down syndrome or open neural tube defects. Low levels of both AFP and UE3 with high amounts of hCG are associated with Down syndrome. Elevated levels of AFP are correlated with neural tube defects. When there is a possibility that a fetus has a genetic disorder, samples of either amniotic fluid (amniocentesis) or chorionic villus cells (chorionic villus sampling, CVS) from the placenta are removed, cultured, and tested for biochemical, cytogenetic, and genetic defects. Both sampling procedures present a risk to the fetus. The rates of miscarriage from amniocentesis and CVS are 0.5% to 1% and 1% to 2%, respectively.

Counseling should precede a prenatal test and provide information about the nature of the presumed disorder, the medical risks to both the fetus and mother posed by the sampling procedure, the significance of negative, ambiguous, and positive results, the likelihood that the fetus will have the disorder, and other pertinent issues. After a positive diagnosis, further consultation entails a discussion of the range of severity of the disorder, the most common symptoms, the potential emotional and economic consequences on the family, and the availability and effectiveness of the recommended treatment. If requested, all aspects pertaining to the termination of the pregnancy are examined. Finally, prenatal testing for adult-onset conditions is not usually sanctioned because there is no immediate medical benefit to be gained by the child.

Bayesian Determination of Recurrent Risks for Genetic Disorders Within Families

One of the most common concerns when a genetic disorder is diagnosed in a family is whether a nonaffected family member will have an affected child. A major task of genetic counseling is to determine the likelihood of this type of occurrence. The principles of Mendelian genetics can be sufficient for calculating such risks. For example, the birth of a child with an autosomal recessive disorder indicates that both parents are heterozygous (carriers) and the probability that the next child will be affected is 25%. Moreover, in this case, each child with a normal phenotype has a 1/3 and 2/3 chance of being homozygous dominant and heterozygous, respectively, because the expected ratio of the former to the latter is 1:2.

Clearly, a gene test that provides definitive information about carrier status makes the determination of risks straightforward. However, identification of heterozygotes is not currently possible for many inherited conditions. Fortunately, the probability that a person has a particular genotype can be calculated by applying the Bayes theorem that was devised by Thomas Bayes (1702–1761), a British clergyman and mathematician. This statistical method uses Mendelian principles to establish an initial (prior) probability. Then, a conditional probability is determined with additional information such as the number of unaffected siblings and/or offspring, age of onset, penetrance, diagnostic test results, and other features. A joint probability is generated by combining the prior and conditional probabilities. A final (posterior) probability is derived by normalizing the joint probabilities on the basis of the different hypotheses that are under consideration. The essence of calculating genetic risks in families with the Bayesian approach is readily conveyed with specific examples.

In Figure 17.6, the occurrence of two affected individuals (II-1, II-2) and their affected uncle (I-3) with the same X-linked recessive disorder indicates that I-2 is a heterozygote. It is also very unlikely that the disorder is due to new mutations because there are three affected individuals in the family. Under these circumstances, II-3 would very much like to know the likelihood that she is a carrier. Because I-2 is a carrier, then II-3 is either heterozygous (carrier) or homozygous dominant (noncarrier) for the X-linked gene. In other words, her prior probability as a carrier is 1/2. Thus the chance that II-3 will have an affected son is the probability of her being a carrier (1/2) times the probability of transmission of the mutant gene to a son (1/2), that is, $1/2 \times 1/2 = 1/4$.

Over time, II-3 of Figure 17.6 has two unaffected sons (Figure 17.7A). Now the Bayesian method can be used to determine how much the prior probability of II-3 as either a carrier or noncarrier has changed as a consequence of the birth of two unaffected sons. The prior probability that II-3 is a carrier is 0.5. If she is a carrier, then the probability of having a normal son is 0.5. In other words, the probability of this condition, that is, the conditional probability, is 0.5. The same conditional probability applies to the second son. Thus the joint probability of II-3 being a carrier and having had two normal sons is $(0.5)(0.5)(0.5) = 0.125$. But II-3 could very well be a noncarrier, that is, homozygous dominant. The prior probability for this genotype is 0.5. With this genotype, all her sons would be normal. Therefore the conditional probability for each unaffected son is 1.0. The joint probability of these occurrences under this hypothesis is $(0.5)(1.0)(1.0) = 0.5$. The difference between the two

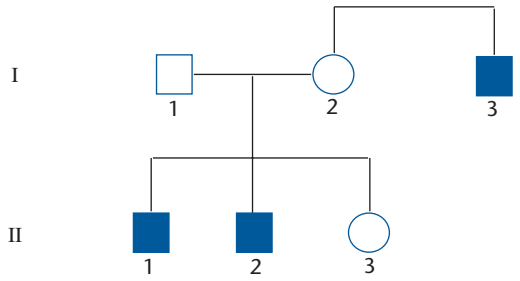
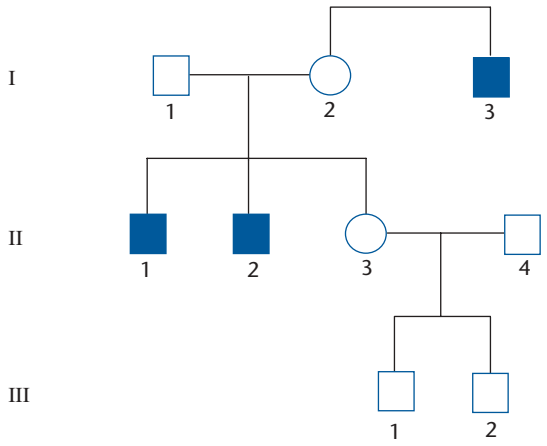


Figure 17.6 Pedigree of a family with an X-linked recessive disorder. Open and solid symbols denote unaffected and affected individuals, respectively.

A



B

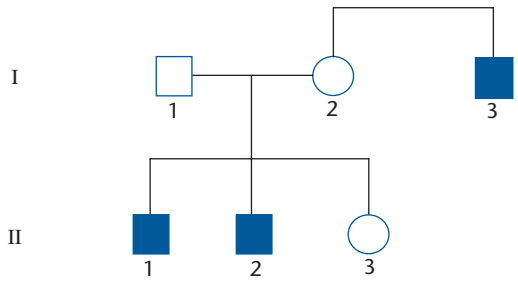
	Carrier	Noncarrier
Prior probability (II-3)	0.5	0.5
Conditional probability		
Unaffected son (III-1)	0.5	1
Unaffected son (III-2)	0.5	1
Joint probability	(0.5)(0.5)(0.5) = 0.125	(0.5)(1)(1) = 0.5
Posterior probability	0.125/0.125+0.5 = 0.2	0.5/0.5 + 0.125 = 0.8

Figure 17.7 Bayesian probability analysis. (A) An expanded pedigree of the family shown in Figure 17.6. (B) Determination of the posterior probabilities of the carrier and noncarrier status of individual II-3 in the pedigree in A.

joint probabilities, that is, 0.5 chance of being a noncarrier versus 0.125 chance of being a carrier, indicates that II-3 is four times more likely to be homozygous dominant than heterozygous. Because the sum of the joint probabilities for the two hypotheses is not unity, they cannot be represented directly as percentages. However, a simple normalization process converts each joint probability to a posterior probability. The joint probability for one hypothesis is divided by the sum of joint probabilities for all hypotheses, which yields a posterior probability. Specifically, for the carrier hypothesis, the posterior probability is $\frac{0.125}{0.125+0.5} = 0.2$ or 20%. And, for the noncarrier hypothesis, the posterior probability is $\frac{0.5}{0.5+0.125} = 0.8$ or 80%. Thus there is a 20% chance that II-3 is a carrier and an 80% probability that she has a homozygous dominant genotype (Figure 17.7B). The chance that her next child, if it is male, will be affected is 10%, that is, $(0.2)(0.5) \times 100$. If her next child is an unaffected son, then the posterior probability that she is a carrier becomes 11% and the probability of the next son being affected is (0.11)(0.5) = 0.055 or 5.5%. Obviously, if II-3 has an affected son, then the nature of her genotype is no longer in question because she would definitely be a carrier.

A conditional probability can be derived from information other than the likelihood of transmission of an allele to an offspring. For example, the results of a diagnostic test for the X-linked disorder that affects her brothers and uncle

A



B

	Carrier	Noncarrier
Prior probability (II-3)	0.5	0.5
Conditional probability		
Test result	0.3	0.95
Joint probability	(0.3)(0.5) = 0.15	(0.5)(0.95) = 0.475
Posterior probability	0.15/0.15 + 0.475 = 0.24	0.475/0.475 + 0.15 = 0.76

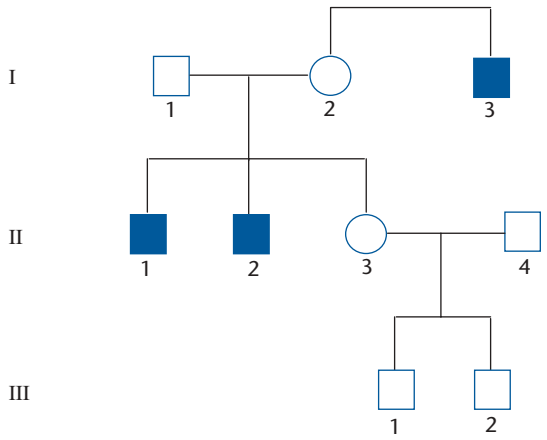
Figure 17.8 Bayesian probability analysis. (A) The same pedigree as in Figure 17.6. (B) Determination of the posterior probabilities of the carrier and noncarrier status of individual II-3 in the pedigree in A considering the results of a particular diagnostic test. The conditional probabilities are based on the test results for heterozygous (0.3) and homozygous dominant (0.95) individuals.

placed individual II-3 (Figure 17.8A) within the limits for homozygous dominant genotypes. However, this test is not conclusive because 30% of the carriers have the same scores as homozygous dominant individuals. Thus, in this case, the conditional probability for inferring the posterior probability that II-3 is a carrier is 0.30. Alternatively, the test is 95% effective for noncarriers, which represents a conditional probability for the hypothesis that II-3 is a noncarrier. With Bayesian analysis, the posterior probability that II-3 is a carrier is 0.24 (Figure 17.8B). The test result reduces her risk from 0.5 to 0.26. The posterior probability that II-3 is a carrier with a test result equivalent to a homozygous dominant genotype and two unaffected sons is 7.3% (Figure 17.9). And, the chance that her third son will be affected is only 3.65%, that is, $(0.5)(0.073) \times 100$.

With additional rules, Bayesian risk calculations can be determined for different members of a family where a prior probability for an offspring is derived from the posterior probability of a parent (Figure 17.10). In this example, the prior probability that III-3 is a carrier is the probability that her mother is a carrier times the probability of transmission of the recessive allele, that is, $(0.073)(0.5) = 0.0365$. Because III-3 can either be a carrier or a noncarrier, then the prior probability that she is a noncarrier is $1 - [(0.073)(0.5)] = 0.9635$. Obviously, there is a small chance that III-3 is a carrier. Moreover, with the birth of an unaffected son and a test result in the range of homozygous dominant individuals, she has an even more remote probability of being a carrier (Figure 17.10C).

Other conditions such as incomplete penetrance and delayed onset of the disorder can be taken into account for risk calculations. For example, the pedi-

A



B

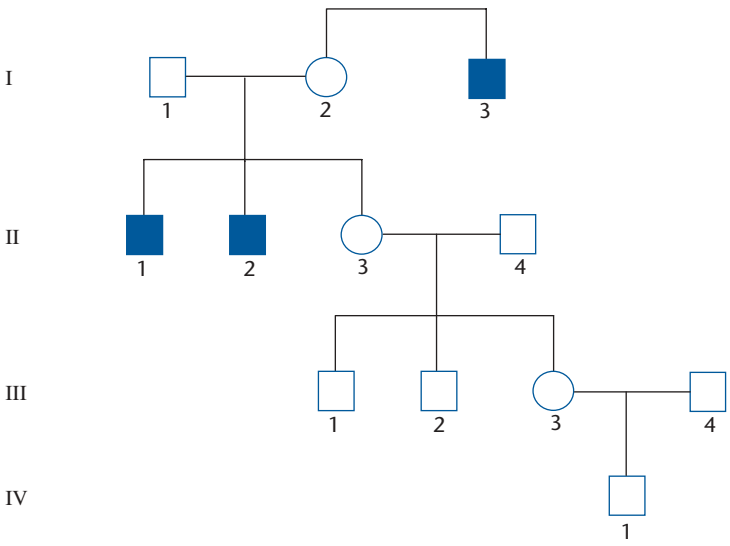
	Carrier	Noncarrier
Prior probability (II-3)	0.5	0.5
Conditional probability		
Unaffected son (III-1)	0.5	1
Unaffected son (III-2)	0.5	1
Test result	0.3	0.95
Joint probability	(0.5)(0.5)(0.5)(0.3) = 0.0375	(0.5)(1)(1)(0.95) = 0.475
Posterior probability	0.0375/0.0375 + 0.475 = 0.073	0.475/0.475 + 0.0375 = 0.927

Figure 17.9 Bayesian probability analysis. (A) Same pedigree as in Figure 17.7A. (B) Determination of the posterior probabilities of the carrier and noncarrier status of individual II-3. The conditional probabilities are based on the test results for heterozygous (0.3) and homozygous dominant (0.95) individuals and two unaffected sons.

gree in Figure 17.11A represents an autosomal dominant disorder that is 80% penetrant. Thus, considering that II-1 has a 50% chance of receiving a dominant allele from I-2 and a 20% chance of incomplete dominance with the assumption that I-1 is homozygous recessive, then there is 16.7% probability that he has the mutant allele (Figure 17.11B). With age of onset as a consideration, then II-1 in Figure 17.12A, who is at an age when 60% of the heterozygotes manifest the onset of a completely penetrant autosomal dominant disorder, has a 28.6% probability of carrying the mutant allele (Figure 17.12B).

In contrast to X-linked recessive disorders, where only the genotype of the mother is relevant, the carrier status of both parents must be considered in the risk calculations for autosomal recessive disorders. The couple II-1 and II-2 in Figure 17.13A want to know the probability that their fifth child will have the same autosomal disorder that affected both II-3 and II-4. Because I-1 and I-2 had two affected offspring with an autosomal recessive disorder, it is highly likely that they are both heterozygotes. Thus the probability that II-2 is heterozygous or homozygous dominant is 2/3 (0.667) or 1/3 (0.333), respectively. Because there is no history of this particular autosomal recessive disorder in her husband's (II-1) family, his risk of being a carrier for the same mutant gene is equal to the frequency of heterozygotes for this disorder in the population at large, say 1 in 80. Accordingly, there are four possible combinations of

A



B

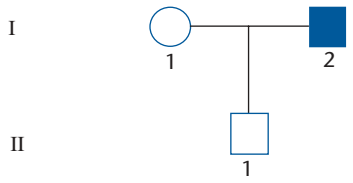
	Carrier	Noncarrier
Prior probability (II-3)	0.5	0.5
Conditional probability		
Unaffected son (III-1)	0.5	1
Unaffected son (III-2)	0.5	1
Test result	0.3	0.95
Joint probability	(0.5)(0.5)(0.5)(0.3) = 0.0375	(0.5)(1)(1)(0.95) = 0.475
Posterior probability	0.0375/0.0375 + 0.475 = 0.073	0.475/0.475 + 0.0375 = 0.927

C

	Carrier	Noncarrier
Prior probability (III-3)	(0.073)(0.5) = 0.0365	1 - (0.073)(0.5) = 0.9635
Conditional probability		
Unaffected son (IV-1)	0.5	1
Test result	0.3	0.95
Joint probability	(0.0365)(0.5)(0.3) = 0.005475	(0.9635)(1)(0.95) = 0.915325
Posterior probability	0.005475/0.005475 + 0.915325 = 0.0059459	0.915325/0.915325 + 0.005475 = 0.9940541

Figure 17.10 Bayesian probability analysis. (A) Same pedigree as in Figure 17.9A with the addition of III-3 and her family. (B) Determination of the posterior probabilities of the carrier and noncarrier status of individual II-3 (see Figure 17.9B). (C) Determination of the posterior probabilities of the carrier and noncarrier status of individual III-3 based on the prior probabilities that she is either a carrier or noncarrier considering the accuracy of the diagnostic test (see Figure 17.8B) and one unaffected son (IV-1).

A



B

	Heterozygous	Homozygous recessive
Prior probability (II-1)	0.5	0.5
Conditional probability		
80% Penetrance	0.2	1
Joint probability	(0.5)(0.2) = 0.1	(0.5)(1) = 0.5
Posterior probability	0.1/0.1 + 0.5 = 0.167	0.5/0.5 + 0.1 = 0.833

Figure 17.11 Bayesian probability analysis. (A) A family with an 80% penetrant autosomal dominant disorder. The solid symbol denotes an affected individual. (B) Determination of the posterior probability that individual II-1 has inherited the disease-causing autosomal dominant gene based on 80% penetrance.

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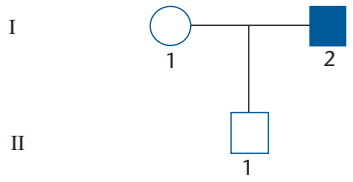


B

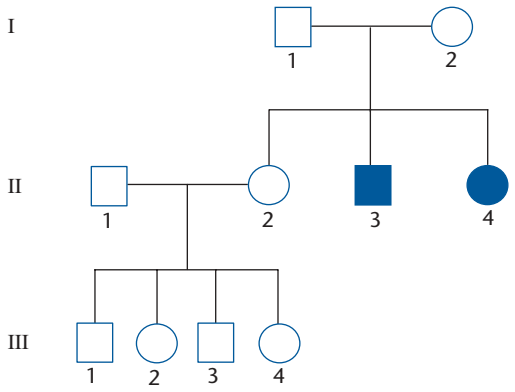
	Heterozygous	Homozygous recessive
Prior probability (II-1)	0.5	0.5
Conditional probability		
60% Onset at age of II-1	0.4	1
Joint probability	(0.5)(0.4) = 0.2	(0.5)(1) = 0.5
Posterior probability	0.2/0.2 + 0.5 = 0.2857	0.5/0.5 + 0.2 = 0.7143

Figure 17.12 Bayesian probability analysis. (A) A family with a late-onset autosomal dominant disorder. The solid symbol denotes an affected individual. (B) Determination of the posterior probability that individual II-1 has inherited the disease-causing autosomal dominant gene based on the information that 60% of the individuals with this gene who are as old as II-1 already have the major symptoms of the disorder.

genotypes with respect to the carrier or noncarrier (i.e., homozygous dominant) status of either II-1 or II-2. They both could be carriers, both noncarriers, or one or the other a carrier or a noncarrier. Remember that 3/4 of the offspring of a carrier-carrier mating for an autosomal recessive disorder are unaffected and every offspring is unaffected if one parent is homozygous dominant. The posterior probability that II-1 and II-2 are both carriers is 0.00265 or about 0.27% (Figure 17.13B). Thus the risk that their next child will be affected is (1/4)(0.0027) = 0.000675, or about 0.07%. In this vein, before the birth of their first child and assuming that II-1 and II-2 were both carriers, the likelihood that they would have an affected child was (1/4)(2/3)(1/80) = 0.0028, or 0.2 %. Obviously, the birth of four unaffected children is important information for determining whether a fifth child is likely to be affected.

Posterior probabilities for primary (parents, offspring, siblings) and secondary (uncles, aunts, grandchildren) relationships within families have been

A



B

Individual II-2	Carrier		Noncarrier	
Prior probability	2/3		1/3	
Individual II-1	Carrier	Noncarrier	Carrier	Noncarrier
Prior probability	1/80	79/80	1/80	79/80
Conditional probability				
Unaffected (III-1)	0.75	1	1	1
Unaffected (III-2)	0.75	1	1	1
Unaffected (III-3)	0.75	1	1	1
Unaffected (III-4)	0.75	1	1	1
Joint probability	$(2/3)(1/80)(0.75)^4 = 0.00264$	$(2/3)(79/80)(1)^4 = 0.65866$	$(1/3)(1/80)(1)^4 = 0.00417$	$(1/3)(79/80)(1)^4 = 0.32917$
Posterior probability	0.00265	0.66221	0.00419	0.33094

Figure 17.13 Bayesian probability analysis. (A) A family with an autosomal recessive disorder. The solid symbols denote affected individuals. (B) Determination of the posterior probabilities of the carrier-carrier and other genotype combinations for II-1 and II-2. The posterior probability for the carrier-carrier combination is $\frac{0.00264}{0.00264 + 0.65866 + 0.00417 + 0.32917} = 0.00265$, and so on for the other possibilities.

assembled into tables as convenient references for genetic counselors. In addition, recurrence risk assessments for complex genetic disorders (e.g., Table 17.3) and other conditions (e.g., Table 17.4) that cannot be readily inferred from pedigrees have been formulated from large population-based data sets and tabulated for use during consultations. Finally, computer programs are available for inferring the genetic status of family members for both simple and complex situations.

Screening Programs for Genetic Disorders

About 4×10^6 newborns (neonates) in the US and millions more around the world are screened annually for various genetic disorders. By definition, a screening test is performed on a large population of individuals who do not have any obvious symptoms to discover those who are likely to develop an inherited disorder. By contrast, a diagnostic test is conducted when there is a higher than average risk that a disease is present to confirm a diagnosis, or to

Table 17.3 Recurrence risks (%) for some complex disorders among various relationships.

Disorder	General population	1st-Degree relatives	2nd-Degree relatives	3rd-Degree relatives
Nonsyndromic cleft lip/palate	0.04	2.94	0.46	0.17
Pyloric stenosis	0.30	7.50	2.00	0.40
Congenital heart disease	0.60	3.50	0.70	0.20
Club foot	0.10	5.00	0.60	0.20
Congenital hip dislocation	0.20	5.00	0.60	0.40
Diabetes mellitus type I	0.20	5.00	2.50	<1.5
Diabetes mellitus type II	3.50	12.50	4.00	<2.00
Manic depressive illness	1.00	15.00	5.00	3.50
Schizophrenia	1.00	13.00	3.00	2.00
Autism	0.05	5.50	0.18	0.12

Table 17.4 Age-specific probabilities of breast cancer of women as a function of the age of onset of breast cancer in a first-degree relative.

	First-degree relative with age of onset (yr)					
Age of Woman (yr)	20–29	30–39	40–49	50–59	60–69	70–79
29	0.007	0.005	0.003	0.002	0.002	0.001
39	0.025	0.017	0.012	0.008	0.006	0.005
49	0.062	0.044	0.032	0.023	0.018	0.015
59	0.116	0.086	0.064	0.049	0.040	0.035
69	0.171	0.130	0.101	0.082	0.070	0.062
79	0.211	0.165	0.132	0.110	0.096	0.088

Derived from Table 2 in Claus, E. B., N. Risch, and W. D. Thompson. 1994. Autosomal dominant inheritance of early-onset breast cancer. Implications for risk prediction. *Cancer* **73**:643–651.

establish the carrier status of a person who could possibly transmit a deleterious gene to an offspring. In most jurisdictions, newborn screening is mandated by law and carried out as a routine procedure after birth. In some regions, a sample from a newborn for genetic testing can only be obtained with the informed consent of the parents.

Traditionally, the essential prerequisite of a screening newborn program was the identification of a serious disorder that would benefit from treatment before the onset of symptoms. However, adherence to this principle is slackening as an increasing number of families want to know whether they have a presymptomatic child with an untreatable disorder. Such information would enable families to be prepared emotionally and practically for a child with an incurable condition and plan future pregnancies. As well, medical researchers could follow the progress of a disease from its earliest stages to learn more about the disorder and use these data to devise therapeutic measures. Screening programs

that test for a large number of disorders will become feasible as new, rapid, and inexpensive assay systems such as tandem MS are put into practice.

In the US, all 50 states screen newborns for phenylketonuria (PKU) and congenital hypothyroidism; a large number for galactosemia (47) and sickle cell anemia (41); some for biotinidase deficiency (22), maple syrup urine disease (20), congenital adrenal hyperplasia (20), and homocystinuria (14); and a few for medium-chain acyl-CoA dehydrogenase deficiency (5), cystic fibrosis (3), and tyrosinemia type 1 (2). Some states test for at least three different genetic disorders, and others for 20 or more. Newborn screening is conducted in most developed countries, although the roster of tests often varies according to the incidence of a disorder. For example, sickle cell anemia in newborns is screened in the US but not in the UK, Australia, or New Zealand. The features of some of the genetic disorders that are detected by screening newborns are described in Table 17.5.

There is no single set of standards for establishing a screening program for newborns. In addition to the requirement that early screening ought to benefit the infant and improve the outcome, the disease should be serious and well-understood both clinically and biochemically and should occur relatively frequently. Each diagnostic test must be safe, simple, and extremely accurate. In

Table 17.5 Genetic disorders that are detected by newborn screening programs.

Disorder	Frequency	Defect	Detection	Symptoms	Treatment	Outcome
Phenylketonuria (PKU)	~1/12,000	Phenylalanine hydroxylase deficiency	Guthrie test; fluorometry; chromatography; tandem MS	Severe mental retardation; seizures; hyperactivity; muscular hypertonicity	Phenylalanine restrictive diet instituted by 3 wk of age; frequent monitoring of phenylalanine blood levels	Early treatment prevents mental retardation
Congenital hypothyroidism	1/4,000	Defective thyroid gland	Radioimmunoassay (RIA) for thyroxine (T4) or thyroid-stimulating hormone (TSH)	At birth: difficulty feeding, constipation, sluggishness, jaundice; neonatal: sparse growth, mild to severe mental retardation, tremors, lack of coordination	Oral doses of thyroxine	Early treatment averts many extreme symptoms
Sickle cell anemia	1/400 among African Americans; <1/50,000 in other populations	Defective β-hemoglobin (*HBB*; Glu6Val)	Electrophoresis; thin-layer isoelectric focusing; DNA test	Severe anemia; severe infections; chronic debilitation; jaundice; recurrent joint pain	Antibiotics; immunization against *Streptococcus pneumoniae* and *Hemophilus influenzae*; symptom-specific procedures as required	Survival beyond adolescence; ongoing treatments; alleviation of pain; partial control over crises

Table 17.5 Genetic disorders that are detected by newborn screening programs (*continued*)

Disorder	Frequency	Defect	Detection	Symptoms	Treatment	Outcome
Congenital adrenal hyperplasia	1/12,500	Enzyme defect in steroid biosynthetic pathway; usually (>90%) 21-hydroxylase deficiency	Radioimmunoassay; dissociation-enhanced lanthamide fluorescence immunoassay (DELFIA); DNA test	Excessive vomiting; diarrhea; dehydration; cardiovascular collapse; excess sodium levels; deficient potassium levels; learning difficulties; genital virilization in females	Hydrocortisone	Many symptoms alleviated; acute crises prevented
Maple syrup urine disease [MSUD; maple syrup disease (MSD)]	1/185,000; 1/176 among Mennonites	Branched-chain 2-keto acid dehydrogenase deficiency	Bacterial inhibition assay; tandem MS	Excess leucine, isoleucine, and valine in the blood and urine; isoleucine metabolites cause maple syrup smell; lethargy; poor sucking; weight loss; neurological damage; convulsions; respiration difficulties; blindness; spasticity; mental deficiency	Reduced dietary intake of branched-chain amino acids; high-energy diet to reduce protein metabolism; dialysis; supportive care	Growth and psychomotor development are often normal if treatment is initiated before 14 days after brith; fatal cerebral edema may occur despite treatment
Homocystinuria	<1/85,000	Cystathionine β-synthase deficiency	Guthrie test; chromatography; tandem MS	Myopia; dislocated lens; skeletal abnormalities; mental retardation; psychiatric disturbances; blood disorders	About 50% of the patients respond to high daily doses of pyridoxine (vitamin B6) plus folic acid; the remaining patients are given a methionine-restricted diet supplemented with betaine and L-cystine	If either treatment is initiated early, most serious symptoms are avoided
Tyrosinemia type 1	1/100,000	Fumarlyacetoacetase deficiency	Biochemical, chromatographic, or fluorescent assay; tandem MS	Acute form: vomiting, diarrhea, lethargy, failure to thrive, liver failure; chronic form: protracted liver damage, kidney	Low tyrosine and phenylalanine diet; liver transplant; 2-(2-nitro-4-trifluoromethyl benzoyl)-1,3-cyclohexanedione (NTBC)	Restricted diet improves kidney function but does not prevent liver deterioration; progression of the disease is

Table 17.5 Genetic disorders that are detected by newborn screening programs (*continued*)

Disorder	Frequency	Defect	Detection	Symptoms	Treatment	Outcome
				failure, fatal by 10 yr of age		slowed but not prevented; if initiated early, NTBC seems to improve health and prevents most of the major symptoms
Medium-chain acyl-CoA dehydrogenase deficiency (MCAD)	1/13,000	Medium-chain acyl-CoA dehydrogenase deficiency	Metabolite assay; tandem MS	Infections; lethargy; vomiting; respiratory arrest; encephalopathy; enlarged liver; seizures; sleep apnea; cardiac failure; muscle weakness	Large intravenous doses of glucose; frequent meals; low fat intake; antibiotics; standard care for apnea, cardiac arrest, and cerebral ecdema	Prognosis good if treatment is initiated early; damage from previous episodes cannot be reversed
Cystic fibrosis	1/2,500 among Europeans	Defective cystic fibrosis transmembrane regulator (*CFTR*) gene	"Sweet" test; immunoreactive trypsin/ trypsinogen (IRT) assay; combined IRT-DNA tests	Neonatal intestinal blockage; absorption difficulties; malnutrition; inflamed airways; chronic lung disease; pancreatic enzyme deficiency	Anti-inflammatory drugs; pancreatic enzyme supplementation; antibiotics; improved nutrition	Fewer chest infections; reduced hospital care; increased quality of life; extension of life expectancy
Galactosemia	1/62,000	Usually galactose-1-uridyl-transferase deficiency; also galactokinase or uridine diphosphate galactose-4-epimerase deficiency	Microbiological, enzyme activity, or chromatographic assays	Jaundice; failure to thrive; enlarged liver and spleen; kidney malfunction; infections; cataracts; speech defects; reduced intellectual capacity	Galactose-free diet; antibiotics	Acute neonatal phase is prevened, but long-term complications are not averted
Biotinidase deficiency	1/61,000	Insufficient biotinidase activity	Enzyme activity assay	Progressive skin infections; rashes; seizures; developmental delays; ataxia; loss of muscle tone; visual and hearing problems	Oral biotin supplement	All symptoms are abolished; consequences, if any, of long-term treatment are not known

this regard, most of the current neonate tests rely on a sample of blood from the heel (heelstick), dorsal vein of the hand, or the umbilical cord. Because newborn tests are concerned with identifying the presence of a disorder and not primarily with the nature of the gene mutation(s) that causes the disorder, assays other than gene tests are often used. This approach is especially helpful if a disease is due to a number of different mutations within a gene that produce the same biochemical phenotype. About 2500 infants with an inherited disorder are identified annually in the US through newborn screening programs. Although it is difficult to assess all the key parameters thoroughly, most economic analyses indicate that the benefits of screening outweigh the costs. Among the positive features, the duration of hospitalization is reduced and the expense of diagnosis after symptoms occur is lessened. But most importantly, a large number of children are spared the full impact of a serious disease. In this context, the best example of the benefits of screening newborns is PKU. Since the 1960s, early treatment has prevented severe mental retardation in more than 20,000 individuals.

The primary targets of newborn screening programs are monogenic disorders. However, as the detection systems become more sophisticated, genetically heterogeneous and complex (multigenic) conditions may become candidates for newborn testing. For example, deafness/hearing loss occurs in 1 in 500 births in the US. And, because at least 60 different genes contribute directly or indirectly to this phenotype, it is difficult to pinpoint the basis of sporadic and familial hearing loss. Regardless of whether deafness has either a genetic or nongenetic origin, detection before 6 mo of age that is followed with the appropriate training leads to normal speech development and academic achievement. Moreover, the need for expensive rehabilitation programs during school years is minimized. Electrophysiological procedures that are painless and noninvasive and require about 5 min to perform determine the extent of hearing in infants. One of these tests entails placing electrodes on the head of a sleeping infant to measure whether sound is received by the brain (auditory brainstem response, ABR). A second strategy involves placing a tiny receiver in the ear of a sleeping infant to detect sounds produced by the inner ear (otoacoustic emissions, OAE). If the brain does not respond to sound in the ABR test or the inner ear emissions are not generated in the OAE test, then hearing is impaired. Although a likely addition to the current newborn screening programs, the feasibility of testing newborns for hearing loss, validity of the diagnostic methods, costs, availability of treatment, and other issues must be resolved before widespread testing can be implemented.

There are no universal screening programs for prevalent adult-onset genetic disorders although population-specific carrier testing of Tay–Sachs disease (TSD) among Ashkenazi Jews, thalassemia in Mediterranean countries, and various conditions in populations with founder effects is performed routinely. Carrier screening of Ashkenazi Jews in the US and Canada over the past 30 years has reduced the incidence of TSD in these populations by about 95%.

A possible candidate for an adult screening program is hereditary hemochromatosis (HHC). This autosomal recessive condition occurs between 40 to 60 yr of age and is characterized by excessive deposition of iron in the liver, heart, pancreas, skin, and joints that causes weakness, lethargy, and weight loss.

If HHC is not treated by the removal of blood (phlebotomy), the combination of diabetes and liver, heart, and other organ damage is usually fatal. Initiation of a regular regimen of blood removal before the onset of the symptoms prevents all complications. Treatment after the disease becomes established is not fully effective in reversing the symptoms. Until the recent development of a DNA-based test for mutations in the *HFE* gene that is responsible for HHC, there was no way of determining which individuals were at risk for the disorder.

At 1 in 300, the incidence of HHC is very high in the US, Ireland, the UK, and parts of northern Europe. With a carrier frequency of 11%, HHC is the most common genetic disorder in the US. From 60% to 90% of HHC patients are homozygous for a single mutation (C282Y/C282Y) in the *HFE* gene, others are homozygous (H63D/H63D) for another *HFE* mutation and some are compound heterozygotes (C282Y/H63D). A DNA test, which is accurate and relatively inexpensive, readily detects these mutations and the corresponding genotypes. Theoretically, a universal adult screening program for *HFE* gene mutations would reveal those individuals who will develop HHC, and, consequently, with the initiation of regular treatments, prevent the appearance of the symptoms. In practice, among other considerations, the administration and laboratory costs may be too high and the extent of participation too low to warrant a nationwide adult HHC screening program. However, family history and individual concern will probably provide sufficient incentive for a large number of people to opt for presymptomatic testing.

Treating Genetic Disorders

Medical management of genetic disorders is invariably challenging because almost every condition has an unique constellation of symptoms. Therapeutic measures range from supportive care dedicated to alleviating pain and enhancing the quality of life as much as possible to cures that abolish all clinical features. In this context, the assertion of the Roman poet Ovid (43 BCE–17 CE) that "a thousand ills require a thousand cures" is more than apt.

Most of the experience of managing genetic diseases has been gathered from inherited inborn errors of metabolism and, to a lesser extent, from disorders with defective structural proteins. In broad terms, the strategies for treating genetic disorders include restrictive or supplemented diets, inhibition of enzyme reactions to prevent the accumulation of toxic molecules, removal of toxic molecules, replacement of defective or absent proteins, restoration of protein activity, selective protein removal, organ and bone marrow transplantation, and nucleic acid-based therapies (Figure 17.14).

Restrictive diets prevent the intake of metabolites that are present in excess in the body as a result of a metabolic disorder. This approach is effective for treating PKU, but not with too many other inherited conditions. Alternatively, for different metabolic disorders, the amount of a toxic substance may be reduced by dialysis, facilitated excretion, or in vivo conversion of the toxic compound to a benign form. When a metabolic deficiency occurs, it can be treated with either a supplemented diet or intravenous administration of the required compound. Enzymatic activity may be restored by ingestion of an essential

Specially formulated diets
- restrictive diet to lower the intracellular level of a toxic molecule
- supplemented diet to replace a metabolic deficiency

Inhibition of enzyme reactions
- enzyme inhibitor to prevent the accumulation of a toxic molecule by blocking a step in a metabolic pathway that precedes the reaction with a defective enzyme

Removal of toxic molecules
- dialysis
- removal of excess cations (chelation)
- facilitation of excretion by binding a toxic molecule to a small molecular weight compound

Replacement of defective or missing product
- enzyme replacement therapy
- protein replacement therapy
- cofactor supplementation

Alteration of defective protein by small molecules
- restoration of partial protein function
- directed proteolytic degradation of a defective protein

Transplantation
- replacement a nonfunctional organ (organ transplantation)
- providing a required protein synthesized by blood cells (bone marrow transplantation)

Gene therapy
- rectification of a genetic defect with a functional gene

Nucleic acid therapy
- blocking translation of mRNA from a mutant gene with an oligonucleotide (antisense, ribozyme)
- correction of a gene mutation with an oligonucleotide

Figure 17.14 Strategies for treating genetic disorders.

cofactor (substitutive therapy). Finally, standard medical treatments such as surgery, blood transfusions, anti-inflammatory agents, and organ and bone marrow transplantation are also part of the care for many genetic diseases.

With the development of recombinant DNA technology, large amounts of enzymes and structural proteins are available for therapeutic use. Infusions of β-glucosidase, α-galactosidase, α-L-iduronidase, and adenosine deaminase have significantly reduced the adverse effects of Gaucher disease, Fabry disease, mucopolysaccharidosis I, and severe combined immunodeficiency disorder (SCID) in clinical trials. This type of treatment, which is called enzyme replacement therapy (ERT), works well when the circulatory system is able to deliver either the enzyme or structural protein (protein replacement therapy) to its biological site of action.

Novel strategies using easily administered low-molecular-weight compounds are being devised to overcome the harmful effects of mutant proteins. For example, depending on the site of binding, a complex is formed between a small molecule and a defective protein that prevents the mutant protein from inhibiting normal functions, restores its function, or marks it for selective proteolytic degradation.

Since the 1940s, when it was discovered that a gene from one strain of bacteria could be transferred to and expressed in another strain, researchers, clinicians, and others have theorized that human genetic diseases might be cured in an analogous manner. Basically, introduction of a normal gene into a cell with a defective gene ought to correct the disorder because the transferred (input) gene provides the required gene product. In 1990, after exhaustive

reviews by many different regulating panels in the US, the first human gene therapy trial was initiated. Two young girls with adenosine deaminase (ADA)-deficient SCID received large doses of their own cells that had been engineered to carry a functional adenosine deaminase gene. In both instances, the adverse symptoms were alleviated, indicating that this form of therapy was feasible. One of the patients has been free of the SCID phenotype for more than 10 years. The second ADA-deficient patient from the original trial and others from additional experiments have not had permanent cures. As well, most of the gene therapy trials with other disorders such as cystic fibrosis have not been particularly successful. Notwithstanding the general lack of effective cures, these various trials provided a great deal of information about the protocols of gene delivery systems, duration of gene expression, and other technical features of this form of therapy. Generally, this research was safe despite the failure to correct any genetic disorder with an exogenous functional gene. However, in September 1999, the attitude toward gene therapy dramatically changed. Jesse Gelsinger, a healthy 18-year-old with ornithine transcarbamylase (OTC) deficiency, was given a large dose of a virus carrying the *OTC* gene as part of a clinical trial. Tragically, he died 4 days later of a massive immune response. As a consequence, although this disastrous outcome was not predictable, the rigorous requirements for human gene therapy experiments became even more stringent. Researchers, in addition, became disinclined to initiate new trials.

In 2000, with a new set of protocols, two infants with an X-linked form of SCID (SCID-X1) were successfully treated with the gene encoding the subunit (γc) that is part of various cytokine receptors. These patients were free of symptoms for 10 months and are being monitored to determine whether the correction is permanent. Also, hemophilic patients expressed an input gene encoding the blood coagulation factor IX for long periods that, importantly, reduced the extent of the condition from severe to mild. Although promising, it is unlikely the gene-based therapies will be available on a regular basis in the next decade. Similarly, oligonucleotide-based therapies may be problematic. In these cases, preliminary research has shown that oligonucleotides could be used to either prevent the translation of mRNA from a mutant gene or correct specific disease-causing mutations.

Clinical Features, Diagnosis, and Treatment of Some Genetic Disorders

Specialized handbooks, comprehensive review articles in medical journals, and authoritative essays posted at Internet sites (e.g., http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM, http://www.geneclinics.org) are the common sources of information about the distinctive features, definitive diagnostic tests, and effective treatments of inherited conditions. Here, by way of illustration, brief characterizations of the clinical aspects of some genetic disorders are presented.

Williams Syndrome

Williams syndrome [WS, Williams-Beuren syndrome (WBS)] is a multisystem disorder that affects about 1 in 20,000 individuals worldwide. It is

inherited as an autosomal dominant disorder. Most of the cases are sporadic rather than familial.

Clinical Features. Cardiovascular disease is a prevalent component of WS that often entails the narrowing of arteries, especially the aortic artery above the aortic valve (supervalvar aortic stenosis, SVAS). Connective tissue abnormalities are common and include hoarse voice; abnormal joint movement; soft, flexible skin; groin and abdominal ruptures; and protrusions (diverticulae) of the bladder and bowel that extend through the surrounding layers. There is excess calcium in the blood and urine. Feeding problems, colic, and constipation curtail growth and weight gain during the first 4 years after birth. The facial features (facies) are distinctive with a broad forehead; narrowing at the temples; epicanthal folds; crossed eyes (strabismus); full cheeks and lips; a rounded tip of the nose; puffiness around the eyes; prominent ear lobes; small chin and jaw; small, widely spaced teeth; a longer than normal groove (philtrum) in the midline of the upper lip and a starlike pattern in the iris (Figure 17.15).

Among the very specific cognitive abilities and personality traits are mild to severe mental retardation; very sensitive hearing; excellent memory for information processed by the auditory system (auditory rote memory); grammatically complex vocabulary; very expressive story telling; proficient use of language; excellent musical skills; extreme friendliness; short attention span (attention-deficit disorder); extremely poor visual-spatial comprehension; and difficulty with drawing, mathematics, writing, abstract thinking, and sequential actions such as dressing.

Diagnosis. About 99% of WS patients are heterozygous for a large deletion at 7q11 that covers about 1.5 Mb and removes at least 20 genes. The WS

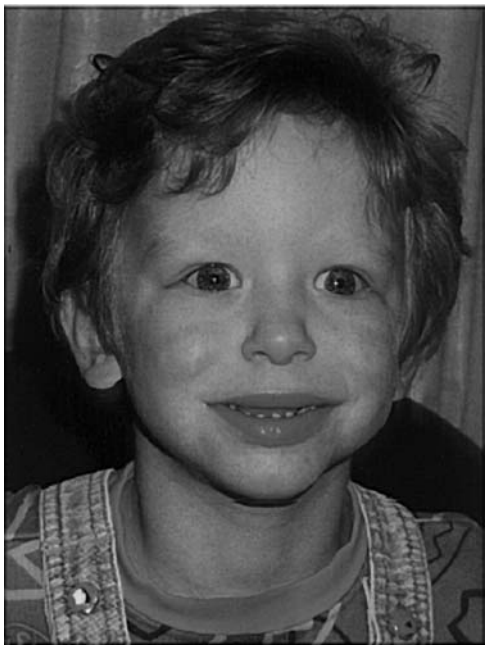


Figure 17.15 Facial features of a child with Williams syndrome. From Figure 2 in Morris, C. A. and C. B. Mervis. (2000). Williams syndrome and related disorders. *Annu Rev Genomics Hum Genet* **1**:461–484.

deletion is detected by fluorescent in situ hybridization (FISH) with a 180-kb probe that carries the elastin gene (*ELN*; 7q11.23). The cardiovascular and connective tissue abnormalities associated with WS are likely due to hemizygosity of the *ELN* gene.

Treatment. The care of WS patients is multifaceted and based on the symptoms of each individual. Usually, management of the disorder entails special education programs; psychological and psychiatric counseling; lifelong monitoring of the cardiovascular system; corrective surgery for arterial stenosis, hernias, and diverticulae; reduced-calcium diet; orthodontic correction of dental misalignment; and medication to relieve chronic abdominal pain, constipation, and infections of both the gastrointestinal and urinary tracts.

Norrie Syndrome (Norrie Disease)

Norrie syndrome (NS) is a rare recessive condition due to mutation of the *NDP* gene at Xp11.4 that affects fewer than 1 in 200,000 worldwide.

Clinical Features. Fibrous and vascular abnormalities of the retina are present to the same extent in both eyes at birth. The deterioration of the eye progresses through adolescence and adulthood. Grayish-yellow masses (pseudogliomas) are present behind each lens in newborns. By 10 years of age, there is cataract formation; corneal clouding; reduction of the anterior chamber; shrinking of the globe; and other damage to the eye. Complete blindness is common. The less severe forms of NS have a fibrous stalk extending from the optic disk to the posterior lens capsule (persistent hyperplastic primary vitreous, PHPV); no blood vessels at the periphery of the retina; and/or retinal folding (familial exudative vitreoretinopathy, FEVR). Mental retardation and hearing loss affect about 35% to 50% of NS patients.

Diagnosis. Point mutations of the *NDP* gene, which account for about 70% of all the occurrences of, NS, are unique. Consequently, single-strand conformation polymorphism (SSCP) analysis, or less often, DNA sequencing of the complete coding region of the *NDP* gene is carried out to determine the presence of a mutation. Submicroscopic deletions of the *NDP* gene occur in about 24% of cases of NS and are detected by either a change in the size or absence of an expected PCR fragment.

Treatment. There is no treatment that will either restore or prevent the loss of sight in severe cases. Consequently, care is supportive and addresses specific symptoms. Retinal surgery may be beneficial for mild cases. Hearing is monitored regularly. When required, hearing aids effectively overcome NS-specific deafness. Special education programs and counseling provide strategies for maximizing the quality of life.

Achondroplasia

Achondroplasia is an autosomal dominant disorder that produces short stature due to deficient bone growth. Affected adult males and females reach about 4 feet 4 inches (131 cm) and 4 feet 1 inch (124 cm) in height, respectively. The worldwide incidence is about 1 in 25,000.

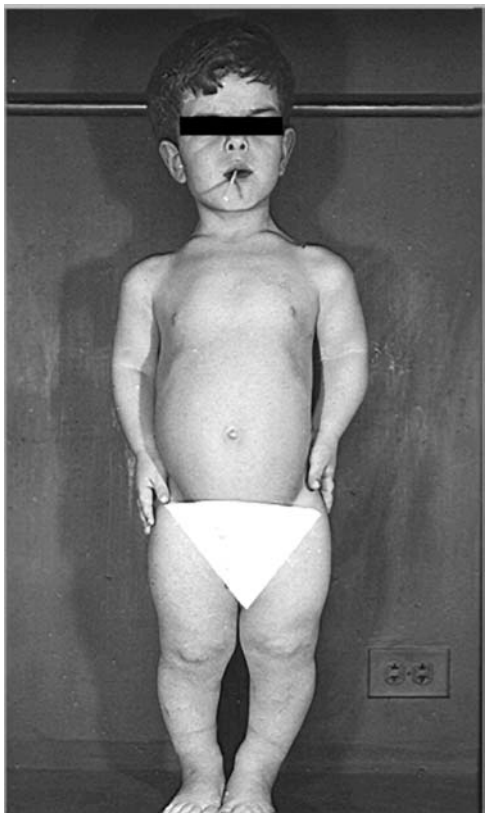


Figure 17.16 Body structure of a child with achondroplasia. From http://matweb.hcuge.ch/matweb/Selected_images/Developmental_genetic_diseases/achondroplasia.htm

Clinical Features. Achondroplasia can be noted by physical appearance at birth and during infancy. Generally, the phenotype includes a long, narrow body with short arms and legs; disproportionately shortened upper arms and thighs in comparison to distal limb segments (Figure 17.16); a large head; prominent forehead (frontal bossing); underdeveloped midface; flat nasal bridge; divergence between the second and third fingers that gives the hand a three-pronged appearance (trident hand); hyperextendable knee joints; inflexible elbows and hips; decreased muscle tone; and a small prominence of the mid-to-lower back region. Intelligence is completely normal despite developmental delays. The spine is often S-shaped with inward (lordosis) and outward (kyphosis) curvatures in the lumber and upper regions, respectively. The legs bow outward and create a gait with a distinctive swaying motion.

Diagnosis. The physical features are usually sufficient for an accurate diagnosis of achondroplasia. Radiology (X rays), ultrasound, or other imaging techniques can be used to substantiate the skeletal defects. A DNA-based test is available for newborns before the onset of the symptoms and for atypical cases. About 98% of those with achondroplasia have a point mutation (G1138A) in the *FGFR3* gene at 4p16.3 that encodes fibroblast growth factor receptor 3.

Treatment. All current treatments for achondroplasia are supportive in nature, with specific requirements for infants, children, and adults. Because of both the large size of the head and the weakness of the neck muscles,

positions that could block breathing of newborns must be avoided. Infections of the inner ear are common in infants and children and must be treated as soon as possible to avert hearing loss. Surgery may be needed to prevent sudden and possibly fatal cessation of breathing (apnea) during sleep. Excessive weight gain occurs frequently during childhood and, if not controlled, exacerbates skeletal and joint problems. Growth hormone therapy and surgical elongation of limbs have been attempted to increase height. However, the effectiveness of either of these procedures has not yet been established. Among adults, painful narrowing of the spinal canal is corrected by surgery (laminectomy). Orthopedic care may be required to correct extreme bowing of the legs and excess spinal curvature. Affected individuals and their families may seek various types of counseling to help with issues concerning socialization, the psychological impact of the physical consequences of the disorder, and how to cope with everyday activities in a society that is designed for taller people.

Familial Mediterranean Fever

Familial mediterranean fever (FMF) is an autosomal recessive disorder that results from mutations of the *MEFV* gene at 16p13.3. The frequency of FMF is 1 in 200 or greater in indigenous Mediterranean populations including Turks, Armenians, Arabs, and Sephardic Jews. A mild form of FMF is found among Ashkenazi Jews. The *MEFV* gene encodes the protein pyrin, which, in all likelihood, constrains the inflammatory response in differentiated granulocytes. Otherwise, the inflammatory response would be excessive and prolonged.

Clinical Features. Familial mediterranean fever has two major aspects. First, the primary symptom is recurring, high-fever (104˚F, 40˚C) inflammatory episodes that are accompanied by severe pain in the abdominal area (peritonitis), joints (synovitis, arthritis) especially in one hip, knee, or ankle, and the thoracic cavity including the lungs (pleuritis). Peritonitis, synovitis, and pleuritis occur in about 90%, 75%, and 45% of FMF patients, respectively. An episode usually lasts from 24 to 72 hours and occurs every 2 to 4 weeks. None of the extreme discomfort is present between attacks. The duration and intensity of the episodes and the interval between them are variable. A rash (erysipeloid erythema) may develop on the lower leg during an attack. The indicators of an acute inflammatory phase, namely, neutrophils, fibrinogen, C-reactive protein, and β-2-microglobulin, are elevated during each crisis. These reactants return to normal levels when an attack subsides. The first episode often occurs between 5 to 10 years of age, although infants may have attacks. In 90% of the cases, there is at least one severe occurrence before 20 years of age.

The second notable characteristic is the accumulation of amyloid A in the kidneys and, to a lesser extent, spleen, liver, and gut. Amyloidosis may be the only FMF symptom in some individuals. If untreated, the accumulated amyloid causes fatal kidney failure at a young age, or, more frequently, kidney damage by age 40. Amyloid A is derived from serum amyloid A protein, which is a component of the acute inflammatory response system.

Diagnosis. There is no definitive laboratory test for FMF. Diagnosis depends on the clinical features of a patient. A DNA test for the more common

from the HUMAN GENETICS files

Protecting Genetic Privacy and Preventing Genetic Discrimination

With the proliferation of genetic testing, concerns have been raised about protecting the confidentiality of the results (genetic privacy) and preventing them from being used unfairly (genetic discrimination). Although personal health records cannot be accessed without formal authorization, some scientists and bioethicists have argued that DNA-based information is unique and deserves special safeguards. Some of the features that make DNA different from other types of medical samples include the vast amount of information that can be generated from a single sample, its long-term stability and ease of storage that enable additional analyses as new gene tests become available, and the simplicity of obtaining a DNA sample from hair or saliva without consent. Many elected officials throughout the world have accepted the premise that genetic and conventional medical information are not equivalent, and, consequently, about 22 states in the US and various countries in Europe and Asia have initiated genetic privacy legislation. There is no uniformity among these laws. Most of them forbid releasing the results of gene tests to third parties without explicit consent. In some cases, a DNA sample must be destroyed promptly after a test is completed. Hopefully, in the places without genetic privacy laws, traditional norms of confidentiality are sufficient to keep personal genetic information secret.

In addition to anxiety about genetic privacy, there are concerns that insurance companies and employers might use genetic information to discriminate against policyholders and workers. Insurance offers financial reimbursement for an unanticipated event to a member of a large group of people who have each made a modest contribution to the pool. In other words, there is a communal sharing of risk. Health, disability and life insurance are for illness, work-related injuries including loss of employment income, and death, respectively. In some countries, government programs provide health and disability insurance for all their citizens; in others, there is a mixture between public and private schemes; and in some, such as the, US, health-related insurance is predominantly private. The cost of premiums for a private health insurance plan is based on risk assessment that considers, among other criteria, family history, medical status, results of various tests, lifestyle, and age. On the basis of this information, an applicant is allocated to a particular risk category and the cost of the policy is determined. Although not an issue in the past because illness due to a late-onset inherited disorder was considered an unexpected event, now, with presymptomatic diagnostic tests, a number of questions must be considered. Should an asymptomatic individual who tests positive for a late-onset disorder be barred from conventional insurance coverage? Will the fear of being uninsurable or the cost of insurance discourage some individuals from undergoing tests despite the benefits of presymptomatic diagnosis? Will those who test positive load up on insurance at low rates to receive large payments when the illness strikes? The insurance industry calls the latter phenomenon adverse selection. If adverse selection becomes widespread, the financial viability of many insurance companies could be jeopardized. Although it is difficult to determine whether adverse selection will be rampant, in one study, women who tested positive for the *BRCA1* mutation that is associated with early-onset breast cancer did not purchase any more insurance than relatives or nonrelatives who were not at risk for breast cancer.

Genetic discrimination may occur in the workplace. Traditionally, employment was based on the health status of a candidate and whether he/she could perform the required tasks without presenting a risk to others. Clearly, it is not desirable to have narcoleptic airline pilots who might, unpredictably, fall asleep for various durations during a flight. In the US, employers are usually responsible for the health insurance of their employees. Thus, although discriminatory, it is in the economic interest of employers not to hire individuals who might, in the future, develop a genetic disorder. In other words, of two equally qualified candidates, the one with a positive gene test result would probably not be offered the job.

The potential controversy over genetic discrimination by insurance companies and employers has been forestalled by the enactment of specific laws by many US state legislatures. These 'genetic rights' statutes vary in scope. In New Jersey, for example, employers and insurers cannot require a gene test or other genetic information as a condition for employment or insurability. In addition, a gene test cannot be used by an employer to either dismiss or block the promotion of an employee or by an insurance company to prevent renewal of an existing policy. In some states, the statutes are either less detailed or nonexistent, whereas, in others, they are highly specific. In Alabama, health insurers cannot deny coverage to those individuals with sickle cell anemia or use gene testing to determine a predisposition for cancer. Outside the, US, many countries have enacted comprehensive legislation that both preserves genetic privacy and prevents genetic discrimination.

MEFV gene mutations is available to confirm the diagnosis. Rare *MEFV* mutations are not detected by the current assay systems. There a number of disorders that resemble FMF. Consequently, a correct diagnosis is essential, especially because the specified treatment for FMF is extremely effective.

Treatment. Oral doses of colchicine, an alkaloid, abort attacks, reduce recurrences, and prevent amyloidosis in about 85% of the FMF patients. The drug regimen depends on the severity of the condition. The mode of action of colchicine in alleviating FMF is unknown.

Peutz–Jeghers Syndrome

Peutz–Jeghers syndrome (PJS) is an autosomal dominant condition that affects about 1 in 25,000 worldwide. About 70% of the familial cases and 50% of the sporadic occurrences have a mutation in the *STK11* gene at 16p13.3 that encodes serine/threonine kinase 11. The relationship between *STK11* gene mutations and PSJ is unknown.

Clinical Features. Patients with PJS develop dozens to thousands of benign growths (polyps, hamartomas) in the small intestine and, to a lesser extent, in the stomach and bowel. These gastrointestinal polyps cause abdominal pain and internal bleeding. In some cases, the small intestine folds in on itself and obstructs the bowel. Also, PJS patients have an increased risk of internal malignancies as well as breast, testicular, and pancreatic cancer. The second key characteristic of PJS is highly pigmented dark-brown or dark-blue spots (mucocutaneous macules) on the lips and gums; inside of the mouth; and around the mouth, eyes, and nostrils (Figure 17.17). This freckling begins during early childhood and, except for the spots on the lips and gums and in the mouth, fades over time.

Diagnosis. Generally, both gastrointestinal polyps and pigmented spots in and around the mouth are sufficient for a diagnosis of PJS. X-irradiation of the abdomen or endoscopy detects the polyps. Microscopic examination of biopsy material establishes the assessment because PJS polyps have a distinctive shape and histological composition. A DNA test is available for asymptomatic individuals who have a closely related family member with PJS.

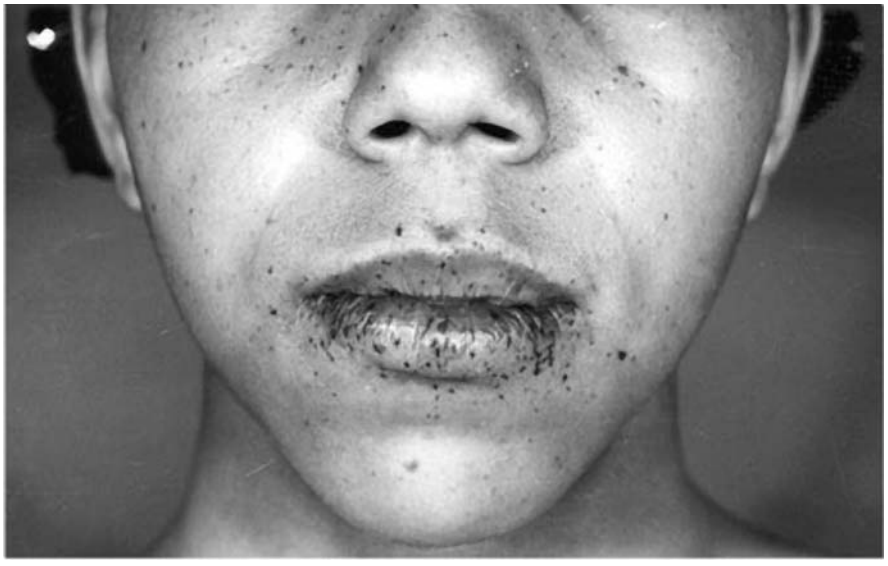


Figure 17.17 Freckling pattern associated with Peutz–Jeghers syndrome. From http://www.macgn.org/cases/p_pjs_freckling.html

Treatment. Large (>1.5 cm) polyps are removed surgically and the smaller ones by less invasive procedures such as electrocautery snare. Regular examinations for polyps and early signs of cancer are recommended.

Smith–Lemli–Opitz Syndrome

Smith–Lemli–Opitz syndrome (SLOS) is an autosomal recessive disorder with multiple malformations at birth and mental retardation later. Initially, SLOS was designated the RHS syndrome, with the initials representing the surnames of the first three patients who were used to establish the syndrome. The incidence of SLOS is 1 in 20,000 or greater in central European populations and their descendants. It is rare among Africans and Asians. Mild to severe forms of SLOS result from mutations of the *DHCR7* gene at 11q12–13 that encodes sterol-Δ^7-reductase (7-dehydrocholesterol-Δ^7reductase, Δ^7-dehydrocholesterol reductase, 3β-hydroxysterol-Δ^7-reductase). A defective sterol-Δ^7-reductase causes both an accumulation of 7-dehydrocholesterol and a deficiency of cholesterol (Figure 17.18). The severity of SLOS is negatively correlated with the endogenous concentration of cholesterol. Cholesterol is an important component of cell membranes and the myelin sheath. As well, it is the principal

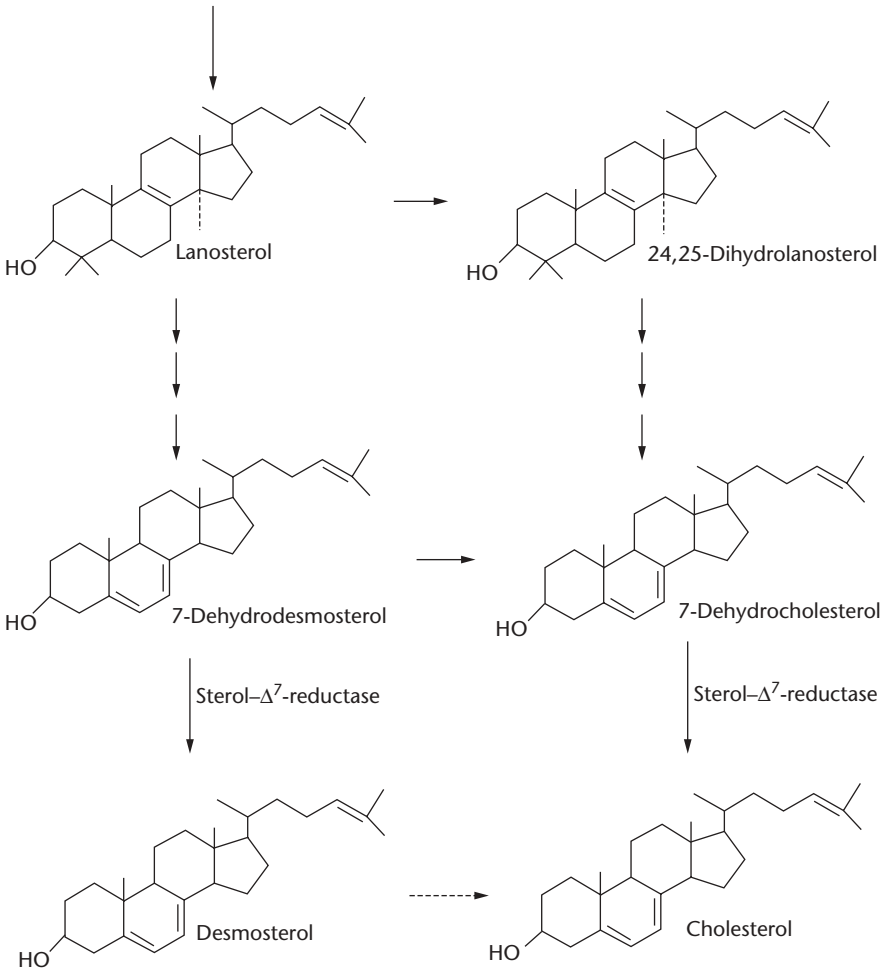


Figure 17.18 Final steps (solid arrows) of cholesterol biosynthesis. The reactions catalyzed by sterol-Δ^7-reductase are labeled. The dotted arrow denotes a presumed reaction.

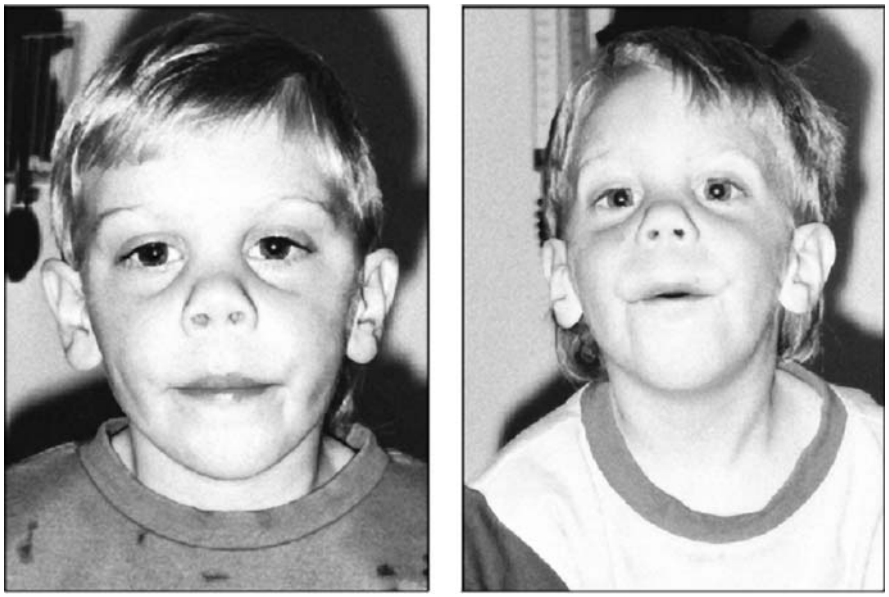


Figure 17.19 Facial features of two boys with Smith–Lemli–Opitz syndrome. From Figure 3 in Nowaczyk, M. J., D. T. Whelan, T. W. Heshka, and R. E. Hill. 1999. Smith–Lemli–Opitz syndrome: a treatable inherited error of metabolism causing mental retardation. *Can Med Assoc J* **161**:165–170.

precursor of steroid hormones such as progesterone and androstenedione and bile salts that participate in the uptake of fat-soluble vitamins and digestion of fat.

Clinical Features. The anomalies in 50% or more of the individuals with SLOS are mild to profound mental retardation (100% affected); small brain at birth (microcephaly, >90%); fusion of the second and third toes (syndactyly, ~98%); genital abnormalities in males (>50%); and muscle weakness (hypotonia, ~50%). A distinctive set of facial characteristics include a high, broad forehead; narrow temples; downward-slanting eyelids; epicanthal folds; short nose with upward-pointing nostrils (anteverted nares); drooping eyelids (blepharoptosis); prominent cheek bones; broad nasal bridge; low-set, large ears; and small jaw (micrognathia) and tongue (Figure 17.19). Cleft palate; cataracts; extra fingers or toes (polydactyly); deformity of the feet (talipes equinovarus); and abnormalities of the heart, lungs, liver, kidneys, urogenital system, and digestive tract are present in about 10% to 50% of the cases. Vomiting and untreatable colonic blockage often cause infant mortality. Speech usually does not develop, although some patients have good comprehension. Sensitivity to sunlight is common. The behavior phenotype is complex and has many aspects of autism including, to varying degrees, repeated self-injury, prolonged temper tantrums, destruction of property, violent outbursts, inability to interact with other individuals, hyperactivity, and sleep disturbances.

Diagnosis. Determination of an elevated level of 7-dehydrocholesterol (7-DHC) in the plasma is the best way of detecting an individual with SLOS. Because the treatment of SLOS is helpful, it is recommended that individuals with unexplained (idiopathic) mental retardation should be tested for plasma concentration of 7-DHC to exclude the possibility of SLOS. Prenatal elevation of 7-DHC can be measured from amniocentesis and chorionic villus samples. A DNA-based test must be used for carrier detection because the

levels of plasma 7-DHC in heterozygotes and homozygous dominant individuals are not significantly different.

Treatment. Care for individuals with SLOS is supportive. Many of the congenital abnormalities are corrected by surgery. Initial studies of dietary cholesterol supplementation with either food-grade cholesterol or cholesterol-rich foods such as eggs and cream showed an increase in growth of children and adolescents, improved general health, and a reduction in the number of major negative behavioral episodes. Adults also benefit from the additional intake of dietary cholesterol.

Wilson Disease

Wilson disease (WD; hepatolenticular degeneration) is a progressive autosomal recessive disorder with damage to the liver, kidneys, brain, and spleen resulting from an accumulation of copper. The incidence of WD is 1 in 30,000 worldwide, and it is it fatal if untreated. Copper is required for the activity of a number of enzymes including cytochrome oxidase, superoxide dismutase, ceruloplasmin, tyrosinase, and dopamine β-hydroxylase. Under normal conditions, excess copper is processed in the liver, transferred to the bile, and excreted in the urine. The role of the liver in the removal of copper is defective in WD patients. An overload of nonexcreted copper causes degradation of liver cells and the release of copper into the bloodstream. Over time, copper becomes concentrated in the lenticular area of the brain, cornea of the eye, and a number of other sites. The effects of chronic copper poisoning are usually evident between the ages of 6 to 25 years. Mutations of the *ATP7B* gene at 13q14–21 that encodes a copper-binding P-type adenosine triphosphatase (ATPase) are responsible for WD. This ATPase is probably localized in the membrane of late endosomes and presumably is the major transporter of copper from liver cells to the bile. Over 200 *ATP7B* gene mutations have been identified. Of these, H1069Q and R778L occur frequently in European and Asian populations, respectively.

Clinical Features. Liver disease, neurological abnormalities, and psychiatric disturbances, either together or separately, are indicators of WD. Copper toxicity initially causes hepatitis with recurring jaundice, fatigue, abdominal pain, hemolytic anemia, and loss of appetite. If untreated, cirrhosis or necrosis of the liver occurs with other complications such as kidney failure. Among the neurological effects are facial and muscular rigidity, unsteady gait, loss of fine motor control, and tremors. Psychiatric disturbances consist of depression, anxiety, and antisocial behavior. Poor memory and impaired abstract thinking diminish intellectual performance. A common, although not unique, sign of WD is the formation of a rusty-brown ring of pigment at the periphery of the cornea (Kayser–Fleischer ring) due to the deposition of copper in Descemet's membrane (Figure 17.20). Generally, 50% of WD patients have only liver disease and 20% to 40% have psychiatric and neurological symptoms.

Diagnosis. Wilson disease is not easily identified because liver damage is a feature of many different illnesses. However, the combination of liver damage,

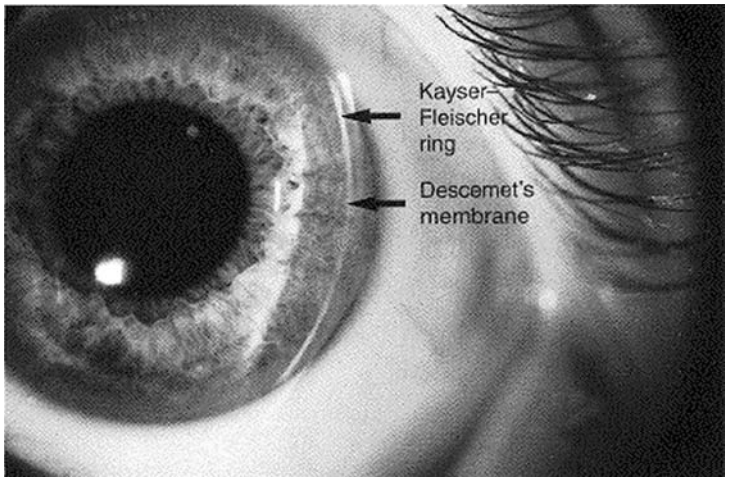


Figure 17.20 Kayser–Fleischer ring of the cornea associated with Descemet's membrane in an adult with Wilson disease. From http://matweb.hcuge.ch/matweb/Selected_images/Developmental_genetic_diseases/Wilson_disease.htm

neurological and psychiatric symptoms, and Kayser–Fleischer rings is a strong indication of WD. Although not definitive, the association of low levels of both ceruloplasmin, a blood copper-binding dehydrogenase, and serum copper combined with a high copper concentration in the urine and liver are expected with WD. A DNA test is available for asymptomatic individuals who have a family history of WD.

Treatment. Effective treatment of WD depends on both the removal of copper from tissues and organs and prevention of the recurrence of copper accumulation. Daily oral doses of penicillamine, a penicillin derivative that binds (chelates) heavy metals such as copper, is effective in reversing the symptoms of WD and impeding its progression. Unfortunately, 30% of the individuals treated with penicillamine develop anemia, kidney damage, severe rashes, and other side effects. Trientine, another chelator, is used as a substitute for penicillamine. A very different strategy for treating WD entails oral doses of zinc acetate. In this case, the ingested zinc stimulates the synthesis of the protein metallothionein that sequesters copper inside intestinal cells. The bound copper is retained and, subsequently, as part of the normal process of cell loss, the cells that are loaded with copper are sloughed off and passed out of the body in the stool. This treatment restricts the acquisition of copper from the intestinal cells and, as a result, keeps endogenous copper levels low. Each prescribed treatment is lifelong, and failure to comply can be fatal. Individuals with WD are also advised to avoid eating liver, shellfish, chocolate, mushrooms, and nuts; taking multivitamin and mineral supplements; and drinking tap water delivered in copper pipes.

key terms

allele-specific oligonucleotide assay
amniocentesis
apnea
asymptomatic
Bayes theorem
carrier test
chelation
chorionic villus sampling
conditional probability
diagnosis
diagnostic test
diverticulum
Down syndrome
–emia
enzyme replacement therapy
false negative
false positive
first-degree relative
forward dot blot
gene therapy
genetic counseling
genetic test
Guthrie test
hamartoma
hernia
joint probability
multiplex assay
neonate
nuchal translucency
oligonucleotide ligation assay
oligonucleotide ligation reaction
palliative
philtrum
phlebotomy
polyp
posterior probability
predictive test
preimplantation diagnostic test
prenatal test
presymptomatic
prior probability
prognosis
protein replacement therapy
recurrence risk
reverse dot blot
screening test
second-degree relative
sensitivity
specificity
substitutive therapy
symptom
syndrome
tandem mass spectrophotometry
third-degree relative
true negative
true positive
–uria

summary

Unless there is a family history, the occurrence of a genetic disorder comes as a complete shock. Once a genetic condition appears, family members have a myriad of questions. What is the nature of the disorder? How severe will it be? Is there treatment for the disorder? How effective is the treatment? Who will administer the treatment? Is there any way of determining whether a fetus has the disorder? What is the chance that another child in the family will have the defect? Can unaffected adults develop the condition? In addition to queries about the facts of the disorder, a genetic disorder has a significant psychological impact on each family member that must be addressed. In particular, anxiety is a serious concern. Professional genetic counselors are specifically trained to handle these complex and sensitive issues.

Basically, genetic counseling is a process of communication that conveys relevant, accurate, and detailed information about genetic disorders in clear and understandable terms; describes the full set of medical options that are available to patients and nonaffected family members; provides, in a nonbiased way, strategies for decision making; helps families to cope with the information; and directs clients to support groups and associations that deal specifically with the disorder. Genetic counselors advise clients about prenatal tests; screening of newborns for genetic disorders; genetic testing of couples to determine whether they both have a recessive allele for a serious genetic disorder; and testing of adults to discover whether they carry a gene for a late-onset condition. They recommend and explain the features of diagnostic tests. However, it is the client's decision whether to take a test. If a client does undergo testing, a genetic counselor plays an important role in explaining the meaning of the results. The discussions between a genetic counselor and a client are confidential and shielded from others. In some cases, in addition to a genetic counselor, a primary health provider will dispense information and help patients when they are confronted with difficult decisions. Many universities have genetic counseling training programs at the graduate and postgraduate levels. As more inherited disorders are diagnosed, there will be a greater need for specialized health care workers who can inform, educate, and counsel patients and clients about the biological and emotional consequences of genetic disorders.

Highly accurate clinical tests are essential for diagnosing genetic disorders. A required treatment will not be initiated if a test result is a false negative and will be unnecessarily invoked if the result is a false positive. Two of the parameters that describe the effectiveness of a diagnostic test are specificity and sensitivity that are defined as $\frac{\text{true positive}}{\text{true positive} + \text{false negative}}$ and $\frac{\text{true negative}}{\text{true negative} + \text{false positive}}$, respectively.

Biochemical assays are important for determining the nature of many inherited metabolic disorders. However, with the advent of PCR and the characterization of many disease-causing genes, mutation detection systems have become the preferred way of determining the molecular basis of inherited conditions. Indirect methods such as single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and denaturing high-performance liquid chromatography (DHPLC) are used to detect any mutation within a gene. Alternatively, a large number of allele-specific techniques are in use including assays that score the mutational gain or loss of a restriction endonuclease site, oligonucleotide ligation protocols, allele-specific oligonucleotide arrays, and DNA sequencing. Multiplex tests detect simultaneously either mutant sites throughout a gene or mutations among a number of genes.

A genetic test is designed to determine changes at the DNA level and can be used for specific purposes including establishing the basis of an existing disorder (diagnostic testing), determining the presence of a genetic condition when there are no obvious symptoms (predictive testing), identifying heterozygotes (carrier testing), and assessing a fetus for abnormalities (prenatal testing). When genotype determination is not possible, Bayesian analysis can be used to calculate the likelihood of carrier status and, accordingly, the probability of the birth of an affected child. The power of Bayesian statistics is its capability of inferring a final (posterior) probability based on an initial probability along with quantified contingencies (conditional probabilities) such as the number of unaffected offspring or siblings in a family, extent of penetrance, age of onset, and so on.

Screening programs entail testing a large sample of asymptomatic individuals to determine those who at risk for a genetic disorder. Testing all newborns for inherited conditions is commonly practiced, although the number of tests and the targets vary from one jurisdiction to another. The traditional requirement for implementing a screening test was that early diagnosis allows beneficial treatment to be initiated before the onset of symptoms.

In addition to conventional medical remedies such as surgery, palliative prescriptions, or transplantation, the treatment of genetic disorders tends to be specific for each disease. Of the various strategies, restrictive diets are used to lower the intracellular level of a toxic molecule; supplemented diets to replace a metabolic deficiency; dialysis, chelation, or other means to remove toxic compounds; and replacement of both missing or defective molecules with a functional counterpart. In the future, gene and other nucleic acid therapies will supply a missing protein or correct deleterious gene mutations.

References

Andrews, L. B. et al. 1994. *Assessing Genetic Risks: Implications for Health and Social Policy*. National Academy Press, Washington, DC.

Baker, D. L., J. L. Schuette, and W. R. Uhlmann. 1998. *A Guide to Genetic Counseling*. Wiley-Liss, New York.

Bennett, R. L. 1999. *The Practical Guide to the Genetic Family History*. Wiley-Liss, New York.

Bridge, P. J. 1997. *The Calculation of Genetic Risks: Worked Examples in DNA Diagnostics*. The Johns Hopkins University Press, Baltimore, MD.

British Medical Association 1998. *Human Genetics: Choice and Responsibility*. Oxford University Press, Oxford.

Cavazzana-Calvo, M. et al. 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* **288**:669–672.

Charrow, J. et al. 2000. Tandem mass spectrometry in newborn screening. *Genet Med* **2**:267–269.

Clarke, J. T. R. 1996. *A Clinical Guide to Inherited Metabolic Diseases*. Cambridge University Press, Cambridge, UK.

Claus, E. B., N. Risch, and W. D. Thompson. 1994. Autosomal dominant inheritance of early-onset breast cancer. Implications for risk prediction. *Cancer* **73**: 643–651.

Cullen, L. M. et al. 1999. Genetics of hemochromatosis. *Annu Rev Med* **50**:87–98.

Desnick, R. J. and M. M. Kaback. (eds.) 2001. *Tay-Sachs Disease*. Academic Press, San Diego, CA.

Eng, C. M. et al. 2001. A phase 1/2 clinical trial of enzyme replacement in Fabry disease: Pharmacokinetic, substrate clearance, and safety studies. *Am J Hum Genet* **68**:711–722.

Flodman, P. and S. E. Hodge. 2002. Determining complex genetic risks by computer. *J Genet Couns* **11**:213–230.

Hodge, S. E. 1998. A simple, unified approach to Bayesian risk calculations. *J Genet Couns* **7**:235–259.

Hook, E. B. 1981. Rates of chromosome abnormalities at different maternal ages. *Obstet Gynecol* **58**:282–285.

Hook, E. B., P. K. Cross, and D. M. Schreinemachers. 1983. Chromosomal abnormality rates at amniocentesis and in live-born infants. *JAMA* **249**:2034–2038.

Kakkis, E. D. et al. 2001. Enzyme-replacement therapy in mucopolysaccharidosis I. *N Engl J Med* **344**:182–188.

Kay, M. A. et al. 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* **24**:257–261.

Khoury, M. J., L. L. McCabe, and E. R. McCabe. 2003. Population screening in the age of genomic medicine. *N Engl J Med* **348**:50–58.

Levy, H. L. and S. Albers. 2000. Genetic screening of newborns. *Annu Rev Genomics Hum Genet* **1**:139–177.

Mahowald, M. B., M. S. Verp, and R. R. Anderson. 1998. Genetic counseling: Clinical and ethical challenges. *Annu Rev Genet* **32**:547–559.

Morris, C. A. and C. B. Mervis. 2000. Williams syndrome and related disorders. *Annu Rev Genomics Hum Genet* **1**:461–484.

Mulholland II, W. F. and A. S. Jaeger. 1999. Genetic privacy and discrimination: a survey of state legislation. *Jurimetrics J* **39**:317–326.

Nowaczyk, M. J. et al. 1999. Smith-Lemli-Opitz syndrome: a treatable inherited error of metabolism causing mental retardation. *Can Med Assoc J* **161**:165–170.

Pastinen, T. et al. 1997. Minisequencing: a specific tool for DNA analysis and diagnostics on oligonucleotide arrays. *Genome Res* **7**:606–614.

Petticrew, M. P. et al. 2000. False-negative results in screening programmes: systematic review of impact and implications. *Health Technol Assess* **4**:1–120.

Pollitt, R. J. et al. 1997. Neonatal screening for inborn errors of metabolism: cost, yield and outcome. *Health Technol Assess* **1**:1–202.

Roche, P. A. and G. J. Annas. 2001. Protecting genetic privacy. *Nat Rev Genet* **2**:392–396.

Rothstein, M. A. 1997. *Genetic Secrets: Protecting Privacy and Confidentiality in the Genetic Era*. Yale University Press, New Haven.

Seymour, C. A. et al. 1997. Newborn screening for inborn errors of metabolism: a systematic review. *Health Technol Assess* **1**:1–95.

Shuber, A. P. et al. 1997. High throughput parallel analysis of hundreds of patient samples for more than 100 mutations in multiple disease genes. *Hum Mol Genet* **6**:337–347.

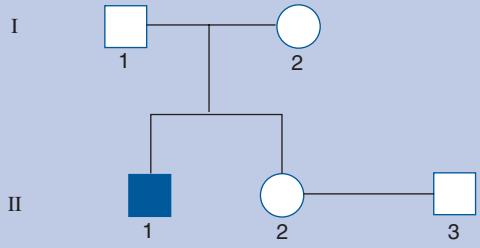
Tassabehji, M. 2003. Williams-Beuren syndrome: a challenge for genotype-phenotype correlations. *Hum Mol Genet* **12**:229–237.

Yan, H., K. W. Kinzler, and B. Vogelstein. 2000. Genetic testing—present and future. *Science* **289**:1890–1892.

Yu, H. et al. 2000. Spectrum of Δ^7-dehydrocholesterol reductase mutations in patients with the Smith-Lemli-Opitz (RSH) syndrome. *Hum Mol Genet* **9**:1385–1391.

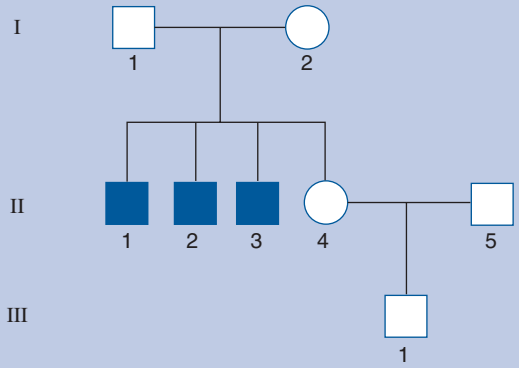
review questions

1. Describe the fundamental features of the genetic counseling process.
2. With regard to a diagnostic test, what do specificity and sensitivity signify?
3. What is an ARMS test? Describe in detail how this procedure detects mutations.
4. What are the accepted conditions for establishing a universal screening program for newborns?
5. How are PKU, congenital adrenal hyperplasia, and Wilson disease treated? What is the rationale for each of these treatments?
6. Summarize the clinical features, diagnosis, and treatment of two genetic disorders that are not discussed in this chapter. Clinical descriptions of a large number of genetic disorders can be found at http://www.geneclinics.org/.
7. In a central European family, individual II-1 (pedigree A) has Smith–Lemli–Opitz syndrome (SLOS). There is no history of SLOS in II-3's family. What is the probability that II-2 and II-3 will have a child with SLOS? What is the probability that this couple will have an affected child after the births of two unaffected sons? What is the probability that II-2 and II-3 are both noncarriers?



Pedigree A

8. For the X-linked disorder in pedigree B, what is the probability that the next son of II-4 and II-5 will be affected?



Pedigree B

9. A man with a rare X-linked disorder marries a woman without any family history of this disorder. What is the probability that they will have an affected son?
10. What is the likelihood that a 34-year-old woman who has a 44-year-old sister with breast cancer will also develop breast cancer?

Glossary

3′ extension A short single-stranded nucleotide sequence on the 3′-hydroxyl end of a double-stranded DNA molecule. *Also called* 3′ protruding end, 3′ sticky end, 3′ overhang.

5′ extension A short single-stranded nucleotide sequence on the 5′-phosphate end of a double-stranded DNA molecule. *Also called* 5′ protruding end, 5′ sticky end, 5′ overhang.

A Adenine residue in either DNA or RNA.

absolute linkage *See* complete linkage.

acentric A chromosome or chromosome fragment without a centromere.

acrocentric chromosome A chromosome with a centromere close to one end.

action potential A strong electric signal generated and propagated along the cell membrane of an axon and other excitable cell membranes by cycles of membrane depolarization and repolarization. *Also called* nerve impulse, nerve signal.

activation Induction of transcription.

adaptive evolution Selection favoring a particular trait or gene. *Also called* positive selection, directional selection.

adaptor (1) A synthetic double-stranded oligonucleotide with one blunt end and a cohesive extension at the other end. (2) A synthetic double-stranded oligonucleotide with cohesive ends on opposite strands.

adaptor-linker PCR Chemically synthesized DNA sequences ligated to the ends of DNA fragments to provide primer sites for polymerase chain reaction.

additive gene effect A large number of genes, each with a small effect, that form the basis of a phenotype.

adenine One of the organic bases found in either DNA or RNA.

admixture The amalgamation of genetically different populations. *Also called* genetic admixture.

afferent neuron A nerve cell that carries impulses from a sensory receptor to the central nervous system.

affinity purification Selective isolation of a tagged molecule caused by the specific binding of the tag (e.g., biotin) to another molecule (e.g., avidin, streptavidin).

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algorithm Precise procedure for solving a problem that is usually implemented by a computer program. Bioinformatics algorithms are developed to process, store, analyze, and visualize biological data.

alignment Unit-by-unit lining up of two sequences.

aliquot Commonly, one or more samples drawn from a primary sample.

allele frequency In a large population, the ratio of the occurrence of one specific allele at a locus to all alleles of the locus.

allele One of a number of possible alternative forms of genetic information at a gene locus.

allele-sharing tests Various methods for testing whether a gene locus is associated with a biological condition.

allelic heterogeneity Disparate phenotypes produced by different mutations in the same gene.

allogenic Cell types with different antigens. *Also called* allogeneic.

alternative splicing Cell-specific removal of an exon during processing of a primary transcript.

***Alu* sequence** A human repetitive DNA family of approximately 500,000 members, each approximately 294 base pairs long, scattered at approximately 1000 base pair intervals throughout the genome.

***Alu*-PCR** Polymerase chain reaction (PCR) amplification between *Alu* sequences using *Alu*-specific primers.

***Alu*-vector PCR** Polymerase chain reaction (PCR) with vector- and *Alu*-specific primers for amplification across the insertion sites of a cloned piece of DNA.

amino acid The repeating unit of a protein.

amniocentesis Surgical removal of a small amount of amniotic fluid and cells for biochemical, chromosome, and/or DNA testing to determine disorders in a human fetus.

amplicon A plasmid vector carrying *Herpes simplex* virus Type 1 genes. *Also called* amplicon plasmid.

amyloid An abnormal fibrillar aggregation of proteins, which, after staining with Congo Red, produces a green color under polarized light. *Also called* amyloid body.

amyloidogenesis The formation of amyloid.

amyloidosis The formation of amyloid bodies.

anabolism The intracellular synthesis of organic compounds.

anaphase A stage of mitosis of the cell division cycle during which sets of chromosomes move to opposite spindle poles. Mechanistically, anaphase I and anaphase II of the meiotic process are the same as a mitotic anaphase.

aneuploidy An abnormal chromosome complement resulting from either the absence of a chromosome(s) or the presence of an additional chromosome(s).

aniridia The absence of all or part of the iris of the eye.

annealing The process of heating (denaturation) and slowly cooling a double-stranded nucleic acid to allow complementary regions to form base pairs.

annotated database Computer-stored data that are supplemented with additional information such as detailed descriptions, comments, and references.

anticipation An earlier onset and greater severity of a disorder in successive generations. *Also called* genetic anticipation, Sherman paradox.

anticodon Three contiguous nucleotides in a tRNA molecule that are complementary to three contiguous nucleotides in an mRNA.

antisense RNA An RNA sequence that is complementary to mRNA.

antisense strand A transcribed DNA strand. *Also called* anticoding strand.

antisense therapy In vivo treatment of a disease by blocking translation of a protein with an RNA sequence that is complementary to a specific mRNA.

apnea Cessation of breathing.

aptamer A protein-binding synthetic nucleic acid.

arrayed library The maintenance of clones of a library in assigned wells of multiwell culture plates.

association A statistically significant relationship either between an allele and a disorder or two alleles at different loci.

assortative mating *See* nonrandom mating.

astrocyte A type of glial cell.

asymmetric PCR A polymerase chain reaction with a primer ratio >50:1 for producing one strand in excess of the other.

asymptomatic Without any signs of a disease.

atrophy Deterioration of a cell, tissue, or organ.

autologous cells One's own cells.

autoradiography A technique designed to capture the image formed in a photographic emulsion after the emission of either light or radioactivity from a labeled component.

autosomal dominant inheritance A phenotype produced by a single allele of a gene pair on a chromosome other than the sex chromosomes.

autosomal recessive inheritance A phenotype produced by two alleles of a gene pair on homologous chromosomes other than sex chromosomes.

autosome Any chromosome other than a sex chromosome. Human somatic cells have 22 pairs of autosomes.

axial An organ, appendage, or outgrowth located on the axis of an organism.

axon A single cellular extension emanating from the cell body of a neuron that transmits a nerve impulse to the synaptic bulb. *Also called* nerve fiber.

axon hillock The wide portion of the axon emanating directly from the cell body.

BAC *See* bacterial artificial chromosome.

back mutation Spontaneous change of a defective to a normal allele.

background selection Removal of a neutral allele(s) linked to a negatively selected gene in a region of low recombination.

bacterial artificial chromosome A vector system based on the *E. coli* F factor plasmid for cloning large (100–300 kb) DNA inserts. *Also called* BAC.

bacteriophage A virus that specifically infects a bacterium.

balanced translocation *See* translocation.

balancing selection Greater reproductive fitness of the heterozygous genotype over both homozygous genotypes.

basal ganglia (*singular*, ganglion) Clusters of nerve cell bodies within the cerebral hemispheres.

base pair The hydrogen-bonded combination of either adenine with thymine or guanine with cytosine in duplex DNA. *Also called* nucleotide pair.

base pair substitution Permanent replacement in DNA of a nucleotide pair with another nucleotide pair.

Bayesian analysis For geneticists, a mathematical procedure for inferring the genetic status of a family member.

biallelic expression Transcription of both alleles of a locus in a cell or tissue.

bioinformatics Research, development and application of computational tools to acquire, store, organize, analyze, and visualize data for the biological, medical, behavioral, and health sciences.

biotinylation The incorporation of a biotin-containing nucleotide into a nucleic acid molecule.

bivalent A synapsed homologous chromosome.

BLAST (basic local alignment search tool) A computer program for determining a match between a query sequence and a sequence(s) in a database. BLASTn and BLASTp compare a DNA and an amino acid query sequence with a DNA and a protein database, respectively.

blood-brain barrier The wrapping of astrocytes around the blood vessels of the brain, which prevents the passage of many compounds directly from the blood to neurons.

blot *See* Southern blotting, Northern blotting, Western blotting.

blunt end The end of a duplex DNA molecule in which neither strand extends beyond the other. *Also called* flush end.

blunt-end cut The cleavage of the phosphodiester in the backbone of duplex DNA between the corresponding nucleotide pairs on opposite strands, producing no nucleotide extensions on either strand. *Also called* flush-cut.

blunt-end ligation Joining (ligating) of the nucleotides that are at the ends of two DNA duplex molecules.

bottleneck A drastic reduction in population numbers with the survival of a small random sample of the original population.

branch Linear representation of a divergence in a phylogenetic tree.

breeding The controlled sexual reproduction of plants and animals.

bystander effect Cell death as a result of a cytotoxic substance acquired by cell-to-cell contact.

C Cytosine residue in either DNA or RNA.

cancer A malignant tumor.

candidate gene A gene that might be responsible for an inherited disorder.

candidate gene cloning A strategy for isolating a disease gene, based on an informed guess about the possible gene product.

cardiac muscle Muscle tissue found only in the heart.

cardiocyte A heart muscle cell.

cardiomyopathy Disease of heart muscle.

carrier test An assay that identifies a heterozygous genotype.

cascade mechanism Often a causal catalytic chain, where an extracellular event activates an enzyme, which, in turn, activates another enzyme and so on, until the last activated protein initiates or suppresses a biological response system.

catabolism The intracellular breakdown of organic compounds.

cataract The loss of transparency of the lens of the eye.

cDNA *See* complementary DNA.

cDNA clone A vector carrying a double-stranded DNA molecule that was synthesized in vitro from a messenger RNA sequence with reverse transcriptase and DNA polymerase.

cDNA library A collection of cDNA clones that were generated in vitro from the messenger RNA sequences of a single tissue or cell type.

cell body The part of a neuron that contains the nucleus.

cell division cycle The process that produces two daughter cells, each with a diploid chromosome number, from a single cell. *Also called* mitotic cycle, mitotic process.

cell-free extract The cell components remaining in solution after cell disruption and centrifugation at a low speed.

cell membrane The trilamellar envelope (membrane) surrounding a cell. *Also called* plasma membrane, plasmalemma.

centimorgan A unit of genetic distance between two gene loci, abbreviated as cM. Named after T. H. Morgan (1866–1945), who used the fruit fly *Drosophila melanogaster* to establish the principles of genetic linkage and gene mapping.

centiRay The unit of measure for a radiation hybrid map. *Also called* centiray, cR.

central nervous system All the neurons of the brain and spinal cord. *Also called* CNS.

centriole A cellular organelle responsible for the assembly of spindle fibers during the cell division cycle and meiotic process.

centromere The general location on a chromosome where spindle fibers attach during the cell division cycle and meiotic process. *Also called* primary constriction.

centrosome *See* spindle pole.

CEPH family panel A set of about 65 two- or three-generation families with sibships of eight or more, used for the genetic mapping of human chromosomes. The blood cells from members of these families that provide the source of DNA for genotyping are maintained by the Fondation Jean Dausset-Centre d'Etude Polymorphisme Humain in Paris, France.

cerebral cortex The neuronal layer covering the cerebrum.

cerebrum The portion of the brain in the upper brain cavity that is divided into two hemispheres. The outer cortical layer of the cerebrum is densely packed with neurons and massive numbers of nerve connections. The basal ganglia are found in the interior of the cerebral hemispheres.

channel A protein passageway spanning a cell membrane that selectively allows either an ion or some small molecule to enter or leave the cell. *Also called* channel protein.

charged tRNA A transfer RNA molecule coupled with its specific amino acid. *Also called* aminoacylated tRNA.

chelation Chemical binding of a heavy metal.

chemical mismatch cleavage A mutation detection assay that identifies heteroduplex DNA by chemical modification of mismatched thymidine or cytosine residues and cleavage of the strand with a modified nucleotide.

chiasma (*plural,* chiasmata) The site of overlap of nonsister chromatids of homologous chromosomes during diplotene of meiosis I of the meiotic process, marking the location of a reciprocal exchange of chromosome material.

chimerism (1) The occurrence of two or more noncontiguous chromosome DNA regions in a DNA insert. (2) An in vivo mixture of two genetically different tissues.

chorionic villus sampling Surgical removal of cells of the fetal membrane for biochemical, chromosome, and/or DNA testing to determine disorders in a human fetus. *Also* CVS.

choroid The posterior portion of the middle layer of the eye, containing blood vessels.

choroideremia An X-linked progressive dystrophy of the choroid layer of the eye.

chromatid One of the two chromosome strands of a duplicated chromosome.

chromosome Microscopic threadlike structure in the nucleus of a eukaryotic cell, consisting of a single, intact DNA molecule and associated proteins. Chromosomes carry the genetic information of an organism. In humans, each somatic cell has 46 chromosomes.

chromosome arm The portion of a chromosome extending from the middle of the centromere to one end of the chromosome. *Also called* arm.

chromosome jumping A technique that allows two segments of genomic DNA that are separated by about 200 kb to be cloned together.

chromosome walking A technique that identifies overlapping, contiguous cloned sequences usually from cosmid- or bacteriophage λ-based genomic libraries.

ciliary body The thickened part of the middle layer of the eye, lying between the choroid and the iris. The muscle activity of the ciliary body changes the curvature of the lens.

***cis* configuration** The arrangement of alleles in a double heterozygote, with the dominant alleles of two different gene loci on one chromosome and recessive alleles of the two loci on the homologous chromosome. *Also called cis* phase, coupling, coupling phase.

cleave To break phosphodiester bonds of duplex DNA, usually with a type II restriction endonuclease. *Also called* cut, digest.

clinical heterogeneity Different alleles at a gene locus producing clinically diverse symptoms.

clone (1) A population of genetically identical cells or organisms as a result of asexual reproduction, breeding of pure-bred (isogenic) organisms, and forming genetically identical organisms by nuclear transplantation. (2) A population of host cells with the same DNA construct. (3) To insert a DNA segment into a vector or host chromosome.

cloning In recombinant DNA technology, the insertion and perpetuation of a piece of DNA in a cloning vector.

cloning site The location on a cloning vector into which DNA can be inserted. *Also called* unique site.

cloning vector Any autonomously replicating DNA entity designed to carry an inserted DNA sequence. *Also called* vector, cloning vehicle, vehicle.

closed angle An obstructed iridocorneal angle of the anterior chamber of the eye.

CNS *See* central nervous system.

coalescence time A point in a gene tree where two lineages converge to a common ancestor during a backward analysis and the number of branches is reduced by one.

coalescent A mathematical construct for assigning the distributions of time back to nodes in a gene genealogy.

coding strand The DNA strand of a gene that is not normally used as a template for transcription and, except for T instead of U, has the same sequence as the mRNA.

coding triplet A set of three contiguous nucleotides of the sense DNA strand of the coding region of a structural gene, complementary to a transcribed triplet.

codominance Two different alleles at the same gene locus contribute to the phenotype.

codon A set of three contiguous nucleotides in an mRNA molecule that either specify an amino acid during translation or act as a termination of translation signal.

codon usage The frequencies of the codons of the structural genes of an organism.

cognitive ability The ability to understand, perceive, and formulate complex ideas.

cohesive ends Complementary single-strand extensions on the ends of duplex DNA molecules.

colony (1) A localized population of cells derived from one original cell. (2) A group of organisms of the same species, attached to each other and mutually dependent.

combinatorial pools The combinations of clones used to identify a specific clone from an arrayed library with as few hybridization or PCR reactions as possible. *Also called* hierarchical pools.

complementary base pairs In double-stranded DNA, adenine forms hydrogen bonds with thymine and cytosine forms hydrogen bonds with guanine. In double-stranded regions of RNA molecules, and both RNA-RNA and DNA-RNA strand interactions, adenine forms hydrogen bonds with uracil and cytosine forms hydrogen bonds with guanine.

complementary DNA (1) A single DNA strand synthesized by reverse transcriptase from an mRNA template in vitro. (2) A double-stranded DNA synthesized from mRNA in vitro with reverse transcriptase and DNA polymerase. *Also called* cDNA.

complementation test An assay to determine whether two mutations are allelic.

complete linkage A cluster of gene loci that remains intact through successive generations. *Also called* absolute linkage.

complete penetrance A situation in which all individuals with the same genotype at a gene locus show signs of the same abnormal phenotype.

complex trait A condition not obviously the result of an allele(s) at a single gene locus.

compound heterozygote The genetic condition in which alleles of a locus have mutations at different nucleotide sites.

computational biology Development and application of data analysis, modeling, and simulation techniques to study biological, behavioral, and social systems.

concatemer Tandem array of repeating unit-length DNA elements.

concordance The occurrence of the same trait in both members of a set of twins. *Also called* concordant.

cone A photoreceptor of the retina with a cone-shaped outer segment. *Also called* cone cell, cone photoreceptor.

conformation The shape of a molecule or any other object.

congenital Describing a genetic or nongenetic condition present at birth.

consanguinity Sharing at least one common ancestor in a recent generation.

contagious Transmissable (as a disease) from one person to another by indirect or direct contact.

contig A set of overlapping clones covering a chromosome region or a whole chromosome.

contingency table A set of cells of a table, each containing the joint frequency distribution of a two-way association.

continuous trait A phenotype consisting of a nonoverlapping series of values that usually form a bell-shaped curve when a large number of individuals are sampled.

coretention The presence of two or more sequence tagged sites on a DNA fragment in a radiation hybrid.

cornea The transparent band of tissue at the anterior portion of the outer layer of the eye.

cos ends The 12-base single-strand complementary extensions of bacteriophage λ DNA. *Also called* cos sites.

cosmid A vector system for cloning large (~45 kb) DNA inserts.

Cot-1 DNA A sample that contains most of the repetitive DNA sequences and almost none of the single-copy DNA sequences of the human genome. The average length of the DNA pieces in a Cot-1 DNA sample is about 300 base pairs.

coupling *See cis* configuration.

CpG island A 0.5 to 1 kb region of mammalian DNA with a high density of CpG dinucleotides located upstream of many expressed genes.

cross-fertilization Fusion of gametes from different individuals.

cross-hybridization The determination of reannealing between DNA strands from different clones, chromosomes, or species.

crossing over Reciprocal exchange of genetic material between homologous chromatids during meiosis I.

cross-pollination Transfer of pollen from an anther(s) of one plant to the stigma of the flower of another plant of the same species.

cross-β-structure A complex folded protein conformation with stacked protein sheets in parallel arrays.

crystallin A type of protein found in the lens of the eye. *Also called* lens crystallin.

C-terminus The final amino acid of a protein. *Also called* carboxyl terminus, carboxyl terminal end.

cut *See* cleave.

cytochemistry Microscopic study of cells and cell organelles, using dyes and other reagents to determine the location and quantities of specific cellular components.

cytogenetic map A map of a chromosome based on its banding pattern.

cytogenetics The study of chromosomes, chromosome behavior, and chromosome abnormalities.

cytokinesis The formation of two cells by invagination of the cell membrane, either after the cell division cycle, meiosis I, or meiosis II.

cytosine One of the organic bases found in either DNA or RNA.

cytosol The liquid portion of the cytoplasm.

data mining Use of software applications for locating specified information or relationships within large databases to either validate or formulate hypotheses.

database A file system of formatted information that is readily accessed, retrieved, and updated.

DCO Double crossover.

deletion Loss of part of chromosomal DNA.

dementia Impairment, to various degrees, of short- and long-term memory, abstract thinking, judgment, sentence organization, and/or motor functions.

denaturant A condition or chemical that alters the native conformation of a molecule.

denaturation (1) The separation of duplex nucleic acid molecules into single strands. (2) Disruption of the conformation of a macromolecule without breaking covalent bonds.

denaturing gradient gel electrophoresis A mutation detection assay that depends on the differential denaturation of DNA molecules during electrophoretic migration through a chemical DNA denaturant gradient. *Also called* DGGE.

dendrite One of the many slender, multibranched cellular extensions that emanate from the cell body of a neuron and carry nerve impulses toward the cell body.

deoxyribonucleic acid *See* DNA.

deoxyribose The 5-carbon sugar component of DNA.

depolarization The phase of the action potential when the membrane potential of the cell membrane of a neuron goes from about −70 to about +50 millivolts when both Na^+ and K^+ channels are opened.

deutan An individual with a decreased sensitivity to green light.

deuteranomaly A decreased sensitivity to green light.

deuteranope An individual with no sensitivity to green light.

deuteranopia The total loss of sensitivity to green light.

DGGE *See* denaturing gradient gel electrophoresis.

diagnosis Determination of the cause of a disorder.

diagnostic test An assay that reveals the presence or absence of a specific disorder.

diakinesis A stage during prophase I of meiosis I of the meiotic process, during which chromatids become highly condensed, spindle poles are established, and the nuclear membrane disassembles.

dichotomous trait A polygenic trait with two states. *Also called* binary trait.

dideoxynucleotide A human-made nucleoside triphosphate that lacks hydroxyl groups on both the 2′ and 3′ carbons of the pentose sugar. *Also called* ddNTP.

digenic (1) A trait that is the result of alleles at two different gene loci. (2) Two different gene pairs.

digenic inheritance Alleles at two different loci are necessary for a particular phenotype.

digest *See* cleave.

dihybrid A double heterozygous genotype.

dihybrid cross A mating between individuals who are doubly heterozygous for the same two pairs of genes.

diploid The total complement of chromosomes in the nucleus of a normal somatic cell. The human diploid chromosome number is 46. *Also called* 2N.

diplotene A stage during prophase I of meiosis I of the meiotic process, during which synapsis recedes and chiasmata become evident. *Also called* diplonema.

direct DNA sequencing DNA sequencing without the necessity of cloning the DNA molecule into a DNA sequencing vector.

directional selection Greater reproductive fitness of one of the homozygous genotypes over the other genotypes.

discordance The occurrence of one member of a twin pair having a particular trait and the other not having the trait. *Also called* discordant.

disjunction The separation of chromosomes during anaphase, anaphase I, and anaphase II.

disomy The presence of a pair of homologous chromosomes in the nucleus of a cell.

dispersive process A condition that causes unpredictable allele and genotype frequencies from one generation to another. *Also called* random process, stochastic process.

disruptive selection Greater reproductive fitness of the extreme phenotypes of a quantitative trait.

divergence Separation of a species, population, sequence, haplotype, or gene into two lineages.

diverticulum A sac or pouch protruding from a tubular organ into the surrounding muscle.

dizygotic twins Two offspring produced from two separate fertilizations, who develop together in the same uterus. *Also called* fraternal twins.

DNA Deoxyribonucleic acid. *Also called* genetic material.

DNA codon A set of three contiguous nucleotide pairs of the coding strand of a structural gene.

DNA construct A cloning vector with a DNA insert.

DNA enhancer element A DNA sequence that activates the transcription of a distant gene.

DNA fingerprint A set of DNA fragments that are characteristic for a particular source of DNA.

DNA insert A piece of DNA that has been cloned into a cloning vector.

DNA methylation Addition of a methyl group to the 5 position of cytosine of a CpG dinucleotide in DNA.

DNA polymerase An enzyme that links an incoming deoxyribonucleotide, determined by complementarity to a base in a template DNA strand, with a phosphodiester bond to the 3′-hydroxyl group of the last incorporated nucleotide of the growing strand during replication.

DNA probe *See* probe.

DNA transformation *See* transformation.

DNA typing *See* genotyping.

domain A functional region of a protein.

domestication The process of breeding or training organisms to be of service to humans.

dominant An allele that produces the same phenotype, whether the genotype is heterozygous or homozygous. *Also called* dominance.

dominant negative mutation A mutated gene encoding an altered protein that interacts with a normal protein to cause an abnormal phenotype.

dosage compensation Maintenance of the same level of expression of X-linked genes in XX and XY individuals.

dot blot *See* forward and reverse dot blot.

double heterozygote Two different gene loci, each with two different alleles.

Down syndrome Trisomy 21 or any other chromosome constitution that gives the same phenotype as trisomy 21.

duplex DNA Double-stranded DNA.

dyad A duplicated chromosome in the nucleus of a cell formed by meiosis I.

dystrophy A noninflammatory progressive breakdown of a tissue or organ.

DZ *See* dizygotic twin.

E value Expectation value for a BLAST analysis. The lower the E value, the more significant the alignment score. Represents the number of different alignments with a score equal to or better than S that can be expected to occur simply by chance.

effective population size (N_e) Number of reproducing individuals in a population.

electron transport chain Protein complexes of the mitochondrial inner membrane that generate electrons and pass them onto other protein complexes, which form part of the sequential process responsible for the synthesis of ATP. *Also called* electric transport system.

electrophoresis A method for separating different molecules in a mixture by differential migration, on the basis of size, shape, and electric charge, in an electric field through a porous gel, such as agarose or acrylamide.

elongation Sequential addition of one monomer at a time to a polymer.

—emia A medical suffix for any condition that affects the composition of the blood.

emigration Exit of individuals from a population or country.

encephalomyopathy The occurrence of brain and muscle degeneration as part of the same disorder.

encephalopathy A degenerative disorder of the brain.

endocytosis The transport of materials into a cell via the formation of plasma membrane vesicles.

environment In genetics, all the nongenetic factors that contribute to the determination of a phenotype.

enzyme A protein molecule that catalyzes a biological reaction.

enzyme replacement therapy Treatment of an inherited metabolic defect by the addition of an active protein that facilitates a specific chemical reaction.

ependymal cell A type of glial cell of the central nervous system that secretes cerebrospinal fluid.

epidemiology The study of the factors that determine the frequency, distribution, severity, and other aspects of diseases in animals and humans.

epilepsy Chronic disorder with periodic loss of consciousness, abnormal movements, and sensory disturbances as a result of imbalances of electrical activity in the brain.

epistasis Interaction between genes.

EST *See* expressed sequence tag.

eugenics The genetic improvement of humans by discouraging (negative eugenics) or encouraging (positive eugenics) certain matings.

eukaryotes Organisms, including animals, plants, fungi, and some algae, that have (1) chromosomes enclosed in a membrane-bounded nucleus and (2) functional organelles, such as mitochondria and chloroplasts, in the cytoplasm of their cells.

excitatory response Neurotransmitter stimulation of a nerve impulse in a postsynaptic neuron.

exclusion mapping The failure to observe linkage between a disease-causing gene and a large number of polymorphic loci that fall within a limited chromosome region. In other words, the results "exclude" the disease-causing gene from a particular chromosome region.

exocytosis The transport of materials out of the cell through the plasma membrane by fusion of vesicles with the plasma membrane.

exon A coding region of a gene, retained during the processing of a primary transcript into a functional RNA.

exon skipping Faulty or natural processing of a primary transcript that produces an mRNA without a coded segment(s).

expressed sequence tag A sequence tagged site derived from a cDNA clone. *Also called* EST, expressed sequence tagged site, eSTS.

expression Transcription and translation of a gene.

expression profile Determination of the members of a transcriptome or proteome in a cell, tissue, or organism.

expressivity A range of phenotypes in different individuals with the same allele(s) at a gene locus. *Also called* variability, variable expressivity.

extension Single-stranded region consisting of one or more nucleotides at the end of one of the strands of duplex DNA. *Also called* protruding end, sticky end, overhang, cohesive end.

extracellular matrix A secreted network of collagen, glycoproteins, and other proteins that provides physical support for cells and tissues.

eye The organ of vision.

F_1 The first generation of offspring from a cross. *Also called* first filial generation.

F_2 The second generation of offspring from a cross with at least one F_1 parent. *Also called* second filial generation.

false negative A test result that does not recognize a target when it is present in a sample.

false positive A test result that indicates the presence of a target when it is not in a sample.

familial risk ratio (λ) Frequency of a disorder among individuals of the same genetic relationship (e.g., siblings, first-degree relatives, etc.) divided by the frequency of the disorder in the population.

fascicle A small bundle of muscle or nerve cells.

fatty acid A hydrocarbon chain with a carboxylic acid at one end.

favism Hemolytic anemia in glucose-6-phosphate dehydrogenase-deficient individuals induced by ingestion of broad beans (*Vicia faba*).

female-specific map A linkage map based on the recombination frequencies during meiosis in females.

fertility The ability to produce viable offspring.

fertilization The fusion of a sperm and ovum to produce a zygote.

fetus A human embryo after the formation of the umbilical cord.

fibrillogenic Capable of forming fibrils.

first-degree relatives Parents, siblings, and offspring.

FISH *See* in situ hybridization.

fixation *See* genetic fixation.

flow cytometer Laboratory apparatus that measures the physical or chemical properties of individual items as they pass through a beam(s) in a liquid stream.

flow-sorted chromosome The isolation of a particular chromosome with laser-induced flow cytometry.

flow sorting An additional feature of a flow cytometer, diverting a specified item into a common collecting tube.

fluorescence Emission of a longer wavelength from a compound absorbing energy from radiation with a shorter wavelength.

flush end *See* blunt end.

flush-end cut *See* blunt-end cut.

forward dot blot A nucleic acid hybridization protocol in which a DNA sample(s) is fixed to a solid support and hybridized with a probe(s).

forward mutation Spontaneous change of a common to a rare and, often, deleterious allele.

founder effect The presence of an allele in a large number of individuals that was inherited through a number of generations from a common ancestor who helped establish the original population. *Also called* founder principle.

founder population An isolated population with a high incidence of a genetic disease as the result of an allele that was present in one of the individuals who founded the population.

fovea The region of high density of cone cells in the macula of the retina.

frameshift mutation A change in the reading frame of a gene as a result of an insertion or deletion of one or more base pairs. *Also called* reading frame mutation.

functional gene cloning A strategy that depends on information about a protein to isolate the corresponding gene.

functional genomics Large-scale study of gene expression.

***g* factor** A postulated biological component that plays a key role in determining major cognitive abilities.

G Guanine residue in either DNA or RNA.

G_1 phase The interval of the cell division cycle and meiotic process preceding the S phase. *Also called* G_1, gap 1.

G_1/G_2 phase The interval of meiosis II of the meiotic process, preceding prophase II.

G_2 phase The interval of the cell division cycle and meiotic process following the S phase. *Also called* G_2, gap 2.

gain-of-function mutation A change in a gene, resulting in a gene product that is usually biologically harmful. *Also called* gain-of-dysfunction mutation.

gamete Either a sperm or an ovum. *Also called* sex cell, germ cell.

gametogenesis Formation of sex cells.

ganglion (*plural*, ganglia) A cluster of neuronal cell bodies outside the central nervous system.

gap (1) The loss of one or more nucleotides from one strand of a double-stranded DNA molecule. (2) A missing segment of DNA between two adjacent contigs.

gated channel A transmembrane protein that can be selectively opened or closed to allow passage of an ion or some other molecule in or out of a cell.

G-band A chromosome band observed with a light microscope after treating either metaphase or late prophase chromosomes with trypsin and staining with Giemsa stain. *Also called* Giemsa chromosome band.

GC-clamp A 40-nucleotide GC-rich sequence at the end of one of a pair of PCR primers. The double-stranded GC-rich sequence stabilizes PCR products during a denaturing gradient gel electrophoresis mutation assay.

gene The unit of heredity.

gene cloning Insertion of a gene into a cloning vector. *Also called* recombinant DNA technology, genetic engineering, molecular cloning, cloning, gene splicing.

gene flow Introduction of alleles into a population as a result of migration.

gene locus (*plural*, gene loci) The site on a chromosome occupied by a gene. *Also called* locus (*plural*, loci).

gene map The linear array of genes on a chromosome.

gene pool The complete genetic information of all the members of a population.

gene silencing Inactivation of a gene.

gene test Screening of proteins in large populations for single amino acid changes that alter electrophoretic mobility, based on the premise that each amino acid change represents a point mutation.

gene therapy The use of a gene or cDNA to treat a disease.

genetic counseling A consultative process for informing individuals, couples, or families who are impacted directly or indirectly by a genetic disorder.

genetic diversity The extent of heterozygosity within or between populations. *Also called* gene diversity.

genetic fixation The same allele at a locus carried by all members of a population.

genetic heterogeneity A syndrome, condition, or disease resulting from alleles at different gene loci.

genetic linkage The association of two or more gene loci on the same chromosome. *Also called* linkage.

genetic map A map of the relative locations of sites (loci) on a chromosome, based on recombination frequencies. The distance between sites is measured in centimorgans (cM). *Also called* linkage map, meiotic map.

genetic material *See* DNA.

genetic microdifferentiation Diversity of allele frequencies among small, separated enclaves of a group or tribe.

genetic polymorphism The occurrence of two or more alleles in a population of individuals with a frequency of 1% or greater. In the appropriate context, this is simply called polymorphism.

genetics (1) The term for the scientific study of heredity, coined by W. Bateson (1861–1926). (2) "Genetics is the branch of biology which is concerned with innate differences between similar organisms." J. B. S. Haldane (1892–1964).

genetic screening An assay of a large group of asymptomatic individuals to discover those who may develop an inherited condition.

genetic test An assay that determines whether the cause of a disorder is at the DNA level.

gene tree A phylogenetic tree that is constructed from gene frequencies, haplotypes, or DNA sequences.

genome (1) The entire complement of genetic material of the cell of an organism, unicellular eukaryote, organelle, virus, or bacterium. (2) The haploid set of chromosomes (DNA) of a eukaryotic organism.

genome scan Linkage analysis of either affected individuals or large families with two or more affected individuals with a set of polymorphic DNA markers that are evenly distributed throughout the entire chromosome complement. *Also called* genomewide scan, genomic scan.

genomic imprinting A reversible, inherited modification of an allele that does not alter the nucleotide sequence and is determined by the sex of the parent.

genomics The study and development of genetic and physical maps, large-scale DNA sequencing, gene discovery, and computer-based systems for managing and analyzing genomic data.

genotype (1) The genetic constitution of an organism. (2) The combination of alleles of a gene pair.

genotyping The determination of the alleles of a chromosome of an individual. *Also called* DNA typing, haplotyping.

germ line The cells that give rise to gametes.

germ line therapy Curing a disorder by delivering a therapeutic gene to a fertilized egg or an early embryonic cell so that all the cells of the mature individual, including the reproductive cells, acquire the gene.

Giemsa stain A formulation of methylene blue and eosin used to stain chromosomes and other cellular components.

glaucoma A disease of the eye featuring principally erosion of the optic disk and atrophy of the optic nerve.

glial cell A nonneuronal cell associated with the central nervous system. *Also called* neuroglial cell (plural, neuroglia).

gliosis Accumulation of glial cells at a site of damage in the brain.

gradient A continuous, quantitative change in a system as a function of distance.

grandfather method A technique in which knowledge of the X-linked alleles of a father is used to determine the genetic phase of a daughter whose sons can be examined directly as recombinant or nonrecombinant types for the genetic mapping of X-linked gene loci.

guanine One of the organic bases found in either DNA or RNA.

guanylate cyclase An enzyme that converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP).

Guthrie test A microbial assay for determining phenylketonuria and other inherited metabolic disorders with a blood sample from a newborn.

HAC *See* human artificial chromosome.

hallucination Any persistent or recurrent sense perception, such as a sight, touch, sound, or smell, that is not based on any possible external stimulation.

hamartoma Benign tumorous growth usually composed of one type of mature cell.

haplogroup A set of closely related haplotypes.

haploid The total complement of chromosomes in an egg or sperm. The human haploid chromosome number is 23. *Also called* N.

haploinsufficiency Lowered production of a protein as a result of a mutation or loss of an allele that results in an abnormal phenotype.

haplotype The alleles of the loci of a chromosome. Derived by combining *haplo* from haploid and *type* from genotype.

haplotyping *See* genotyping.

Hardy–Weinberg equilibrium The maintenance of allele and genotype frequencies from one generation to the another in a very large population with random mating, no migration, no mutation, equal reproductive fitnesses for all breeding individuals, and nonoverlapping generations. *Also called* Hardy–Weinberg law, Hardy-Weinberg-Castle law, genetic equilibrium, square law.

hemizygous Single copy of a gene in a genome; usually describes X-linked genes in XY males.

heredity The biological transmission of features determined by genetic information from one generation to the next.

heritability (h^2) The proportion of the total variance of a quantitative trait that is attributed to genetic factors. *Also called* broad-sense heritability, h^2_B.

hernia Protrusion of part of an organ through a tear in a muscle wall.

heterodisomy Two nonidentical chromosomes that originate from one of the parents.

heteroduplex analysis A mutation detection assay that depends on the difference in electrophoretic mobility between a DNA molecule with one or a few nucleotide mismatches (heteroduplex DNA) and one that is fully complementary (homoduplex DNA).

heteroduplex DNA Duplex DNA with one or more mismatched nucleotide pairs.

heterologous probe A DNA probe derived from one organism and used to screen for a similar sequence in a genomic library derived from another organism.

heteromer A protein with two or more different protein chains. *Also called* heteromeric polypeptide, heteromeric protein.

heteroplasmy The occurrence of two or more mitochondrial genomes with different DNA sequences in the same cell or tissue.

heterozygosity The extent of the different alleles at one or more loci in an individual or a population.

heterozygote An individual with different alleles at a gene locus.

heterozygous A genotype with different alleles at a gene locus.

HGP *See* Human Genome Project.

high-resolution map Closely spaced sites throughout a genetic or physical map.

hippocampus A portion of the limbic system of the human brain, controlling memory, learning, emotions, and other functions.

histone(s) A group of low-molecular-weight DNA-binding proteins. The H1 histone binds directly to the DNA of chromosomes. At regular intervals, chromosomal DNA wraps around nuclear bodies (nucleosomes) that each consist of two H2A, H2B, H3, and H4 histone molecules.

holandric Having a male-specific characteristic; usually referring to genes on the Y-specific portion of the Y chromosome.

homeo box An evolutionarily conserved sequence of 180 nucleotides, encoding a DNA-binding amino acid sequence that regulates some aspect of development. *Also called* homeobox.

homeodomain An evolutionarily conserved 60-amino acid segment of a protein that binds to a DNA sequence and regulates some aspect of development.

homeotic gene A DNA sequence encoding a paired domain, homeodomain, or both.

homeotic mutation A change in a gene causing one developmental pattern to be replaced by a different but homologous pattern.

homoduplex DNA A fully complementary double-stranded DNA molecule.

homolog (1) In evolution, an anatomic feature that appears in different organisms and has a common biological origin but not necessarily the same function. (2) The occurrence of a gene in one organism that has a similar nucleotide sequence as a gene in another organism. (3) A chromosome or chromosomal region with the identical genetic loci as another chromosome or chromosomal region.

homologous chromosomes Chromosomes that normally synapse during zygotene.

homology Similarity due to a common origin.

homomer A protein with only one kind of protein chain. *Also called* homomeric polypeptide, homomeric protein.

homoplasmy The occurrence of the same mitochondrial genomes in a cell or tissue.

homozygosity The extent of the same alleles at one or more loci in an individual or a population.

homozygosity-by-descent The occurrence of allelic homozygosity as the result of inheritance of two copies of an allele that was present in a recent common ancestor. *Also called* HBD, allelic identity-by-descent, allelic IBD.

homozygosity mapping A method using genotyping of offspring of consanguineous marriages to localize a mutant allele to a chromosome region.

homozygote An individual with the same alleles at a gene locus.

homozygous A genotype with the same alleles at a gene locus.

homozygous dominant A genotype with two dominant alleles at a gene locus.

homozygous recessive A genotype with two recessive alleles at a gene locus.

housekeeping gene A protein-encoding DNA sequence that is transcribed in many different cell types.

***Hpa*II tiny fragments** Small DNA fragments generated from a genomic clone after treatment with the restriction endonuclease *Hpa*II that cleaves within the CpG islands. *Also called Hpa*II-induced tiny fragments.

HTFs *See Hpa*II tiny fragments.

human artificial chromosome A chromosome manufactured from telomere, centromere, and human genomic DNA sequences. *Also called* HAC.

Human Genome Project An international research effort that successfully sequenced the human genome. *Also called* HGP.

hybrid gene The combination of two genes or the parts of two genes in the correct reading frame that encodes a single protein with amino acid sequences from both genes.

hybridization The process of base pairing of two complementary strands to form a double-stranded region.

hypermethylation High level of 5-methyl cytosine residues in a DNA region that is usually associated with gene silencing.

hyperpolarization The increase in the polarity of the potential difference across the plasma membrane of a nerve cell when the negative charge of the intracellular fluid builds up relative to the positive charge of the extracellular fluid.

hypoacetylation Low level of acetylated histone in a DNA region that is usually associated with gene silencing.

hypoglycemia Abnormally low concentration of glucose in the blood.

hypothalamus The part of the brain beneath the thalamus that regulates body temperature, metabolic processes, and other activities.

hypotonia Reduction of tension of muscle tissue.

iatrogenic An unexpected, adverse condition caused unintentionally by a physician or prescribed therapeutic treatment.

identical-by-descent Formation of a homozygous genotype with copies of an allele that was present in a common ancestor. *Also called* identity by descent, IBD.

identical-by-state Formation of a homozygous genotype with an allele that was not derived from a common ancestor. *Also called* identity by state, IBS.

ideogram A diagrammatic representation of the banding pattern of a chromosome. *Also called* idiogram.

idiopathic Of unknown cause.

immigration Entry of individuals into a population or country.

imprint Transcriptional status of an allele that is established during sex cell formation.

imprinting control region A DNA sequence that governs the transcriptional status of one or more genes on the same chromosome. *Also* ICR.

inbreeding Mating of closely related individuals. *Also called* consanguinity.

inbreeding coefficient (*F*) The probability that an allele from a common ancestor(s) will be identical by descent. *Also called* Wright's inbreeding coefficient.

incest Mating between very closely related individuals.

incidence The number of new cases of a disorder in a population in a given time period.

independent assortment The formation of all possible gene combinations in gametes with genes on different chromosomes, followed by the random fusion of male and female gametes. *Also called* Mendel's Second Law of Inheritance.

infectious Describing (1) the ability of a microorganism to enter the body, multiply, and cause a disease or (2) the indirect transmission of a disease-causing microorganism from an infected to a noninfected person.

infertility The inability to produce viable offspring.

inflammation A complex set of responses in blood vessels and adjacent tissue to injury, leading to redness, increased temperature, swelling, and localized pain.

informatics The study of the management of information, using computer and statistical techniques. The term bioinformatics is used when the information is from a biological source(s).

in-frame The maintenance of a reading frame of a coding region either after a mutation or the ligation of two DNA molecules.

inheritance In genetics, the biological acquisition by an offspring of genetic material from a parent.

inhibitory response Neurotransmitter prevention of the initiation of a nerve impulse in a postsynaptic neuron.

initial segment The portion of an axon that immediately follows the axon hillock.

initiation The start of the biosynthesis of a polymeric macromolecule.

initiator codon The three nucleotides, usually AUG, in an mRNA that facilitate the start of translation. *Also called* initiation codon, start codon, initiating codon.

insert A DNA molecule incorporated into a cloning vector.

insertion The addition of one or more base pairs into chromosomal DNA.

in situ hybridization Complementary base pairing of a labeled probe to the DNA of a denatured chromosome. If the probe is tagged with a fluorescent dye and hybridization is scored by fluorescence microscopy, then the technique is called fluorescence in situ hybridization or FISH.

integrated map The combining of the information from genetic, physical, and other maps of a chromosome into one map.

interactome An extensive set of interacting proteins.

interstitial Located between two structures or sites.

intraclass correlation coefficient The statistical relationship between two data sets where the values from either set cannot be assigned to a particular variable.

intron In a eukaryotic structural gene, the noncoding region that is spliced out of the primary RNA transcript during processing into a functional mRNA.

invagination The infolding of a cell membrane or a sheet of cells.

inversion A chromosome segment that has been rotated 180° with respect to the rest of the chromosome. *Also called* chromosome inversion.

in vitro A reaction or process that takes place in either a laboratory container or culture medium.

in vivo A reaction or process that takes place in a living organism.

in vivo gene therapy Treating a disorder by delivering a therapeutic gene(s) to a tissue or organ.

iridocorneal angle The junction of the iris and the cornea at the base of the anterior chamber of the eye.

iris The anterior portion of the middle layer of the eye that acts as a diaphragm, controlling the amount of light entering the eye. The central opening of the iris is the pupil.

isodisomy Two identical chromosomes that originate from one parent.

isoform One of a group of variant gene products derived from the same gene either by exon skipping or different transcription initiation sites.

karyotype (1) Photomicrographs of metaphase chromosomes from a single cell that are arranged with pairs of homologous autosomes side by side, from the longest to shortest, and with the sex chromosomes grouped separately. (2) A description of a set of chromosomes, including the diploid number, chromosome lengths, and distinguishing morphological features of some of the chromosomes.

Kayser–Fleischer ring A pigmented band encircling the cornea near the junction with the sclera that is often associated with Wilson disease.

kb Abbreviation for kilobase pairs; 1000 base pairs; a unit of length of DNA.

kindred A group of individuals related to each other either genetically or by marriage. *Also called* kinship.

kinetochore The outer surface of the centromere, where spindle fibers directly attach during the cell division cycle and meiotic process.

kuru A fatal central nervous system disorder that was observed among the Fore tribe of New Guinea.

label A compound or atom that is either attached to or incorporated into a macromolecule and used to detect the presence of a compound, substance, or macromolecule in a sample. *Also called* tag.

large ribosomal subunit The larger multiprotein-RNA component of a ribosome.

lens The transparent, biconvex refractive structure of the eye, located behind the pupil of the iris and in front of the vitreous body. Often called crystalline lens to distinguish it from manufactured lenses.

leptotene A stage of prophase I of meiosis I of the meiotic process, during which the chromosomes begin to condense. *Also called* leptonema.

leukocyte A white blood cell.

liability threshold model Postulated basis for polygenic traits with two phenotypes, usually affected and unaffected. Depending on the trait, either below or above a certain biochemical or physiological level leads to the affected phenotype.

library In recombinant technology, a collection of cells that carry different DNA constructs from a specific source. *Also called* clone bank.

ligation Joining of two DNA molecules by the formation of phosphodiester bonds catalyzed by a DNA ligase.

LINE-1 (L1) element A repetitive class of dispersed retrotransposon DNA sequences in mammalian genomes.

linkage disequilibrium Measure of the statistical association of allele frequencies at two or more loci.

linkage equilibrium Random association of two or more loci.

linkage map *See* genetic map.

linker A synthetic double-stranded oligonucleotide that carries the sequence of one or more restriction endonuclease sites.

lipid Fatty acids, glycerides, glyceryl esters, glycolipids, phospholipids, and other cellular substances that are soluble in organic solvents.

lipofection Delivery into eukaryotic cells of DNA, RNA, or other compounds encapsulated in an artificial phospholipid vesicle.

LOD score The logarithm of the ratio of the likelihood of the odds that two loci are linked with a recombination fraction equal to or greater than 0 and less than 0.5 to the likelihood of the odds for independent assortment. *Also called* Lod score, Z, Z score.

long template A DNA strand synthesized during the polymerase chain reaction from an original DNA strand. A long template strand has a primer sequence at one end, and the other end extends beyond the sequence complementary to the second primer.

loss-of-function mutation A change in a gene affecting the amount or function of a gene product.

macromolecule A molecule with a large molecular mass, such as a nucleic acid, protein, or polysaccharide.

macula A small, oval area of the central part of the retina. *Also called* macular region, macula lutea.

male-specific map A linkage map based on the recombination frequencies during meiosis in males.

map distance The number of map units or centimorgans between two gene loci.

map unit A measure of genetic distance between two linked genetic loci; usually 1% recombination corresponds to one map unit.

marker An identifiable DNA sequence on a chromosome. *Also called* marker site, marker locus, genetic marker.

marker density The spacing of marker sites along a chromosome.

mass spectrometry Measurement of mass-to-charge ratio of ions.

maternal inheritance Exclusive transmission of mitochondria from a mother through her oocytes to offspring.

maternally expressed Exclusive transcription of an allele inherited from the female parent.

maternally silenced Establishment of monoallelic inactivation during oogenesis.

mating Sexual reproduction between a specified pair of individuals. *Also called* crossing, cross.

Mb Abbreviation for megabase pairs; a million base pairs; a unit of length of DNA.

MCS *See* polylinker.

meiotic map *See* genetic map.

meiotic process Two successive, specialized divisions of germ cells (meiosis I and meiosis II) that produce cells carrying one member of each pair of chromosomes. *Also called* meiosis, meiotic division.

meristic trait A phenotype that consists of a range of discrete values.

messenger RNA The transcript carrying the code for the sequence of amino acids of a protein. *Also called* mRNA.

metabolome The complete repertoire of metabolites of a cell, tissue, or organism.

metacentric chromosome A chromosome with its centromere at or near the midpoint from both ends.

metaphase A stage of mitosis of the cell division cycle, during which highly condensed chromosomes are aligned at the spindle equator midway between the spindle poles. Mechanistically, metaphase I and metaphase II of the meiotic process are the same as a mitotic metaphase.

metaphase plate *See* spindle equator.

metaphase spread A preparation of dispersed mitotic metaphase chromosomes of a cell on a microscope slide.

microarray Matrix with thousands of bound gene sequences or oligonucleotide probes. *Also called* DNA chip, gene array.

microchromosome *See* human artificial chromosome.

microdeletion Submicroscopic loss of internal chromosome DNA.

microglia A type of glial cell that scavenges (phagocytizes) cell debris and other particulate matter in the central nervous system.

microtubule A long, thin cellular protein cylinder that, among other functions, forms the cytoskeleton of cells and comprises the spindle fibers assembled during the cell division cycle and the meiotic process.

migration Movement of breeding individuals from one population to another.

mismatch The lack of base pairing between one or more nucleotides of two hybridized nucleic acid strands.

missense mutation A nucleotide change in the coding region of a structural gene, resulting in the replacement of the original amino acid by another in the encoded protein. A change from one sense codon to another sense codon.

mitochondrion A two-membrane, two-compartment, semiautonomous cytoplasmic organelle composed of proteins encoded by its own genetic apparatus and by nuclear genes. The principal function of mitochondria is the oxidation of fatty acids and organic acids to generate chemical energy in the form of ATP.

mitosis A phase of the cell division cycle that consists of prophase, metaphase, anaphase, and telophase. *Also called* M phase, mitotic phase.

mitotic process *See* cell division cycle.

M-K test (McDonald–Kreitman test) Test for neutrality that compares the ratios of nonsynonymous and synonymous substitutions within (polymorphic) and between (fixed) species.

modifier gene A gene that alters the phenotypic outcome determined by a major gene.

molecular motor A protein such as kinesin, a kinesin-related protein, or dynein that uses ATP as an energy source to move and, in some cases, translocate specific cellular material along microtubules, microfilaments, or other protein structures.

monoallelic expression Transcription of only one allele of a locus in a cell or tissue.

monochromosomal Refers to the presence of a single human chromosome in a somatic cell hybrid line.

monogenic (1) A trait resulting from an allele(s) at a single gene locus. (2) A single gene pair.

monohybrid A single heterozygous genotype.

monomer A unit of a polymer.

monomorphic probe A DNA sequence used to identify a chromosome site with the same DNA sequence in the genomes of all individuals of a large population.

monomorphic site A chromosome location with the same DNA sequence in the genomes of all individuals of a large population. *Also called* monomorphic locus.

monosomy The loss of one chromosome of a pair from a chromosome set.

monozygotic twins Genetically identical sibs produced by the separation of cells of the early embryo (blastomeres) from a single zygote. *Also called* identical twins.

mores Customs and conventions that are characteristic of a population or community.

mosaicism The tissues or organs of an individual having two or more cell populations with distinct genetic or chromosome constitutions.

most recent common ancestor The single historical entity in a phylogenetic tree that is the origin of all the lineages. *Also called* MRCA.

motor neuron A nerve cell that carries impulses from the central nervous system to a muscle or gland cell. *Also called* efferent nerve.

mRNA *See* messenger RNA.

multigenic inheritance The determination of a phenotype by a large number of different genes. *Also called* quantitative inheritance.

multimer A protein molecule made up of two or more different polypeptide chains.

multiple alleles A set of three or more alleles of a single gene locus.

multiple cloning site *See* polylinker.

multiplex assay Simultaneous determination of a large number of different targets in one reaction vessel or by one analytical device.

multipoint linkage analysis The determination of the order and map distances of many loci on a chromosome at one time. *Also called* multilocus mapping, multipoint mapping, multilocus linkage analysis.

muscle fiber *See* myofiber.

muscular dystrophy Progressive weakening and wasting of muscle tissue.

mutation A change of a nucleotide pair(s) in chromosomal DNA.

mutation detection assay A protocol that identifies the difference of one or a few nucleotide pairs between DNA molecules from different sources.

myelin sheath Multiple layers of cell membrane from an accessory nerve cell wrapped around an axon.

myocardium The middle and thickest layer of muscle of the heart.

myocyte *See* myofiber.

myofiber A mature muscle cell. *Also called* muscle fiber, myocyte.

myofibril The collection of myofilaments of a muscle cell.

myofilament Either the thick or the thin filament of a muscle cell.

myopathy Disease of muscle tissue.

myotube An early cell stage in the development of a mature muscle cell, characterized by a centrally located nucleus.

MZ *See* monozygotic twins.

narrow-sense heritability (h^2_N) The proportion of the total variance of a trait that is attributed to an additive gene effect.

nature In genetics, that component of a trait that is the result of genetic information.

NCO Noncrossover.

negative assortative mating Breeding partners who deliberately choose one another on the basis of a contrasting genetically determined trait. *Also called* disassortative mating.

neonate Newborn infant.

nerve cell *See* neuron.

neuritic plaque A senile plaque surrounded and interspersed with neuronal debris.

neurofibrillary tangles Intraneuronal accumulations of filamentous aggregates of tau and other proteins, often associated with Alzheimer disease. *Also called* NFTs.

neurolemnocyte An accessory nerve cell that forms myelin sheaths around neurons of the peripheral nervous system.

neuron A cell that specializes in initiating and transmitting electrical impulses. *Also called* nerve cell.

neuropathy A condition that affects the functioning of peripheral nerves.

neurotransmitter A chemical compound or peptide that is released by a presynaptic neuron and produces an excitatory or inhibitory response in a postsynaptic neuron.

neutral mutation (1) A nucleotide change that converts a sense codon into a codon for another amino acid with the same physicochemical properties. A neutral mutation does not alter the function of the gene product. (2) A nucleotide replacement that has no effect on reproductive fitness.

neutral theory The concept that most mutations have no effect on reproductive fitness and, consequently, their frequencies in populations are the result of drift and other random processes. *Also called* neutral allele theory, neutral-mutation hypothesis.

neutrophil A phagocytic white blood cell.

nick A break of a phosphodiester bond in the backbone of one of the strands of a duplex DNA molecule.

node The point in a phylogenetic tree that marks a divergence. *Also called* branch point.

nonadditive gene effect The combination of dominant and epistatic genes that contribute to a phenotype.

nonallelic loci Gene sites at different chromosome locations.

nonassortative mating *See* random mating.

nonautologous Originating from a different species or another individual.

noncoding strand The DNA strand of a gene that is used as the template for transcribing mRNA.

nondisjunction The failure of chromatids to be properly distributed to daughter cells during the cell division cycle and meiotic process.

nonhomologous chromosomes Chromosomes that do not normally synapse during zygotene.

nonhomologous recombination The reciprocal exchange of DNA after the mispairing between nonidentical but highly similar chromosomal regions. *Also called* unequal crossing over.

nonparametric method A statistical method that makes no assumptions about the experimental data conforming to a particular probability distribution. Linkage tests, such as sib pair analysis, which make no assumptions about the frequencies of alleles, extent of penetrance, or other parameters, are nonparametric procedures.

nonrandom mating Breeding partners who deliberately choose one another on the basis of a genetically determined trait.

nonsense mutation *See* termination mutation.

nonsister chromatids The chromosome strands of the different members of a bivalent.

nonsynonymous mutation Nucleotide replacement of a codon that alters the encoded amino acid.

nonsystemic A condition that is confined to a specific part of the body or a single organ.

normal distribution Statistical probability plot that forms a bell-shaped curve. *Also called* Gaussian distribution.

norms Accepted rules of behavior in a population or community.

Northern blotting A technique for transferring electrophoretically separated RNA from a gel to a support matrix for a hybridization reaction.

nosology The scientific practice of classifying diseases.

N-terminus The first amino acid of a protein. *Also called* amino terminus, amino terminal end.

nuclear membrane The double-layered envelope enclosing the chromosomes of eukaryotic cells. *Also called* nuclear envelope.

nucleotide diversity (π) Mean number of nucleotide differences per site between pairs of sequences.

nucleotide The repeating unit of a nucleic acid strand consisting of an organic base, a 5-carbon sugar, and phosphorus.

nucleus (1) The organelle of a eukaryotic organism enclosing the chromosomes. (2) A cluster of cell bodies of neurons within the central nervous system. (3) The central portion of an atom containing protons and neutrons.

nurture In genetics, that component of a trait that is the result of nongenetic factors.

oligodendrocyte A type of glial cell that produces myelin sheaths and provides both support and nutrients for neurons of the central nervous system.

oligogenic inheritance The determination of a phenotype by a small number of different genes.

oligonucleotide A short (<100 bases), synthetic molecule of single-stranded DNA. *Also called* oligomer, oligodeoxyribonucleotide, oligo.

oligosaccharide A carbohydrate with only a few monosaccharide units.

oogenesis Formation of unfertilized eggs.

open angle An apparently unobstructed iridocorneal angle of the anterior chamber of the eye.

opsin The protein component of the visual pigments located in the disk membranes of the outer segments of the rods and cones of the retina.

optic disk In the posterior of the eye, a small area through which the retinal artery enters and the nerve fibers of the retinal ganglia exit the eye. *Also called* blind spot.

optic nerve One of a pair of cranial nerves made up of fibers of the retinal ganglia, traveling from the retina to the visual cortex of the brain.

organ A structural part of the body, such as the heart, liver, or spleen, made up of tissues and other cells, that carries out a specific function.

ortholog A sequence in different species that has a common origin.

out-of-frame The loss of a reading frame of a coding region, after either a mutation or the ligation of two DNA molecules.

overdominance Reproductive fitness of a heterozygous genotype is greater than either homozygous genotype. *Also called* heterozygote superiority, heterozygous superiority, heterozygous advantage.

overhang *See* extension.

ovum (*plural*, ova) An unfertilized egg.

oxidation (1) Removal of hydrogen from a compound. (2) Combining of oxygen with another compound with the subsequent release of heat. (3) Loss of electrons from a compound.

oxidative phosphorylation The production of chemical energy (ATP) from the combination of the electron transport chain, which oxidizes various substrates to produce electrons, protons, and water, and the complex (ATP synthase), which synthesizes ATP. *Also called* OXPHOS.

OXPHOS *See* oxidative phosphorylation.

p arm The shorter of the two chromosome arms.

P1 cloning system A vector system based on bacteriophage P1 for cloning large (~100 kb) DNA inserts.

PAC A bacteriophage P1-based vector system for cloning large (~100 kb) DNA inserts.

pachytene A stage of prophase I of meiosis I of the meiotic process, during which synapsis is complete and chromosome condensation continues. *Also called* pachynema.

packaging cell line A cell line designed for the production of replication-defective viral particles.

paired domain An evolutionarily conserved amino acid segment of a protein that binds to DNA and regulates some aspect of development. *Also called* paired box.

paired helical filaments Abnormal accumulation of two intertwined cross-linked protein strands, consisting of monomers of tau protein, within the cell bodies of neurons; often associated with Alzheimer disease.

palliative Providing relief from pain and discomfort.

palsy Partial paralysis.

paracentric inversion A chromosome inversion that includes only material of a chromosome arm.

paralog A sequence that arose by duplication within a species.

paralysis Loss of function over a part of the body as the result of a defect or damage to the nervous system or muscle tissue.

parametric method A statistical method that assumes that the experimental data conform to a particular probability distribution. Linkage tests, such as LOD score determinations, which make assumptions about the frequencies of alleles, extent of penetrance, and other parameters, are parametric procedures.

parental generation The original parents of a genetic cross. *Also called* P generation.

parent-of-origin effect The expression or inactivation of an allele that depends on the sex of the parent.

partial digestion Treatment of a DNA sample with a type II restriction endonuclease under conditions that result in a limited number of cleavages in each DNA molecule to yield all possible combinations of cut pieces in the final sample.

paternally expressed Exclusive transcription of an allele inherited from the male parent.

paternally silenced Establishment of monoallelic inactivation during spermatogenesis.

pathological Leading to or the consequence of a severe disease; often connotes severely diseased.

PCR *See* polymerase chain reaction.

PCR-SSCP *See* polymerase chain reaction–single-strand conformation polymorphism technique.

pedigree A diagrammatic representation of the history of a trait in a multigeneration family.

penetrance The fraction, usually expressed as a percentage, of individuals with a specific genotype at a gene locus who show the expected abnormal phenotype.

peptide bond The covalent bond between the carboxyl group of the α-carbon of one amino acid and the amino group of the α-carbon of an adjacent amino acid in a protein.

pericentric inversion A chromosome inversion that includes the centromere of the chromosome.

peripheral nervous system All the neurons outside the brain and spinal cord.

PERT *See* phenol-enhanced reassociation technique.

PFGE *See* pulse field gel electrophoresis.

phage *See* bacteriophage.

phase-known A double heterozygous genotype known to be in either a *cis* or a *trans* configuration.

phase-unknown The gene arrangement of a double heterozygous genotype that is not known.

phenocopy A nongenetic condition that closely resembles an inherited phenotype.

phenol-enhanced reassociation technique A method using phenol to reduce the aqueous volume of a DNA:DNA hybridization reaction in order to increase the rate of DNA reassociation. *Also called* PERT.

phenotype The observable appearance of a feature (trait) of an individual.

phenylketonuria A human autosomal recessive metabolic disorder resulting from the loss of activity of the liver enzyme phenylalanine hydroxylase, which, in turn, causes the accumulation of phenylalanine. Increased levels of phenylalanine have a neurotoxic effect. *Also called* PKU.

philtrum Vertical groove in the middle portion of the upper lip.

phlebotomy Incision in a vein for removing blood.

phosphodiester bond The linkage of phosphorus via oxygen to a carbon-containing group; the linkage between nucleotides of the same nucleic acid strand.

phosphorothioate linkage An internucleotide linkage similar to a phosphodiester linkage, except the available oxygen of the phosphorus group is replaced with sulfur.

photopigment *See* visual pigment.

photoreceptor A light-sensitive cell, organ, or organelle. For example, the rod and cone cells of the retina of the eye.

phototransduction The conversion of radiant energy (light) into electric signals (nerve impulses).

physical map An ordered set of genomic DNA clones demarcated with restriction endonuclease recognition or sequence tagged sites. The distance between sites is measured in base pairs.

pistil The female reproductive organ of a plant.

plasmid An extrachromosomal, autonomously replicating DNA molecule.

PNS *See* peripheral nervous system.

point mutation A change of a base pair in chromosomal DNA.

polar body A small cell produced during the meiotic process in human and other mammalian females. Three of the four cells produced by the meiotic process are polar bodies.

pollen The microspores of a seed plants. *Also called* pollen grains.

poly(A) tail The sequence of adenine residues at the 3′ end of most eukaryotic mRNAs.

polyadenylation The addition of the adenine residues to the 3′ end of eukaryotic mRNAs. *Also called* poly(A) tailing.

polygenic inheritance *See* multigenic inheritance.

polylinker A synthetic DNA sequence that contains a number of different restriction endonuclease sites. *Also called* multiple cloning site, multiple cloning sequence, MCS.

polymer A macromolecule made up of a series of covalently linked monomers.

polymerase chain reaction A technique for amplifying a specific segment of DNA, using a thermostable DNA polymerase, deoxyribonucleotides, and primer sequences in multiple cycles of denaturation-renaturation-DNA synthesis. *Also called* PCR.

polymerase chain reaction–single-strand conformation polymorphism technique A method for detecting mutations in a gene. *Also called* PCR-SSCP.

polymorphic probe A DNA sequence used to identify a chromosome site with two or more frequently occurring alleles.

polymorphic site A chromosome location with two or more identifiable allelic DNA sequences of which two or more occur with a frequency of 1% (0.01) or greater in a large population. *Also called* polymorphic locus.

polynucleotide Twenty or more nucleotides linked by phosphodiester bonds.

polyp A growth or cell mass protruding from a mucous membrane; small mucosal tumor.

polypeptide A linear series of amino acids linked by peptide bonds. *Also called* polypeptide chain, protein, protein chain.

population A group of contemporaneous individuals of the same species who occupy a particular geographic area.

population stratification A sample of control individuals that contains subgroups with significantly different allele frequencies.

population structure Parameters that determine the frequencies of alleles within a population, such as the extent to which each allele is passed on to the next generation (selection), the magnitude of migration and immigration, the basis for choosing mates, the rates of gene mutation, and the size of the population.

population-mutation parameter ($4N_e\mu$) Mean number of nucleotide substitutions at neutral sites under equilibrium conditions.

positional gene cloning An obsolete strategy for isolating an unknown disease gene that preceded the availability of human genome sequence. The disease gene was mapped to a chromosome site. A contig or genomic clone that covered the site of the disease gene was tested for exons. Examination of exons and mutation detection assays established which gene was the likely disease gene.

positional-candidate gene cloning A strategy for isolating an unknown disease gene based on mapping a disease-causing gene to a chromosome site and testing the likely genes in the mapped region for mutations.

position effect variegation Modification of the expression of a gene as a result of a change in its position relative to nearby genes. *Also called* position effect, PEV.

positive assortative mating Breeding partners who deliberately choose one another on the basis of a shared genetically determined trait.

positive-negative selection A protocol for selecting cells with a DNA insert integrated at a specifically targeted chromosomal location (positive selection) and against cells with a DNA insert integrated at a nontargeted chromosomal site (negative selection).

predictive test An assay that determines whether an asymptomatic individual is at risk for a specific disease.

preimplantation diagnostic test An assay for determining a chromosome abnormality or genetic condition in polar bodies or early embryonic cells before either in vitro fertilization or implantation of an early-stage embryo.

prenatal test An assay that determines the biological or genetic status of a human fetus.

presymptomatic Before the onset of the signs of a disease.

prevalence The number of existing cases of a disorder in a population at a given point in time.

primary constriction *See* centromere.

primary transcript Unprocessed RNA.

primer A short oligonucleotide (~20 bases) designed to base pair with a template strand providing a 3′-hydroxyl group for the initiation of nucleic acid synthesis.

primer walking A method for sequencing long (>1 kb) cloned pieces of DNA.

probe A labeled nucleic acid molecule used to detect complementary sequences as part of a hybridization assay. *Also called* hybridization probe.

processed transcript An RNA molecule after the removal of all introns. *Also called* processed RNA.

prodrug An inactive compound converted into a pharmacological agent by an in vivo metabolic process.

progeny The offspring of a mating.

prokaryotes Organisms, usually bacteria, with neither chromosomes enclosed in a membrane-bounded nucleus nor functional organelles, such as mitochondria and chloroplasts.

promoter A region of DNA to which RNA polymerase binds.

prophase A stage of mitosis of the cell division cycle during which the chromosomes condense, the nuclear membrane disassembles, and the centrioles become oriented opposite each other. Prophase I of the meiotic process consists of five stages, called leptotene, zygotene, pachytene, diplotene, and diakinesis. During prophase I, homologous chromosomes synapse, DNA may be exchanged between nonsister chromatids, and chiasmata are formed. Prophase II of meiosis II resembles the prophase of the cell division cycle.

propositus (*male*), proposita (*female*) Usually an affected individual who is the starting point for assembling a pedigree. *Also called* proband.

protan An individual with a decreased sensitivity to red light.

protanomaly A decreased sensitivity to red light.

protanope An individual with no sensitivity to red light.

protanopia The total loss of sensitivity to red light.

protein *See* polypeptide.

protein truncation test An in vitro transcription-translation assay for detecting nucleotide changes, such as nonsense mutations, in-frame deletions, out-of-frame mutations, and other alterations that shorten a protein. *Also called* PTT, in vitro synthesized protein (IVSP) assay.

proteome Complete repertoire of proteins of a cell, tissue, or organism.

proteomics Study of the structure, function, and interactions of the members of a proteome.

pseudoautosomal region The portion of the X and Y chromosomes that synapse during meiosis I.

pseudogene A DNA sequence in the genome that can neither be transcribed nor translated and is similar to an existing functional gene. There are two categories of pseudogenes: (1) A traditional pseudogene is a duplicate of a functional gene but has accumulated mutations that prevent it from being transcribed or translated. (2) A processed pseudogene is the double-stranded DNA version of a messenger RNA sequence with, in some cases, a portion of the polyA tail.

pseudohypertrophy Increase in the size of an organ or tissue that is not the result of an increase in size or proliferation of the cells of the organ or tissue.

ptosis Loss of muscle control of the upper eyelid.

pulse field gel electrophoresis A method for separating large (100 kb to >1 Mb) DNA molecules in a gel matrix by changing the direction and strength of the electric field.

Punnett square method A system for determining the kinds and frequencies of gametes, genotypes, and phenotypes from crosses. Named after R. C. Punnett (1875–1967), who devised the strategy and was among the first geneticists to study gene linkage. *Also called* checkerboard method.

pupil (1) The central opening of the iris of the eye. (2) A student in grade school.

purine Fusion of a pyrimidine and an iminazole ring, for example, adenine and guanine.

purkinje cells Large nerve cells of the cerebellar cortex.

pyrimidine A heterocyclic ring, for example, thymine, cytosine, and uracil.

q arm The longer of the two arms of a chromosome.

Q-band A chromosome band observed with an ultraviolet microscope after treating either metaphase or late-prophase chromosomes with a fluorochrome dye, such as quinacrine dihydrochloride. *Also called* quinacrine chromosome band.

QTL Quantitative trait locus. *Plural*, quantitative trait loci (QTLs).

quadrivalent Synapsis between four duplicated chromosomes.

query sequence Input sequence for computer analysis, usually for similarity searches.

quinacrine A fluorescent compound that binds to specific chromosome segments.

rad The unit of the dose absorbed by biological tissue from ionizing radiation; 100 ergs per gram of tissue.

radiation hybrid A hybrid cell line that carries a human DNA fragment(s) generated by ionizing irradiation of a donor cell.

radiation hybrid mapping The examination of coretention of two or more marker sites by fragments of DNA that were formed by ionizing irradiation of

donor cells and are maintained as part of rodent chromosomes in hybrid cell lines. The frequency of radiation-induced breakage between two loci is directly related to the distance between the loci. The measure of distance between sites is a centiRay (centiray, cR).

random genetic drift Fluctuation of allele and genotype frequencies from one generation to the next in small populations. *Also called* genetic drift, drift.

random mating Formation of breeding partners based solely on chance. *Also called* panmixia, panmixis.

random primer method A protocol for labeling DNA in vitro.

reading frame A sequence of sense codons.

recessive (1) A phenotype determined only by a homozygous genotype. (2) An allele with no apparent effect on the phenotype when in a heterozygous genotype. *Also called* recessiveness, recessivity.

reciprocal translocation *See* translocation.

recognition site *See* restriction site.

recombinant A new gene arrangement of linked gene loci as the result of crossing over.

recombinant DNA technology *See* gene cloning.

recombination The formation of a new gene arrangement of linked gene loci as the result of crossing over.

recombination fraction The number of meiotic products with a new gene arrangement between two gene loci divided by the total number of meiotic products.

recurrence risk The probability that a harmful event will happen again; often signifies the chance of an inherited condition in either a close relative of an affected individual or future offspring after the birth of an affected child.

refractory period The time span during which a channel cannot respond to a signal that normally elicits a response.

remedial *See* therapeutic.

renaturation The reassociation of two complementary nucleic acid strands after denaturation.

repetitive DNA Nucleotide sequences occurring 10 or more times at different locations in a genome. *Also called* repeat DNA, repeated DNA, repetitious DNA.

replacement therapy The administration of metabolites, cofactors, proteins, or hormones for treating genetic diseases causing biochemical deficiencies.

replication DNA synthesis.

replicative segregation The random separation of different mitochondrial genomes of heteroplasmic cells to descendant cells during mitosis.

repolarization The phase of the action potential of the cell membrane of a neuron when the membrane potential goes from about +50 to about −70 millivolts as a result of the Na^+ channels closing.

repression Inhibition of transcription.

reproductive compensation Increased number of offspring in families who have experienced the death of an infant or young child in comparison to families who have had only viable offspring.

reproductive fitness Relative transmission of a genotype from one generation to another. *Also called* fitness, adaptive value.

repulsion *See trans* configuration.

response element A DNA binding site for a transcription factor. *Also called* initiator element.

restenosis Recurring narrowing of a biological opening, tube, or canal.

resting membrane potential The difference in charge across the cell membrane of a neuron when both Na^+ and K^+ channels are closed and a nerve impulse is not being conducted.

restriction endonuclease (type II) An enzyme that recognizes a specific duplex DNA sequence and cleaves phosphodiester bonds on both strands between specific nucleotides.

restriction endonuclease map The order of the restriction endonuclease sites of a DNA molecule. The distance between sites is measured in base pairs. *Also called* restriction map, restriction enzyme map, recognition site map.

restriction fragment length polymorphism The common occurrence of variations in the lengths of certain DNA fragments produced after cleavage with a type II restriction endonuclease. The differences in the DNA lengths are the result of the presence or absence of a specific restriction endonuclease site(s) and are detected by using DNA hybridization with DNA probes after separation by gel electrophoresis and transfer to a support matrix.

restriction site The sequence of nucleotide pairs in duplex DNA recognized by a type II restriction endonuclease. *Also called* restriction enzyme site, restriction endonuclease site, recognition site.

retention pattern The record of the presence or absence of a number of different sequence tagged sites for each member of a panel of radiation hybrids.

retina The inner layer of the eye, containing photoreceptors that transduce radiant energy into electric impulses and nerve cells that transmit these impulses to the brain.

retinitis pigmentosa A progressive degeneration of the photoreceptors, starting with the rod cells of the retina, that is inherited as an autosomal dominant, autosomal recessive, or X-linked disorder. *Also called* RP.

reverse dot blot A nucleic acid hybridization protocol where the DNA probe(s) is fixed to a solid support and hybridized with a DNA sample(s).

reverse transcriptase An RNA-dependent DNA polymerase that uses an RNA molecule as a template for the synthesis of a complementary DNA strand.

reverse transcription-polymerase chain reaction A combined method for synthesizing cDNA strands from an mRNA sample and then amplifying a specific cDNA strand by polymerase chain reaction. *Also called* RT-PCR.

RFLP *See* restriction fragment length polymorphism.

rhodopsin The light-sensitive photopigment found in the disk membranes of the outer segment of rod cells of the retina.

ribose The 5-carbon sugar component of RNA.

ribosomal RNA RNA molecules that associate with ribosomal proteins and form ribosomal subunits.

ribosome The combination of a large and small ribosomal subunits.

ribozyme A catalytic RNA that cleaves another RNA molecule at a specific nucleotide.

risk The probability of a harmful event.

RNA Ribonucleic acid.

RNA polymerase An enzyme that transcribes RNA from a template DNA strand.

rod A photoreceptor of the retina with a rod-shaped outer segment. *Also called* rod cell, rod photoreceptor.

rolling circle model A mode of DNA replication.

rRNA *See* ribosomal RNA.

RT-PCR *See* reverse transcription-polymerase chain reaction.

S phase The interval in the cell division cycle and meiotic process during which the DNA of each chromosome is precisely replicated. *Also called* DNA replication phase, DNA synthesis phase.

S1 nuclease An enzyme that specifically degrades single-stranded DNA.

sarcolemma The cell membrane of a muscle cell.

sarcoplasm The cytoplasm of a muscle cell.

sclera The posterior portion of the outer layer of the eye.

SCO Single crossover.

screening test An assay of a large group of asymptomatic individuals to discover those who may develop a particular condition.

SDS-PAGE *See* sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

secondary constriction A consistent, narrowed, nonstaining region of a metaphase chromosome.

second degree relatives Grandparents, aunts, uncles, first cousins, nieces, nephews, and grandchildren.

second messenger system Activation of an intracellular factor as a consequence of the response of a cell receptor to an extracellular signal, initiating a series of reactions that eventually trigger a specific cellular response. *Also called* fixed-membrane-receptor mechanism.

segregating site A location in a DNA sequence from different individuals that has different nucleotides.

segregation The distribution of each allele of a gene pair into a separate gamete. *Also called* Mendel's First Law of Inheritance.

selectable marker A gene encoding a product that facilitates detection or isolation of a cell with a DNA construct.

selection A circumstance(s) that influences the transmission of genes from one generation to another.

selective sweep Fixation of a neutral allele(s) linked to a positively selected gene in a region of low recombination.

self-fertilization Fusion of gametes from the same individual.

self-pollination Transfer of pollen from anther to stigma of the same plant.

senile plaque A dense spheroid amyloid body found outside of neurons and often associated with Alzheimer disease.

sense codon A set of three nucleotides in mRNA that pair with an anticodon of a charged tRNA, leading to the placement of a specific amino acid in a protein during translation.

sense strand The nontranscribed DNA strand of a transcribed DNA region. *Also called* coding strand.

sensitivity The ratio of all true positive test results over all positive test results.

sequence tagged site A short (200–500 bp) DNA sequence occurring once in the genome and identified by PCR amplification.

sequence tagged site (STS)-content mapping A strategy for determining common sequence tagged sites among clones of a library. These shared sites are used to assemble contigs.

sequencing Determining the order of nucleotides or amino acids in DNA or protein molecules, respectively.

sex-average map A linkage map based on the mean recombination frequencies in males and females.

sex chromosomes The X and Y chromosomes in humans.

sex determination The process that establishes the sex of an individual.

sex ratio The proportion of males to females in a population.

short tandem repeat A DNA sequence with a sequential repeating set of two (di-), three (tri-), or four (tetra-) nucleotide pairs.

short tandem repeat polymorphism Two or more short tandem repeats at a specific chromosome location that differ from each other by a number of repeated nucleotide units, with each DNA sequence occurring at a frequency of 1% or more in a large population. The difference in the lengths of allelic short tandem repeats is detected by the polymerase chain reaction with primers that are complementary to unique DNA sequences flanking the short tandem repeat.

short template A DNA strand synthesized during the polymerase chain reaction with a primer sequence at one end and a sequence complementary to the second primer at the other end.

sib-pair method A method of testing for linkage between a gene locus and a disease-causing gene.

sibs Brothers and/or sisters who have the same biological parents. *Also called* siblings.

sibship All the siblings in a family who have at least one common biological parent.

signal region *See* response element.

silent mutation A nucleotide change in the coding region of a structural gene, converting a sense codon into another codon that specifies the same amino acid.

similarity Degree of relationship between two sequences.

single-copy DNA Nucleotide sequences that occur twice, once on homologous chromosomes, or less than 10 times in the genome. *Also called* unique DNA.

single-site mutation A change in one base pair in chromosome DNA. *Also called* point mutation.

single-strand conformation analysis A mutation detection assay based on the conformation of single strands of DNA. Electrophoresis is used to detect conformational differences. *Also called* single-strand conformational analysis, single-strand conformation(al) polymorphism, SSCP.

sister chromatids The two chromosome strands of a duplicated chromosome.

site A position on a chromosome demarcated either by physical or genetic mapping. *Also called* locus.

skeletal muscle Muscle tissue that is attached to the skeleton.

small ribosomal subunit The smaller multiprotein-RNA component of a ribosome.

smooth muscle Sheets of muscle tissue in the walls of internal organs and blood vessels.

sodium dodecyl sulfate-polyacrylamide gel electrophoresis A technique for separating proteins. Sodium dodecyl sulfate (SDS) is an anionic detergent that

binds to a protein and breaks noncovalent bonds to form a random coil. Sodium dodecyl sulfate treatment ensures that conformation does not have any effect on the rate of electrophoretic migration of a protein in a polyacrylamide gel. *Also called* SDS-PAGE.

somatic cell In a multicellular eukaryotic organism, any cell that is not a germ line cell.

somatic cell hybrid A cell line that maintains rodent chromosomes and one or a few human chromosomes. *Also called* cell hybrid, hybrid cell line.

somatic cell hybrid panel A set of derived chromosome-specific hybrid cell lines, each carrying a different portion of a particular chromosome. The members of such a panel have chromosomal deletions and, in some cases, carry translocated chromosomes that retain a segment of a particular chromosome. Ideally, the retained portions of the cell lines of a panel cover the entire chromosome. *Also called* somatic cell hybrid mapping panel.

Southern blotting A technique for transferring electrophoretically separated DNA from a gel to a support matrix for a hybridization reaction. *Also called* DNA blotting.

species A set of organisms that are reproductively isolated and able to produce fertile offspring.

specificity The ratio of all true negative test results over all negative test results.

spermatogenesis Formation of sperm.

spermatozoon (*plural*, spermatozoa) Mature, motile male gamete. *Also called* sperm.

spindle The array of microtubular filaments generated by centrioles during the cell division cycle and meiotic process.

spindle equator The region that lies midway between the spindle poles around which the metaphase chromosomes are arranged. *Also called* metaphase plate.

spindle fiber A set of the microtubular filaments generated by a centriole during the cell division cycle and meiotic process.

spindle pole The region adjacent to and including the centriole. *Also called* centrosome, pole.

splice site The nucleotides at (1) the end of an exon and the beginning of an intron or (2) the end of an intron and the beginning of the next exon, required for the joining of two exons and removal of an intron.

splice site mutation Loss or gain of a functional splice site.

spontaneous mutation A DNA change that cannot be attributed to any particular treatment or agent.

sporadic Usually pertains to a disease condition, affecting only one person in a family.

staggered cuts Cleavage of phosphodiester bonds on complementary strands of duplex DNA, but not opposite each another.

stenosis Narrowing of a biological opening, tube, or canal.

sticky ends *See* cohesive ends.

stop codon Termination of translation signal in mRNA consisting of UAA, UAG, or UGA.

STR *See* short tandem repeat.

strain A genetic variant of a standard parental microorganism or multicellular organism.

strand A linear series of nucleotides linked to each other by phosphodiester bonds.

stringency Experimental conditions such as temperature, ionic strength, and concentration of an organic solvent for determining the extent of allowable nucleotide pair mismatching between reannealed strands for a nucleic acid hybridization reaction. High-stringency conditions do not allow significant base pair mismatching between two DNA strands. Low-stringency conditions allow considerable base pair mismatching between two DNA strands.

STRP *See* short tandem repeat polymorphism.

STS *See* sequence tagged site.

subband An additional chromosome band within a previously described chromosome band revealed by higher-resolution staining.

subcloning Inserting a fragment from a cloned piece of DNA into another cloning vector.

substitutive therapy Treatment of an inherited disorder with a cofactor that restores enzyme function.

suicide gene A gene that kills its own cell under specified conditions.

symptom Any sign of a disease.

synapse A specialized electrochemical junction between a neuron and another neuron, muscle, or gland cell.

synapsis Side-by-side pairing of homologous chromosomes during zygotene of prophase I of meiosis I of the meiotic process.

synaptic bulb The enlargement at the terminus of an axon or axonal branch. *Also called* synaptic terminus.

syndrome The set of signs that characterize a specific disease or condition.

synonymous mutation Nucleotide replacement of a codon that does not alter the encoded amino acid.

synteny (1) Set of genes on a chromosome. (2) Chromosome arrays of orthologous genes in different species.

systemic Usually pertains to a disease condition, occurring throughout the body or affecting more than one tissue or organ.

systemic process A condition that causes predictable allele and genotype frequencies from one generation to another. *Also called* deterministic process, vectorial process.

T Thymine residue in DNA.

T4 DNA ligase An enzyme from bacteriophage T4-infected *E. coli* that catalyzes the formation phosphodiester bonds between adjacent 5′-phosphate and 3′-hydroxyl groups of double-stranded DNA molecules.

T4 DNA polymerase end-labeling A technique for labeling the ends of DNA molecules.

tag *See* label.

Tajima's *D* statistic Test for neutrality that scores the difference between nucleotide diversity (π) and segregating sites per nucleotide adjusted for sample size (K/a_n) divided by standard deviation of $\pi - K/a_n$.

tandem array Usually refers to similar sets of DNA sequences repeated two or more times in series.

tandem mass spectrometry (MS/MS) Initial mass analysis of ions (precursor ions) followed by a second mass analysis of the daughter ions of a selected precursor ion.

target gene A descriptive term for a gene either to be cloned or specifically mutated.

TDT Transmission/disequilibrium test.

telomere The terminal region of each arm of a chromosome. *Also called* terminus, chromosome terminus.

telophase A stage of mitosis of the cell division cycle, during which sets of chromosomes congregate near the spindle poles and become enveloped by a reassembled nuclear membrane and the cell divides into two daughter cells. Mechanistically, telophase I and telophase II of the meiotic process are the same as a mitotic telophase.

template strand The polynucleotide strand used by a polymerase that determines the sequence of nucleotides of a newly synthesized strand.

termination The cessation of the biosynthesis of a polymeric macromolecule.

termination mutation A change of a sense codon to a stop codon. *Also called* nonsense mutation, stop mutation.

thalamus A cluster of neurons of the diencephalon.

THE-1 element A moderately repetitive human DNA sequence.

therapeutic Pertaining to treatment of a disease.

third-degree relatives Great-grandparents, great-aunts, great-uncles, second cousins, children of first cousins, grand-nieces, grand-nephews, and great-grandchildren.

three-point cross A mating determining the linkage relationships among three nonallelic loci.

threshold The minimum level of stimulus or accumulation of material necessary to generate a biological response.

threshold potential The extent of membrane depolarization required to initiate an action potential.

thymine One of the organic bases found in DNA.

tip An extant member of a phylogenetic tree.

tissue A set of similar cells collectively performing a specific function.

tonicity The normal level of tension maintained by muscle tissue.

topology The overall branching pattern of a phylogenetic tree.

trait A specific feature of an individual. *Also called* character.

***trans* configuration** In a double heterozygote, one chromosome has one dominant allele at one gene locus and a recessive allele at another gene locus and the homologous chromosome has a recessive allele and dominant allele at these loci, respectively. *Also called trans* phase, repulsion, repulsion phase.

transcribed triplet A set of three contiguous nucleotides of the transcribed DNA strand within the coding region of a structural gene that determine a codon in an mRNA.

transcription RNA synthesis.

transcription factor A protein that regulates RNA synthesis by binding to a specific DNA sequence or another transcription factor.

transcriptional mapping Assigning cDNA clones or expressed sequence tags to specific chromosome regions by fluorescence in situ hybridization, hybridization, PCR analysis of somatic cell hybrid mapping panels, or other strategies. *Also called* transcript mapping.

transcript map *See* transcriptional mapping.

transcriptome Complete repertoire of RNA molecules of a cell, tissue or organism.

transfection (1) Introduction of DNA into cultured eukaryotic cells. (2) Transfer of nucleic acid from a virus to a cell.

transfer RNA An RNA molecule that base pairs with an mRNA codon during translation and specifies the incorporation of an amino acid into a growing protein chain.

transformation (1) The introduction of DNA into a prokaryotic or single-celled eukaryotic organism. (2) The conversion of normal plant and animal cells to cells that show distinctive growth properties in culture, such as loss of contact inhibition.

transgenic animal A fertile animal carrying an introduced gene(s) in its germ line.

transition Permanent replacement of a purine with another purine or a pyrimidine with another pyrimidine in chromosomal DNA.

translation Protein synthesis.

translocation A chromosome abnormality resulting from a terminal chromosome segment of one chromosome becoming the terminal chromosome segment of a nonhomologous chromosome. Reciprocal (balanced) translocation is the interchange of terminal chromosome segments between nonhomologous chromosomes. A nonreciprocal translocation is the one-way transfer of a terminal chromosome segment from one chromosome to a nonhomologous chromosome.

transmission/disequilibrium test A statistical analysis to substantiate linkage between a disease gene and a marker locus when the two loci are associated. *Also called* TDT.

transversion Permanent replacement of a purine with a pyrimidine or a pyrimidine with a purine in chromosomal DNA.

TRED *See* trinucleotide repeat expansion disorder.

trigenic Three different gene pairs.

trihybrid A triple heterozygous genotype.

trinucleotide repeat expansion disorder An inherited condition resulting from an increase in length of a trinucleotide repeat in the coding or noncoding region of a gene.

trisomy Three homologous chromosomes in the nuclei of an individual instead of two.

trisomy rescue Restoration of disomy as a result of the loss of one member of a set of three homologous chromosomes during early embryonic development.

tritan An individual with a decreased sensitivity to blue light.

tritananomaly A decreased sensitivity to blue light.

tritanope An individual with little or no sensitivity to blue light.

tritanopia The total or extreme loss of sensitivity to blue light.

tRNA *See* transfer RNA.

true negative A test result that indicates the absence of a target when it is not present in a sample.

true positive A test result that recognizes a target when it is present in a sample.

truncated protein A shortened protein resulting from either a frameshift or nonsense mutation producing a stop codon within the coding region of a gene.

truncating mutation A nucleotide change creating a termination codon within the coding region of a gene.

two-hybrid system Assay for identifying pairwise protein-protein interactions.

two-point cross A mating determining the linkage relationship between two nonallelic loci.

U Uracil residue in RNA.

underdominance Reproductive fitness of a heterozygous genotype is less than either homozygous genotype. *Also called* heterozygote inferiority, heterozygous inferiority.

uniparental disomy A pair of chromosomes that originate from one parent.

uracil One of the organic bases found in RNA.

—uria A medical suffix for a condition that affects the composition of the urine.

uveitis An inflammation of the middle layer of the eye.

vaccine A suspension of an antigen(s) which, after introduction into a human or other mammal, induces the production of a specific antibody(ies).

variance The mean of the squared deviations of a set of observations.

variation Differences among members of a strain or species excluding those that are the result of age and gender.

vector *See* cloning vector.

vehicle *See* cloning vector.

ventricle (1) A cavity in the brain, filled with cerebrospinal fluid. (2) The left and right lower chambers of the mammalian heart.

viability Maintenance of life.

visual pigment A light-sensitive protein. *Also called* photopigment.

voltage-gated channel A transmembrane ion channel protein that is either opened or closed by a specific level of charge in its vicinity. *Also called* voltage-sensitive channel.

Western blotting A technique for transferring electrophoretically separated proteins from a gel to a support matrix for an immunological assay.

wild type Pertaining to a commonly observed phenotype considered to be the norm for a species.

X chromosome One of the sex chromosomes in humans. Human females have two X chromosomes and human males have one.

X chromosome inactivation Selective silencing of most of the genes on one of the two X chromosomes in mammalian females.

xenogenic Cells or tissues from a different species or another individual. *Also called* xenogeneic.

XIST (X-inactivation specific transcript) A *cis*-acting, noncoding RNA produced by an X-linked gene (*XIST*) that inactivates most of the genes on an X chromosome.

X-linked A gene locus on the X chromosome. The term sex linkage, which has been used to describe X-linked genes, is misleading and should be abandoned. *Also called* X-linkage.

X-ray radiation Ionizing electromagnetic radiation.

YAC *See* yeast artificial chromosome.

Y chromosome The sex chromosome occurring only in human males.

yeast artificial chromosome A yeast-based vector system for cloning large (>100 kb) DNA inserts. *Also called* YAC.

Z *See* LOD score.

Z_{max} The maximum LOD score for the complete range of recombination fractions.

zoo blot Hybridization of a cloned human DNA sequence to DNA from various vertebrate organisms to determine whether the cloned DNA contains an evolutionarily conserved coding region.

zygosity (1) The genetic relationship between same-birth individuals with respect to origin from a single egg or different eggs. (2) The genotypic status of a locus (loci) in a zygote.

zygote A fertilized egg.

zygotene A stage of prophase I of meiosis I of the meiotic process, during which homologous chromosomes synapse. *Also called* zygonema.

θ The Greek letter theta, representing the recombination fraction in studies of human gene linkage and the frequency of breakage in radiation hybrid mapping.

Index

NOTE: Page numbers followed by f refer to figures, page numbers followed by t refer to tables.

An Introduction to Human Molecular Genetics, Second Edition, by Jack J. Pasternak
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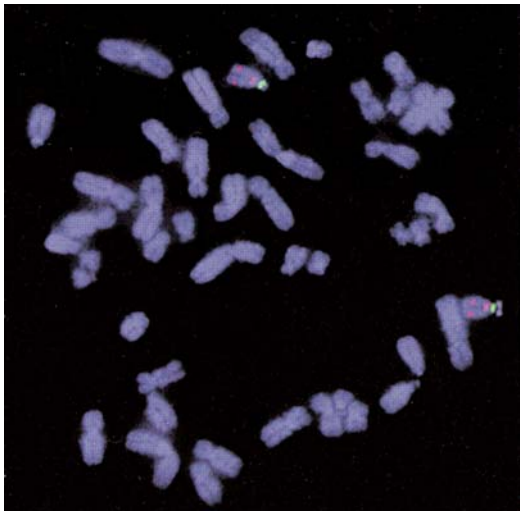


Plate 1 Fluorescence in situ hybridization. A set of human metaphase chromosomes, ie, metaphase spread, was hybridized with labeled probes that are specific for the centromere of chromosome 15 (green); the small ribonucleoprotein N (*SNRPN*) gene at 5q12 (pink) near the centromere; and a control gene (pink) near the terminal end of 15q. A single fluorescent band is observed with the SNRPN probe because the chromosome site is located in a part of the chromosome that does not have separate chromatids. The chromosomes were stained blue with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). The image was kindly provided by Applied Imaging Corporation, Santa Clara, CA.

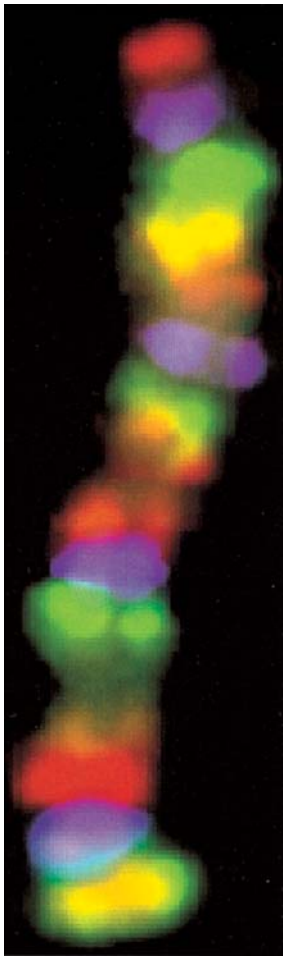


Plate 2 Chromosome rainbow. Fluorescence in situ hybridization of a metaphase spread with an ordered set of 16 YAC- and P1-based probes that are specific for chromosome 10. From the end of the short arm, four consecutive sets of four probes each were labeled with signals that are detected as red, purple, green, and yellow, respectively. The fluorescence from one chromosome 10 is shown. The image was recorded by B. O'Brien and H.-Ulli Weier and kindly provided by H.-Ulli Weier, U.C. Lawrence Berkeley National Laboratory, Berkeley, CA.

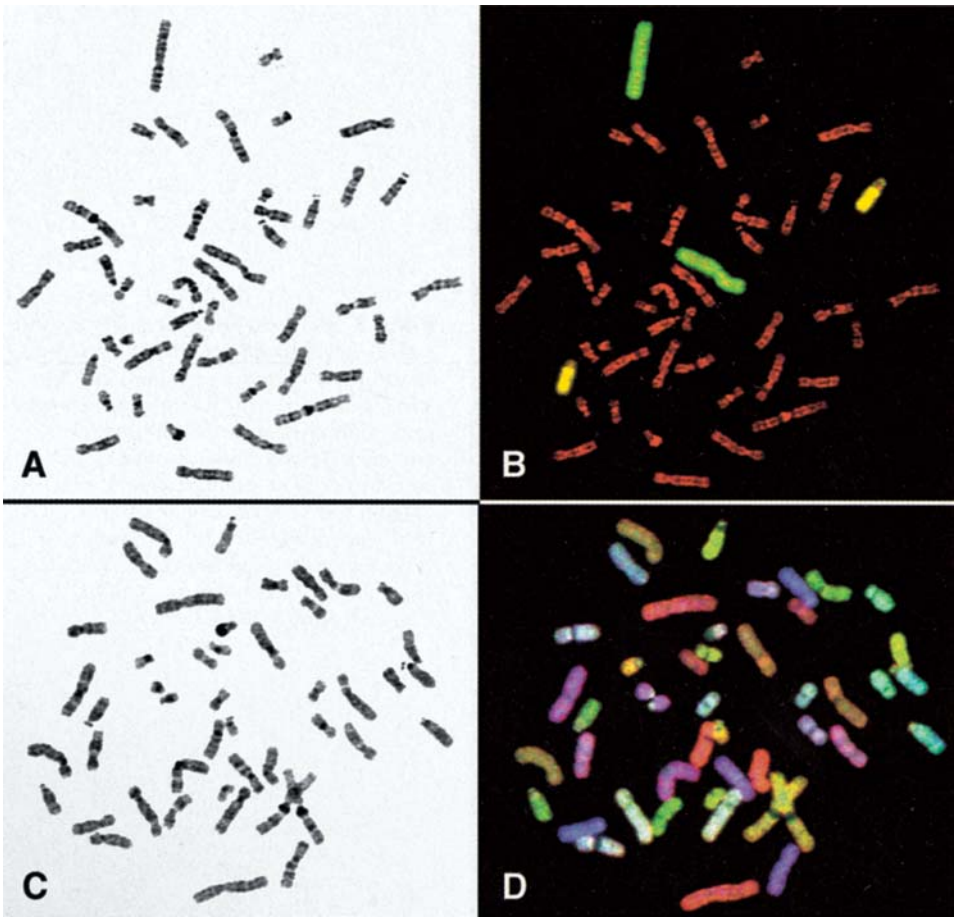


Plate 3 Chromosome and spectral karyotype (SKY) paints. **A.** An inverted-DAPI stained metaphase spread from a normal human male (46, XY). The banding is comparable to G-banding. **B.** The metaphase spread in A after hybridization with chromosome-painting probes for chromosomes 2 (yellow) and 15 (green). The other chromosomes are pseudocolored red. **C.** An inverted-DAPI stained metaphase spread from a normal human male (46, XY). **D.** The results of hybridizing the metaphase spread in C with a SKY probe, ie, 24 different sets probes. Each set of probes produces a distinctive color for a human chromosome and its homolog. The images were kindly provided by H. Padilla-Nash, K. Hesselmeyer-Haddad, and T. Ried, Genetics-FISH Laboratory, National Cancer Institute, NIH, Bethesda, MD.

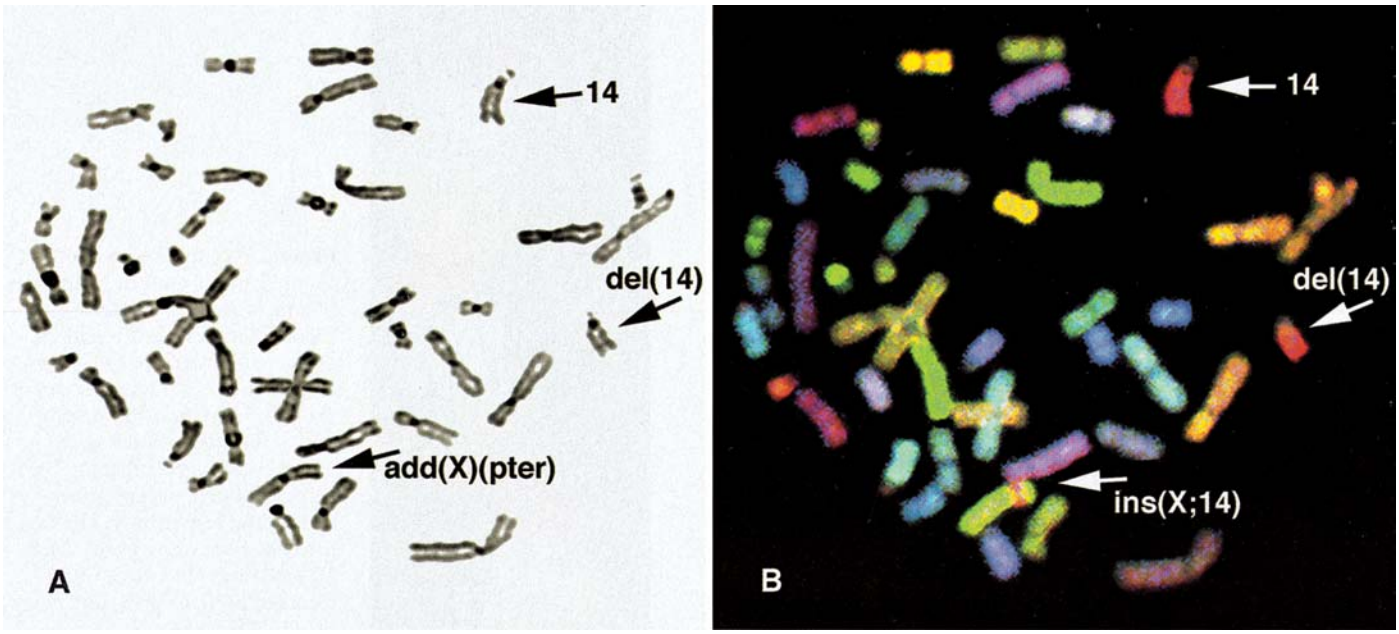


Plate 4 SKY analysis of a non-Hodgkin's lymphoma cell. **A.** An inverted-DAPI stained metaphase spread from a patient with non-Hodgkin's lymphoma. The arrows indicate a deletion in chromosome 14 [del(14)] and an addition of chromosome material to the short arm of chromosome X [add(X)(pter)]. Under these conditions, it is impossible to determine the origin of the added material to chromosome X. **B.** Following the hybridization of the metaphase spread in A with a SKY probe, the chromosome material added to chromosome X (green) is from chromosome 14 (yellow). The derivative chromosome X is actually an ins(X; 14) chromosome. The images were kindly provided by H. Padilla-Nash, K. Hesselmeyer-Haddad, and T. Ried, Genetics-FISH Laboratory, National Cancer Institute, NIH, Bethesda, MD.

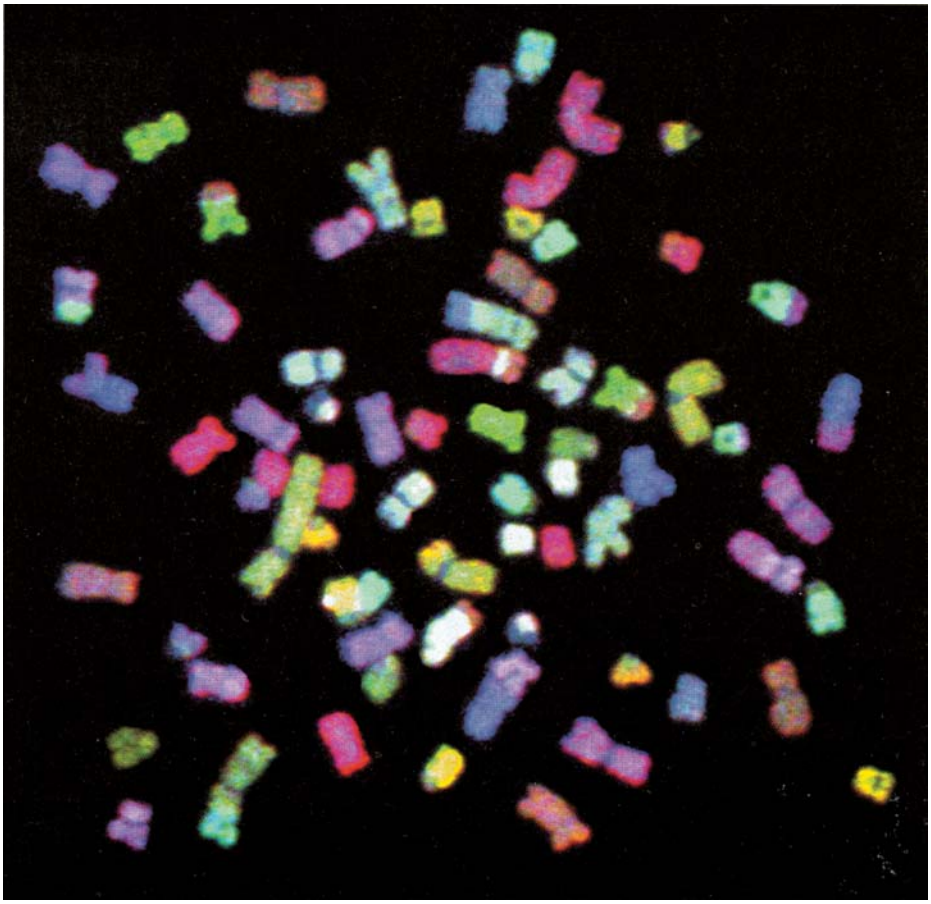


Plate 5 SKY analysis of a bladder carcinoma cell line. Hybridization of a metaphase spread from a bladder carcinoma cell line with SKY probe, which labels each chromosome a different color, reveals multiple chromosome aberrations. Chromosomes with two or more colors represent unbalanced translocations. The image was kindly provided by H. Padilla-Nash, K. Hesselmeyer-Haddad, and T. Ried, Genetics-FISH Laboratory, National Cancer Institute, NIH, Bethesda, MD.

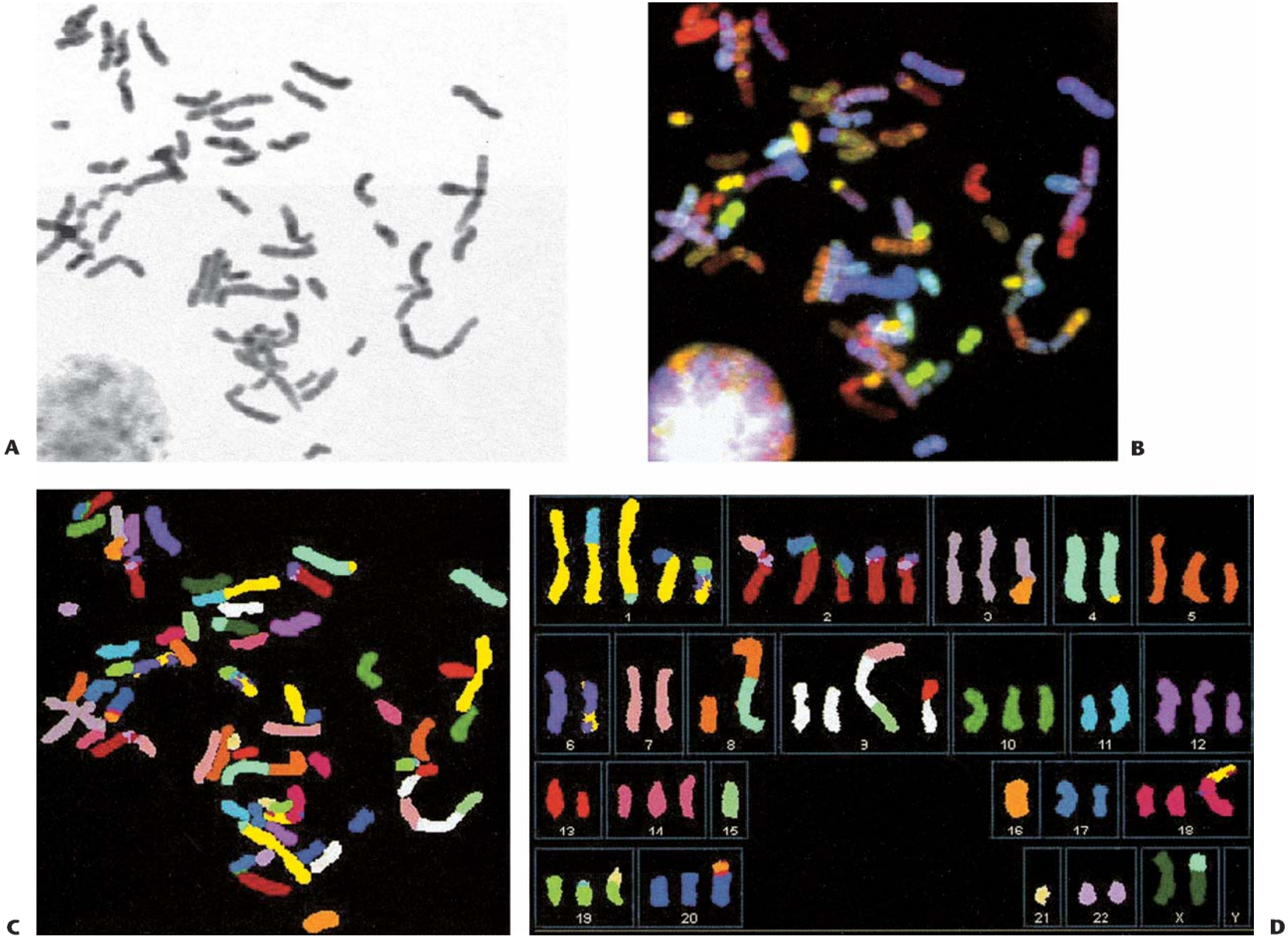
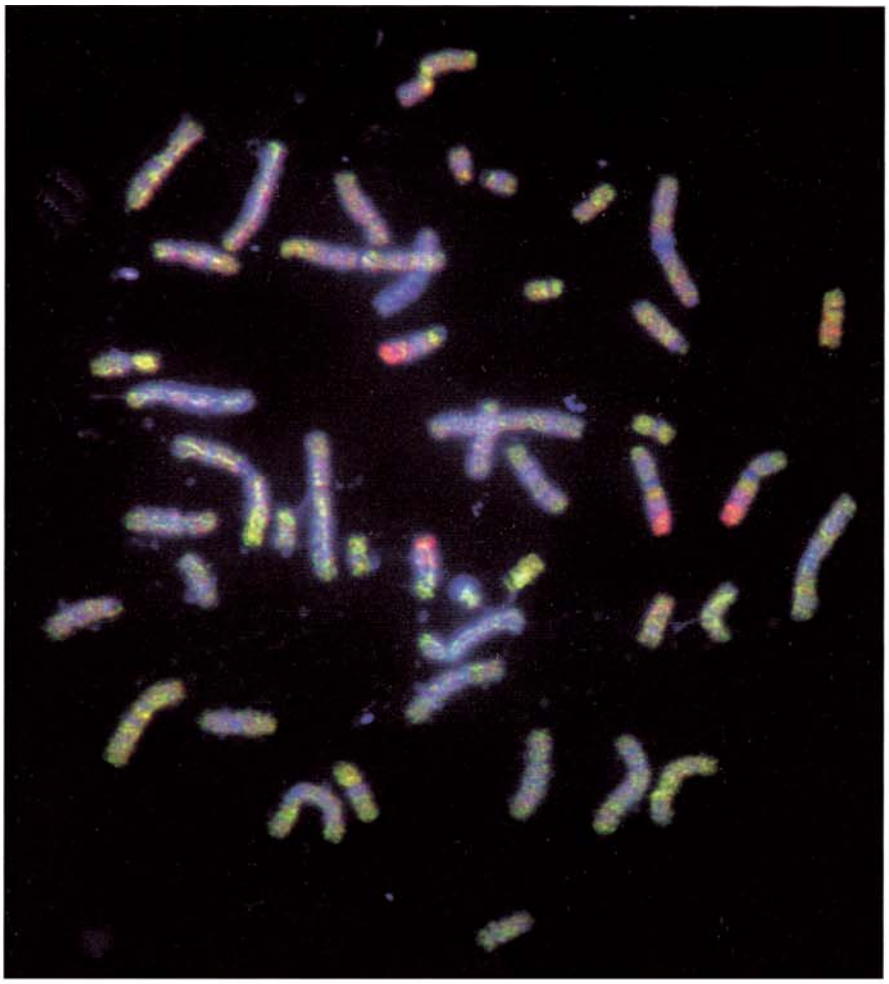
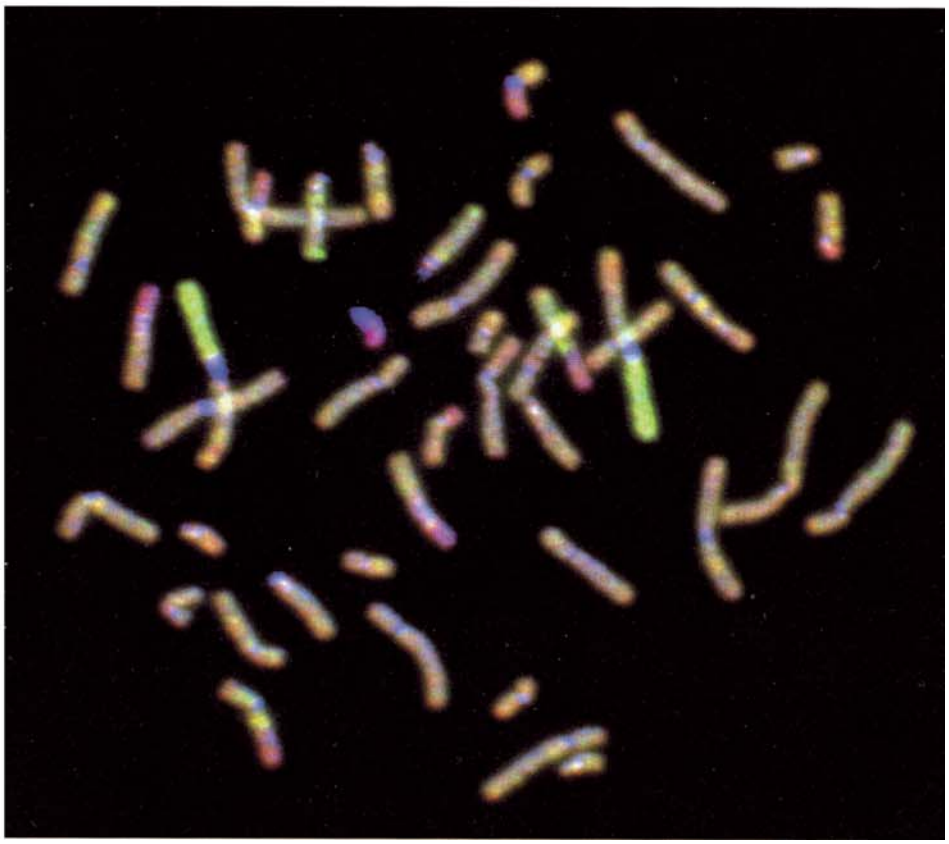


Plate 6 SKY of a thyroid cancer cell. **A.** An inverted-DAPI stained metaphase spread from a patient with thyroid cancer. **B.** SKY analysis of the chromosomes in A. **C.** Computer-assigned color for each chromosomes in A. **D.** In combination with the DAPI-banding patterns, a spectral karyotype is formed by arranging the chromosomes in C in order from chromosome 1 to chromosome X. Interchromosomal rearrangements and aneuploidy are readily detected. Images were recorded by H. Zitzelsberger, J. Fung, and H.-Ulli Weier, and kindly provided by H.-Ulli Weier, U.C. Lawrence Berkeley National Laboratory, Berkeley, CA.



A



B

Plate 7 Comparative genomic hybridization. **A.** A normal human DAPI-stained metaphase spread was co-hybridized with tumor and normal cell DNA that was labeled with a green and red fluorescent dye, respectively. The sites of red or green fluorescence identify regions that are either missing or amplified, respectively, in the tumor cell chromosomes. Other fluorescent colors are the result of various combinations of the green and red fluorescence and denote tumor cell chromosome regions that have not undergone any obvious quantitative changes. **B.** Similar to A except a DNA sample from a different tumor cell was used. The images were kindly provided by Precision Scientific Instruments, Inc., League City, TX.