

Additional Reading

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Topology: Functional Deformations of DNA



Carlos Bustamante [Source: Courtesy of Carlos Bustamante.]

Moment of Discovery

There was an experiment I had wanted to do for many years, but I could never convince anyone to try it. *The idea was to measure the elastic properties of DNA directly using a single molecule of DNA tethered between two opposing pipette tips, such that it could be exquisitely controlled by rotating one pipette tip relative to the other to twist the DNA to different extents.* In solution, DNA can twist on its long axis, and it can also *writhe* by coiling around itself—and it can be very hard

to decouple twisting from writhing by measuring properties of DNA in bulk.

After many students turned me down, eventually two students, Zev Bryant and Michael Stone, were intrigued to try the DNA twisting experiment. These guys worked very hard setting up the technical aspects of the experiment. They figured out how to tether the ends of a nicked fragment of DNA to two opposing pipette tips, and they coupled a readily visualized small bead (the “rotor”) to an internal position in the nicked DNA duplex. They finally got everything working late one night. Zev and Michael started using a hand crank to introduce a specific number of twists into the tethered piece of DNA. But they were so tired that they would get up to, say, 345 turns, and then they weren’t sure if it was 345 or 346! They were determined to do the experiment accurately, so they had to untwist the DNA and start over. But Jan Liphardt, another student in the lab, had an idea. He offered to bring in a small motor from his Lego set at home to rotate the pipette tip, and thus twist the DNA, automatically. So Jan ran home and got the motor, hooked it up to the system, and it worked beautifully. All the data we ultimately published were measured using the Lego motor (we even listed it in the Methods section of the paper). And we learned that DNA is about 50% stiffer than had been previously estimated from bulk solution experiments!

—**Carlos Bustamante**, on discovering the elasticity of DNA

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In all free-living organisms—all bacteria, archaea, and eukaryotes—genomic information comes in the form of DNA; RNA genomes occur only in some classes of viruses. Genomic nucleic acids are large. In fact, they are *much* (orders of magnitude) longer than the biological packages—cells, organelles, or virus particles—that contain them. Most human cells are 7 to 30 μm (micrometers, also called microns) in diameter. The nuclei that house the DNA molecules are rarely more than 10 μm in diameter. The shortest human chromosome (chromosome 21), at just under 47 million base pairs, would be about 16 mm long if stretched out in a line, or more than a thousand times longer than the nucleus. If all of the chromosomes in a diploid human cell were laid end to end, they would stretch for nearly 2 m. It quickly becomes apparent, then, that genomic DNA must be compacted to fit inside the cell.

The compaction (also called condensation) of DNA is extensive, but never random. The genome is restricted to a limited cellular space, but the cell must retain access to the information contained within the DNA. The enzymes that carry out DNA replication, repair, recombination, and transcription must all have ready access to their sites of action. Regulatory proteins must have access to the specific sequences to which they bind. In all cells, a range of DNA-binding proteins contributes to compaction and to regulation of the function of chromosomal DNA. The compaction is both orderly and dynamic.

As we'll see, DNA is compacted primarily by coiling. The process is somewhat analogous to rolling up a length of garden hose or a spool of electrical wire. However, the coiling of DNA occurs in the context of structural constraints peculiar to this nucleic acid, constraints that are dealt with by a unique set of proteins and enzymes.

In this chapter, we shift our focus from the secondary structure of DNA (see Chapter 6) to the extraordinary degree of organization required for the tertiary packaging of DNA into chromosomes. We explore the principles related to the compaction of DNA, beginning with a review of the structural elements that make up viral and cellular chromosomes, and consider chromosomal size and organization in more detail. We then discuss DNA topology for a quantitative description of the coiling and supercoiling of DNA molecules. We conclude with a discussion of the key enzymes, found in all cells, that are involved in creating and maintaining a very high order of compaction. As in all other areas of molecular biology, this information is not merely of academic interest. Many of these enzymes are important targets of antibiotics and other medicines. In Chapter 10, we expand on this discussion of tertiary

packaging by examining the complete structure of chromosomes in the context of the structural DNA-binding proteins unique to eukaryotes and bacteria.

9.1 The Problem: Large DNAs in Small Packages

A great deal of information is packed in the chromosomes: they contain the blueprints for an organism. The genes in each chromosome constitute only part of that information. The chromosomes themselves are macromolecular entities that must be synthesized, packaged, protected, and properly distributed to daughter cells at cell division. Significant segments of every chromosome are dedicated to these functions. All aspects of chromosome function are affected by the reality of chromosome size.

Chromosome Function Relies on Specialized Genomic Sequences

The chromosomes of cells and viruses come in several forms. Bacterial chromosomes are often circular (in the sense of an endless loop rather than a perfect circle). Most eukaryotic chromosomes are linear. In viruses we find additional variations, including both single-stranded and double-stranded forms, as well as RNA genomes. Each type of chromosome structure imposes a unique set of demands on the mechanisms for replicating and transmitting the genome from one generation to the next.

Genes provide the information to specify all the RNAs and proteins produced in a given cell, but other sequences are dedicated to the maintenance of the chromosome itself: initiation and termination of replication, segregation during cell division, and, where necessary, protection and maintenance of the chromosome ends. In bacteria, origins of replication provide start sites for chromosomal replication (see Figure 8-1). Specialized replication-termination regions also exist in most known bacterial species. Within or near these regions, additional sequences serve as binding sites for proteins that ensure the faithful segregation of replicated chromosomes to daughter cells. Eukaryotic chromosomes, too, contain sequences that are critical to chromosome maintenance. Unlike bacteria, eukaryotic chromosomes often have many replication origins. (The structure and function of replication origins are described in Chapter 11.) Eukaryotic chromosomes also have specialized sequences called centromeres and telomeres.

The **centromere** is a segment of each eukaryotic chromosome that functions during cell division as an

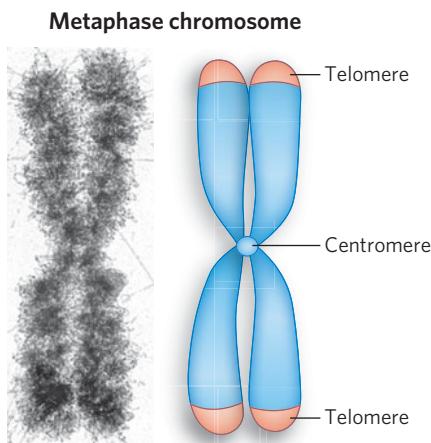
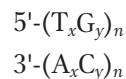


FIGURE 9-1 Linked and condensed sister chromatids of a human chromosome. The products of chromosomal replication in eukaryotes are linked sister chromatids. These are fully condensed at metaphase, during mitosis. The point where they are joined is the centromere. Telomeres are sequences at the ends of the chromatids. [Source: Photo from G. F. Bahr/Biological Photo Service.]

attachment point for proteins that link the chromosome to the mitotic spindle at metaphase (Figure 9-1). This attachment is essential for the equal and orderly distribution of chromosome sets to daughter cells. (See Chapter 2 for a review of the events of mitosis.) The centromeres of *Saccharomyces cerevisiae* have been isolated and studied. The sequences essential to centromere function are about 130 bp long and very rich in A=T pairs. The centromere sequences of higher eukaryotes are much longer and, unlike those of yeast, generally contain regions of simple-sequence DNA consisting of thousands of tandem copies of one or a few 5 to 10 bp sequences. This DNA serves as a binding site for centromere-binding proteins, or cen proteins. The centromere is also the site of kinetochore assembly. Built up on each centromere, the kinetochore anchors

the spindle fibers as chromosomes are segregated into daughter cells during mitosis. Centromeres thus play a key role in stable chromosome segregation during cell division.

Telomeres are sequences at the ends of eukaryotic chromosomes that add stability by protecting the ends from nucleases and providing unique mechanisms for the faithful replication of linear DNA molecules. DNA polymerases cannot synthesize DNA to the very ends of a linear chromosome (see Chapter 11). Solving the end-replication problem is one key function of telomeres, which are replicated by the enzyme telomerase. Telomeres end with multiple repeated sequences of the form



where x and y are generally between 1 and 4 (Table 9-1) and the number of telomere repeats, n , is in the range of 20 to 100 for most single-celled eukaryotes, and generally exceeds 1,500 in mammals. As in centromeres, the telomere repeats serve as binding sites for specialized proteins that are part of telomere function. These proteins package the telomeres and help maintain them in actively dividing cells (see Chapter 11).

Artificial chromosomes have been constructed as a means of better understanding the functional significance of many structural features of eukaryotic chromosomes. A reasonably stable artificial linear chromosome requires only three components: a centromere, a telomere at each end, and an appropriate number of replication origins. Yeast artificial chromosomes (YACs) have been developed as a research tool in biotechnology (see Figure 7-7). YACs have also been useful in confirming the critical functions of centromeres and telomeres. Building on this foundation, human artificial chromosomes (HACs) are now being developed. HACs are reasonably stable when introduced into a human tissue culture cell line, if they include human

Table 9-1 Telomere Sequences

Species	Telomere Repeat Sequence	n^*
<i>Homo sapiens</i> (human)	(TTAGGG) $_n$	800-2,500
<i>Tetrahymena thermophila</i> (ciliated protozoan)	(TTGGGG) $_n$	40
<i>Saccharomyces cerevisiae</i> (yeast)	((TG) $_{1-3}$ (TG) $_{2-3}$) $_n$	50-75
<i>Arabidopsis thaliana</i> (plant)	(TTTAGGG) $_n$	300-1,200

*Number of telomere repeats. Telomere length is longer and fluctuates over a wider range in multicellular eukaryotes. In vertebrates, including humans, telomere length declines with the age of the organism in most cells, but not in germ-line cells.

centromere and telomere sequences in addition to active replication origins.

Continued development of HACs, particularly in their efficient introduction into human cells, may eventually provide new avenues for the treatment of genetic diseases. Most genetic diseases can be traced to an alteration in a particular gene that changes or eliminates that gene's function. The process of correcting disease-causing genetic errors in somatic cells is termed somatic gene therapy. Efforts to directly remove such genes and replace them with normal, functional versions at the correct chromosomal locus have met with limited success in human cells. A simpler technique is to introduce the functional genes into random locations on chromosomes through recombination mechanisms (see Chapters 13 and 14). However, this technique has its own set of problems. The inserted gene can run afoul of regulatory mechanisms that suppress gene expression over large segments of a chromosome, effectively silencing any new gene that is inserted there. Random integration can also result in insertion into the coding sequence of another gene, inactivating that gene. If the inactivated gene has a role in the regulation of cell division, uncontrolled cell division and tumor development can result. The introduction of functional gene copies on stable HACs may eventually circumvent these problems. Success will depend on further advances in clarifying the mechanisms by which chromosomes are stably maintained in cells, and on the development of more efficient procedures for introducing large DNAs into the nuclei of a large number of cells in a living human being.

Chromosomes Are Longer Than the Cellular or Viral Packages Containing Them

The observation that genomic DNAs are orders of magnitude longer than the cells or viruses that contain them applies to every class of organism and viral parasite. Lengths of double-stranded nucleic acids are often described in terms of contour length, or the length measured along the axis of the double-helical DNA. For a closed-loop DNA, contour length is the circumference it would have if laid out in a perfect circle. Lengths are more difficult to describe for a single-stranded nucleic acid, particularly when segments of that nucleic acid fold up into secondary structures. These lengths are sometimes approximated by assuming that the single strand is arrayed in the helical path that would be described by one strand of a double helix, then measuring along the resulting axis.

Given the magnitude of the one-dimensional length of a typical chromosome, how can it be accommodated

within the three-dimensional volume of a virus particle, cell, or nucleus? The compaction mechanisms required for this are highly conserved across the spectrum of living systems. Compaction entails the coiling and structural organization of the chromosome resulting from the action of enzymes; the structural organization is maintained by DNA-binding proteins, including the histones of eukaryotic chromosomes (see Chapter 10), the DNA-binding proteins of bacteria, and the coat proteins of viral particles.

KEY CONVENTION

Molecular biology involves structures with dimensions that are small fractions of a meter. One-thousandth of a meter is 1 millimeter (mm); 1 mm = 1,000 μm (micrometer, or micron) = 1,000,000 nm (nanometer) = 10,000,000 Å (angstrom). Nucleotides, segments of chromosomes, and cells are most often discussed in terms of angstroms, nanometers, and micrometers, respectively.

Viruses Viruses are not free-living organisms; they are infectious parasites that use the resources of a host cell to carry out many of the processes they require to propagate. Many viral particles consist of no more than a genome (usually a single RNA or DNA molecule) surrounded by a protein coat.

Almost all plant viruses and some bacterial and animal viruses have RNA genomes, and they are quite small. For example, the genomes of mammalian retroviruses, such as HIV, have about 9,000 nucleotides, and the genome of the bacteriophage Q β has 4,220 nucleotides. However, even these small nucleic acids have total lengths of about 3 and 1.4 μm , respectively. In comparison, the protein coat of HIV is about 100 nm in diameter, and that of Q β is about 26 nm, so the RNAs are 30 to 50 times longer than their viral protein coats. Both types of virus have linear, single-stranded RNA genomes. Some of the viral coat proteins are effectively RNA-binding proteins, and they enforce a highly compacted folding arrangement of the RNA within the viral particle. An example can be seen in the tobacco mosaic virus (TMV), a pathogen of tobacco plants. The single-stranded RNA genome of TMV, 6,400 nucleotides long, is wound into a tight left-handed helix by its packaging within the rodlike helical protein coat ([Figure 9-2a](#)).

The genomes of DNA viruses vary greatly in size (see Table 8-1). Many viral DNAs are circular for at least part of their life cycle. During viral replication inside a host cell, specific types of viral DNA, called replicative forms, may appear; for example, many linear DNAs

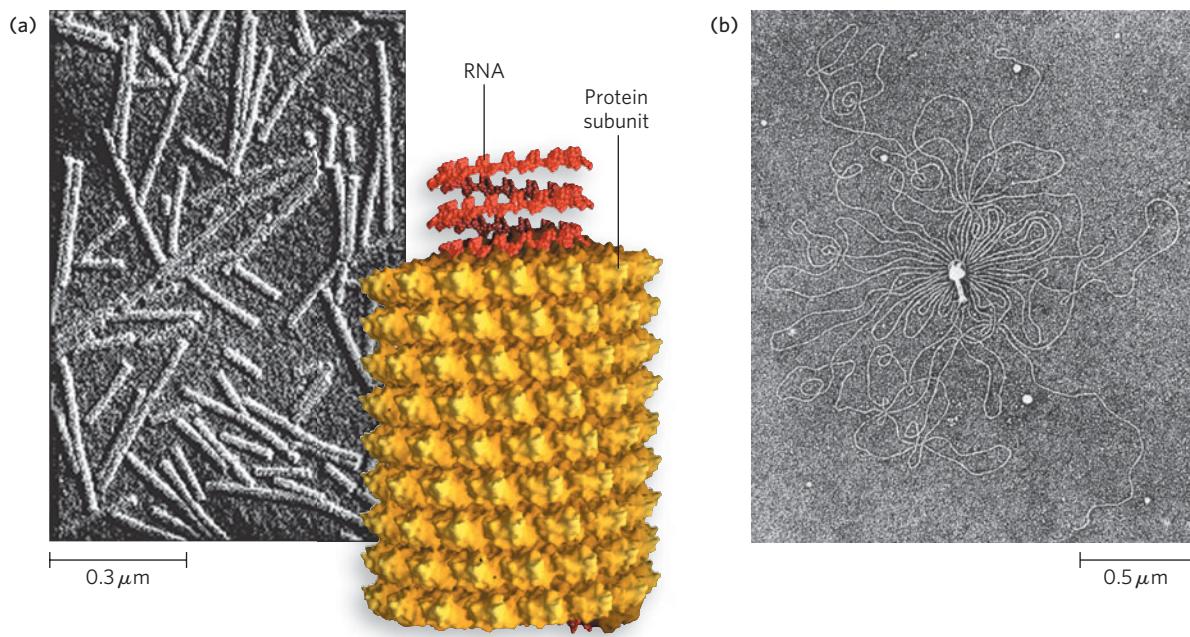


FIGURE 9-2 Genome packaging in a virus. (a) The tobacco mosaic virus has an RNA genome coiled inside a rod-shaped viral coat by RNA-binding proteins, as shown in an electron micrograph and molecular model. (b) A bacteriophage T2 particle was lysed and its DNA allowed to spread on the

surface of distilled water in this electron micrograph. All the DNA shown here is normally packaged inside the phage head. [Sources: (a) Science Source/Photo Researchers; PDB ID 1VTM. (b) From A. K. Kleinschmidt et al., *Biochim. Biophys. Acta* 61:857–864, 1962.]

become circular, and all single-stranded DNAs become double-stranded. Bacteriophage T2 has a double-stranded DNA genome of 160,000 bp, a molecule more than 50 μm long that must be packaged into a virus head about 100 nm across in its longest dimension (Figure 9-2b).

Table 9-2 summarizes the genome and particle dimensions for several DNA viruses. A typical medium-sized DNA virus is bacteriophage λ (lambda), which infects *Escherichia coli*. In its replicative form inside cells, λ DNA is a circular double helix. This double-

stranded DNA contains 48,502 bp and has a contour length of 17.5 μm . Bacteriophage φ X174 is a much smaller DNA virus; the DNA in the viral particle is a single-stranded circle, and the double-stranded replicative form, in the host cell, has 5,386 bp.

Bacteria A single *E. coli* cell contains almost 100 times more DNA than a bacteriophage λ particle. The chromosome of the most common *E. coli* strain studied in laboratories worldwide (K-12 MG1655) is a single double-stranded, circular DNA molecule (Table 9-3).

Table 9-2 The Sizes of DNA and Viral Particles for Some Bacterial Viruses (Bacteriophages)

Virus	Number of Base Pairs in Viral DNA*	Length of Viral DNA (nm)	Long Dimension of Viral Particle (nm) [†]	Chromosome Form
φ X174	5,386	1,939	25	Circular
T7	39,936	14,377	78	Linear
λ (lambda)	48,502	17,460	190	Linear
T4	168,889	60,800	210	Linear

*Data are for the replicative form (double-stranded). The φ X174 chromosome is single-stranded inside the viral particle. The bacteriophage λ chromosome is circularized after it enters a host cell. Calculation of contour length assumes 3.4 Å per base pair (see Figure 6-14).

[†]This measurement includes the head and the tail where relevant.

Table 9-3 DNA, Gene, and Chromosome Content in Some Genomes

Species	Total DNA (bp)	Chromosomes*	Genes
<i>Escherichia coli</i> (bacterium)	4,600,000	1	~4,300
<i>Drosophila melanogaster</i> (fruit fly)	180,000,000	18	~13,600
<i>Arabidopsis thaliana</i> (plant)	125,000,000	10	~25,500
<i>Oryza sativa</i> (plant)	480,000,000	24	~57,000
<i>Mus musculus</i> (mouse)	2,500,000,000	40	~26,000-29,000
<i>Saccharomyces cerevisiae</i> (yeast)	12,068,000	16 [†]	~5,800
<i>Caenorhabditis elegans</i> (nematode)	97,000,000	12 [‡]	~19,000
<i>Homo sapiens</i> (human)	~3,200,000,000	46	~25,000

*Diploid chromosome number for all eukaryotes except yeast.

[†]Haploid chromosome number; wild yeast strains generally have eight (octoploid) or more sets of chromosomes.

[‡]Number for females, with two X chromosomes; males have an X but no Y, for 11 total.

Its 4,639,221 bp have a contour length of about 1.7 mm, some 850 times the length of the *E. coli* cell, 2 μm. In addition to the very large, circular DNA chromosome, many bacteria contain one or more **plasmids**, much smaller circular DNA molecules that are free in the cytosol (Figure 9-3; see also Chapter 7). Most plasmids are only a few thousand base pairs long, but some have more than 100,000 bp. They carry genetic information and undergo replication to yield daughter plasmids, which pass into the daughter cells at cell division. The spread of bacterial plasmids (and transposons) that

confer antibiotic resistance among pathogenic bacteria has reduced the utility of standard antibiotics in medicine and agriculture (Highlight 9-1).

Eukaryotes A yeast cell, one of the simplest eukaryotes, has 2.6 times more DNA in its genome than an *E. coli* cell (see Table 9-3). Cells of *Drosophila melanogaster*, the fruit fly used in classical genetics studies, contain more than 35 times as much DNA as *E. coli* cells, and human cells have almost 700 times as much. The cells of many plants and amphibians contain even

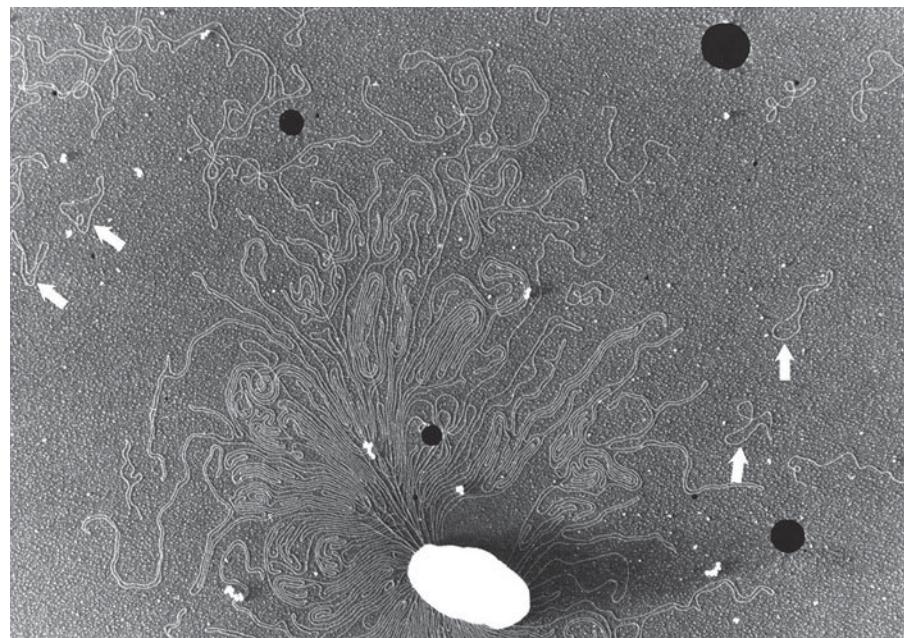


FIGURE 9-3 DNA from a lysed *E. coli* cell. In this electron micrograph, white arrows indicate several small, circular plasmid DNAs. The black spots and white specks are artifacts of the preparation. [Source: Huntington Potter and David Dressler, Department of Neurobiology, Harvard Medical School.]

HIGHLIGHT 9-1 MEDICINE

The Dark Side of Antibiotics

Over the course of the twentieth century, the average life expectancy for people in the developed countries increased 10 years, and the development of antibiotics for the treatment of infectious diseases was a major contributor to this improved longevity. Ironically, an overuse of antibiotics is now leading to their demise as useful therapeutics, as bacterial pathogens evolve resistance to them.

The most common vehicles for transmitting antibiotic-resistance elements between bacterial populations are plasmids, and large numbers are present in the environment. Some plasmids confer no obvious advantage on their host, and their sole function seems to be self-propagation. However, many plasmids carry genes that are useful to the host bacterium. These may include genes that extend the range of environments that can be exploited by the host, such as conferring resistance to naturally occurring antibiotics, new metabolic properties, or the ability to synthesize toxins or agents that facilitate tissue colonization—and thus make the host bacterium pathogenic to other organisms. Given that most antibiotics are natural products (e.g., penicillin is derived from the mold *Penicillium notatum*), it is not surprising that genes conferring antibiotic resistance occur in natural bacterial populations.

Plasmids include a range of sequences involved in their own propagation. These sequences often function in several related bacterial species, and the host range can increase with the aid of small numbers of mutations. When genes conferring antibiotic

resistance are integrated into a plasmid, the resistance element is more readily transferred from one bacterium to another, and even between species. For example, plasmids carrying the gene for the enzyme β -lactamase confer resistance to β -lactam antibiotics, such as penicillin, ampicillin, and amoxicillin. Horizontal gene transfer also can occur, in which plasmids pass from an antibiotic-resistant cell to an antibiotic-sensitive cell of the same or another bacterial species (see Figure 1-11). This can occur when cells of a resistant strain die and rupture, releasing DNA into the environment. If an antibiotic-sensitive strain or species takes up the DNA, it may acquire the antibiotic resistance. When an antibiotic-resistance gene occurs on a conjugational plasmid, its transfer between bacteria becomes particularly efficient. Many antibiotic-resistance elements are further harbored in transposons, which can move from cellular chromosomes to plasmids and back again, further facilitating the dispersal of these elements.

Under the strong selective pressure brought about by widespread antibiotic treatments, bacterial pathogens can acquire antibiotic resistance rapidly. The extensive use of antibiotics in some human populations has encouraged the spread of antibiotic resistance-coding plasmids (as well as transposable elements that harbor similar genes) in disease-causing bacteria. Physicians are becoming increasingly reluctant to prescribe antibiotics unless a clear medical need is confirmed. For similar reasons, the widespread use of antibiotics in animal feeds is being curbed.

more. All of this DNA must fit into a eukaryotic cell that is typically 10 to 20 μm across (although size can vary greatly even within a single organism). The genetic material of eukaryotic cells is apportioned into multiple chromosomes, the diploid ($2n$) number depending on the species. A human somatic cell, for example, has 46 chromosomes (Figure 9-4). Each chromosome of a eukaryotic cell contains a single, very large, duplex DNA molecule. The DNA molecules in the 24 different types of human chromosomes (22 matching pairs plus the X and Y sex chromosomes) vary in length over a 25-fold range. Each type of chromosome in eukaryotes carries a characteristic set of genes.

Eukaryotic cells also have organelles that contain DNA. Mitochondria and chloroplasts carry their own ge-

nomic DNAs (Figure 9-5). The evolutionary origin of mitochondrial and chloroplast DNAs has been the sub-

ject of much speculation. A widely accepted hypothesis, proposed by Lynn Margulis, is that they are vestiges of the chromosomes of ancient bacteria that gained access to the cytoplasm of host cells and became the precursors of these organelles (see Figure 8-17a). Mitochondrial DNA (mtDNA) codes for mitochondrial tRNAs and rRNAs and a



Lynn Margulis [Source: Courtesy of Lynn Margulis.]



FIGURE 9-4 Eukaryotic chromosomes. This is a complete set of chromosomes from a leukocyte of one of the authors. There are 46 chromosomes in every normal human somatic cell. [Source: G. F. Bahr/Biological Photo Service.]

few mitochondrial proteins; more than 95% of mitochondrial proteins are encoded by nuclear DNA. Mitochondria and chloroplasts divide when the cell divides. Their DNA is replicated before and during cell division, and the daughter DNA molecules pass into the daughter organelles.

Mitochondrial DNA molecules are much smaller than nuclear chromosomes. In animal cells, mtDNA contains fewer than 20 kbp (16,569 bp in human

mtDNA) and is a circular duplex. Each mitochondrion typically has 2 to 10 copies of the mtDNA, but the number can be much higher: hundreds in muscle cells, and 100,000 in a mature oocyte. In a few organisms (e.g., trypanosomes), each mitochondrion contains thousands of copies of mtDNA organized into a complex interlinked matrix known as a kinetoplast. Plant cell mtDNA is much larger than that in animal cells, ranging from 200 to 2,500 kbp. Chloroplast DNA (cpDNA) exists as circular duplexes of 120 to 160 kbp. Organelle DNA, like nuclear DNA, undergoes considerable compaction: DNA molecules 5 to 500 μm long must be accommodated in organelles about 1 to 5 μm in diameter.

Some eukaryotes also contain plasmids; they have been found in yeast and some other fungi.

SECTION 9.1 SUMMARY

- All cellular chromosomes contain sequences required for chromosome function, including replication origins. Bacterial chromosomes also contain termination sequences and other sequences necessary for chromosomal segregation during mitosis.
- Eukaryotic chromosomes contain centromeres, which are attachment points for the mitotic spindle, and telomeres, specialized sequences at the ends of a chromosome that protect and stabilize the entire chromosome.
- All genomic DNA and RNA molecules are longer—often orders of magnitude longer—than the viral coats, organelles, and cells in which they are packaged. Special mechanisms for the compaction/condensation of the nucleic acids are employed in chromosomal packaging.
- Viral genomes vary in nucleic acid (DNA or RNA), structure (single-stranded or double-stranded), and length.
- Bacterial cells contain both genomic DNA (usually circular) and plasmids; both types are compacted in the cell and replicated and segregated into daughter cells at cell division.
- Eukaryotic chromosomes are linear and vary in number, depending on the species. Humans have 46 chromosomes, varying in length and condensed to fit into the cell nucleus. Mitochondria and chloroplasts contain their own circular genomes, in numbers ranging from several copies to hundreds of thousands of copies per organelle, depending on cell type.

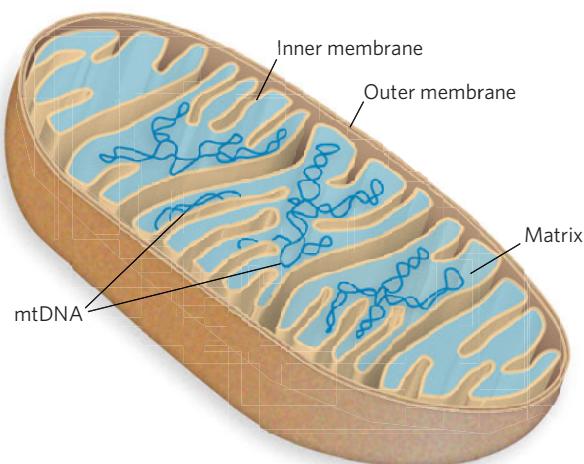


FIGURE 9-5 Mitochondrial DNA. Some mitochondrial proteins and RNAs are encoded by the multiple copies of mtDNA in the mitochondrial matrix. The mtDNA is replicated each time the organelle divides, before cell division.

9.2 DNA Supercoiling

Cellular DNA, as we have seen, is a very long molecule that must somehow be made to fit inside the cell, implying a high degree of structural organization. The folding mechanism must not only pack the DNA but permit access to the information in the DNA, in processes such as replication and transcription. Any consideration of how this is accomplished requires an understanding of an important property of DNA structure known as supercoiling.

Supercoiling simply means the coiling of a coil. An old-fashioned telephone cord, for example, is typically a coiled wire. The path taken by the wire between the base of the phone and the receiver often includes one or more supercoils (Figure 9-6). DNA is coiled in the form of a double helix, with both strands coiling around an axis. **DNA supercoiling** is the further coiling of that axis upon itself (Figure 9-7). As described below, **supercoiled DNA** is generally a manifestation of structural strain. When there is no net coiling or bending of the DNA axis upon itself, the molecule is referred to as **relaxed DNA** (see How We Know). Supercoiling occurs in all chromosomal DNAs in all cells, as well as in viruses that have a double-stranded DNA genome or

generate double-stranded DNA as a replication intermediate.

We might have predicted that DNA compaction involved some form of supercoiling. Perhaps less predictable is that replication and transcription of DNA also affect, and are affected by, supercoiling. Both processes require a separation of DNA strands, which is complicated by the helical interwinding of the strands (Figure 9-8). As we'll see in Section 9.3, specialized enzymes found in all organisms alleviate the stress of replication and transcription by introducing or relaxing supercoils.

Inside the cell, a DNA molecule must bend, and it inevitably becomes supercoiled. However, even when extracted and purified, many circular DNA molecules remain highly supercoiled, even in the absence of protein and other cellular components. This indicates that

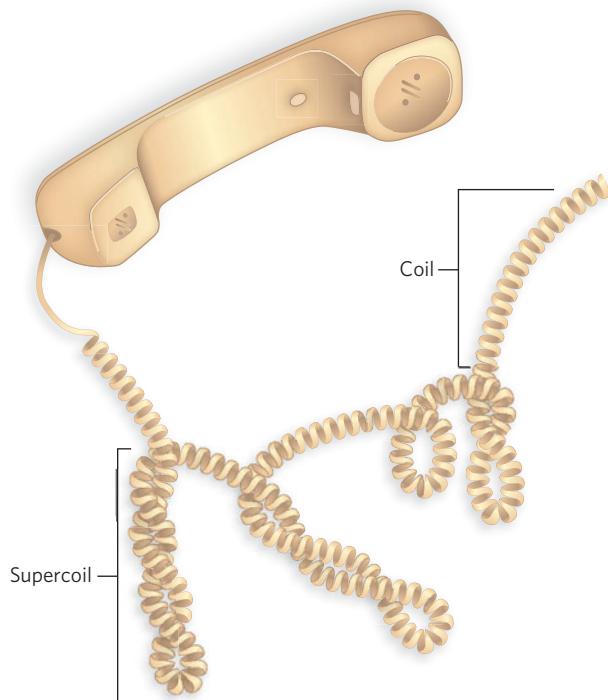


FIGURE 9-6 Supercoils. An old-fashioned phone cord is coiled like a DNA helix, and the coiled cord can supercoil.

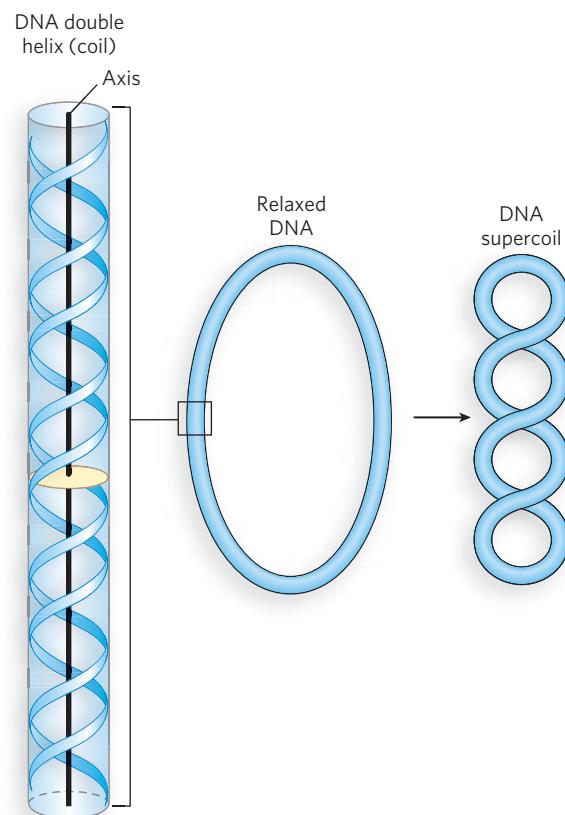


FIGURE 9-7 DNA supercoiling. When the DNA double helix is coiled on itself, it forms a new helix, or superhelix. The DNA superhelix is usually referred to as a supercoil. [Source: Adapted from N. R. Cozzarelli, T. C. Boles, and J. H. White, in *DNA Topology and Its Biological Effect* (N. R. Cozzarelli and J. C. Wang, eds), Cold Spring Harbor Laboratory Press, 1990, pp. 139–184.]

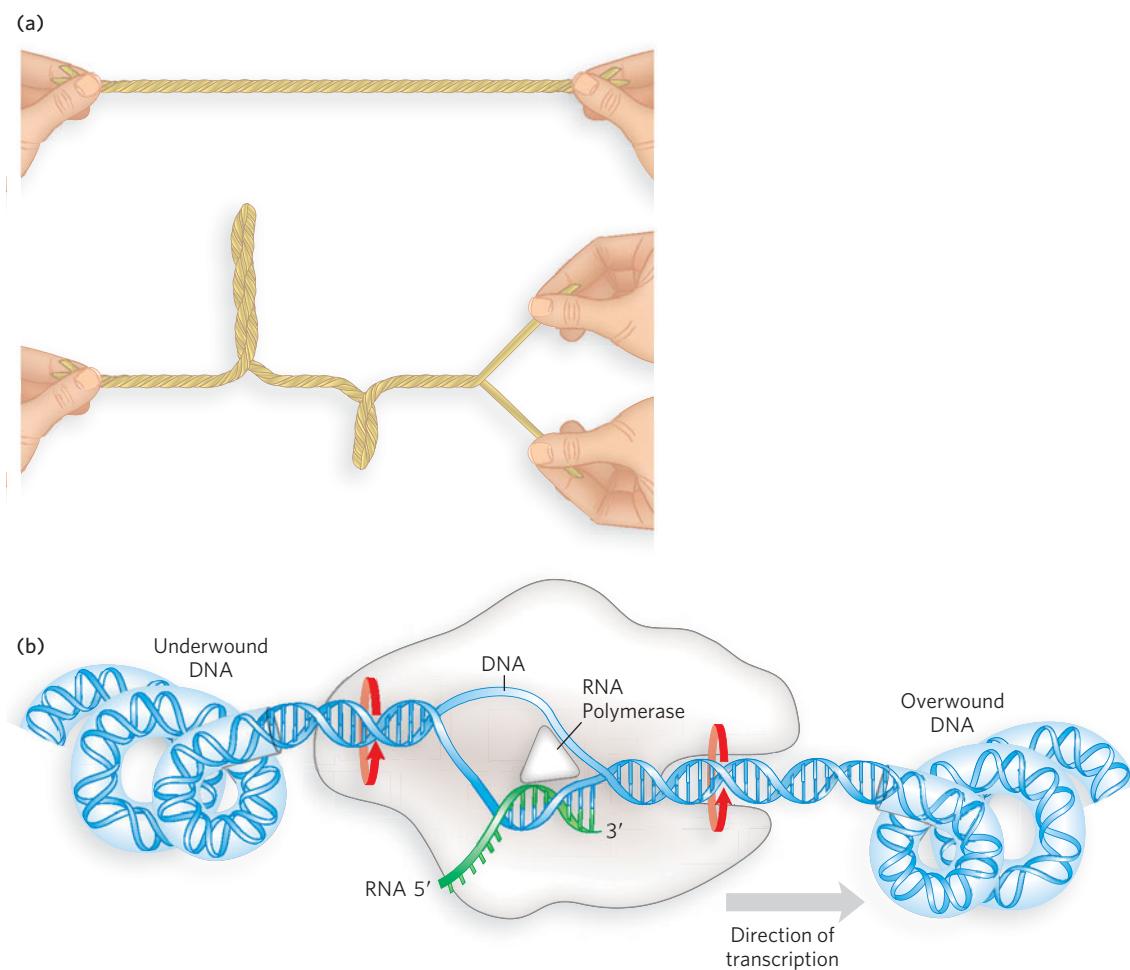


FIGURE 9-8 The effects of replication and transcription on DNA supercoiling. Because DNA is a double-helical structure, strand separation leads to added stress and supercoiling if the DNA is constrained (not free to rotate) ahead of the strand separation. (a) The general effect can be illustrated by twisting two strands of a rubber band about each other to form a double helix. If one end is constrained, separating the two strands at the other end will lead to

twisting. (b) In a DNA molecule, the progress of a DNA polymerase or RNA polymerase (as shown here) along the DNA involves separation of the strands. As a result, the DNA becomes overwound ahead of the enzyme (upstream) and underwound behind it (downstream). Red arrows indicate the direction of winding. [Sources: (a) Adapted from W. Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag, 1984, p. 452.]

supercoiling is an intrinsic property of DNA tertiary structure, as opposed to an incidental result of spatial constriction. Supercoiling is the direct result of structural strain caused by the underwinding of the DNA—that is, the removal of helical turns relative to the most stable structure of B-form DNA. DNA underwinding is catalyzed by enzymes called topoisomerases, and the degree of DNA underwinding is highly regulated in every cell.

Several measurable properties of supercoiling have been established, and the study of supercoiling has provided many insights into DNA structure and function. This work has drawn heavily on concepts

derived from topology, a branch of mathematics that studies the properties of an object that do not change under continuous deformations. In the context of **DNA topology**, continuous deformations include conformational changes due to stretching, thermal motion, or interaction with proteins or other molecules. The twisting experiments in the Bustamante lab (see Moment of Discovery) provide an example of continuous deformation. Discontinuous deformations involve DNA strand breakage. Topological properties of DNA can be changed only by the breakage and rejoining (ligation) of the backbone of one or both DNA strands.

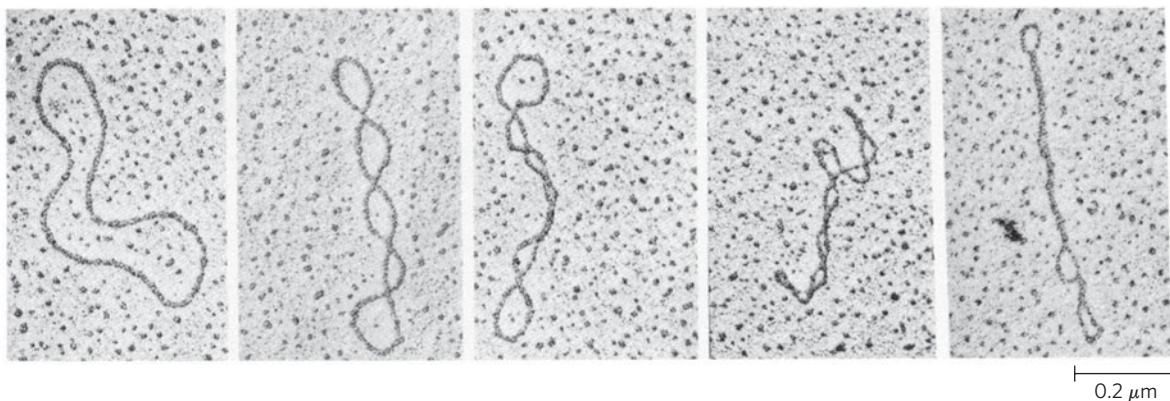


FIGURE 9-9 Relaxed and supercoiled closed-circular plasmid DNAs. The scanning electron micrograph on the far left shows relaxed DNA. Increased supercoiling is shown, from left to right. [Source: Laurien Polder, from A. Kornberg, *DNA Replication*, W. H. Freeman, 1980, p. 29.]

Most Cellular DNA Is Underwound

To understand supercoiling, we must first focus on the properties of small circular DNAs, such as plasmids and small, double-stranded viral DNAs. When these DNA molecules have no breaks in either strand, they are referred to as **closed-circular DNAs**. If the DNA of a closed-circular molecule conforms closely to the B-form structure (see Figure 6-17), with one turn of the double helix per 10.5 bp, it is relaxed rather than supercoiled (Figure 9-9). Supercoiling results when DNA is subject to some form of structural strain. Purified closed-circular DNA is rarely relaxed, regardless of its biological origin. Furthermore, all DNA derived from a given cellular source has a characteristic degree of supercoiling. DNA structure is therefore strained in a manner that is regulated by the cell to induce the supercoiling.

In almost every instance, the strain is a result of underwinding of the DNA double helix in the closed circle. In **DNA underwinding**, the molecule has *fewer* helical turns than would be expected for the B-form structure. Consider, for example, an 84 bp segment of a circular DNA in the relaxed state: it would contain eight double-helical turns, one for every 10.5 bp (Figure 9-10a). If one of these turns were removed, there would be $(84 \text{ bp})/7 = 12.0 \text{ bp}$ per turn, rather than the 10.5 found in B-DNA (Figure 9-10b). This is a deviation from the most stable DNA form, and the molecule would be thermodynamically strained as a result. Generally, much of this strain would be accommodated by coiling the axis on itself, forming a supercoil (Figure 9-10c). Some of the strain in this 84 bp segment would simply become dispersed in the untwisted structure of the larger DNA molecule. In principle, the strain could also be accommodated by separating the two DNA strands over a distance of about

10 bp (Figure 9-10d). In isolated closed-circular DNA, strain introduced by underwinding is generally accommodated by supercoiling rather than strand separation, because coiling the axis of the DNA usually requires less energy than breaking the hydrogen bonds that stabilize paired bases. Note, however, that the underwinding of DNA *in vivo* eases the separation of the DNA strands and thus access to the information they contain.

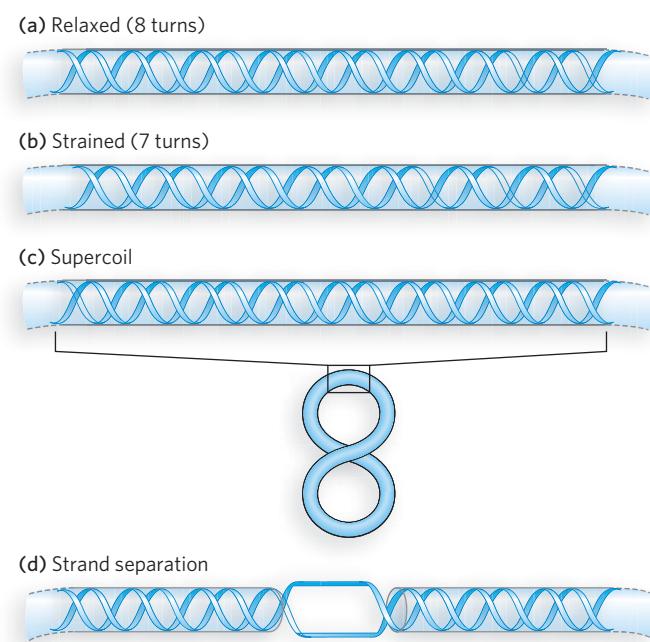


FIGURE 9-10 The effects of DNA underwinding. See text for details. [Source: Adapted from N. R. Cozzarelli, T. C. Boles, and J. H. White, in *DNA Topology and Its Biological Effect* (N. R. Cozzarelli and J. C. Wang, eds), Cold Spring Harbor Laboratory Press, 1990, pp. 139–184.]

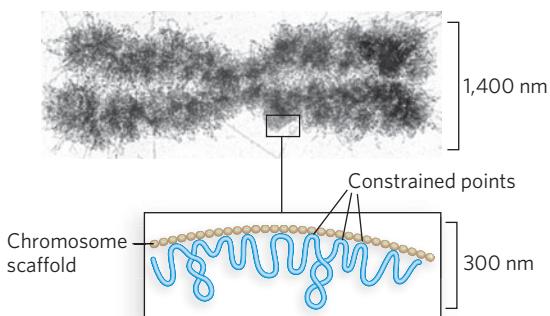


FIGURE 9-11 Loops in a eukaryotic chromosome constrained by scaffold proteins. The chromatin scaffold is attached to the chromosome at intervals, with the DNA between the attachment points defining loops that are topologically constrained. [Source: Photo from G. F. Bahr/Biological Photo Service.]

Every cell actively underwinds its DNA with the aid of enzymes (see Section 9.3), and the resulting strained state represents a form of stored energy. Underwinding thus accomplishes two things. First, cells maintain DNA in an underwound state to promote its compaction by coiling. Second, underwinding facilitates strand separation and enzymatic access to the encoded information. DNA underwinding is thus important to the enzymes of DNA replication and transcription, which must bring about strand separation as part of their function.

The underwound state can be maintained only if the DNA is a closed circle or, if linear, is bound and stabilized by proteins so that the strands are not free to rotate about each other. If there is a break in one strand of an isolated, protein-free circular DNA, free rotation at that point will cause the underwound DNA to revert spontaneously to the relaxed state. In a closed-circular DNA molecule, however, the number of helical turns cannot be changed without at least transiently breaking one of the DNA strands. The number of helical turns in a DNA molecule therefore provides a precise description of supercoiling. In the linear chromosomes of eukaryotic cells, DNA underwinding is maintained by bound proteins that constrain the DNA in an elaborate structure called chromatin. In chromatin, large loops of DNA are constrained at their base, such that each loop is topologically fixed as if it were circular (Figure 9-11; we discuss this further in Chapter 10).

DNA Underwinding Is Defined by the Topological Linking Number

The **linking number (Lk)** is a topological property of double-stranded DNA—that is, it does not vary when

the DNA is bent or deformed. To define linking number, imagine the separation of the two strands of a double-stranded circular DNA. If the two strands are joined as shown in Figure 9-12a, they are effectively linked by a topological bond. Even if all hydrogen bonds and base-stacking interactions were removed so that the strands were not in physical contact, the two strands would still be linked. If we think of one of the circular strands as the boundary of a surface (such as the soap film on the loop of a bubble wand before you blow a bubble), the linking number can be defined as the number of times the second strand pierces this surface (Figure 9-12b). The linking number for a closed-circular DNA is always an integer. By convention, if the DNA strands are interwound in a right-handed helix, the linking number is positive (+); for strands interwound in a left-handed helix, the linking number is negative (-). Negative linking numbers are, for all practical purposes, not encountered in DNA.

Consider a closed-circular DNA with 2,100 bp (Figure 9-13a). When the molecule is relaxed, the linking

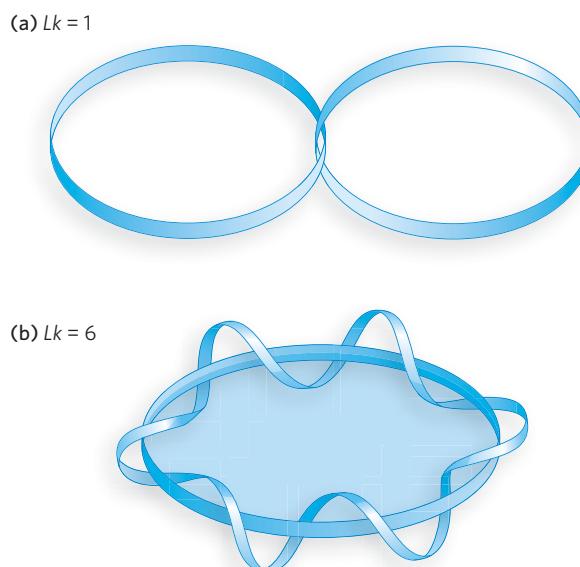


FIGURE 9-12 Linking number (Lk). Each blue ribbon represents one strand of a double-stranded DNA molecule. (a) The linking number of the molecule is 1. (b) The linking number is 6. One of the strands is kept untwisted for illustrative purposes, to define the border of an imaginary surface (solid blue oval). The number of times the twisting strand (its relative length exaggerated here) penetrates this surface is the linking number. [Source: Adapted from N. R. Cozzarelli, T. C. Boles, and J. H. White, in *DNA Topology and Its Biological Effect* (N. R. Cozzarelli and J. C. Wang, eds), Cold Spring Harbor Laboratory Press, 1990, pp. 139–184.]

number is equal to the number of base pairs divided by the number of base pairs per turn, 10.5; in this case, $Lk = 200$. As noted above, DNA can have a topological property such as a linking number only if both strands are intact. If there is a break in either strand, the strands can, in principle, be unraveled and separated; in this case, no topological bond exists and Lk is undefined ([Figure 9-13b](#)).

We can now describe DNA underwinding in terms of changes in the linking number. The linking number in relaxed DNA, Lk_0 , is used as a reference. For the molecule in Figure 9-13a, $Lk_0 = 200$; if two turns are removed from this molecule by breaking a strand, unwinding, and joining the ends back together, $Lk = 198$ (Figure 9-13c). The change can be described by the equation:

$$\Delta Lk = Lk - Lk_0 \quad (9-1)$$

For our example, $\Delta Lk = 198 - 200 = -2$.

We can also express the change in linking number independent of the length of the DNA molecule. This quantity, usually called the **superhelical density** (σ), is a measure of the number of turns removed relative to the number present in relaxed DNA:

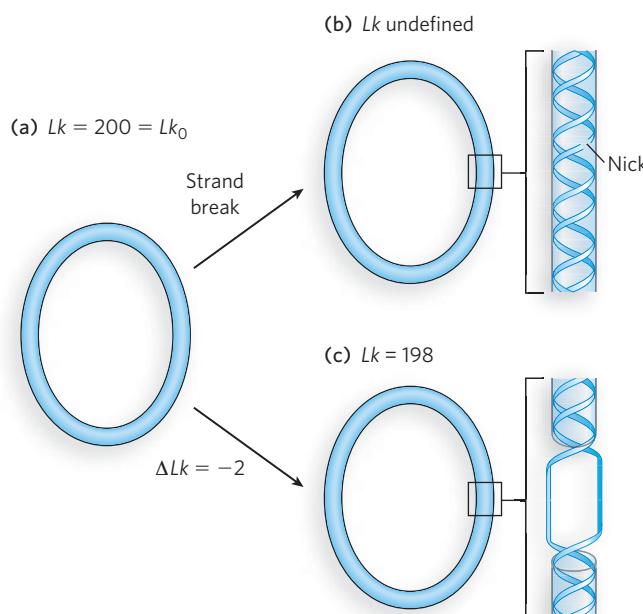


FIGURE 9-13 Linking number of closed-circular DNAs. A 2,100 bp molecule is shown in three forms: (a) relaxed, $Lk = 200$; (b) relaxed with a nick in one strand, Lk undefined; (c) underwound by two turns, $Lk = 198$. The underwound molecule is generally supercoiled, but underwinding also facilitates the separation of DNA strands.

$$\sigma = \frac{\Delta Lk}{Lk_0} \quad (9-2)$$

In the Figure 9-13c example, $\sigma = -0.01$, which means that 1% (2 of 200) of the helical turns present in the DNA (when it is relaxed, in its B form) have been removed. The degree of underwinding in cellular DNAs generally falls in the range of 5% to 7%; that is, $\sigma = -0.05$ to -0.07 . The negative sign indicates that the change in the linking number is due to underwinding of the DNA. The supercoiling induced by underwinding is therefore defined as **negative supercoiling**. Conversely, under some conditions DNA can be overwound, resulting in **positive supercoiling**.

Notice that negative supercoiling results in a twisting of the axis of the DNA to form a right-handed superhelix, and positive supercoiling results in a left-handed superhelix ([Figure 9-14](#)). Supercoiling is not a random process; it is largely prescribed by the torsional strain imparted to the DNA by decreasing or increasing the linking number relative to that of B-DNA.

The linking number can be changed by ± 1 by breaking one DNA strand, rotating one of the ends 360° about the unbroken strand, and rejoining the broken ends. This reaction is catalyzed by topoisomerases (see Section 9.3). The change in linking number has no effect on the number of base pairs or the number

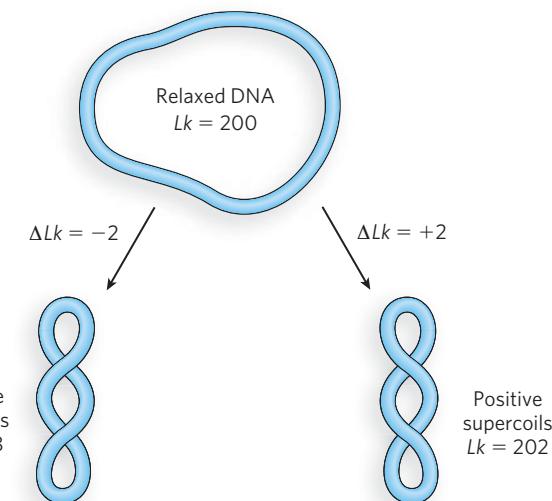


FIGURE 9-14 Negative and positive supercoils. For the relaxed DNA in Figure 9-13a, underwinding or overwinding by two helical turns ($\Delta Lk = \pm 2$) produces negative or positive supercoiling, respectively. Notice that the DNA axis in the two forms twists in opposite directions.

of atoms in the circular DNA molecule. Two forms of a circular DNA that differ only in a topological property such as linking number are referred to as **topoisomers**.

We can break down the linking number into two structural components, writhe (Wr) and twist (Tw) (Figure 9-15). **Writhe (Wr)** is a measure of the coiling of the helical axis, and **twist (Tw)** describes the local twisting or spatial relationship of neighboring base pairs. When the linking number changes, some of the resulting strain is usually compensated for by writhe (supercoiling) and some by changes in twist, giving rise to the equation:

$$Lk = Tw + Wr \quad (9-3)$$

Tw and Wr need not be integers. Twist and writhe are geometric rather than topological properties, because they may be changed by deformation of a closed-circular DNA molecule. Tw and Wr may change in a reciprocal manner without altering Lk .

In addition to causing supercoiling and making strand separation somewhat easier, the underwinding of DNA facilitates structural changes in the molecule. Although these changes are of less physiological importance, they help illustrate the effects of underwinding. Recall that a cruciform generally contains a few unpaired bases (see Figure 6-20b); DNA underwinding helps maintain the required strand separation in regions where palindromic sequences allow cruciform formation (Figure 9-16). Underwinding of a right-handed DNA helix also facilitates the formation of short stretches of left-handed Z-DNA in regions where

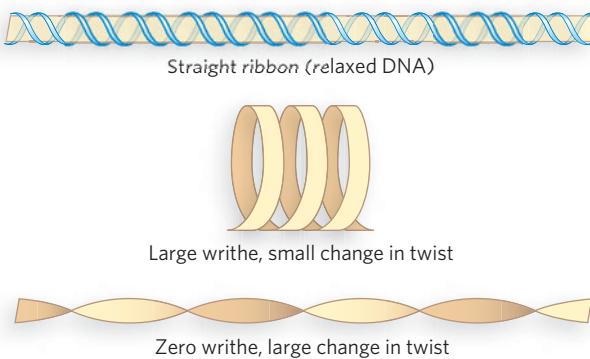


FIGURE 9-15 Writhe and twist. The beige ribbon represents the axis of a relaxed DNA molecule. Strain from underwinding of the DNA can manifest as writhe or twist. Topological changes in the linking number are usually accompanied by geometric changes in both writhe and twist.

the base sequence is consistent with the Z form (see Chapter 6).

DNA Compaction Requires a Special Form of Supercoiling

All supercoiled DNA molecules are similar in several respects. The supercoils are right-handed in a negatively supercoiled DNA molecule, and they tend to be extended and narrow rather than compacted, often with multiple branches (Figure 9-17a). At the superhelical densities normally encountered in cells, the length of the supercoil axis (the axis about which the supercoils turn), including branches, is about 40% of the length of the DNA. This type of supercoiling is referred to as **plectonemic supercoiling** (Figure 9-17b). This term can be applied to any structure with strands intertwined in some simple and regular way, and it is a good

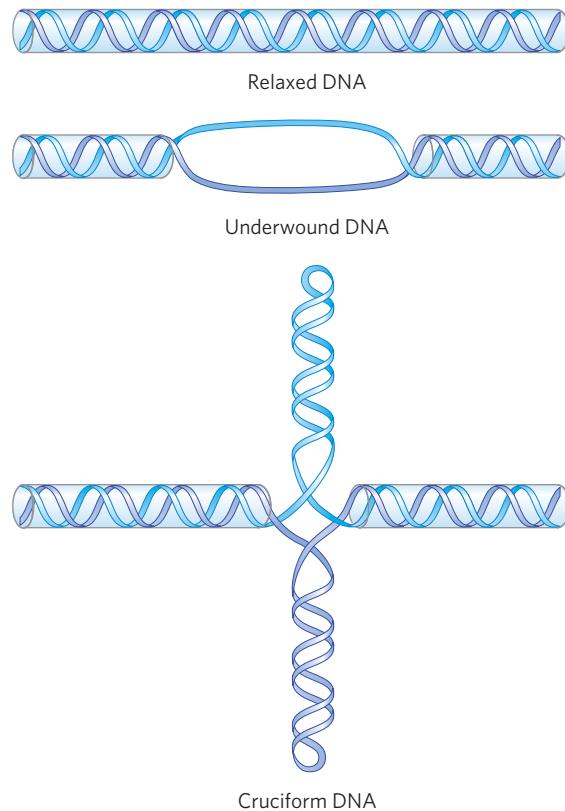


FIGURE 9-16 Promoting cruciform structures by DNA underwinding. Cruciforms can form at palindromic sequences, but they seldom occur in relaxed DNA because linear DNA accommodates more paired bases than the cruciform structure. DNA underwinding facilitates the partial strand separation required for promoting cruciform formation at appropriate sequences.

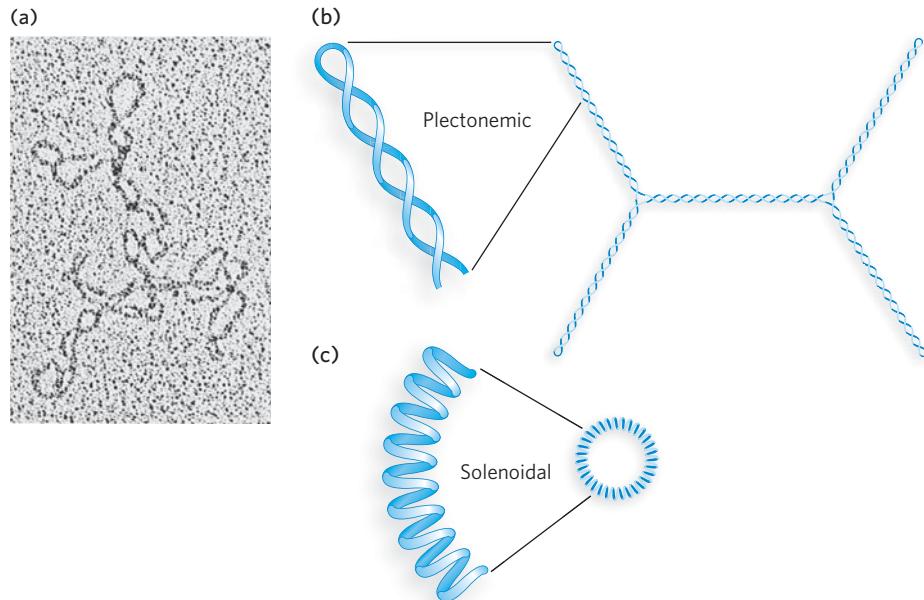


FIGURE 9-17 Plectonemic and solenoidal supercoiling.

(a) Electron micrograph of plectonemically supercoiled plasmid DNA. (b) Plectonemic supercoiling consists of extended right-handed coils. (c) Solenoidal supercoiling of the same DNA molecule depicted in (b), drawn to scale. Solenoidal negative

supercoiling consists of tight left-handed turns about an imaginary tubelike structure. Solenoidal supercoiling provides a much greater degree of compaction. [Source: (a) James H. White, T. Christian Boles, and N. R. Cozzarelli, Department of Molecular and Cell Biology, University of California, Berkeley.]

description of the general structure of supercoiled DNA in solution.

Plectonemic supercoiling, the form observed in isolated DNAs in the laboratory, does not produce sufficient compaction to package DNA in the cell. A second form, **solenoidal supercoiling**, can be adopted by an underwound DNA (Figure 9-17c). Instead of the extended right-handed supercoils characteristic of the plectonemic form, solenoidal supercoiling involves tight left-handed turns, much like a garden hose neatly wrapped on a reel. Although their structures are very different, plectonemic and solenoidal supercoiling are two forms of negative supercoiling that can be adopted by the *same* segment of underwound DNA. The two forms are readily interconvertible. The plectonemic form is more stable in solution, but the solenoidal form can be stabilized by protein binding and provides a much greater degree of compaction. Solenoidal supercoils are formed when DNA is wrapped around the nucleosomes that make up eukaryotic chromatin (see Chapter 10). Similarly, in bacteria, the tight wrapping of DNA around a variety of DNA-binding proteins gives rise to solenoidal supercoils. Solenoidal supercoiling is the primary mechanism by which underwinding contributes to DNA compaction.

SECTION 9.2 SUMMARY

- Most cellular DNAs are supercoiled. Underwinding decreases the total number of helical turns in the DNA relative to the relaxed, B form. To maintain an underwound state, DNA must be either a closed circle or, if linear, bound to protein.
- Underwinding is quantified by the linking number (Lk), a topological parameter that describes the number of times two DNA strands are intertwined.
- Underwinding is measured in terms of σ , the superhelical density; $\sigma = (Lk - Lk_0)/Lk_0$. For cellular DNAs, σ is typically -0.05 to -0.07 , which means that about 5% to 7% of the helical turns in the DNA have been removed. DNA underwinding facilitates strand separation by enzymes of DNA metabolism.
- Plectonemic supercoiling includes right-handed branches and is the most common type of supercoiling in isolated DNA. Solenoidal supercoiling, an alternative form that produces a greater degree of compaction, is characterized by tight left-handed turns that are stabilized by wrapping the DNA around proteins; this occurs in eukaryotic and bacterial chromosomes.

9.3 The Enzymes That Promote DNA Compaction

DNA supercoiling—or, more specifically, DNA underwinding—is a precisely regulated process that influences many aspects of DNA metabolism. As we've seen, it allows access to DNA during replication and transcription, and it contributes to DNA condensation during mitosis. The underwinding and relaxation of DNA are catalyzed by DNA topoisomerases, enzymes that break one or both DNA strands to allow a topological change, and then religate them. Additional condensation of cellular DNA is facilitated by SMC proteins, a class of enzymes that reversibly form protein loops that link DNA segments, affecting both condensation/compaction of chromosomes and cohesion of daughter DNA molecules for periods following replication. The maintenance of the underwound and condensed state of chromosomes by structural DNA-binding proteins such as histones is discussed in Chapter 10.

Topoisomerases Catalyze Changes in the Linking Number of DNA

All cells, from bacteria to eukaryotes, have enzymes with the sole function of underwinding and relaxing DNA. **Topoisomerases** increase or decrease the extent of DNA underwinding by changing the linking number. They play an especially important role in the complex changes in DNA topology during replication and DNA packaging.

There are two classes of topoisomerases (Table 9-4). **Type I topoisomerases** break one of the two DNA strands, pass the unbroken strand through the break,

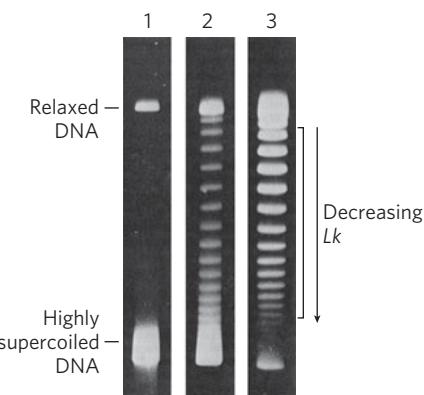


FIGURE 9-18 Visualizing topoisomers. In this experiment, DNA molecules (plasmids) have an identical number of base pairs but differ in the degree of supercoiling. In lane 1, highly supercoiled DNA migrates as a single band. Lanes 2 and 3 show the effect of treating supercoiled DNA with a type I topoisomerase; the DNA in lane 3 was treated for a longer time than the DNA in lane 2. Each individual band in the bracketed region of lane 3 contains DNA plasmids with the same linking number; Lk changes by 1 from one band to the next. [Source: W. Keller, Proc. Natl. Acad. Sci. USA 72:2553, 1975.]

and ligate the broken ends; they change Lk in increments of 1. **Type II topoisomerases** break both DNA strands and change Lk in increments of 2. The DNA is never released from the enzyme during these topological transactions, so uncontrolled relaxation of the DNA does not occur.

The activity of these enzymes can be observed with agarose gel electrophoresis, which separates DNA species according to their topoisomeric form (Figure 9-18).

Table 9-4 Topoisomerases in Bacteria and Eukaryotes

	Class	Function
Bacteria		
Topoisomerase I	Type I	Relaxes negative supercoils
Topoisomerase II (DNA gyrase)	Type II	Introduces negative supercoils
Topoisomerase III	Type I	Specialized functions in DNA repair and replication
Topoisomerase IV	Type II	Decatenation of replicated chromosomes
Eukaryotes		
Topoisomerase I	Type I	Relaxes negative supercoils, especially during DNA replication
Topoisomerase II α	Type II	Relaxes positive or negative supercoils; functions in chromatin condensation, replication, transcription
Topoisomerase II β	Type II	Relaxes positive or negative supercoils; functions in chromatin condensation, replication, transcription
Topoisomerase III	Type I	Specialized functions in DNA repair and replication

A population of identical plasmid DNAs with the same linking number migrates as a discrete band during electrophoresis. DNA topoisomers that are more supercoiled are more compact and migrate faster in the gel. Topoisomers with Lk values differing by as little as 1 can be separated by this method, so the changes in linking number induced by topoisomerases are readily detected.

E. coli has at least four individual topoisomerases, I through IV. Topoisomerases I and III are of type I, and they generally relax DNA by introducing transient single-strand breaks to remove negative supercoils (increasing Lk). Figure 9-19 shows the steps in the reaction catalyzed by bacterial type I topoisomerases (also see How We Know). A DNA molecule binds to the topoisomerase, and one DNA strand is cleaved (step 1). The enzyme changes conformation (step 2), and the unbroken DNA strand moves through the break in the first strand (step 3). Finally, the DNA strand is ligated and released (step 4). ATP is not used in this reaction. The enzyme promotes the formation of a less strained, more relaxed state by removing supercoils.

The topoisomerase must both cleave a DNA strand and ligate it again after the topological change is complete. The phosphodiester bond is not simply hydrolyzed, because this would entail loss of a high-energy bond, and an activation step would then be required to promote the subsequent ligation. Instead, a nucleophile on the enzyme (usually a Tyr residue, as in the case of *E. coli* topoisomerase I) attacks the phosphodiester bond, displacing the 3' hydroxyl and forming a covalent 5'-phosphotyrosine linkage with the DNA strand at the break. Strand passage brings about the topological change. The broken strand is then ligated by means of a direct attack of the free 3'-hydroxyl group on the phosphotyrosyl linkage. In this scheme, one high-energy bond is replaced by another at each chemical step. The resulting conservation of energy allows strand ligation without an activation step that would otherwise consume ATP.

The Two Bacterial Type II Topoisomerases Have Distinct Functions

Bacterial topoisomerase II, also known as DNA gyrase, can introduce negative supercoils (decrease Lk). This enzyme cleaves both strands of a DNA molecule (thus is a type II topoisomerase) and passes another duplex through the break (see How We Know). It uses the energy of ATP to drive key conformational changes that counteract the thermodynamically unfavorable introduction of negative supercoils that the gyrase activity

brings about. Bacterial DNA gyrases are the only topoisomerases known to actively introduce negative supercoils.

Gyrase is composed of two types of subunits, GyrA and GyrB, functioning as a GyrA₂GyrB₂ heterotetramer (Figure 9-20a). GyrB interacts with DNA and ATP, and catalyzes ATP binding and hydrolysis. Parts of GyrB form the entry point for DNA, called the N-gate. The DNA exits through a domain in GyrA called the C-gate. A separate domain of GyrA binds DNA and promotes DNA wrapping. Reaction steps are detailed in Figure 9-20b. To introduce negative supercoils, a gyrase complex first binds to a DNA segment via the N-gate (step 1), and wraps the DNA around itself (step 2). ATP is bound and both strands of the DNA are cleaved by active-site Tyr residues (step 3), forming two 5'-phosphotyrosine intermediates. ATP hydrolysis is coupled to the passage of a second region of DNA through the cleaved DNA strands, entering at the N-gate and exiting at the C-gate. To complete the reaction (step 4), the DNA strands are ligated by attack of the free 3'-hydroxyl groups on the phosphotyrosine intermediates. The complex is then poised to initiate another reaction cycle. The degree of supercoiling of bacterial DNA is maintained by regulation of the net activity of topoisomerase I, which increases Lk , and DNA gyrase, which decreases Lk .

Immediately following replication, the circular daughter chromosomes of bacteria are topologically intertwined. Circular DNAs that are intertwined in this way are called **catenanes** (Figure 9-21). The second bacterial type II topoisomerase, DNA topoisomerase IV, has a specialized function in unlinking the catenated daughter chromosomes, allowing their proper segregation at cell division. Unlike DNA gyrase, this enzyme does not use ATP and does not introduce negative supercoils.

Eukaryotic Topoisomerases Have Specialized Functions in DNA Metabolism

Eukaryotic cells also have type I and type II topoisomerases. The type I enzymes are called topoisomerases I and III. They function primarily in relieving tension and resolving topological problems in DNA during replication and repair. The type II enzymes are topoisomerases II α and II β (see Table 9-4). Eukaryotic type II topoisomerases cannot underwind DNA (introduce negative supercoils), but they can relax both positive and negative supercoils. They function in all aspects of eukaryotic DNA metabolism, resolving a range of topological problems that occur during replication, transcription, and repair. They play an especially important

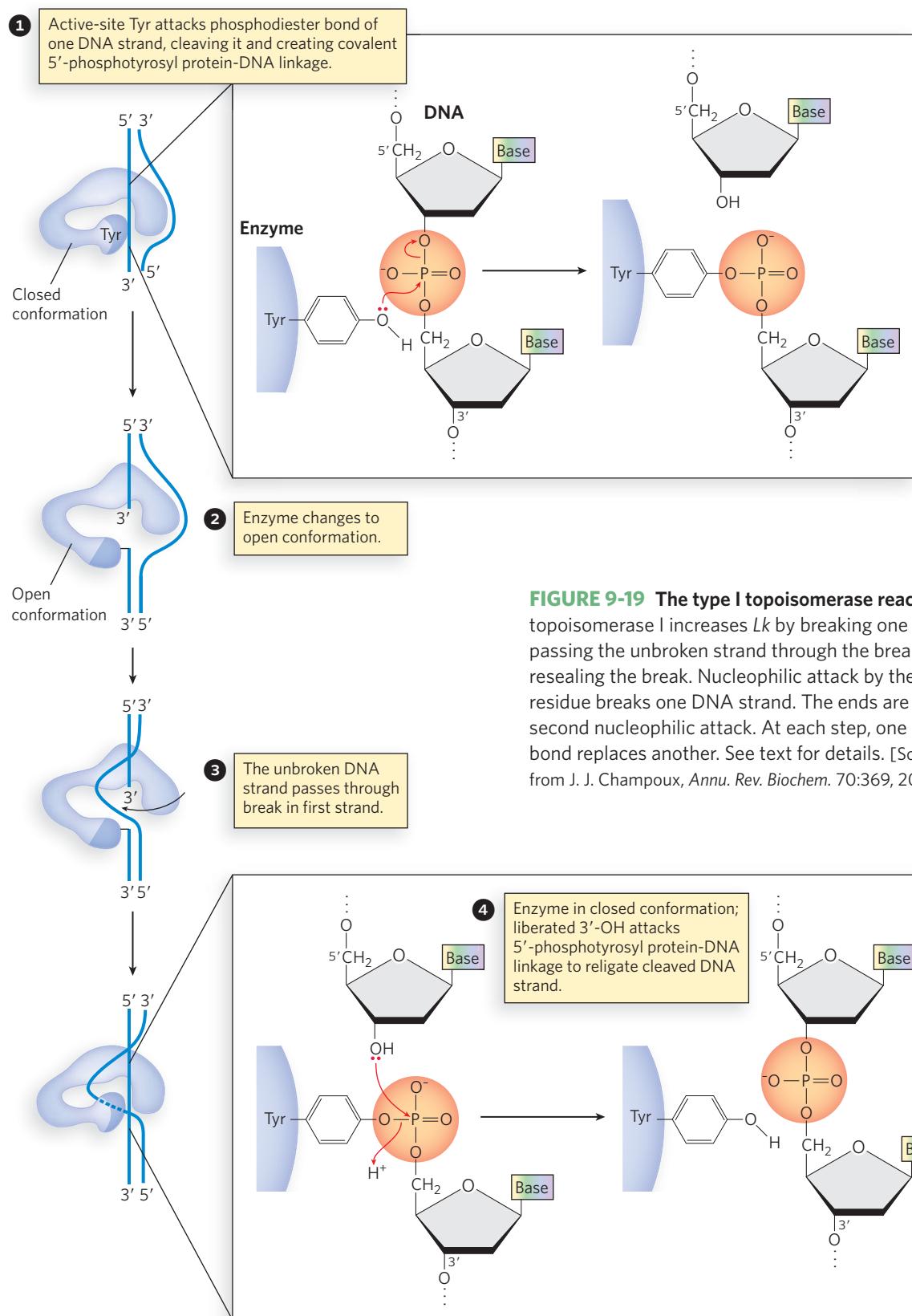


FIGURE 9-19 The type I topoisomerase reaction. Bacterial topoisomerase I increases Lk by breaking one DNA strand, passing the unbroken strand through the break, then resealing the break. Nucleophilic attack by the active-site Tyr residue breaks one DNA strand. The ends are ligated by a second nucleophilic attack. At each step, one high-energy bond replaces another. See text for details. [Source: Adapted from J. J. Champoux, *Annu. Rev. Biochem.* 70:369, 2001, Fig. 3.]

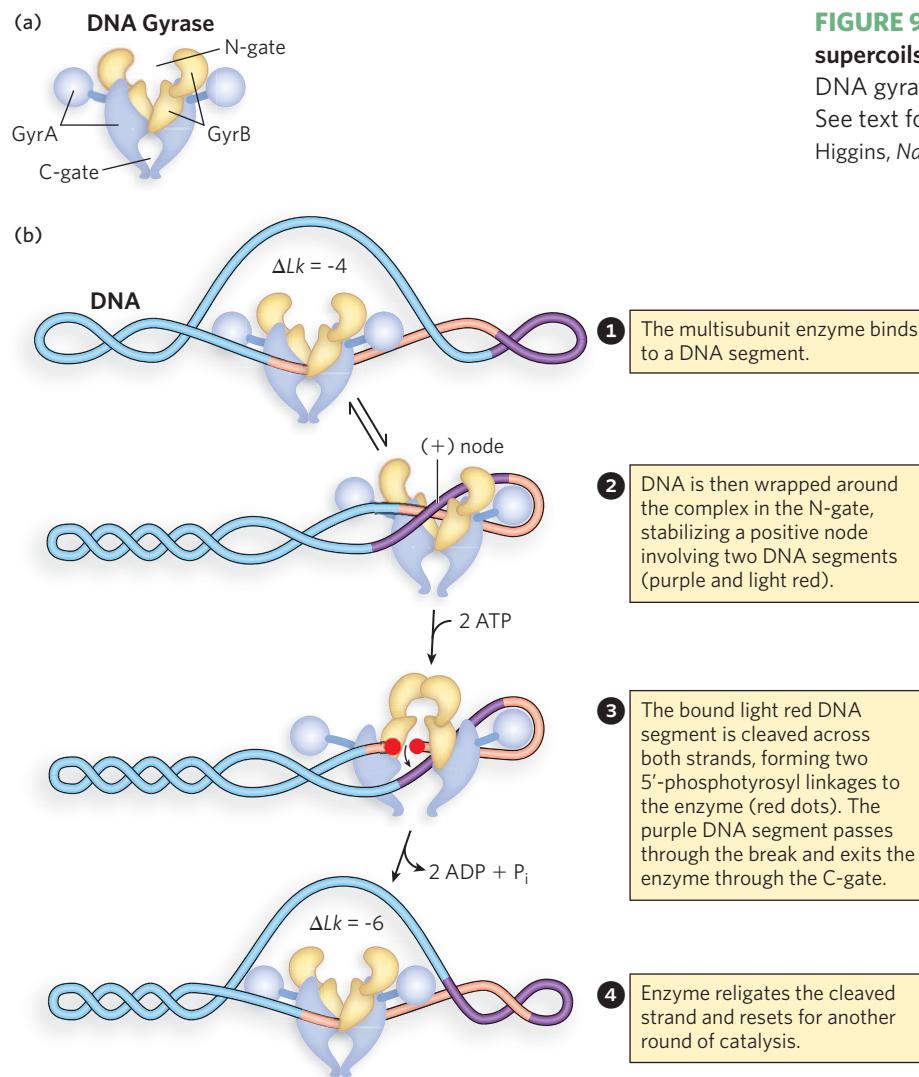


FIGURE 9-20 Introduction of negative DNA supercoils by DNA gyrase. (a) The structure of DNA gyrase. (b) The mechanism of gyrase action. See text for details. [Source: Adapted from N. P. Higgins, *Nat. Struct. Mol. Biol.* 14:264–271, 2007.]

role in the condensation of chromosomes into highly structured chromatin.

Although eukaryotes do not have an enzyme that can introduce negative supercoils into DNA, when a circular DNA is isolated from a eukaryotic cell (e.g., a plasmid from yeast), it is negatively supercoiled. This reflects the generally underwound state of cellular DNA in eukaryotic cells. One probable origin of negative supercoils in eukaryotic DNA is the tight wrapping of the DNA around a nucleosome in chromatin, which introduces a negative solenoidal supercoil (see Chapter 10). In the absence of any change in Lk , a positive supercoil must form elsewhere in the DNA to compensate (Figure 9-22). The type II topoisomerases can relax the unbound positive supercoils that arise in this way. The bound and stabilized negative supercoils are left behind,

conferring a net negative superhelicity on the DNA. Next to the histones that make up the nucleosomes, type II topoisomerases are the most abundant proteins in chromatin.

Figure 9-23 shows the reaction catalyzed by eukaryotic type II topoisomerases. The multisubunit enzyme binds a DNA molecule (step 1). The gated cavities above and below the bound DNA are the N-gate and C-gate, respectively. The second segment of the same DNA is bound at the N-gate (step 2). Both strands of the first DNA are now cleaved (step 3; the chemistry is similar to that in Figure 9-19), forming phosphotyrosine intermediates. The second DNA segment passes through the break in the first segment (step 4), and the broken DNA is ligated and the second segment released through the C-gate (step 5). Two ATPs are bound and

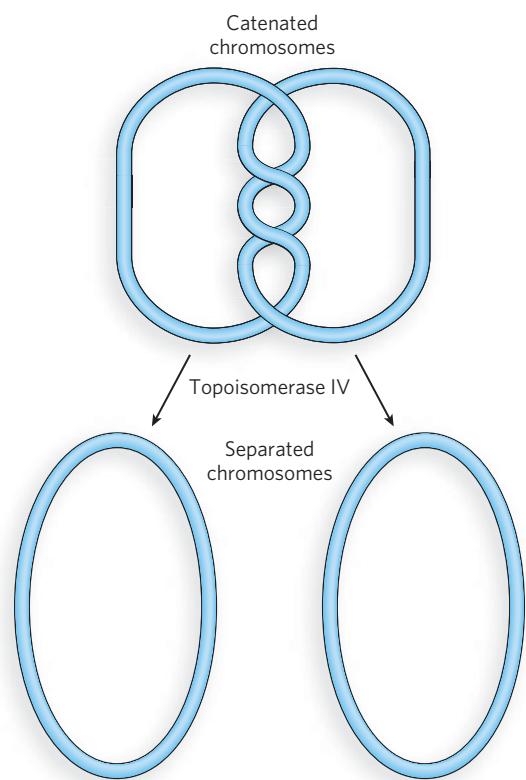


FIGURE 9-21 Solving topological problems with type II topoisomerases. Type II topoisomerases resolve knots and catenanes that arise in DNA by passing one duplex through a transient double-strand break in another duplex.

hydrolyzed during this cycle; it is likely that one is hydrolyzed in the step leading to the complex in step 4. Additional details of the ATP hydrolysis have yet to be worked out.

As we'll show in later chapters, topoisomerases are crucial to every aspect of DNA metabolism. As a consequence, they are important drug targets for the treatment of bacterial infections and cancer (Highlight 9-2).

SMC Proteins Facilitate the Condensation of Chromatin

Whereas topoisomerases influence supercoiling by changing the linking number of chromosomes, **SMC proteins** (structural maintenance of chromosomes) promote chromosome condensation by creating physical contact between segments of DNA that may otherwise be quite distant in the chromosome, or even on different chromosomes. These enzymes have integral roles in DNA condensation and chromosome segregation during mitosis, as well as in DNA repair. They perform their tasks by lining up

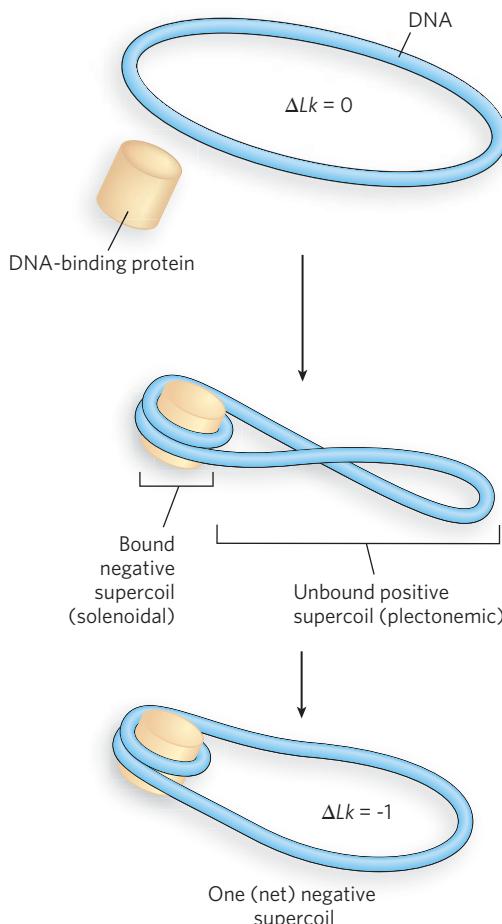


FIGURE 9-22 The origin of negative supercoiling in eukaryotic DNA. When DNA is wrapped tightly around a DNA-binding protein or protein complex, a solenoidal negative supercoil is fixed in the DNA. In a constrained DNA molecule, positive supercoils must develop elsewhere to compensate for the resulting strain. Relaxation of unbound positive supercoils by topoisomerases leads to development of a net negative superhelicity in the DNA.

along the DNA and binding to each other, providing a link between distant parts of the chromosome.

SMC proteins have five distinct domains (Figure 9-24a). The amino-terminal (N) and carboxyl-terminal (C) domains each contain part of an ATP-hydrolytic site, and they are connected by two regions of α -helical coiled-coil motifs (see Figure 4-16b) joined by a hinge domain. With bending at the hinge, the N and C domains come together to form a head structure at one end with a complete ATP-binding site. SMC proteins are generally dimeric, forming a V-shaped complex linked through the hinge domains (Figure 9-24b). Thus the dimeric SMC complex contains two head domains and two ATP-binding sites. ATP is not hydrolyzed until the two heads come together. Although

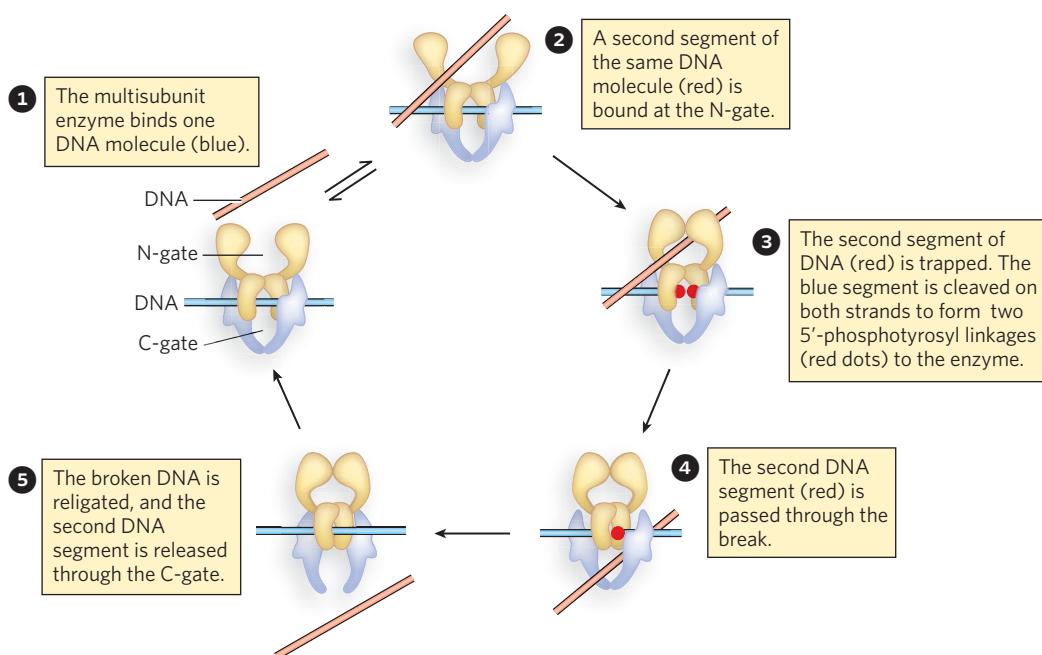


FIGURE 9-23 Alteration of the linking number by eukaryotic type II topoisomerases. The general mechanism is similar to that of the bacterial DNA gyrase (see Figure 9-20b), with one intact duplex DNA segment passed through a transient double-

strand break in another segment. The enzyme structure and use of ATP are distinct to this reaction. See text for details. [Source: Adapted from J. J. Champoux, *Annu. Rev. Biochem.* 70:369, 2001, Fig. 11.]

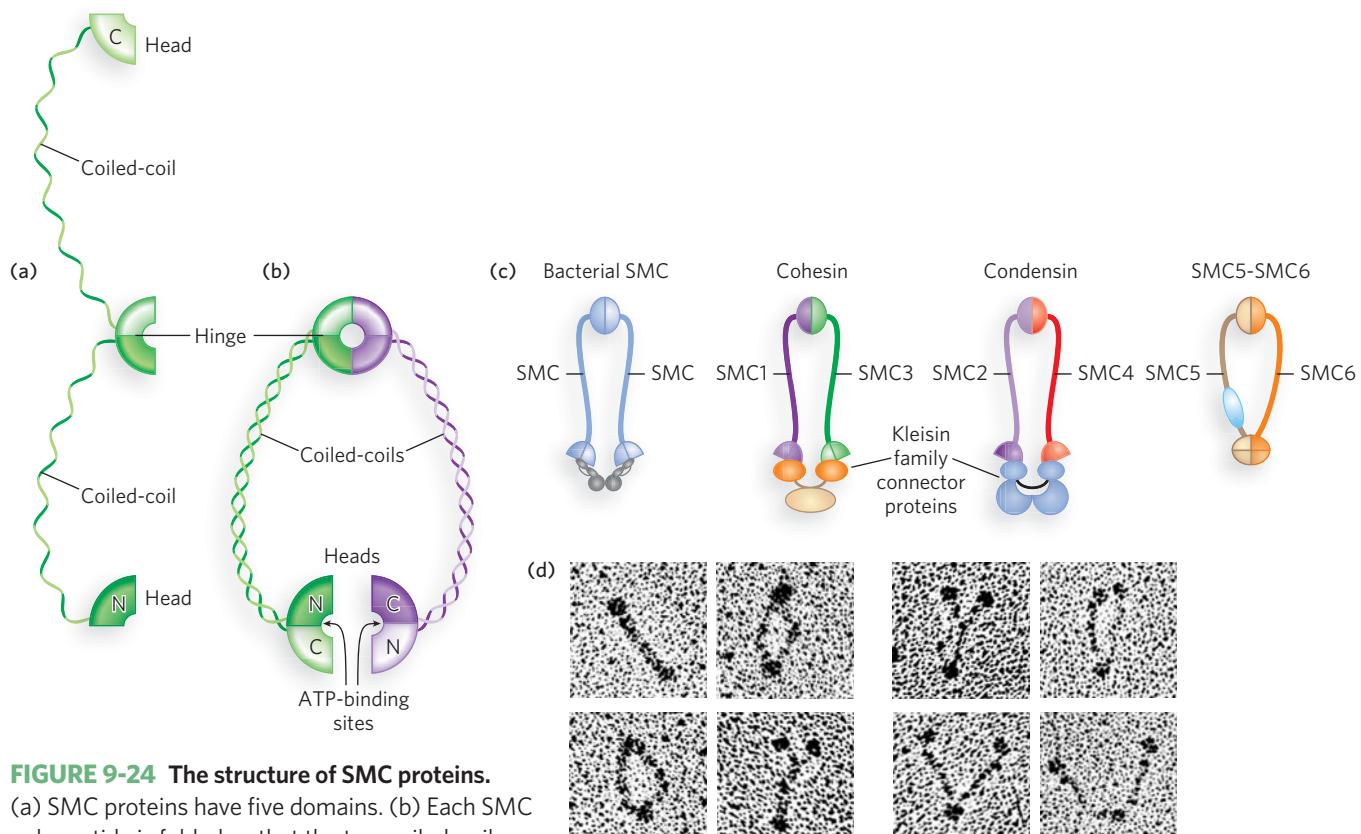


FIGURE 9-24 The structure of SMC proteins. (a) SMC proteins have five domains. (b) Each SMC polypeptide is folded so that the two coiled-coil domains wrap around each other and the N and C domains come together to form a complete ATP-binding site. Two polypeptides are linked at the hinge region to form the dimeric V-shaped SMC molecule. (c) Bacterial SMC proteins form a homodimer. The three different eukaryotic SMC proteins form heterodimers. Cohesins are made up of SMC1-SMC3 pairs, and condensins consist of SMC2-SMC4 pairs. (d) Electron micrographs of SMC dimers. [Source: (d) Courtesy of Harold Erickson.]

HIGHLIGHT 9-2 MEDICINE

Curing Disease by Inhibiting Topoisomerases

The topological state of cellular DNA is intimately connected with its function. Without topoisomerases, cells cannot replicate or package their DNA, or express their genes—and they die. Inhibitors of topoisomerases have therefore become important pharmaceutical agents, targeted at infectious organisms and malignant cells.

Two classes of bacterial topoisomerase inhibitors have been developed as antibiotics. The coumarins, including novobiocin and coumermycin A1, are natural products derived from *Streptomyces* species. They inhibit the ATP binding of the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV. These antibiotics are not used to treat infections in humans, but research continues to identify clinically effective variants.

The quinolone antibiotics, also inhibitors of bacterial DNA gyrase and topoisomerase IV, first appeared in 1962 with the introduction of nalidixic acid (Figure 1). This compound had limited effectiveness and is no longer used clinically in the United States, but the continued development of this class of drugs eventually led to the introduction of the fluoroquinolones, exemplified by ciprofloxacin (Cipro). The quinolones act by blocking the last step of the topoisomerase reaction in bacteria, the resealing of the DNA strand breaks. Ciprofloxacin is a broad-spectrum antibiotic that works on a wide range of disease-causing bacteria. It is one of the few antibiotics reliably effective in treating anthrax in-

fections and is considered a valuable agent in protection against possible bioterrorism. Quinolones are selective for the bacterial topoisomerases, inhibiting the eukaryotic enzymes only at concentrations several orders of magnitude greater than the therapeutic doses.

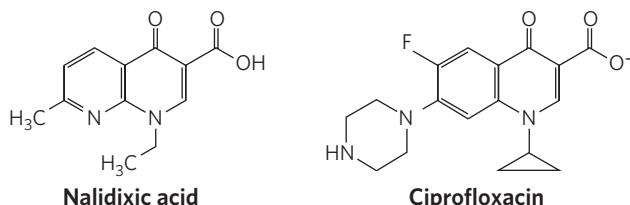


FIGURE 1 Inhibitors of bacterial type II topoisomerases.

Some of the most important chemotherapeutic agents used in cancer treatment are inhibitors of human topoisomerases. Tumor cells generally contain elevated levels of topoisomerases, and agents targeted to these enzymes are much more toxic to the tumors than to most other tissue types. Inhibitors of both type I and type II topoisomerases have been developed as anticancer drugs.

Camptothecin, isolated from a Chinese ornamental tree and first tested clinically in the 1970s, is an inhibitor of eukaryotic type I topoisomerases. Clinical trials indicated limited effectiveness, however, despite its early promise in preclinical work on mice. Two effective derivatives were developed in the 1990s: irinotecan (Campto) and topotecan (Hycamtin), used to treat colorectal cancer and ovarian cancer, respectively (Figure 2). Additional derivatives are likely to be approved for clinical use in the

many details of SMC protein function have yet to be elucidated, the head-head association between the two subunits seems to be critical.

Proteins in the SMC family are found in all types of organisms. All bacteria have at least one SMC protein that functions as a homodimer to assist in compacting the genome, whereas eukaryotes generally have six SMC proteins, functioning in defined pairs as heterodimers with different functions (Figure 9-24c). The SMC1-SMC3 and SMC2-SMC4 pairs have roles in mitosis, and the SMC5-SMC6 pair is involved in DNA repair, but its molecular role is not well understood. All these complexes are bound by regulatory

and accessory proteins. The interactions with DNA involve patches of basic amino acid residues near the hinge regions of the SMC proteins. Electron microscopy reveals the flexible V shape of these proteins (Figure 9-24d).

The SMC1-SMC3 pair forms a functional unit called a **cohesin**. During mitosis, cohesins link two sister chromatids immediately after chromosomal replication and keep them together as the chromosomes condense to metaphase (Figure 9-25). Additional proteins, particularly proteins in the kleisin family such as SCC1, bridge the cohesin head units to form a ring (see Figure 9-24c). The ring wraps around the sister chromatids, tying them

coming years. All of these drugs act by trapping the topoisomerase-DNA complex in which the DNA is cleaved, inhibiting religation.

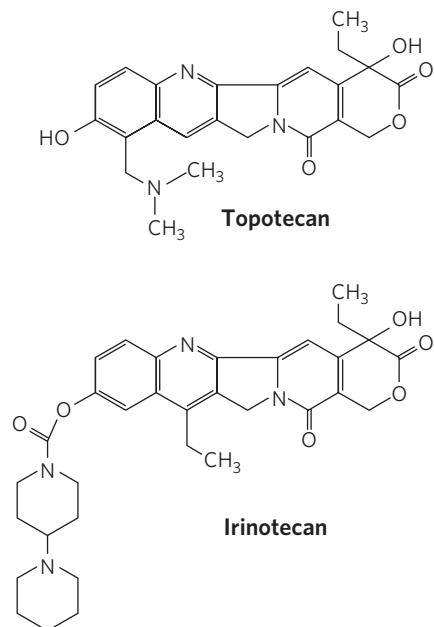


FIGURE 2 Inhibitors of eukaryotic topoisomerase I that are used in cancer chemotherapy.

The human type II topoisomerases are targeted by a variety of antitumor drugs, including doxorubicin (Adriamycin), etoposide (Etopophos), and ellipticine (Figure 3). Doxorubicin, effective against several kinds of human tumors, is in clinical use. Most of these drugs stabilize the covalent topoisomerase-cleaved DNA complex.

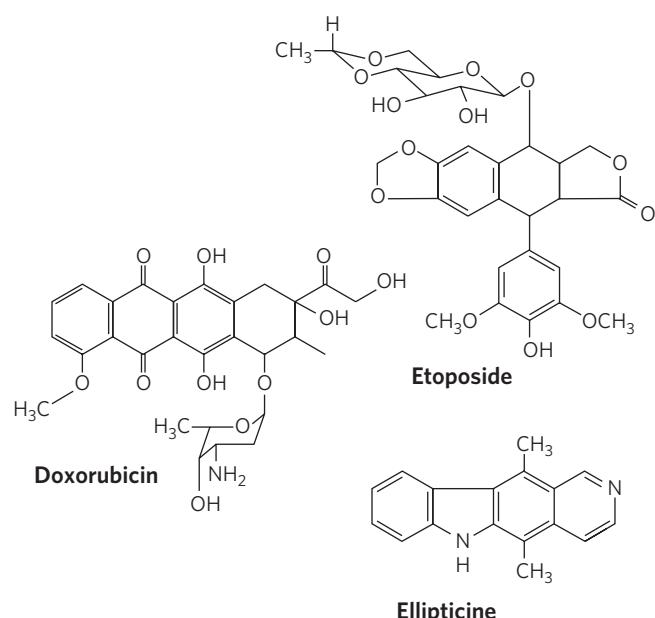


FIGURE 3 Inhibitors of human topoisomerase II that are used in cancer chemotherapy.

All of these anticancer agents generally increase the levels of DNA damage in targeted, rapidly growing tumor cells, but noncancerous tissues can also be affected, leading to a more general toxicity and unpleasant side effects that must be managed during therapy. As cancer therapies become more effective and survival statistics for cancer patients improve, the independent appearance of new tumors is becoming a greater problem. In the continuing search for new cancer therapies, the topoisomerases are likely to remain prominent targets.

together until separation is required at cell division. The ring may expand and contract in response to ATP hydrolysis. As chromosome segregation begins, the cohesin tethers are removed by enzymes known as separases.

Head-to-head engagement of SMC proteins has the potential to produce several different architectures, such as rings, rosettes, and filaments (Figure 9-26). It is not yet clear whether the ringed cohesin tethers around sister chromatids are intra- or intermolecular. The associated proteins may modulate intermolecular interactions, or, for intramolecular rings, they may perform a gatekeeping function in bringing DNA molecules into the ring.

The SMC2-SMC4 complex is called a **condensin**. The bacterial SMC proteins are most closely related to condensins. The condensins are critical to chromosome condensation as cells enter mitosis (see Figure 9-25). In the laboratory, condensins bind DNA to create positive supercoils; that is, condensin binding causes the DNA to become overwound, in contrast to the underwinding induced by the binding of nucleosomes. Figure 9-27 shows a current model of how condensins may interact with DNA to promote chromosome condensation. The condensin complexes (SMC2-SMC4 plus associated proteins) first bind to the DNA in a closed form. ATP hydrolysis then opens the intramolecular ring and

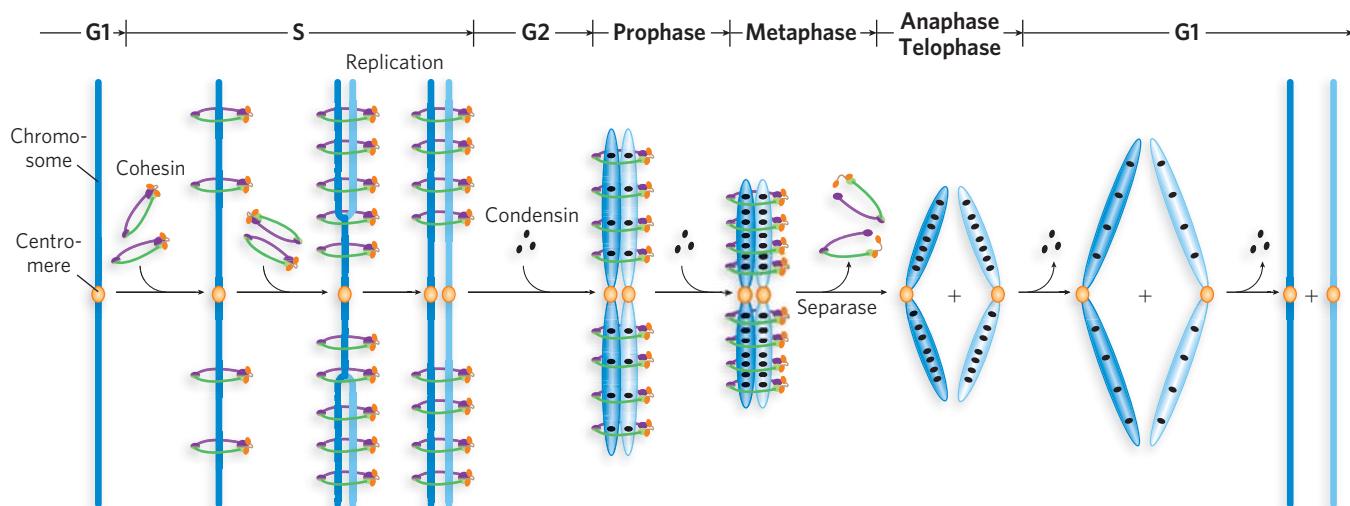


FIGURE 9-25 The roles of cohesins and condensins in the eukaryotic cell cycle. Cohesins are loaded onto the chromosomes during G1 (see Section 2.2), tying the sister chromatids together during replication. At the onset of mitosis, condensins bind and maintain the chromatids in a

condensed state. During anaphase, the enzyme separase removes the cohesin links. Once the chromatids separate, condensins begin to unload and the daughter chromosomes return to the uncondensed state. [Source: Adapted from D. P. Bazett-Jones, K. Kimura, and T. Hirano, *Mol. Cell* 9:1183, Fig. 5.]

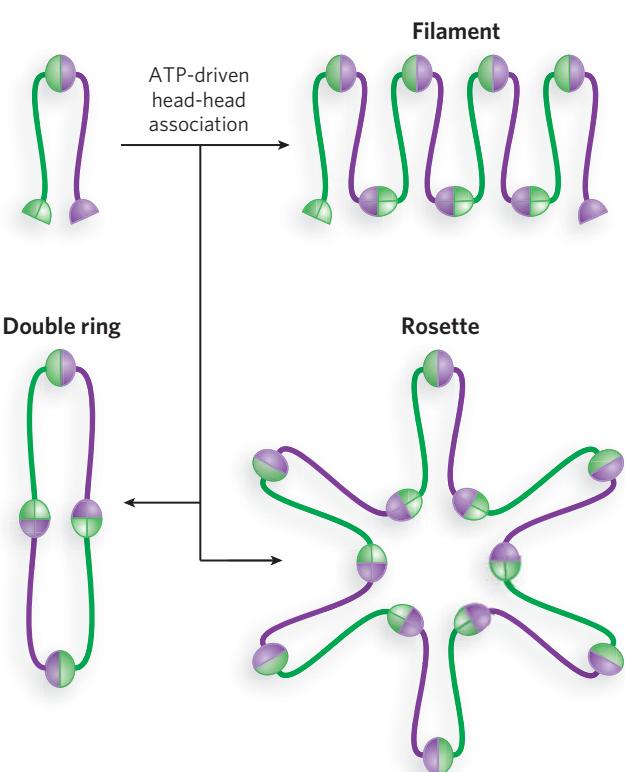


FIGURE 9-26 Potential architectural arrangements of SMC proteins. Head-to-head association results in the formation of ring structures, rosettes, or filaments. [Source: Adapted from T. Hirano, *Nat. Rev. Mol. Cell. Biol.* 7:311–322, 2006.]

brings the DNA inside. Head-to-head association creates a structure that traps DNA with a positive superhelical tension. Finally, aggregation of the condensins into rosettes forms a condensed chromatid with a defined architecture.

The topoisomerases and SMC proteins enable cells to deal with the complex topological changes occurring as DNA strands separate during replication, repair, and transcription, and the extraordinary degree of DNA compaction required in every cell. The compaction is maintained by additional specialized DNA-binding proteins, and we turn to these proteins, and their organization and function, in Chapter 10.

SECTION 9.3 SUMMARY

- Topoisomerases catalyze the underwinding and relaxation of DNA. On a molecular level, topoisomerases catalyze changes in the linking number.
- The two classes of topoisomerases, type I and type II, change Lk in increments of 1 or 2, respectively, per catalytic event.
- The reactions catalyzed by DNA topoisomerases involve the formation of transient covalent DNA-enzyme intermediates, usually in the form of a phosphotyrosyl linkage.
- Bacterial DNA gyrases introduce negative supercoils.

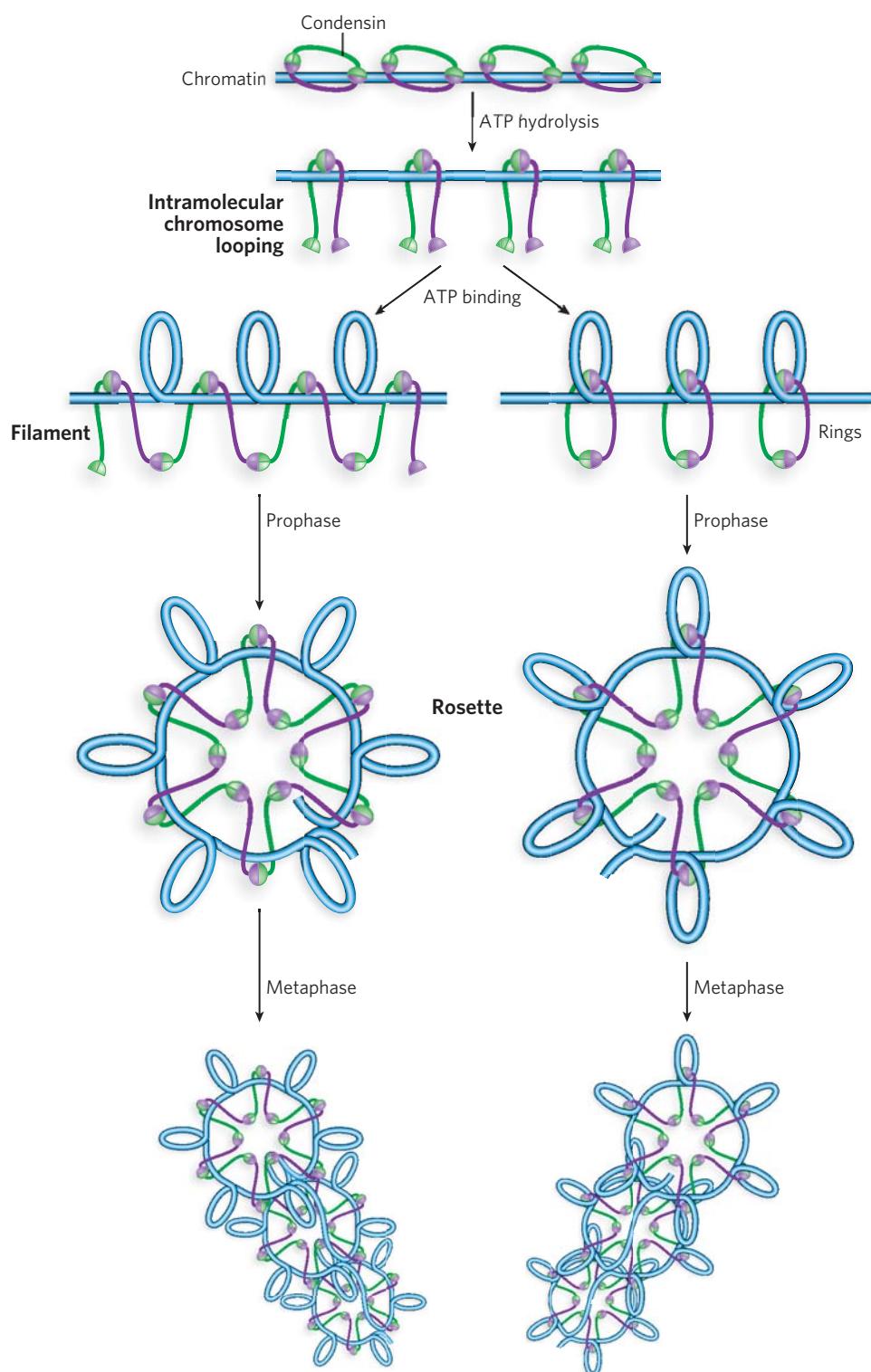


FIGURE 9-27 The possible role of condensins in chromatin condensation. Initially, the DNA is bound at the hinge region of the SMC protein, in the interior of what can become an intramolecular SMC ring. ATP binding leads to head-to-head

association, forming supercoiled loops in the bound DNA. Subsequent rearrangement of the head-to-head interactions to form rosettes condenses the DNA. [Source: Adapted from T. Hirano, *Nat. Rev. Mol. Cell. Biol.* 7:311-322, 2006.]

- Topoisomerases have functions specific to DNA metabolism, such as unlinking catenated bacterial DNA after replication or relaxing supercoils formed by unwinding during replication and transcription.
- Condensation of cellular chromosomes is facilitated by SMC proteins, including cohesins and condensins. Cohesins tether the sister chromatid products of DNA replication, and condensins provide a general structural scaffold for chromosome condensation.

Unanswered Questions

In large chromosomes, with their DNA highly complexed with proteins and thereby constrained, topological challenges accompany every process in DNA metabolism. The simple movement of a DNA polymerase through the DNA during replication (defining a structure called a replication fork) leads to overwinding ahead of the fork and underwinding behind it. Some of the challenges are extraordinary, such as when two replication forks converge in a eukaryotic chromosome, or when intertwined chromosomal DNA must be separated at cell division. A complete understanding of DNA metabolism in cells will require more detailed information on how every system interfaces with the proteins that solve topological problems.

1. **What is the role of bacterial SMC proteins?** Bacteria generally have at least one, and sometimes several, SMC proteins. Mutational loss of the major bacterial

SMC protein usually leads to defects in the condensation and segregation of chromosomes at cell division. Recent work indicates that this SMC protein is recruited to the daughter replication origins and participates in the mechanism that ensures proper segregation. However, much remains to be learned about this process.

2. **What is the function of the eukaryotic SMC5-SMC6 proteins?** The most enigmatic of the eukaryotic SMC proteins is the SMC5-SMC6 pair. So far, we know that this protein pair functions primarily in processes such as DNA recombination and repair, but its precise contributions remain unknown.
3. **How does topoisomerase III function in DNA metabolism?** In both bacteria and eukaryotes, topoisomerase III is closely tied to the function of helicases in the RecQ family. In humans, defects in the genes encoding RecQ family helicases lead to genetic diseases, including Bloom and Werner syndromes, that are characterized by genomic instability and a greater propensity to develop cancer. These enzymes are essential to many aspects of DNA metabolism. For example, the topoisomerase III–RecQ pairing in bacteria plays a critical role in resolving topological problems that accompany the convergence of replication forks. Again, much remains to be learned about the mechanics of these complicated transactions.

How We Know

The Discovery of Supercoiled DNA Goes through Twists and Turns

Lebowitz, J. 1990. Through the looking glass: The discovery of supercoiled DNA. *Trends Biochem. Sci.* 15:202-207.

Vinograd, J., J. Lebowitz, R. Radloff, R. Watson, and P. Laipis. 1965. The twisted circular form of polyoma viral DNA. *Proc. Natl. Acad. Sci. USA* 53:1104-1111.

By 1962, the double-helical structure of DNA was established, but little was known about the detailed structure of chromosomes. Researchers had been surprised by reports that the *E. coli* chromosome was a continuous circle. Were circular chromosomes unusual, or were they widespread? Two research groups, led by Renato Dulbecco and Jerome Vinograd, both at the California Institute of Technology, took up the problem of DNA structure, using the mammalian polyoma virus. The experiments with polyoma DNA led the Vinograd group to the concept of supercoiling.

Analytical ultracentrifugation measures the migration of molecules through a density gradient when ultracentrifugal force is applied (see Figure 16-2); molecules that migrate farther through the gradient have a larger sedimentation coefficient (*S*). Using this and other methods, researchers found three forms of polyoma DNA in the isolated preparations that migrated with sedimentation coefficients of 20S, 16S, and 14S. Because the 20S (form I) and 16S (form II) forms were most abundant, the researchers focused on those two. They had the same molecular weight, so the different sedimentation velocities had to involve different conformations. Form I could be isolated in almost pure preparations, if care was taken while preparing the DNA.

Both the Dulbecco and the Vinograd groups published reports indicating that form I could be converted to form II by the addition of reagents that promoted DNA strand cleavage. The researchers developed a model in which they assumed form I to be circular and

form II to be linear. The observed kinetics suggested that the conversion occurred in a single step. This challenged the model, because two strands would have to be cleaved to generate the linear molecule, and both would have to be broken at the same position.

The Vinograd group decided to examine all three forms of polyoma DNA by electron microscopy. Philip Laipis carried out the first experiments. His examination of forms I and II yielded the unexpected result that both were circular (**Figure 1**). Laipis was a relatively inexperienced undergraduate, and some of the other researchers in the lab initially assumed he had made a mistake in preparing the samples. However, several repetitions yielded the same result. Only form III (14S) was linear. Careful controls eliminated any possibility that the result reflected a selective elimination of linear forms during preparation of the DNA for examination; when the researchers premixed measured amounts of forms III and II and then examined them, the linear and circular DNAs were always there in the expected proportions. Additional kinetic studies showed that only one strand break was needed to convert form I to form II. Cleaving form I with endonuclease I (which cleaves both strands) produced only the form III (14S) DNA, with no form II. Forms I and II also had identical buoyant densities, making it unlikely that some non-DNA mass was removed in converting form I to form II.

The important clue lay in the electron micrographs. The form I molecules appeared much more twisted on themselves than the form II molecules. Denaturation experiments showed that the strands of form II could be separated, but those of form I could not. The researchers gradually established that form II was a nicked DNA circle. Understanding form I required a little more work. A comment from colleague Robert Sinsheimer led Vinograd to focus on the twisted nature of the form I DNA. His subsequent modeling with phone cords was documented in 1990 in a delightful retrospective article by Jacob Lebowitz, one of the authors on the 1965 paper. Although the term “supercoiling” was not yet in common use, the discovery of the supercoiled nature of polyoma DNA opened an entirely new field of investigation.

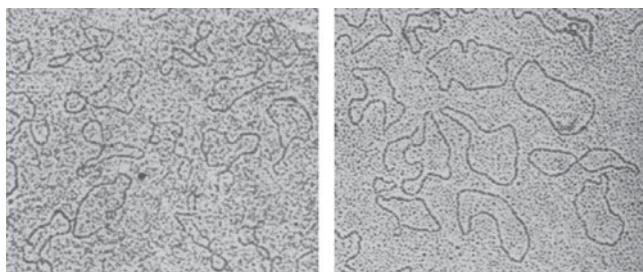


FIGURE 1 These electron micrographs of polyoma virus DNA, form I (20S) at left and form II (16S) at right, show the unexpected circular patterns. [Source: J. Vinograd et al., *Proc. Natl. Acad. Sci. USA* 53:1104-1111, 1965.]

The First DNA Topoisomerase Unravels Some Mysteries

Wang, J.C. 1971. Interaction between DNA and an *Escherichia coli* protein ω . *J. Mol. Biol.* 55:523–533.

Over the course of the twentieth century, life scientists were getting used to a fundamental idea: if a change occurs in any cellular structure, there is at least one enzyme that catalyzes it. The first discovery of an enzyme involved in DNA supercoiling was made by James Wang and reported in 1971.

Working at the University of California, Berkeley, Wang had initiated studies of superhelicity in small DNAs that could be isolated from bacteria. Focusing on the DNA from bacteriophage λ , he noticed that *E. coli* extracts contained a macromolecule that converted the superhelical form of the circular DNA to a relaxed form. He did what any good molecular biologist would do: he purified the macromolecule. The result was a protein that he dubbed the ω (omega) protein. Later, it became known as DNA topoisomerase I.

Initially, the purification was incomplete. However, Wang could establish that the macromolecule was a protein because its activity was not lost after extended dialysis (using a membrane that allowed small molecules to escape but retained larger proteins); activity was lost when the preparation was heated to 50°C (a temperature that denatures most proteins). Wang demonstrated that the conversion of a DNA circle with 150 superhelical turns to a relaxed circle did not happen in one step. Using sedimentation velocity studies and

electron microscopy, he showed that the change was progressive, with one or a few superhelical turns lost in each catalytic step. The activity affected only negative, not positive, superhelicity.

Wang concluded that the enzyme had two activities: a nicking activity and a strand-joining activity. He proposed that the ω protein acted by transiently introducing a break into one strand, creating a swivel in the unbroken strand that would allow the removal of superhelical turns. The reaction required no enzymatic cofactors, suggesting that the reaction pathway featured a transient covalent intermediate. His speculation, shown in **Figure 2**, proved to be largely correct. Overall, the study produced a remarkable set of insights that thoroughly framed the subsequent study of this and related topoisomerases.

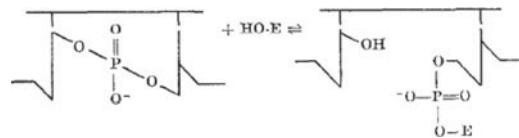
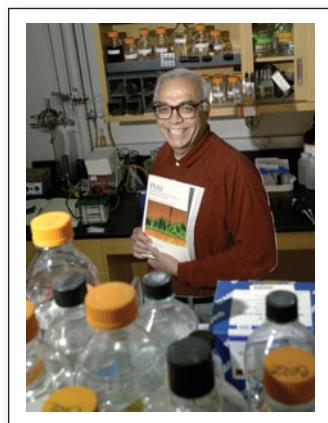


FIGURE 2 Wang proposed this simple reaction mechanism for the chemistry of DNA strand nicking and closing by the ω protein. E-OH represents a hydroxyl group on the enzyme (ω protein) [Source: J. C. Wang, *J. Mol. Biol.* 55:523–533, 1971.]

DNA Gyrase Passes the Strand Test

Brown, P.O., and N.R. Cozzarelli. 1979. A sign inversion mechanism for enzymatic supercoiling of DNA. *Science* 206:1081-1083.



Nicholas Cozzarelli, 1938–2006 [Source: Courtesy of University of California, Berkeley, Office of Media Relations.]

In 1976, Martin Gellert and colleagues reported the discovery of a second topoisomerase in *E. coli*. The enzyme, DNA gyrase, had the novel property that it could introduce negative supercoils into DNA, hydrolyzing ATP in the process. DNA gyrase was quickly shown to be critical to DNA replication and other processes, and there was great interest in determining how it worked. Many researchers expected that DNA gyrase generated a net negative superhelicity by relaxing positive supercoils, using a mechanism much like that exhibited by the ω protein, with the creation of a break in one strand and rotation of that strand about the other.

Nicholas Cozzarelli and colleagues, at the University of Chicago, began to focus on experimental observations that did not fit this scheme. First, when active DNA gyrase was acting on a DNA and the gyrase-DNA combination was treated with a protein denaturant, double-strand breaks were introduced into the DNA. Gyrase molecules were covalently linked to the 5'-phosphoryl groups in the DNA on *both* ends of the break. This implied that the normal mechanism of gyrase action involves an intermediate in which both strands, not just one, are cleaved. The research group also noticed that gyrase has the unusual capacity to catenate (interlink) two DNA circles. Such a

reaction would require the formation of at least a transient double-strand break in one of the DNAs.

Pooling this and other information, Cozzarelli proposed a very different mechanism for gyrase action, one he dubbed “sign inversion” (Figure 3). He imagined that in a circular DNA, gyrase would bind to two crossing segments, thereby stabilizing a positive crossover, or node. The creation of such a node would necessarily create a compensating negative node elsewhere in the DNA molecule. Gyrase would then invert the sign of the bound node by breaking both DNA strands, passing the unbroken DNA segment through the break, and resealing the break on the other side. This would change the sign of the node to negative and effectively fix two negative supercoils in the DNA.

The sign inversion model made a novel and unique prediction. DNA gyrase would do something very different from the ω protein: it would change superhelicity in increments of 2 rather than increments of 1. This prediction was not trivial to test. Supercoiled circular DNA (such as plasmid DNA) is isolated from cells as a heterogeneous mixture of topoisomers with a roughly Gaussian distribution of linking numbers. Gyrase could shift the center of that distribution, but highlighting individual reaction steps to observe the predicted Lk increments of 2 would be difficult. Cozzarelli and his student, Patrick O. Brown, found a way to overcome the problem.

They initially focused on a particularly small circular DNA, a plasmid of about 2,400 bp called p15. Such a small DNA limited the total number of topoisomers in the Gaussian distribution and facilitated the separation of one topoisomer from another on an agarose gel. Using the ω protein, Brown and Cozzarelli took a naturally supercoiled preparation of p15 DNA and relaxed it

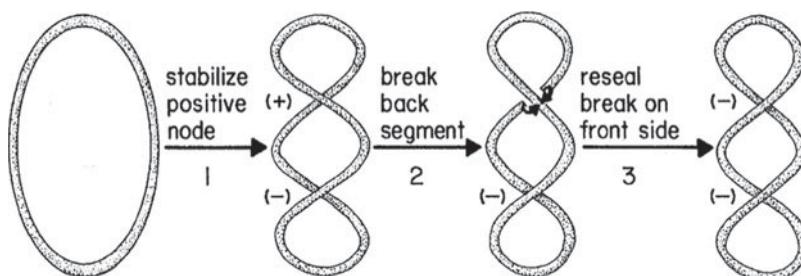


FIGURE 3 The sign inversion model for the generation of negative supercoils by DNA gyrase. [Source: P. O. Brown and N. R. Cozzarelli, *Science* 206:1081-1083, 1979.]

completely. They then ran the DNA on an agarose gel under conditions in which the topoisomers of p15 were well separated. They cut the most abundant topoisomer out of the gel and extracted it, effectively isolating a DNA preparation with one topoisomer only.

With a topologically pure DNA in hand, the key experiment could be done. The researchers added enough DNA gyrase (about two heterotetramers per DNA molecule) to ensure that essentially every DNA circle had a gyrase bound. After incubating the DNA and gyrase for 3 minutes, they added ATP, but only enough ($30 \mu\text{M}$, or about one-tenth of the K_m) to support a slow reaction. The results, shown in **Figure 4**, are most striking at the 5 second time point. The major product is a species with a change in linking number (ΔLk) of -2 . A little DNA with a ΔLk of -4 is also evident. Markers showing topoisomers differing by a ΔLk of 1 are shown in the lanes marked MW. At later time points, the DNA becomes more supercoiled, but topoisomers with odd-numbered ΔLk 's do not appear.

Brown and Cozzarelli carried out one additional test. After 5 minutes, the p15 DNA was highly supercoiled. They then added novobiocin, an antibiotic that inhibits supercoiling but not relaxation by gyrase (see Highlight 9-2). After another 30 minutes of incubation, much of the DNA had been substantially relaxed (see Figure 4). The topoisomers present included species with superhelicity changes of 0 , -2 , and -4 relative to the starting material. This demonstrated that gyrase promoted both supercoiling and relaxation of DNA in increments of 2. The result fulfilled a key expectation of any enzymatic reaction—that the reaction pathway is the same in the forward and reverse directions. Overall, the experiments constituted a compelling case for the sign inversion model and provided the impetus to eventually define two separate classes of topoisomerase.

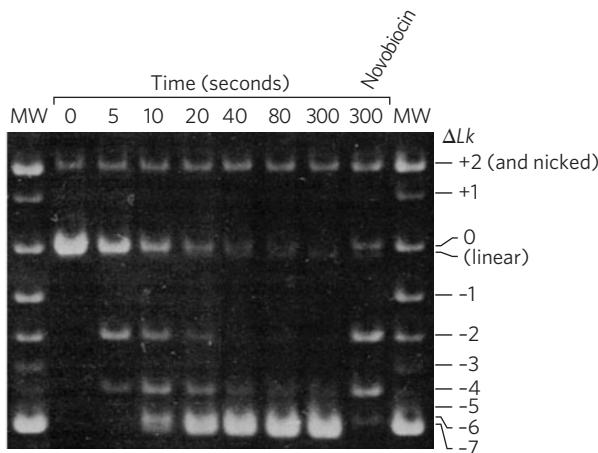


FIGURE 4 The p15 plasmid DNA was topologically pure; the minor band at the top of the $t = 0$ column is a small amount of nicked DNA, due to damage inflicted during purification. The p15 plasmid was mixed with gyrase for 5 to 300 seconds. After 300 seconds, a sample of DNA was treated with novobiocin and incubated for another 30 minutes. Marker lanes (MW) show the p15 plasmid DNA with change in linking number (ΔLk) in increments of 1. [Source: P. O. Brown and N. R. Cozzarelli, *Science* 206:1081-1083, 1979.]

These advances helped explain the mechanism of action of a range of important antibiotics and antitumor drugs (see Highlight 9-2). They were among a string of important contributions from the Cozzarelli lab, first at Chicago and later at the University of California-Berkeley. Imbued with an ebullient personality and a creative intellect, Cozzarelli inspired a generation of scientists as a mentor and a colleague. Cozzarelli succumbed to complications of a treatment for Burkitt's lymphoma in 2006 at the age of 67, but his lab motto—Blast ahead—lives on.

Key Terms

centromere, p. 298
 telomere, p. 299
 plasmid, p. 302
 DNA supercoiling, p. 305
 supercoiled DNA, p. 305
 relaxed DNA, p. 305
 DNA topology, p. 306
 closed-circular DNA, p. 307
 DNA underwinding, p. 307

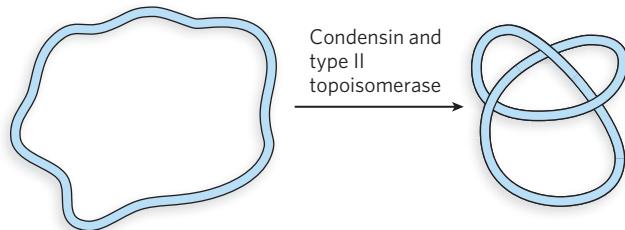
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Problems

- What is the superhelical density (σ) of a closed-circular DNA with a length of 4,200 bp and a linking number (Lk) of 374? What is the superhelical density of the same DNA when $Lk = 412$? In each case, is the molecule negatively or positively supercoiled?
- The T4-like bacteriophage JS98 has a DNA of molecular weight 1.11×10^8 contained in a head about 100 nm long.
 - Calculate the length of the DNA (assume the molecular weight of a nucleotide pair is 650) and compare it with the length of the JS98 head.
 - Consult the online database Entrez Genome. What is the exact number of base pairs in the JS98 genome?
- The base composition of phage M13 DNA is A, 23%; T, 36%; G, 21%; C, 20%. What does this tell you about the DNA structure of phage M13?
- The complete genome of the simplest bacterium known, *Mycoplasma genitalium*, is a circular DNA molecule with 580,070 bp. Calculate the molecular weight (assume the molecular weight of a nucleotide pair is 650) and contour length (when relaxed) of this molecule. What is Lk_0 for the *Mycoplasma* chromosome? If $\sigma = -0.06$, what is Lk ?
- A closed-circular DNA molecule in its relaxed form has an Lk of 500. Approximately how many base pairs are in this DNA? How does the linking number change (increases, decreases, doesn't change, becomes undefined) in each of the following situations?
 - A protein complex binds, wrapping the DNA around it to form a solenoidal supercoil.
 - One DNA strand is broken.
 - DNA gyrase and ATP are added to the DNA solution.
 - The double helix is denatured by heat.
- In the presence of a eukaryotic condensin and a type II topoisomerase, the Lk of a relaxed closed-circular DNA molecule does not change. However, the DNA becomes highly knotted, as shown in the next column. The formation of knots requires breakage of the DNA, passage of a segment of DNA through the break, and religation by the topoisomerase. Given that every reaction of the

topoisomerase would be expected to result in a change in linking number, how can Lk remain the same?

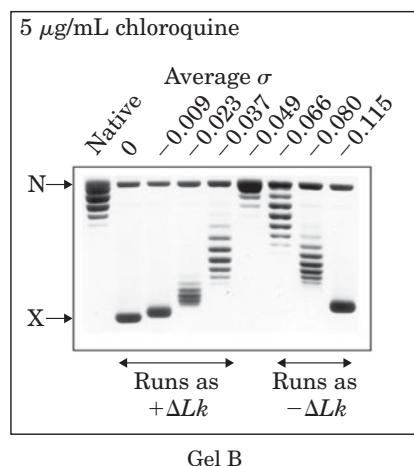
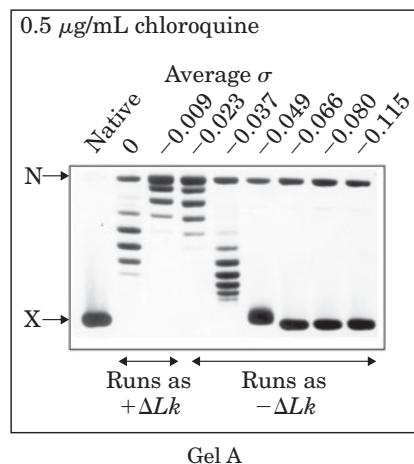


- Bacteriophage λ infects *E. coli* by integrating its DNA into the bacterial chromosome. The success of this recombination depends on the topology of the *E. coli* DNA. When the superhelical density (σ) of the *E. coli* DNA is greater than -0.045 , the probability of integration is $<20\%$; when σ is less than -0.06 , the probability is $>70\%$. Plasmid DNA isolated from an *E. coli* culture is found to have a length of 13,800 bp and an Lk of 1,222. Calculate σ for the plasmid DNA (which reflects the superhelical density of all DNA in the cell, plasmid and chromosome), and predict the likelihood that bacteriophage λ will be able to infect this culture.
- (a) What is the Lk of a 5,250 bp circular duplex DNA molecule with a nick in one strand?
 (b) What is the Lk of the molecule in (a) when the nick is sealed (relaxed)?
 (c) How would the Lk of the molecule in (b) be affected by the action of a single molecule of *E. coli* topoisomerase I?
 (d) What is the Lk of the molecule in (b) after eight enzymatic turnovers by a single molecule of DNA gyrase in the presence of ATP?
 (e) What is the Lk of the molecule in (d) after four enzymatic turnovers by a single molecule of bacterial type I topoisomerase?
 (f) What is the Lk of the molecule in (d) after binding of one protein that wraps DNA around it to form a solenoidal supercoil, with no other changes in the DNA?

- 9.** Explain how the underwinding of a B-DNA helix might facilitate or stabilize the formation of Z-DNA.
- 10. (a)** Describe two structural features required for a circular DNA molecule to maintain a negatively supercoiled state.
- (b)** List three structural conformations that become more favorable when a DNA molecule is negatively supercoiled.
- (c)** What enzyme, with the aid of ATP, can generate negative supercoiling in DNA?
- (d)** Describe the physical mechanism by which this enzyme acts.
- 11.** YACs are used to clone large pieces of DNA in yeast cells. What three types of DNA sequence are required to ensure proper replication and propagation of a YAC in a yeast cell, and what is the function of each?
- 12.** When DNA is subjected to electrophoresis in an agarose gel, shorter molecules migrate faster than longer ones. Closed-circular DNAs of the same size but with different linking numbers also can be separated on an agarose gel; topoisomers that are more supercoiled, and thus more condensed, migrate faster through the gel. In the gel shown below, purified plasmid DNA has migrated from top to bottom. There are two bands, with the faster band much more prominent.
- (a)** What are the DNA species in the two bands?
- (b)** If topoisomerase I is added to a solution of this DNA, what will happen to the upper and lower bands after electrophoresis?
- (c)** If DNA ligase is added to the DNA, will the appearance of the bands change?
- (d)** If DNA gyrase plus ATP is added to the DNA after the addition of DNA ligase, how will the band pattern change?



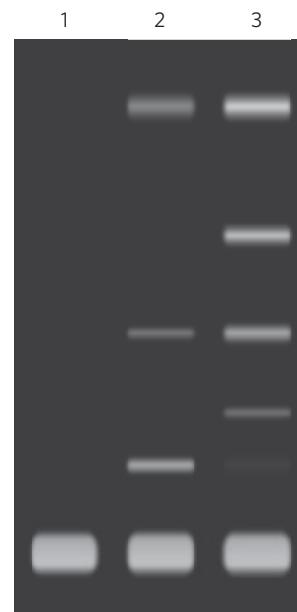
- 13.** When DNA is subjected to electrophoresis in an agarose gel, shorter molecules migrate faster than longer ones. Closed-circular DNAs of the same size but different linking numbers also can be separated on an agarose gel; topoisomers that are more supercoiled, and thus more condensed, migrate faster through the gel—from top to bottom in the gels shown below. The dye chloroquine was added to these gels. Chloroquine intercalates between base pairs and stabilizes a more underwound DNA structure. When the dye binds to a relaxed, closed-circular DNA, the DNA is underwound where the dye binds, and unbound regions take on positive supercoils to compensate. In the experiment shown here, topoisomerases were used to make preparations of the same closed-circular DNA with different superhelical densities (σ). Completely relaxed DNA migrated to the position labeled N (for nicked), and highly supercoiled DNA (above the limit where individual topoisomers can be distinguished) migrated to the position labeled X.



Source: Photos from R. P. Bowater, in *Encyclopedia of Life Sciences*, John Wiley & Sons, Inc./Wiley Interscience, 2005.

- (a) In gel A, why does the $\sigma = 0$ lane (i.e., DNA prepared so that $\sigma = 0$, on average) have multiple bands?
- (b) In gel B, is the DNA from the $\sigma = 0$ preparation positively or negatively supercoiled in the presence of the intercalating dye?
- (c) In both gels, the $\sigma = -0.115$ lane has two bands, one a highly supercoiled DNA and one relaxed. Propose a reason for the presence of relaxed DNA in these lanes (and others).
- (d) The native DNA (leftmost lane in each gel) is the same closed-circular DNA isolated from bacterial cells and untreated. What is the approximate superhelical density of this native DNA?
14. In the early electron microscopy experiments of Vinograd and colleagues (see How We Know), the polyoma virus DNA was clearly circular in both form I and form II. However, the DNA in form II tended to be spread out on the electron microscope grid, whereas the DNA in form I tended to twist on itself, often repeatedly. Explain these observations.
15. A small plasmid DNA is isolated, and one negatively supercoiled topoisomer is purified (see How We Know). A small amount of bacterial topoisomerase III or topoisomerase II (DNA gyrase) plus ATP is added to the plasmid DNA in two separate experiments, allowing for limited

reaction of the topoisomers. The experiments yield the DNA banding patterns shown below. Which pattern is produced by DNA topoisomerase III, and which by DNA gyrase?



Data Analysis Problem

Boles, T.C., J.H. White, and N.R. Cozzarelli. 1990. Structure of plectonemically supercoiled DNA. *J. Mol. Biol.* 213:931–951.

16. The structural parameters of plectonemically supercoiled DNA in solution were first examined by Cozzarelli and coworkers. Using a combination of electron microscopy and gel electrophoresis, they examined the properties of plasmid DNAs with different superhelical densities (σ). DNAs were spread for electron microscopic examination, as shown in the figure. DNA twists on itself to form a superhelix. The length of the superhelical axes of the entire molecule can be determined by measuring the lengths of the axes of all segments and branches, and summing. In the example shown, there are five segments with three branch points. The superhelix is seen as the crossing of the DNA as it winds about itself, with the crossing points on the DNA defined as nodes.

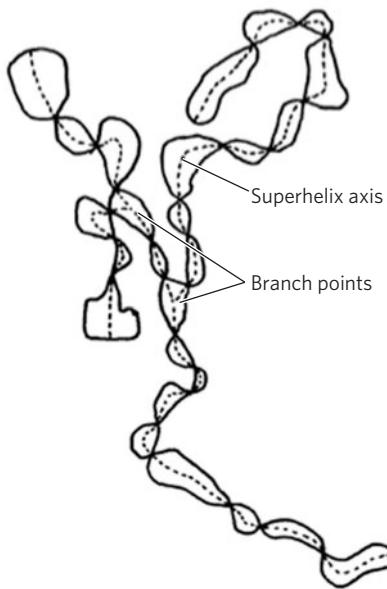
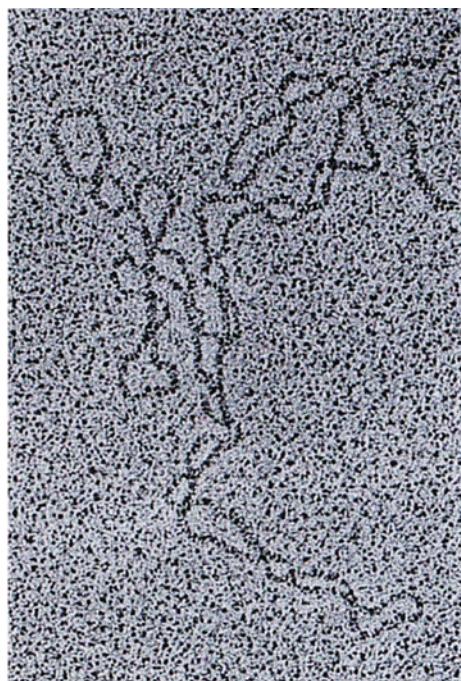
(a) How many nodes are there in this DNA? Each node is roughly equivalent to a supercoil.

(b) If one assumes that each supercoil results from an underwinding of the DNA by one turn, and the DNA has a length of 7,000 bp, calculate σ .

The investigators found a linear relationship between the number of nodes (n) and ΔLk , such that $n = -0.89 \Delta Lk$ (implying that most, but not all, of the change in linking number results from changes in the writhing or twisting of the DNA helix axis on itself).

(c) With this in mind, calculate the σ for this molecule.

(d) Is there any evidence of solenoidal supercoiling?



Additional Reading

General

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Nucleosomes, Chromatin, and Chromosome Structure



Jonathan Widom [Source: Jill Carlson for Martin Woods Image Consulting, LLC.]

nucleosomes. We published this finding a decade ago. During the following years, we solved the nucleosome sequence alignment problem, but we still didn't understand how nucleosome sequences worked in genomes.

I took a sabbatical at Rockefeller University, where I met Eran Segal, a visiting postdoctoral fellow. We both had the same vision of getting a quantitative understanding of how nucleosomes and other regulatory proteins compete for access to the genome. So the second key moment came from working with Eran. We first developed theoretical models, using available experimental data as well as laws of physical chemistry, to predict nucleosome positions within chromosomal DNA. We addressed the problem of avoiding physical overlap of nucleosomes while maximizing their preferred positions in the genome in a thermodynamic equilibrium. Segal wrote a computer program to solve this mathematical problem, and we compared the resulting predictions to experimentally determined nucleosome binding sites—and they matched very well! Many things immediately fell into place that previously had not made sense, such as why few nucleosomes bind near promoters, and why nucleosomes tend to cluster near transcription start sites (now explained by DNA sequences found in promoters and at transcription start sites). We continue to make new discoveries about how the evolution of the genome has been constrained by the need to position nucleosomes for optimal control of gene expression. The work is at least as exciting now as it was a few years ago!

—**Jonathan Widom**, on discovering the code for genome-wide nucleosome organization

10.1 Nucleosomes: The Basic Units of DNA Condensation 332

10.2 Higher-Order Chromosome Structure 338

10.3 The Regulation of Chromosome Structure 343

Eukaryotes contain thousands of times more DNA than do bacteria, and as a result, the DNA-condensation problems of eukaryotes—compacting the DNA so that it fits in the cell nucleus—are more complex than those of bacteria. In Chapter 9 we introduced DNA topoisomerases, enzymes that can untwist DNA and keep the long DNA molecules within cellular chromosomes from becoming intertwined. We also discussed the ring-shaped condensins and cohesins that encircle DNA segments to hold them tightly together in loops, thus increasing compaction. This chapter focuses on a specific DNA-condensation particle of eukaryotes—the nucleosome—around which DNA is wrapped. Bacteria do not contain nucleosomes, although they have small, basic (positively charged) proteins that are involved in condensing their DNA.

DNA compaction must be dynamic, because changes in the degree of condensation must occur quickly and when needed, as the cell passes through the stages of the cell cycle (see Figure 2-10). Furthermore, when in its most highly compacted form, DNA is not accessible to transcription or replication enzymes, so it must be able to rapidly expose regions containing genes that are required at any given moment, and then condense again. Changes in DNA compaction in a cell can occur on a global level (such as during mitosis or replication) or a local level (such as giving access to specific genes for transcription regulation). To accommodate these essential activities, modification enzymes have evolved that alter the state of DNA condensation by various means, and these enzymes can target their activity to specific regions of the chromosome that must be transcribed or replicated.

We explore how nucleosome units are arranged in higher levels of chromosome structure, and see how the cell manipulates nucleosomes in many ways to achieve dynamic changes in DNA condensation. For example, the cell contains large protein machineries that move nucleosomes on DNA to assist in the regulation of gene expression. Nucleosomes are also modified by enzymes that attach a variety of small molecules to the nucleosome's protein subunits. These alterations regulate genes by controlling the state of the chromatin. In fact, these modifications are inherited and encode information that is passed down from one cell generation to the next. This is especially important during an organism's development, to maintain the transcriptional program of differentiated cell types. Genetic information that is not coded by the DNA itself is referred to as epigenetic information, and defects in epigenetic pathways are closely associated with cancer.

10.1 Nucleosomes: The Basic Units of DNA Condensation

Scientists have been fascinated with the structure and behavior of chromosomes for more than 100 years. Chromosomes were first made strikingly visible through the use of dyes that stain specific subcellular structures. The deeply stained colored bodies (thus the name “chromosomes”) appeared in pairs and separated into two new daughter cells at cell division, correlating with Mendel’s observations that the particles of heredity come in pairs. Scientists eventually came to understand that DNA is the hereditary substance inside chromosomes (see Chapter 2).

The material of chromosomes, both protein and DNA, is often referred to as **chromatin**. The protein component is about equal in mass to the DNA component. Some chromatin proteins are SMC proteins (see Chapter 9), topoisomerases, and transcription regulatory molecules; however, histones constitute the largest protein component of chromatin. **Histones** are highly conserved, basic proteins that assemble into octameric complexes containing two each of four different histone subunits. DNA wraps around the histones to form condensed **nucleosomes**. Beginning with nucleosomes, eukaryotic chromosomal DNA is packaged into a succession of higher-order structures that ultimately yield the compact chromosome seen under the light microscope.

Histone Octamers Organize DNA into Repeating Units

The first evidence that DNA is packaged into regularly organized units came from studies in which chromosomal DNA was treated with a nonspecific DNA nuclease, such as micrococcal nuclease, that cuts DNA wherever it is not associated with proteins. The digested DNA fragments were then analyzed for size in an agarose gel. If DNA is packaged by proteins into units of a particular size, the nuclease would cleave the DNA between these units, and the protected DNA segments would migrate in the gel as a ladder of unit-sized bands. If there were no regular repeating unit of protein-DNA packaging, protein would be distributed on DNA in a random way, and nuclease digestion would produce a smear of DNA fragments with no regular pattern. The results of such experiments revealed a series of regularly spaced DNA bands about 200 bp apart, indicating that DNA is packaged by proteins into units that encompass approximately 200 bp (Figure 10-1).

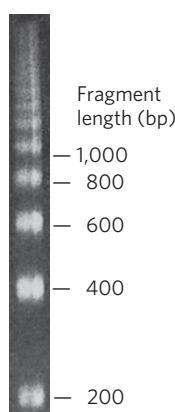


FIGURE 10-1 The earliest evidence of DNA packaging.

Carefully isolated chromatin was treated with micrococcal nuclease and analyzed by agarose gel electrophoresis. The result was a DNA ladder of fragments that differed in length by 200 bp, suggesting that DNA packaging involves a repeat unit of 200 bp. [Source: Roger Kornberg, MRC Laboratory of Molecular Biology.]

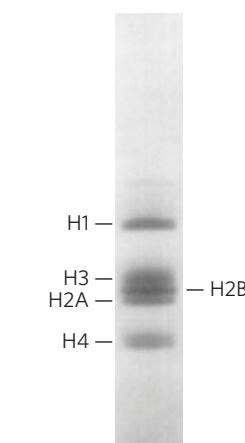


FIGURE 10-2 Histone representation in nucleosomes.

Histones within nucleosomes were separated by SDS-polyacrylamide gel electrophoresis. Measurement of the band intensity showed histones H2A, H2B, H3, and H4 present in equal stoichiometry, and histone H1 at about half the level of the other histones. [Source: S. Panyim and R. Chalkley, *Archiv. Biochem. Biophys.* 130:337–346, 1969.]

When the protein-DNA units, referred to as nucleosomes, were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), four histone proteins (designated H2A, H2B, H3, and H4) were found in approximately equimolar ratios (Figure 10-2). A fifth histone (H1) was present in about half the amount relative to the other four histones. The five histones have molecular weights (M_r) between 11,000 and 21,000. Histones are rich in the basic amino acids arginine and lysine, which together make up about 25% of the amino acid residues in any given histone protein (Table 10-1). Histone proteins are highly conserved among eukaryotic cells. Histones H3 and H4 are nearly identical in all eukaryotes, suggesting strict conservation of their functions. For example, only 2 of the 102 residues

differ between the H4 histones of peas and cows, and only 8 residues differ between the H4 histones of humans and yeast. Histones H1, H2A, and H2B show less sequence similarity, but on the whole, they are more conserved than other types of proteins. Eukaryotes also have several variant forms of certain histones, notably histones H2A and H3, which, as we'll see later in the chapter, have specialized roles in DNA metabolism.

To understand how the histones are organized within the nucleosome, the native state of the nucleosome unit must be preserved. Early studies of nucleosomes used denaturing methods of extraction that disrupted their native state. Later, by extracting chromatin with mild salt solutions (2 M sodium chloride

Table 10-1 Types and Properties of Histones

Histone	M_r	Number of Amino Acid Residues	Content of Basic Amino Acids (% of total)	
			Lys	Arg
H1	21,130	223	29.5	1.3
H2A	13,960	129	10.9	9.3
H2B	13,774	125	16.0	6.4
H3	15,273	135	9.6	13.3
H4	11,236	102	10.8	13.7

Note: For H1, H2A, and H2B, sizes vary from species to species; the data here are for bovine histones, except H1, which is from rabbit.

and 50 mM sodium acetate), researchers kept nucleosomes intact and could investigate the composition and organization of the nucleosome unit.

Some of the key studies involved protein **cross-linking**. In this technique, a chemical with two reactive groups is used to react with a protein complex. Because the chemical has two reactive groups it can covalently attach to two proteins, but because the chemical is a small molecule, it can only react with two proteins that are close together. Thus, identification of the cross-linked proteins reveals which proteins are next to each other in an oligomer. Based on the findings from these procedures, Roger Kornberg proposed how histones are organized within the nucleosome (see How We Know).

Kornberg suggested that most of the 200 bp of DNA in a protein-DNA unit is wrapped around a **histone octamer** composed of two copies each of histones H2A, H2B, H3, and H4. These four histones have come to be known as the **core histones** (Figure 10-3a). The remainder of the DNA serves as a linker between nucleosomes, to which histone H1 binds. Visualization of nucleosomes in the electron microscope was consistent with the histone octamer hypothesis, revealing a structure in which the DNA is bound tightly to beads of protein, often

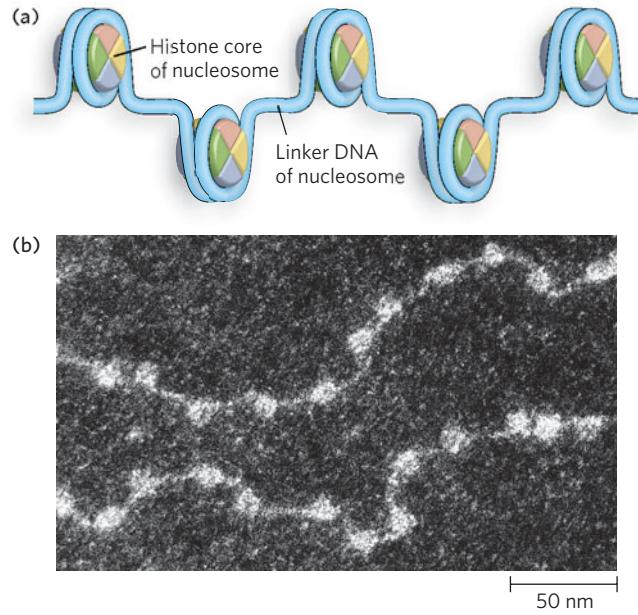


FIGURE 10-3 Nucleosomes as beads on a string.

(a) Regularly spaced nucleosomes consist of core histone proteins bound to DNA. (b) In this electron micrograph, the DNA-wrapped histone octamers are clearly visible, with linker DNA between them. [Source: (b) Ada L. Olins and Donald E. Olins, Oak Ridge National Laboratory.]

regularly spaced like beads on a string (Figure 10-3b). One bead plus the connecting (linker) DNA that leads to the next bead forms the nucleosome unit.

Under physiological conditions, formation of the histone octamer from individual histone proteins requires the presence of DNA. In the absence of DNA, the highly conserved H3 and H4 subunits form a tightly associated heterotetramer that contains two of each subunit, and the less conserved H2A and H2B subunits form a heterodimer. Without DNA, these components do not assemble into a histone octamer, unless incubated under nonphysiological conditions at high salt concentrations. In the presence of DNA, however, two H2A-H2B heterodimers assemble with one H3-H4 heterotetramer and the DNA to form the nucleosome.

DNA Wraps Nearly Twice around a Single Histone Octamer

The crystal structure of a histone octamer in a complex with 146 bp of DNA reveals the detailed architecture of a nucleosome particle (Figure 10-4). The most striking

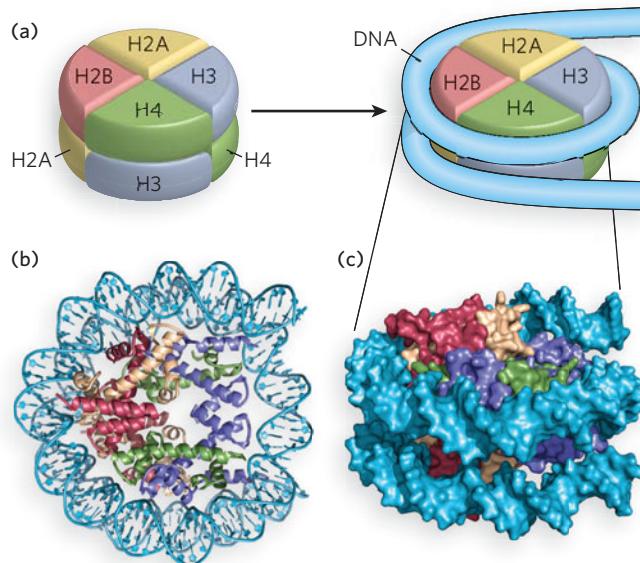


FIGURE 10-4 Crystal structure of the nucleosome. (a) The simplified structure of a nucleosome octamer (left), with DNA wrapped around the histone core (right). (b) A ribbon representation of the structure of the nucleosome from the African frog *Xenopus laevis*, with different colors representing the five histones, matching the colors in (a). (c) Surface representation of the nucleosome. The view in (c) is rotated relative to the view in (b), to match the orientation shown above, in (a). A 146 bp segment of DNA binds in a left-handed solenoidal supercoil that circumnavigates the histone complex 1.67 times. [Source: (b) PDB ID 1AOI.]

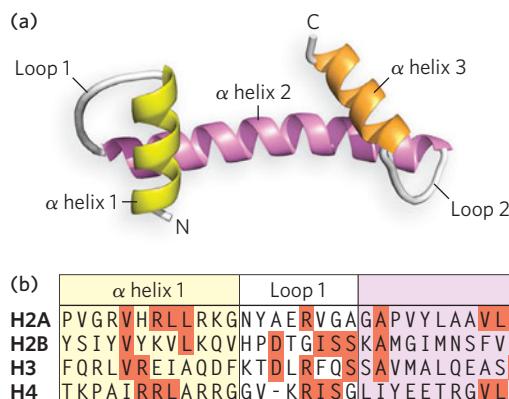


FIGURE 10-5 The histone-fold motif. (a) This internal structure in each of the core histones is formed from three α helices connected by two loops. (b) The amino acid sequences of the histone fold of the four core histones. Residues that are identical among the different histone subunits are shaded in red. [Sources: (a) PDB ID 1AOI. (b) Adapted from V. Ramakrishnan, *Annu. Rev. Biophys. Biomol. Struct.* 26:83–112, 1997.]

feature is the tight wrapping of DNA around the octamer in the form of a left-handed solenoidal supercoil. Overall, the supercoil arrangement of DNA on the nucleosome compacts the DNA six- to sevenfold. The DNA is not uniformly bent, but instead follows a pattern of relatively straight 10 bp segments joined by bends.

Each histone contains a **histone-fold motif**, three α helices linked by two short loops (Figure 10-5). The elemental structural unit of the nucleosome is a head-to-tail dimer of histone-fold motifs of either the H3-H4 pair or the H2A-H2B pair. Each histone-fold dimer forms a V-shaped structure that contains three DNA-binding sites (Figure 10-6a). The octamer structure shows that the connections between each dimer (the two H3-H4 pairs and two H2A-H2B pairs) of the four core histones are also mediated mainly by the highly conserved histone fold (Figure 10-6b).

The contacts between histones and DNA are mainly between the conserved histone fold and the phosphodiester backbone or minor groove of the DNA, in keeping with the relatively nonspecific binding of nucleosomes to DNA. Approximately half of the 142 hydrogen bonds of the histone fold occur between the DNA and peptide backbone atoms, rather than amino acid side chains. This seems counterintuitive, given the many basic side chains of histones. A possible explanation is that amino acid side chains are not as rigidly held in place as is the peptide backbone, and therefore hydrogen bonding of DNA to peptide backbone atoms more firmly secures the DNA to the protein. The basic histone side chains are needed to neutralize the negative charge of the DNA's phosphodiester backbone. Charge neutralization is important in DNA condensation, especially in the further stages of compaction.

The average DNA twist when wrapped around the histone octamer is 10.2 bp per turn, compared with the 10.5 bp per turn of unrestricted DNA, and therefore the DNA structure must adapt to the octamer. This

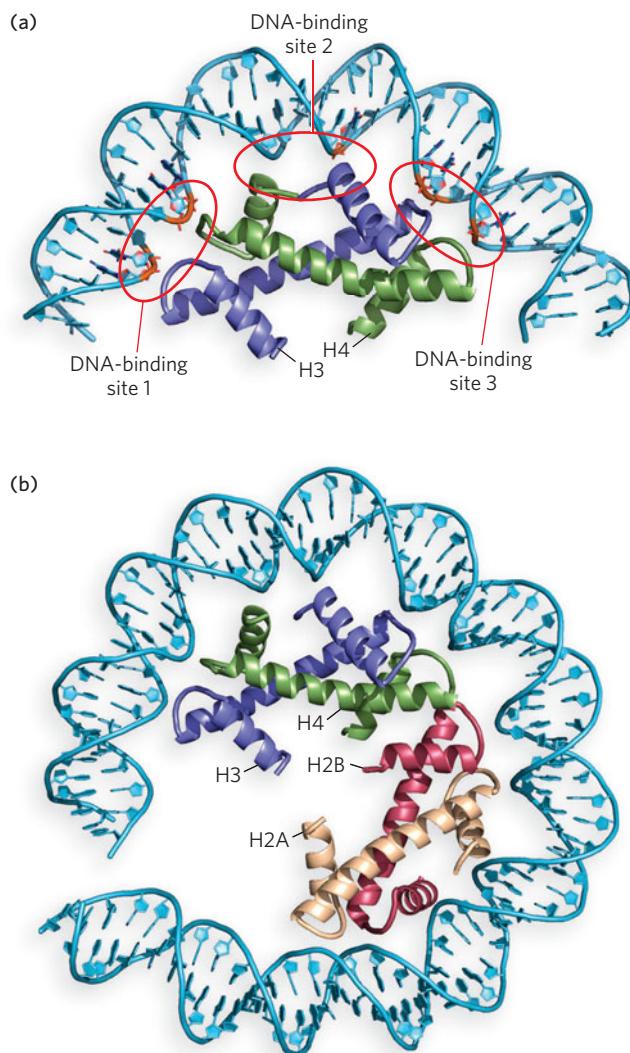


FIGURE 10-6 The histone-fold dimer. (a) The figure shows a ribbon representation of the histone folds of only one pair of H3-H4 subunits within the octamer, bound to DNA. DNA is in contact with the three DNA-binding sites formed by the two histone folds (i.e., the histone-fold dimer). (b) One face (one half) of the nucleosome contains two histone-fold dimers. [Source: PDB ID 1AOI.]

overtwisting of DNA results in a narrowing of the minor groove. The change in shape of the DNA as it binds the histone octamer through bending and overtwisting implies that the octamer is more likely to bind DNA sequences that readily conform to such changes. For example, a local abundance of A=T base pairs in the minor groove of a DNA helix, where it is in contact with the histones, facilitates the compression of the minor groove that is needed for tight wrapping of DNA around the histone octamer (Figure 10-7). In fact, histone octamers assemble particularly well with sequences where two or more A=T bp are staggered at 10 bp intervals, because DNA is naturally bent at these sequences, and when two or more consecutive A=T bp are spaced along the same face of the helix, the DNA bends into a circle. Tracts of G≡C bp have the opposite effect, preventing compression of the minor groove, and thus are preferred at positions not facing nucleosomes.

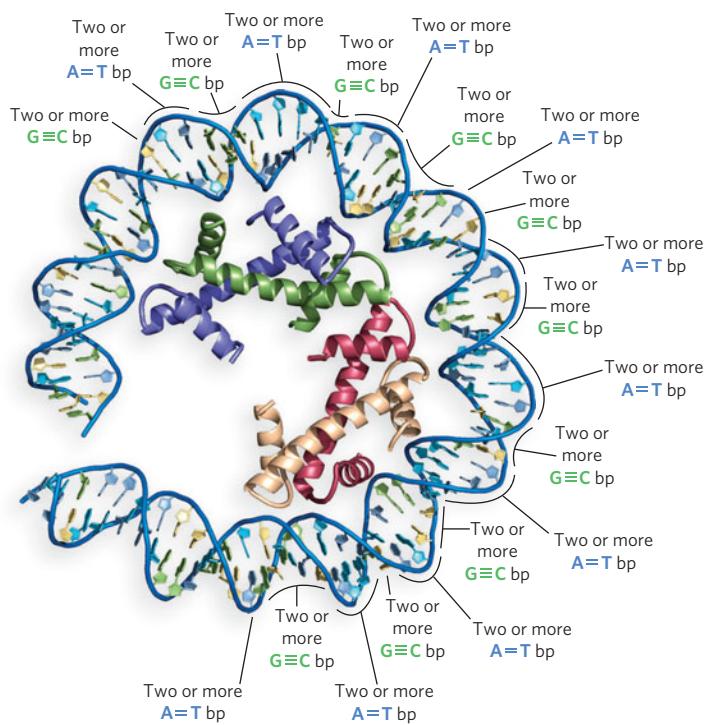


FIGURE 10-7 The effect of DNA sequence on nucleosome binding. Runs of two or more adjacent A=T base pairs facilitate the bending of DNA, whereas runs of two or more G≡C base pairs have the opposite effect. When spaced at about 10 bp intervals, consecutive A=T base pairs help bend DNA into a circle. When consecutive G≡C base pairs are spaced 10 bp apart, and offset by 5 bp from runs of A=T base pairs, the DNA binds tightly to the nucleosome.

[Source: PDB ID 1AOI.]

The left-handed solenoidal supercoil of the 146 bp duplex that winds almost twice around the nucleosome reveals why eukaryotic DNA is underwound, even though eukaryotic cells lack topoisomerases that underwind DNA. Recall that the solenoidal wrapping of DNA is but one form of supercoiling that can be taken up by underwound (negatively supercoiled) DNA (see Chapter 9). The tight wrapping of DNA around the histone core requires the removal of about one helical turn in the DNA. When the histone core of a nucleosome binds *in vitro* to a relaxed, closed-circular DNA, the binding introduces a negative supercoil. Because binding in this fashion does not break DNA or change the linking number, formation of the negative supercoil around the histones must be accompanied by a compensatory positive supercoil in the unbound region of the DNA (see Figure 9-22). As described in Chapter 9, eukaryotic topoisomerases can relax positive supercoils and are required for the assembly of chromatin from purified histones and closed-circular DNA *in vitro*. Relaxing the unbound positive supercoil leaves the negative supercoil fixed, through its binding to the nucleosome histone core. Overall, this results in a decrease in linking number.

Histone Tails Mediate Internucleosome Connections That Regulate the Accessibility of DNA

Most of the mass in the histone octamer forms a tightly packed particle, but the N-termini of the histones protrude from the core particle and are less ordered (Figure 10-8). These N-terminal **histone tails** are flexible and therefore mostly disordered in the crystal structure. The parts of the histone tails that are visible in the electron microscope appear as irregular chains extending outward from the nucleosome disk. The tails exit the DNA superhelix through channels formed by the alignment of minor grooves of adjacent DNA helices every 20 bp. The histone tails do not contribute much strength to DNA binding, but they form intermolecular contacts with adjacent nucleosome particles and organize nucleosomes into a higher-order chromatin structure (Figure 10-9).

The histone tails are at the heart of the dynamic regulation of chromatin structure, because they are the target of numerous chemical modifications that control the access of regulatory proteins to DNA. These modifications affect the net electrical charge, shape, and other properties of histones, which in turn affect the structural and functional properties of chromatin. Histone tails can also be recognized by particular enzymes. As we'll see, these modifications play important roles in the regulation of transcription, replication, and repair.

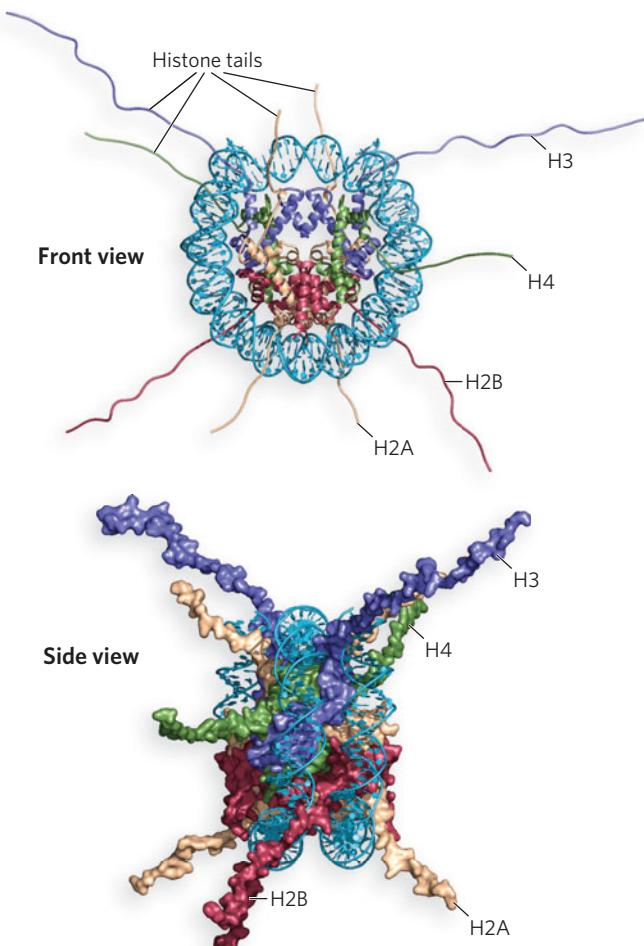


FIGURE 10-8 Histone tails. Two views of histone N-terminal tails protruding from between the two DNA duplexes that supercoil around the nucleosome. (a) Ribbon representation, looking at the circular face of the nucleosome. (b) Space-filling representation of the nucleosome as viewed from the side. Some tails pass between the supercoils through holes formed by alignment of the minor grooves of adjacent helices. The H3 and H2B tails emerge between the DNA wrapped around the histone; the H4 and H2A tails emerge between adjacent histone cores. [Source: PDB ID 1AOI.]

Histone tail modifications alter the molecular interactions between adjacent nucleosomes, thereby changing the level of chromatin compaction and thus the access of enzymes to the DNA in the altered chromatin structure. The tighter the internucleosome connections mediated by the histone tails, the less accessible is the DNA to transcription factors and other proteins. Histone H1 also plays a role in DNA sequestration. Histone H1 is not as extensively modified by chemical groups as the core histones, but it facilitates the general repressive

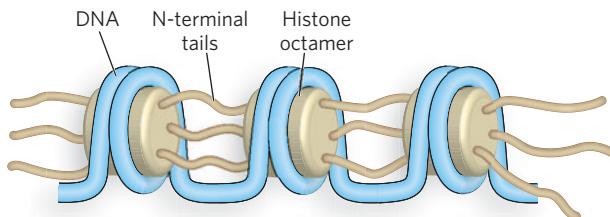


FIGURE 10-9 Internucleosomal contacts through N-terminal tails. The N-terminal tails of the histones of one nucleosome extrude from the particle and interact with adjacent nucleosomes, resulting in higher-order DNA packaging.

effect of histones on transcription. This effect can be demonstrated experimentally in an *in vitro* assay that uses a transcription extract, a cellular extract prepared such that transcription can proceed when DNA templates are added. As shown in Figure 10-10, a plasmid DNA containing the *kruppel* gene of *Drosophila melanogaster* is transcribed in the absence (lanes 1 and 2) or presence (lanes 3 to 10) of histones. Adding the core histone proteins results in decreased RNA synthesis (compare lanes 1 and 3). Adding the linker histone H1 also has a profound effect. As histone H1 is titrated into

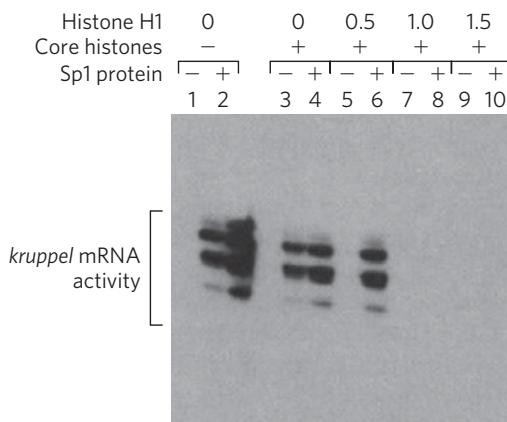


FIGURE 10-10 The repression of transcription by histones. An *in vitro* transcription extract is used to monitor transcription from a promoter in the presence or absence of histone proteins and the transcription activator Sp1. The first two lanes are controls in the absence of histones: transcription is optimal and Sp1 stimulates. Lanes 3 and 4 are also controls, showing results with core histones present but no H1. In lanes 5 to 10, histone H1 was added in concentrations of 0.5, 1.0, or 1.5 H1 molecules per nucleosome. Sp1 prevents gene repression by binding specific sequences in the promoter. See text for details. [Source: P. J. Laybourne and J. T. Kadonaga, *Science* 254:238–245, 1991, Fig. 7B.]

the assay (as shown along the top of Figure 10-10), transcription is diminished even further (compare lanes 1, 3, and 5). This indicates that H1 further compacts the chromatin and represses transcription.

Transcription factors that bind specific sites on DNA can modulate the repressive effect of histones on transcription. The site-specific DNA-binding protein Sp1, which enhances transcription, protects against histone-mediated transcription repression (compare lanes 5 and 6). One may infer that Sp1 binds DNA and blocks the binding of histones to DNA in its immediate vicinity. However, Sp1 is not effective in the presence of higher levels of H1 (compare lanes 6, 8, and 10).

SECTION 10.1 SUMMARY

- Chromatin is the sum of the protein and nucleic acid that comprise the material of chromosomes.
- The basic unit of DNA packaging is the nucleosome, composed of about 200 bp of DNA wrapped around a histone octamer.
- The histone octamer contains a tetramer of histones H3-H4 and two dimers of histones H2A-H2B. The amino acid sequences of the histones are highly conserved among eukaryotes.
- Nucleosomes are linked together by DNA with one bound molecule of H1. The spacing of nucleosomes along DNA results in a beads-on-a-string appearance in the electron microscope.
- DNA is wrapped around the histone octamer in a left-handed solenoidal supercoil, making almost two turns. Histones contain a conserved histone-fold motif, which makes most of the contacts with the DNA, mainly with the phosphodiester backbone and minor groove.
- N-terminal histone tails are flexible and can be modified to affect DNA transcription. These modifications probably alter the molecular interactions of adjacent nucleosomes, thereby changing the access of enzymes to DNA in the altered chromatin structure.

10.2 Higher-Order Chromosome Structure

As we've seen, the nucleosome compacts DNA six- to sevenfold, yet condensing a 2 m thread of DNA into a 5 μm diameter nucleus ultimately requires a 10,000-fold reduction in length. This remarkable level of condensation is achieved in stages, none of which, unfortunately,

are defined at high resolution, as is the DNA wrapping in the nucleosome. Here we explore what is known about these processes of higher-level DNA packaging and some of the current hypotheses that explain them.

Histone H1 Binds the Nucleosome to Form the Chromatosome

Treating chromatin with a nonspecific nuclease to digest all naked DNA—DNA not protected by protein—yields a segment of about 168 bp to which all five histones are bound, the four core histones plus histone H1. This structure is called a **chromatosome**. More extensive nuclease digestion releases H1, leaving the core histone octamer bound to about 147 bp of DNA (i.e., the nucleosome). Indeed, the addition of H1 to a nucleosome results in protection of an additional 20 to 22 bp of linker DNA adjacent to the nucleosome, and thus H1 is often referred to as the **linker histone**. Only one H1 subunit is present per chromatosome, unlike the core histones, which are present in two copies each.

H1 consists of a short, 20 to 35 residue N-terminal region, a central globular domain of about 80 amino acid residues, and a long C-terminal region of about 100 residues. DNA binding is intrinsic to the central globular region, which contains two DNA-binding sites. It was originally thought that the two DNA-binding sites in H1 were used to bind each of the two linker DNA strands at the sites where DNA enters and exits the nucleosome. However, more recent studies indicate that H1 binds only one of the linker DNA strands, and the second DNA site in histone H1 binds to the central region of the DNA supercoil in the nucleosome (Figure 10-11).

By binding an additional 20 bp of DNA, histone H1 alters the DNA entry and exit angles, facilitating the packing of DNA into higher-order chromatin structures

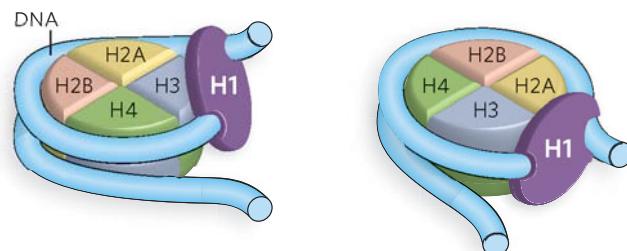


FIGURE 10-11 The binding of DNA by histone H1. Two views of the nucleosome containing histone H1 are shown. H1 has two DNA-binding sites, through which it makes contact with one arm of linker DNA and the central region of the DNA wrapped around the histone octamer.

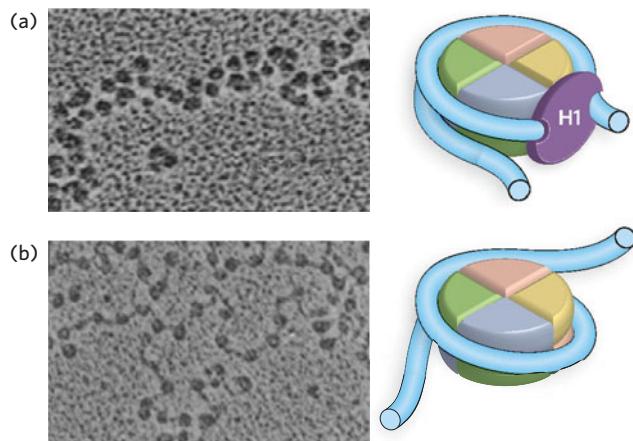


FIGURE 10-12 The zigzag appearance of nucleosomes in the presence of histone H1. Electron micrographs of nucleosomes (a) in the presence of H1 and (b) in the absence of H1. Histone H1 increases the zigzag appearance by decreasing the DNA entry/exit angles, as shown in the cartoon models to the right of each micrograph. Scale bar = 1,000 Å. [Source: Photos © F. Thoma et al., 1979. Originally published in *The Journal of Cell Biology* 83:403–427.]

(Figure 10-12). When H1 is extracted from chromatin, DNA seems to enter and exit nucleosomes at different places, thus creating the beads-on-a-string appearance (see Figure 10-3b). The presence of H1 causes DNA to enter and exit the nucleosome at nearly the same place, resulting in a zigzag pattern with a DNA entry/exit angle between 40° and 100°, depending on the conditions of sample preparation. Overall, the level of condensation provided by H1 is six to seven times that of the nucleosome, for a total length reduction of 35- to 40-fold. H1 helps nucleosomes condense into a higher level of packaging, the 30 nm filament (described below).

Nucleosomes generally repress transcription by sterically preventing the access of regulatory proteins to promoter sequences, and H1 participates in this activity by stabilizing nucleosomes on the DNA and promoting higher-order chromatin structure that further compacts nucleosomal DNA (see Figure 10-10). Indeed, most regions of actively transcribed DNA are known to be devoid of histone H1. Compared with the core histones, H1 is more variable in sequence. Most organisms even have multiple H1 subtypes. For example, mammals have eight H1 variants that differ in their ability to condense chromatin. The H1 subtypes are expressed at different times of development or are present in different cell types. The avian counterpart of H1 is known as H5.

Chromosomes Condense into a Compact Chromatin Filament

Under certain experimental conditions, such as increased ionic strength or the presence of particular divalent cations, nucleosomes condense into a compact filament with a width of about 30 nm, referred to as the **30 nm filament** (Figure 10-13a). Although histone H1 promotes condensation into the 30 nm filament, it is not essential for forming the filament. In contrast, the N-terminal tails of the core histones are absolutely required, suggesting that the tails provide important nucleosome-nucleosome contacts needed for 30 nm filament formation. Recall that the crystal structure of the nucleosome shows that the terminal tails of H4, H3, and H2A make contact with adjacent nucleosomes, and these contacts may be involved in the condensation of nucleosomes into a filament.

The exact arrangement of nucleosomes in a 30 nm filament is still unclear, although two main models prevail. Any model should accommodate the following observations: (1) neutron diffraction studies place histone H1 in the center of the filament, (2) linker DNA should also be placed in the center of the filament, because H1 binds linker DNA, and (3) electron microscopy and x-ray diffraction studies indicate that the nucleosome units in the 30 nm filament are arranged with a helical pitch of about 11 nm, the width of a nucleosome.

The two most widely accepted models for nucleosome arrangement in the 30 nm filament fit these criteria, and both are supported by substantial experimental evidence. The two models may, in fact, be alternative ways in which nucleosomes pack in different areas of the same 30 nm filament. In the **solenoid model** (also called the one-start helix model), the nucleosome array adopts a spiral shape, in which the flat sides of adjacent nucleosome disks are next to each other (Figure 10-13b). The linker DNA is presumed to bend inside the center of the filament to account for the observed constant thickness of the fiber with different linker lengths.

The second model for the 30 nm filament is the **zigzag model** (also called the two-start helix model), in which zigzag histone pairs stack on each other and twist about a central axis (Figure 10-13c). The zigzag model was inspired by the zigzag appearance of chromatin under the electron microscope (see Figure 10-12). This model is supported by the crystal structure of a tetranucleosome, in which four nucleosomes are bound to one DNA molecule (Figure 10-14a). The structure shows two histone-pair zigzags stacked on top of each other, with the linker DNA passing through the central axis connecting two nucleosomes on opposite sides of the filament. A zigzag model of a chromatin filament of

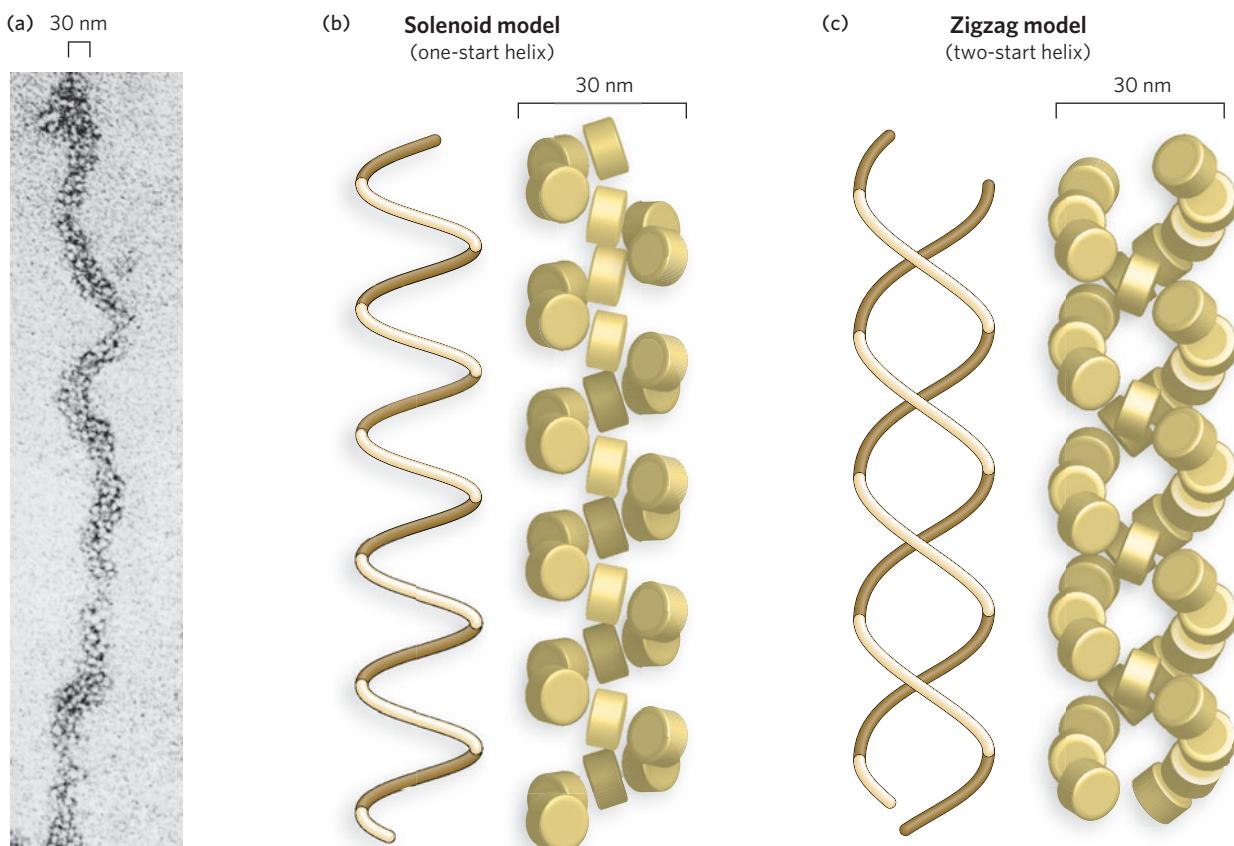


FIGURE 10-13 The 30 nm filament, a higher-order organization of nucleosomes. The compact filament is formed by the tight packing of nucleosomes. (a) The 30 nm filament as seen by electron microscopy. Two proposed models of filament structure are (b) the solenoid model and

(c) the zigzag model. DNA is not shown, for clarity of histone visualization. The two models are described more fully in the text. [Source: (a) Barbara Hamkalo, Department of Molecular Biology and Biochemistry University of California, Irvine.]

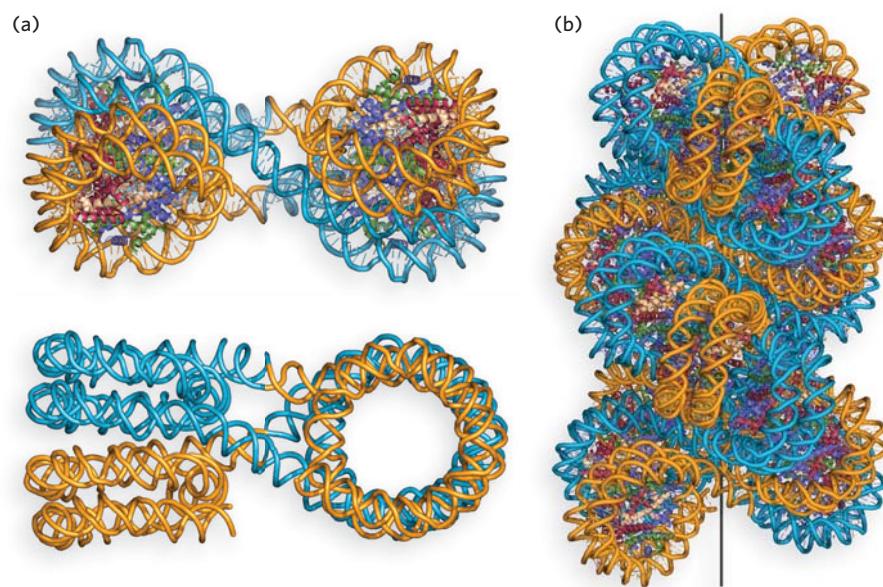


FIGURE 10-14 The crystal structure of a tetranucleosome. (a) The four nucleosomes are arranged in two zigzag pairs that stack on top of each other. Shown here are the DNA and histone octamers (top) and the DNA only (bottom), in orientations that illustrate the geometry of the two stacks. (b) A model of the 30 nm filament made by repeating the tetranucleosome in a continuous zigzag configuration. [Source: Adapted from T. Schalch et al., *Nature* 436:138, 2005, Figs. 1, 3.]

many nucleosomes, based on the tetranucleosome structure, is shown in **Figure 10-14b**.

The two models for nucleosome stacking in a 30 nm filament are fundamentally very different, so it might seem they would be easy to distinguish experimentally. The inability of investigators to resolve this issue reflects the many irregularities in natural chromatin fibers, leading to poor-quality experimental data. However, as noted above, both types of nucleosome packing might occur in the same chromatin filament.

Higher-Order Chromosome Structure Involves Loops and Coils

Inside chromosomes, DNA is much more highly condensed than in the 30 nm filament. Treating chromosomes with a low-salt buffer causes them to expand, and the edges of these swollen chromosomes reveal 30 nm filaments that appear to be organized in loops estimated at 40 to 100 kbp long (**Figure 10-15a**). The existence of loops of DNA as a substructure within chromosomes is also supported by electron microscopic studies of histone-depleted chromosomes. Histones can be selectively extracted from chromosomes by treatment with negatively charged polymeric chemicals, such as heparin and dextran sulfate, which compete with histones for binding DNA. After histone extraction, a proteinaceous residue remains that retains the size and shape of the original chromosome. This residue is called the **chromosomal scaffold** (**Figure 10-15b**). One of the major components of the chromosomal scaffold is SMC proteins that hold DNA

strands together, keeping eukaryotic chromosomes topologically constrained (see Figure 9-11). The histone-depleted DNA can be seen to form large loops anchored in the scaffold (**Figure 10-15c**), which is consistent with DNA looping as a higher-order arrangement of DNA in chromosomes.

Although very little is known about further steps of DNA condensation beyond the 30 nm filament, there are probably several more layers of organization in eukaryotic chromosomes, each dramatically enhancing the degree of compaction. Many speculative models have been proposed, one of which is shown in **Figure 10-16**. In this model, loops of the 30 nm filament are connected to the scaffold in a radial fashion. One radial turn forms a rosette composed of six loops, held together by SMC proteins (see Figure 9-27). A spiral of 30 rosettes per turn forms a coil, and a chromosome consists of several coils.

In reality, the higher-order structure of chromatin probably varies from chromosome to chromosome, from one region to the next in a single chromosome, and from moment to moment in the life of a cell. No single model can adequately describe these structures. Nevertheless, the principle is clear: DNA condensation in eukaryotic chromosomes probably involves coils upon coils upon coils.

Bacterial DNA, Like Eukaryotic DNA, Is Highly Organized

Bacterial DNA is compacted in a structure called the **nucleoid**, which can occupy a significant fraction of the

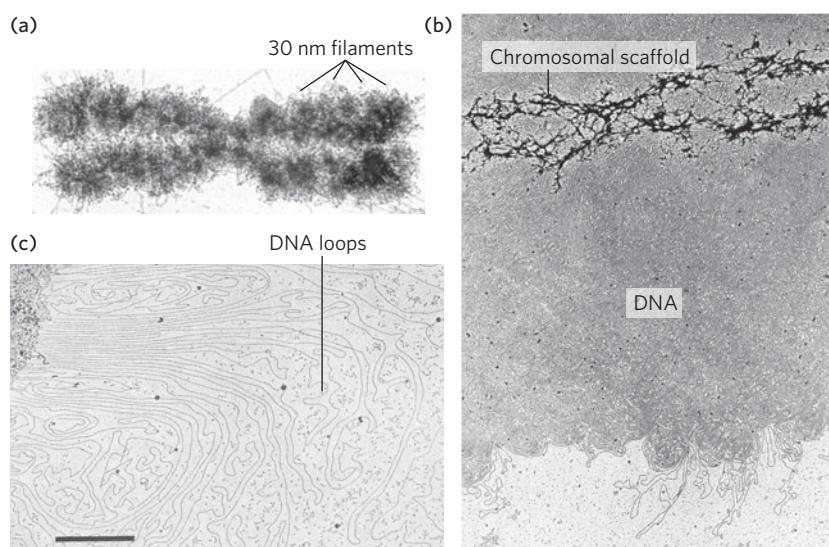


FIGURE 10-15 Loops of DNA attached to a chromosomal scaffold. (a) A swollen chromosome, produced in a buffer of low ionic strength, as seen in the electron microscope. Notice the appearance of 30 nm filaments (chromatin loops) at the margins. (b) Extraction of the histones leaves a proteinaceous chromosomal scaffold surrounded by naked DNA. (c) The DNA appears to be organized in loops attached at their base to the scaffold in the upper left corner. Scale bar = 1 μm . The three images are at different magnifications. [Sources: (a) G. F. Bahr/Biological Photo Service. (b) and (c) U. K. Laemmli et al., *Cold Spring Harb. Symp. Quant. Biol.* 42:351, 1977.]

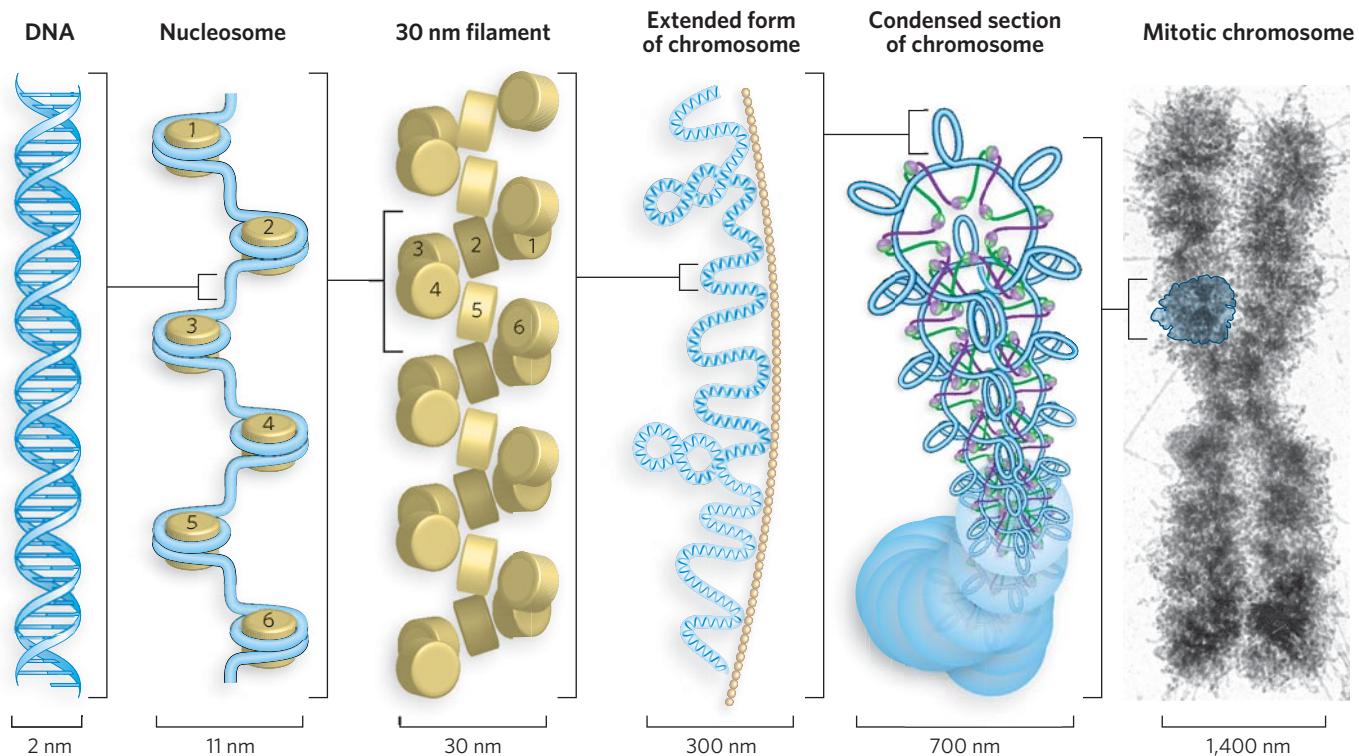


FIGURE 10-16 Higher-order DNA compaction in a eukaryotic chromosome. This model shows the levels of organization that could provide the observed degree of DNA compaction in the chromosomes of eukaryotes. First the DNA is wrapped around histone octamers, then H1 stimulates formation of the 30 nm filament. Further levels of organization are not well understood but seem to involve

further coiling and loops in the form of rosettes, which also coil into thicker structures. Overall, progressive levels of organization take the form of coils upon coils upon coils. It should be noted that in cells, the higher-order structures (above the 30 nm filament) are unlikely to be as uniform as depicted here. [Source: Photo from G. F. Bahr/Biological Photo Service.]

cell volume (Figure 10-17a). The DNA seems to be attached at one or more points to the inner surface of the cytoplasmic (plasma) membrane. Much less is known about the structure of the nucleoid than of eukaryotic chromatin. Bacteria contain SMC proteins, and studies in *E. coli* reveal a scaffoldlike structure that seems to organize the circular chromosome into a series of about 500 looped domains, each encompassing 10 kbp, on average (Figure 10-17b). Like the looped domains in eukaryotic chromosomes, the looped DNA domains in the bacterial chromosome are topologically constrained. For example, if the DNA is cleaved in one domain, only the DNA in that domain becomes relaxed. However, bacterial DNA does not seem to have any structure comparable to the local organization provided by nucleosomes in eukaryotes.

Although bacteria lack nucleosomes, they do contain abundant histonelike proteins. A well-studied example is the two-subunit protein HU (M_r 19,000). Bacterial histonelike proteins do not seem to form stable oligomeric structures, and this may reflect the need

for bacteria to respond very rapidly to their environment and thus to have more ready access to their genetic information. Consider, for example, that the bacterial cell division cycle can be as short as 15 minutes, whereas a typical eukaryotic cell may not divide for hours or even months. In addition, a much greater fraction of bacterial DNA than eukaryotic DNA is used to encode protein or functional RNA. Furthermore, higher rates of cellular metabolism in bacteria mean that a much larger proportion of the DNA is being transcribed or replicated at a given time than in most eukaryotic cells.

SECTION 10.2 SUMMARY

- The histone octamer and associated DNA that form the nucleosome combine with histone H1 to form the chromatosome. H1 binds additional DNA, altering the entry and exit angles of the DNA so that the chromatosomes pack in a zigzag pattern.

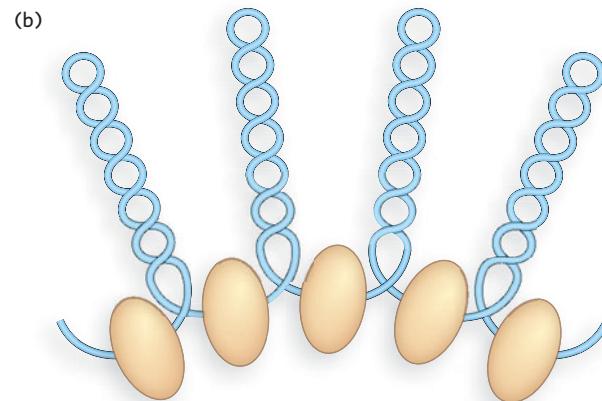
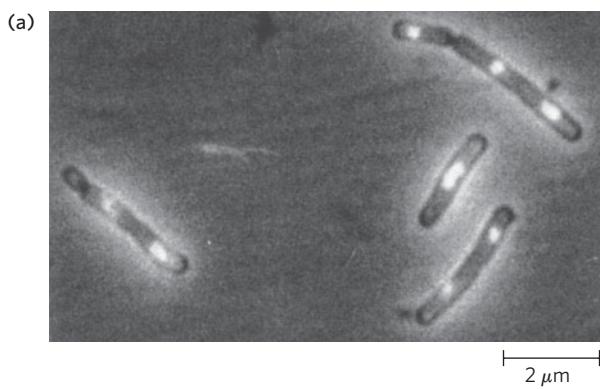


FIGURE 10-17 Highly condensed bacterial DNA. (a) The DNA is stained with a dye that fluoresces when exposed to UV light. The light areas define the nucleoids. Notice that some cells have replicated their DNA but have not yet

undergone cell division and hence have multiple nucleoids. (b) Looped domains of DNA in the bacterial chromosome are separated by points of connection to the scaffold, shown as beige ovoids.

- Two models describe how nucleosomes might pack into a 30 nm filament. In the solenoid model, nucleosome disks are next to one another, forming a spiral shape. In the zigzag model, pairs of nucleosomes stack upon each other and twist about a central axis.
- Higher-order chromatin structure is largely undetermined, but most likely involves loops that form topologically constrained domains. Looped domains form rosettes, and these form coils. Chromosome shape is determined by a rigid proteinaceous scaffolding containing SMC proteins, among other components.
- Bacteria lack nucleosomes, but the chromosomal DNA is compacted into looped domains.

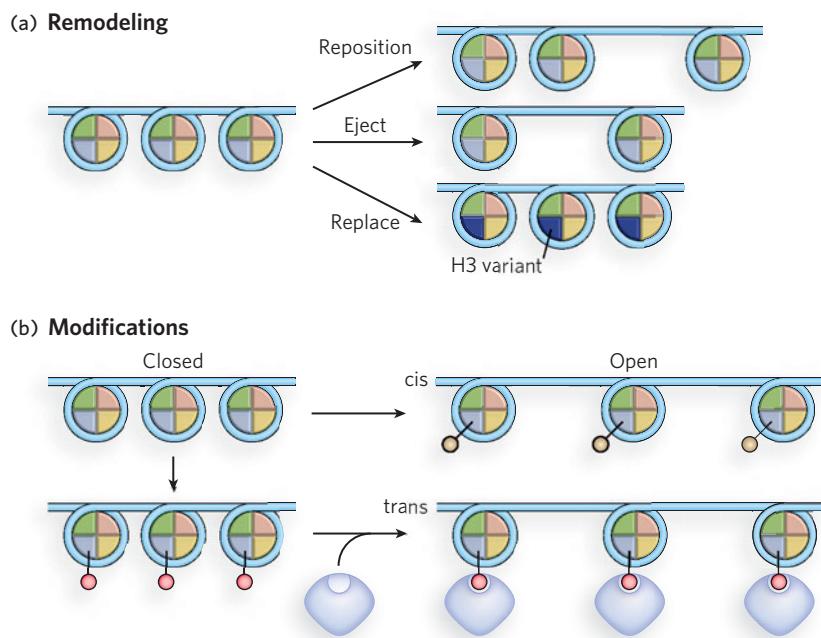
10.3 The Regulation of Chromosome Structure

Nucleosomes control the accessibility of DNA to decoding proteins such as RNA polymerase and to transcription activators and repressors. For example, nucleosome arrangements that lead to a more “open” chromatin state are associated with active gene transcription, whereas a more “closed” chromatin state typically represses transcription. The arrangement of nucleosomes on DNA is regulated by two main classes of enzymes, whose activity usually depends on other functional subunits within a multiprotein complex.

One class of proteins that alter nucleosome arrangement comprise the **chromatin remodeling complexes**,

which slide the nucleosome to a different location, or eject it from the DNA, or replace it with a new nucleosome that contains a variant histone subunit (Figure 10-18a). Variant histone subunits impart special properties to the chromatin. The other class is the **histone modifying enzymes**, which covalently modify the N-terminal tails of histones (Figure 10-18b). Histone modifications that affect chromatin structure solely through the modification itself are referred to as *cis*-acting. A *cis*-acting histone modification may result in opening or closing of the chromatin by tightening or loosening of the arrangement of nucleosomes along the DNA. Histone modifications that are *trans*-acting attract other proteins, such as transcription factors or chromatin remodeling factors, that produce the chromatin change.

Enzymes that modify histones are often contained in a multiprotein complex, along with transcription activators or repressors. Nucleosome modifying enzymes and chromatin remodeling complexes can also work together to alter specific regions of chromatin in response to environmental signals. The control of DNA accessibility by altering the structure of nucleosome-DNA complexes is essential to all types of genetic processes, including gene expression, replication, repair, and recombination. Histone modifications are also inheritable, which is especially important during an organism’s development, when a cellular program to form a specific cell type or tissue must be propagated over numerous cell divisions. Inheritance of genetic properties that are not encoded in the DNA sequence is referred to as **epigenetic inheritance**. Histone variants, along with the many covalent modifications that histones undergo, define and stabilize the chromatin state in localized

**FIGURE 10-18** Modification of nucleosome arrangements.

(a) Nucleosome position can be altered by chromatin remodeling complexes that move, eject, or replace nucleosomes.

(b) Histone modifying enzymes attach chemical groups to specific amino acid residues of nucleosome subunits, as indicated by small circles attached to histones. If the outcome is a direct result of the modification, such as making the chromatin structure more open, the modification is acting in *cis*. If the modification attracts another protein (blue) that performs the histone modifying function, it is acting in *trans*.

regions throughout the genome. They mark the chromatin, facilitating or suppressing specific functions such as transcription, replication, DNA repair, and chromosome segregation.

Nucleosomes Are Intrinsically Dynamic

Given the numerous contacts between nucleosomes and DNA, and the high level of DNA compaction, it seems reasonable to imagine that the condensed DNA is no longer available for other proteins to bind. However, for promoters to become accessible, the chromatin structure within the promoter region must become accessible. Molecular interactions that hold nucleosome arrays together in the 30 nm filament are likely to be relatively weak, in which case regions within nucleosome arrays will be dynamic, with hydrogen bonds between the histones and DNA breaking and re-forming quite easily.

Clever experimental designs have allowed measurements of the force on the individual molecules in chromatin. Single chromatin fibers can be isolated and chemically modified at either end, and the two ends attached to different polystyrene beads (Figure 10-19a). The bead at one end is held in a laser optical trap, and the bead at the other end is captured by a micropipette. The force required to stretch the chromatin fiber and thus disrupt the nucleosome array can be measured by moving the optical trap with a known force. The stretching force on a chromatin fiber produces a structural transition at just 5 pN (piconewton) (Figure 10-19b)—

substantially less than the 10 to 15 pN needed to unzip double-stranded DNA, and much less than the 1,600 pN required to break a covalent bond. Once the chromatin fiber is stretched, the force can be relieved and the fiber then contracts to its original length, thus demonstrating that condensation of the nucleosome array is spontaneous and reversible.

ATP-Driven Chromatin Remodeling Complexes Can Reposition Nucleosomes

Although nucleosomes can bind almost any DNA sequence, the position of nucleosomes in the genome is not entirely random. Some DNA sequences, as noted earlier, are preferentially bound by nucleosomes, but nucleosome positioning is also dictated by chromatin remodeling complexes, which use the energy of ATP to move nucleosomes around on the DNA. Chromatin remodeling complexes consist of 2 to 17 subunits and can be divided into three main classes: SWI/SNF (switch-sniff, the first remodeling complex discovered), ISWI (imitation switch), and Mi2/NURD (Table 10-2). All chromatin remodeling complexes contain a conserved ATPase subunit and use the energy from ATP hydrolysis to disrupt the many contacts between nucleosomes and DNA, allowing nucleosomes to be ejected or repositioned on the DNA. Although we lack details about the mechanisms, all chromatin remodeling complexes can be thought of as mobilizing nucleosomes on DNA. The activity of chromatin remodeling complexes can result in gene activation or repression. In general, the

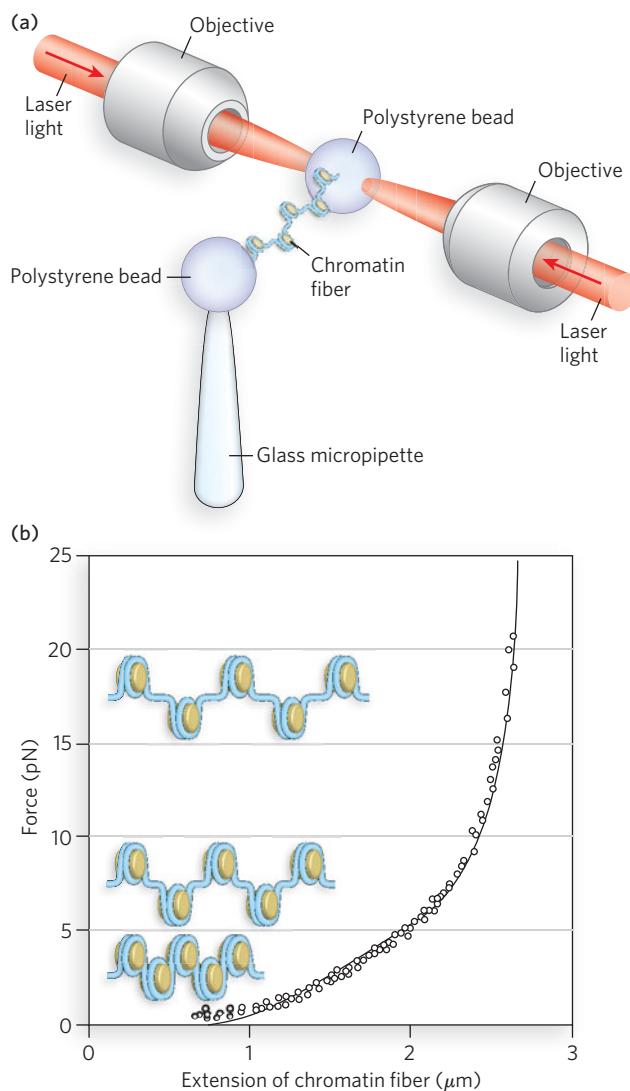


FIGURE 10-19 Weak internucleosomal interactions in chromatin revealed by optical trapping. (a) The experimental setup involves DNA packaged into chromatin, with a bead coupled to each end of the DNA. One bead is held in place with a micropipette, and the other is held by an optical trap formed by a focused laser beam. The optical trap can be moved to apply a specific force on the DNA, and the length to which the DNA is stretched is measured directly. (b) Fiber length is measured (from the known magnification) at different applied forces (in piconewtons), and the process can be reversed, showing that chromatin can stretch and snap back to its original structure. [Source: Adapted from Y. Cui and C. Bustamante, *Proc. Natl. Acad. Sci. USA* 97:127-132, 2000, Fig. 1.]

SWI/SNF class is associated with gene activation, and the ISWI and Mi2/NURD classes with gene repression.

The different chromatin remodeling complexes seem to have distinct mechanisms of action. Some mobilize nucleosomes by forming a DNA loop within the

nucleosome (Figure 10-20a). The loop is propagated around the histone octamer, with the net effect that the nucleosome slides to a new segment of the DNA. This can result in enhanced DNA accessibility—for example, by exposing a promoter that was previously occluded by the nucleosome. Alternatively, the same mechanism can reposition a nucleosome over a previously exposed promoter region, thereby repressing gene transcription. Likewise, repositioning nucleosomes into a regular array tends to condense the DNA, making it less accessible, whereas disrupting regularity in nucleosome arrays decondenses chromatin and thus facilitates transcription. Some chromatin remodeling complexes can also eject a histone octamer to generate a nucleosome-free region for transcriptional activation.

The mechanistic details of how chromatin remodeling complexes function are largely unknown, and this is an exciting area of research. The low-resolution structure of a remodeling complex of yeast, for example, reveals a complex containing a jaw sufficiently wide to accommodate an entire nucleosome (Figure 10-20b).

The position of nucleosomes within a genome can be determined through the use of large-scale array technologies, such as the ChIP-Seq and ChIP-Chip techniques (Figure 10-21a). Briefly, cells are treated with formaldehyde to covalently cross-link nucleosomes to DNA. The cells are then disrupted and genomic DNA is digested with micrococcal nuclease. An antibody to a histone is then used to immunoprecipitate the nucleosome-DNA complex. Any DNA not bound to the histone is digested and washed away, the protein-DNA cross-links are broken, and the released DNA is sequenced. This technique of chromatin immunoprecipitation followed by DNA sequencing is known as **ChIP-Seq**. Alternatively, the released DNA is labeled and used to probe a microarray representing the genomic sequences of that particular cell type. The pattern of hybridization on the microarray reveals the DNA sequences that associate with the nucleosomes. Because microarrays are often referred to as chips, this technique is called a **ChIP-Chip** experiment.

Application of the ChIP-Seq technique has defined the position of every nucleosome in the genomes of some species of yeast, worms, fruit flies, and humans. Combining the genome-wide data into a consensus shows a surprisingly well-defined pattern of nucleosome position at active promoter elements (Figure 10-21b). Transcriptionally active promoters are usually free of bound nucleosomes and are flanked on either side by a nucleosome with a well-defined position. This arrangement facilitates transcription by making the promoter region accessible to transcription factors. The upstream nucleosome is the most well defined, and the DNA

Table 10-2 Chromatin Remodeling Complexes

Class	Chromatin Remodeling Complex	Organism	ATPase	Number of Subunits	Domain That Associates with Histones	Effect on Transcription
I. SWI/SNF	SWI/SNF	<i>S. cerevisiae</i>	Swi2/Snf2	11	Bromodomain	Activation
	RSC	<i>S. cerevisiae</i>	Sth1	15	Bromodomain	Activation
	Brahma	<i>D. melanogaster</i>	Brahma	Unknown	Bromodomain	Activation
	SWI/SNF	<i>H. sapiens</i>	hBRM	10	Bromodomain	Activation
	NRD	<i>H. sapiens</i>	CHD4	18	Bromodomain	Activation
II. ISWI	ISWI	<i>S. cerevisiae</i>	ISWI	4	—	Repression
	ACF	<i>D. melanogaster</i>	ISWI	2	—	Repression
	NURF	<i>D. melanogaster</i>	ISWI	4	—	Repression
	CHRAC	<i>D. melanogaster</i>	ISWI	5	—	Activation
III. Mi2/NURD	Mi2/NURD	<i>H. sapiens</i>	Mi2	8-10	Chromodomain	Repression

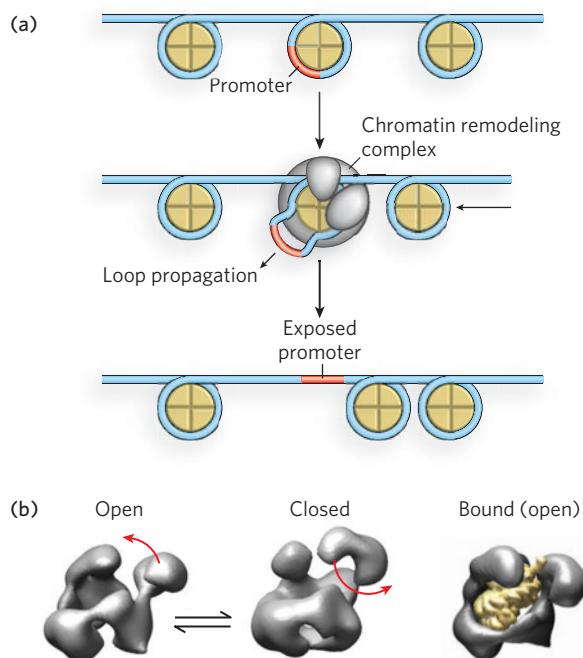


FIGURE 10-20 A mechanism for chromatin remodeling complexes. (a) When a nucleosome covers a promoter, a chromatin remodeling complex can expose the promoter by pulling the DNA out of the nucleosome into a loop. The loop can be propagated around the nucleosome, with the net effect of moving the nucleosome away from the promoter. (b) The shape of the chromatin remodeling complex RSC of yeast, based on three-dimensional image reconstruction of numerous images taken in the electron microscope with different views and tilts. By grouping the different shapes observed in the electron microscope into distinctive categories, different conformations can be deduced, as shown here. [Source: (b) Adapted from A. E. Leschziner et al., Proc. Natl. Acad. Sci. USA 104:4913-4918, © 2007 National Academy of Sciences, U.S.A.]

sequence often conforms quite well to sequences known to bind nucleosomes, as determined by Widom and other researchers (see Figure 10-7 and Moment of Discovery). The position of the downstream nucleosome is also well defined; nucleosome positions further downstream are less defined and become irregular. This pattern is observed in the genomes of all eukaryotes.

Variant Histone Subunits Alter DNA-Binding Affinity

Eukaryotes contain several variants of H2A and H3 that differ in their N- and C-terminal sequences and replace the wild-type subunit; these changes confer special properties on the chromatin structure (Figure 10-22). The primary difference in the histone variants that replace H3 is the availability of residues in the N-terminal tail to modifications such as methylation and phosphorylation. In contrast, variants of H2A differ primarily in the C-terminal tail region, which can recruit various proteins to the nucleosome. We don't yet know how histone variants function, but genetic models have linked each histone variant with a specific cellular process.

The wild-type H3 subunit is replaced by the H3.3 variant in regions where active gene expression is occurring (Figure 10-23a). The H2A variant H2AZ is also associated with nucleosomes located at actively transcribed genes. Based on these findings, H2AZ and H3.3 are thought to stabilize the open state of chromatin, thereby facilitating access of the transcriptional machinery to DNA in actively transcribed regions.

The H3 histone variant CENPA is associated with the repeated DNA sequences in centromeres and contains a large extension that connects to the kinetochore

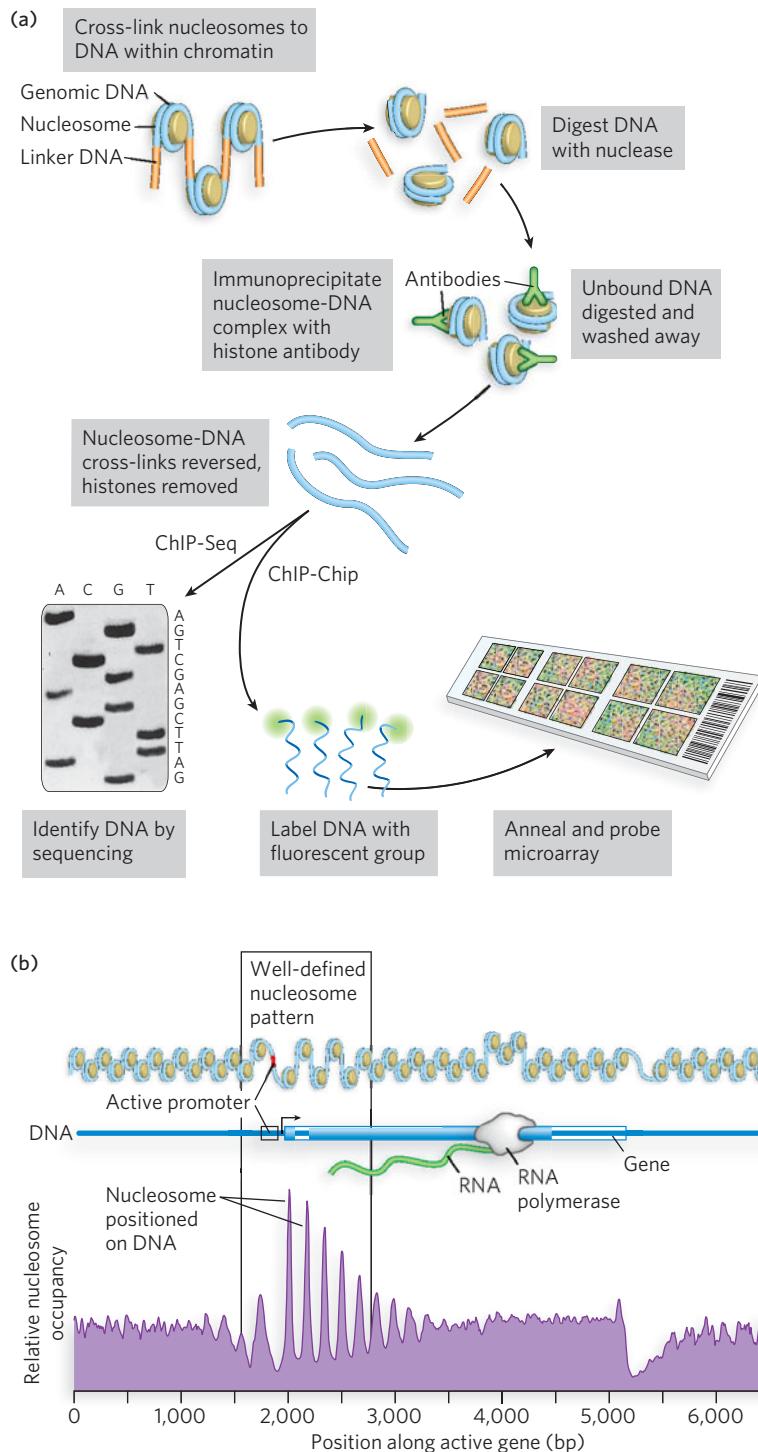
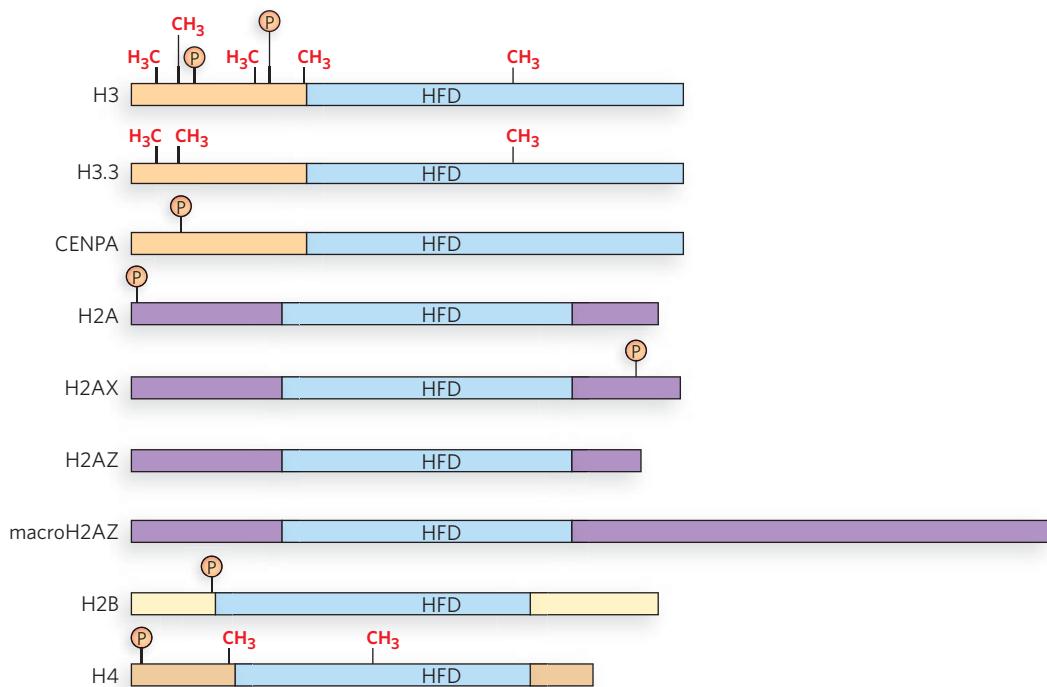


FIGURE 10-21 Determining the position of nucleosomes on genomic DNA by ChIP techniques. (a) The ChIP-Chip and ChIP-Seq techniques. (b) Genome-wide analysis of the position of histones at all active promoter elements of yeast by the ChIP-Seq technique reveals a pattern of DNA accessibility at the promoter region. Peaks indicate regions of DNA bound to histones, and valleys indicate regions of DNA relatively free of histones. [Source: (b) Adapted from T. N. Mavrich et al., *Genome Res.* 18:1073–1083, 2008.]

(Figure 10-23b), where spindle fibers attach and pull chromosomes apart during cell division (see Chapter 2). Modification of the H2A variant H2AX is associated with DNA repair and genetic recombination (Figure 10-23c). Modest amounts of H2AX seem to be scattered throughout the genome. When a double-strand break occurs, nearby molecules of H2AX become

phosphorylated at Ser¹³⁹ in the C-terminal region, attracting DNA repair proteins. If this phosphorylation is blocked experimentally, formation of the protein complex necessary for DNA repair is inhibited. Another H2A variant, macroH2A, is abnormally large and contains a unique C-terminal domain. MacroH2A is involved in X chromosome inactivation, a fascinating process

**FIGURE 10-22 Wild-type histones and variant histones compared.**

compared. Sites of Lys residue methylation (CH_3) and Ser residue phosphorylation (P) are indicated. HFD is the histone-fold domain, a structural motif common to all core

histones. H3.3 and CENPA are H3 variants; H2AX, H2AZ, and macroH2A are H2A variants. Regions of different color indicate differences in sequence among histone subunits.

that shuts down one of the two X chromosomes in the cells of female mammals (Highlight 10-1).

Nucleosome Assembly Requires Chaperones

Isolated histones are difficult to assemble onto DNA in vitro; only low yields are obtained in the absence of other factors. In cells, **histone chaperones** are required to assist the assembly of histone octamers onto DNA. These histone chaperones are acidic proteins that bind either the H3-H4 tetramer or the H2A-H2B dimer. Histone chaperones also function in assembling new histones onto DNA during DNA replication. Chaperone-mediated nucleosome assembly occurs in two steps, as shown in Figure 10-24. First, the CAF-1 chaperone deposits an H3-H4 tetramer onto the DNA; second, the NAP-1 chaperone assembles two H2A-H2B heterodimers with the H3-H4 heterotetramer to form the complete nucleosome.

Similar chaperones assemble the nucleosomes containing histone variants. Nucleosomes containing histone H3.3, for example, are deposited by a complex in which CAF-1 is replaced by the protein HIRA (the name is derived from a class of proteins called HIR, for histone

repressor). HIRA is a chromatin remodeling complex but can also be considered a histone chaperone, helping to ensure the proper assembly and placement of nucleosomes.

Modifications of Histone Tails Alter DNA Accessibility

For many years, scientists thought that histones performed only a structural role—condensing DNA. We now know that chromatin structure regulates essentially all genetic transactions and that modification of the N-terminal tails of histones plays a major role in altering chromatin structure. The amino acid residues of histone tails can be chemically modified in many ways; the most intensively studied are the acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines, and ubiquitination of lysines (Figure 10-25).

Acetylation of Lys residues is performed by enzymes called **histone acetyltransferases**, or **HATs** (see How We Know). Several different types of HATs modify histone subunits, and most often they acetylate specific residues in a histone tail. Acetylation is generally associated with enhanced accessibility to

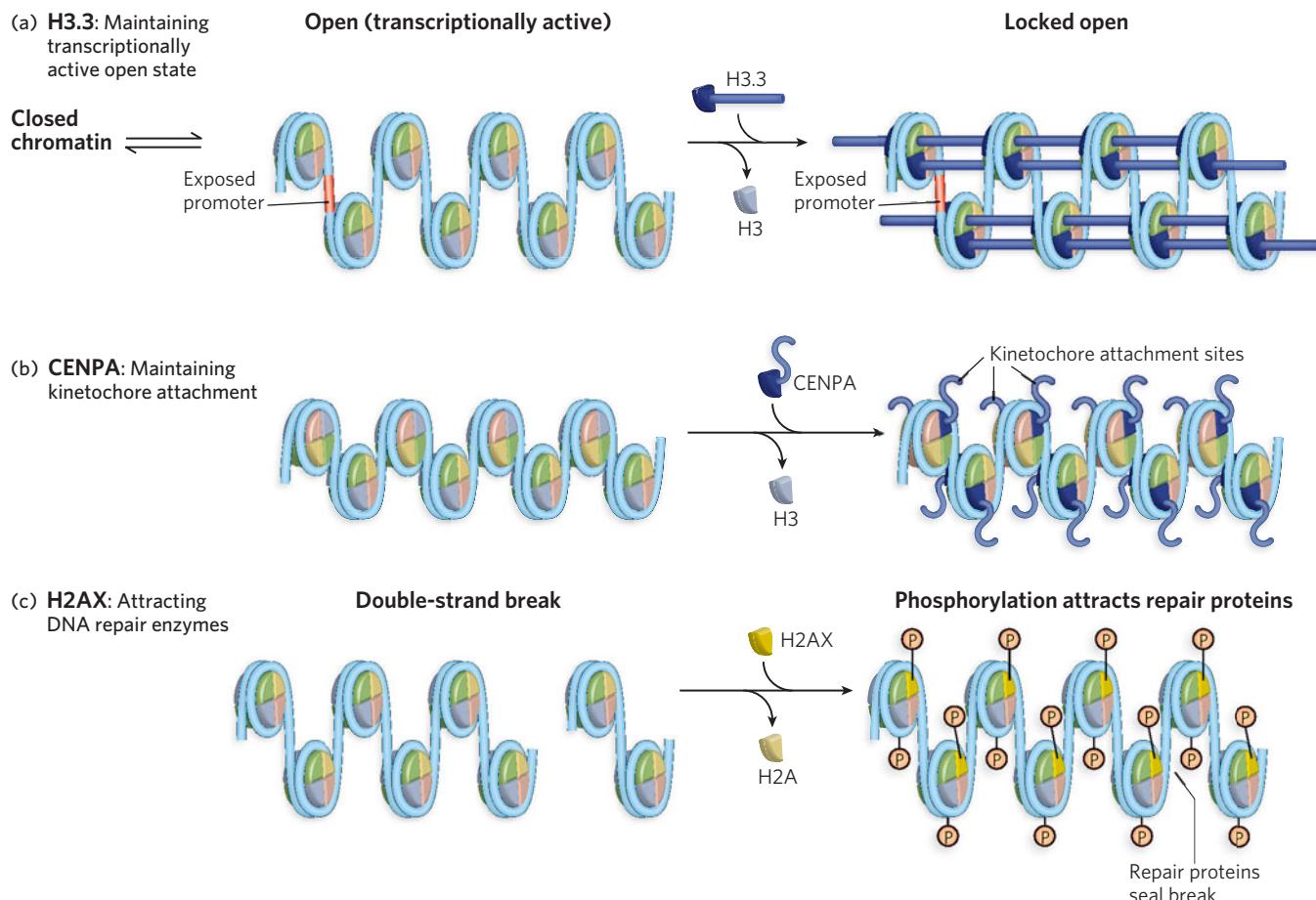


FIGURE 10-23 Proposed mechanisms for the roles of three histone variants. (a) The H3.3 variant is remodeled into octamers at sites of active transcription, where it helps stabilize chromatin in an open, transcriptionally active form. (b) The CENPA variant is localized to the

centromere region; it contains a unique extension that may help attach the chromosome to the kinetochore. (c) H2AX localizes to sites of double-strand breaks, where it becomes phosphorylated on Ser¹³⁹ and attracts DNA repair complexes to the site.

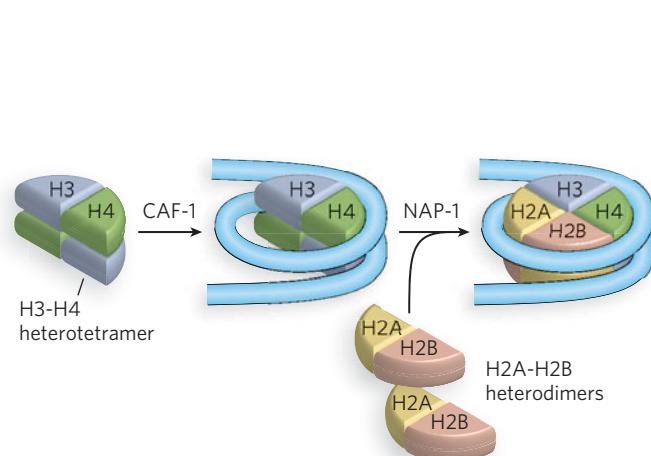


FIGURE 10-24 Chaperone-mediated nucleosome assembly. The H3-H4 heterotetramer is placed on DNA by the CAF-1 chaperone. Two H2A-H2B heterodimers then assemble in a reaction promoted by NAP-1.

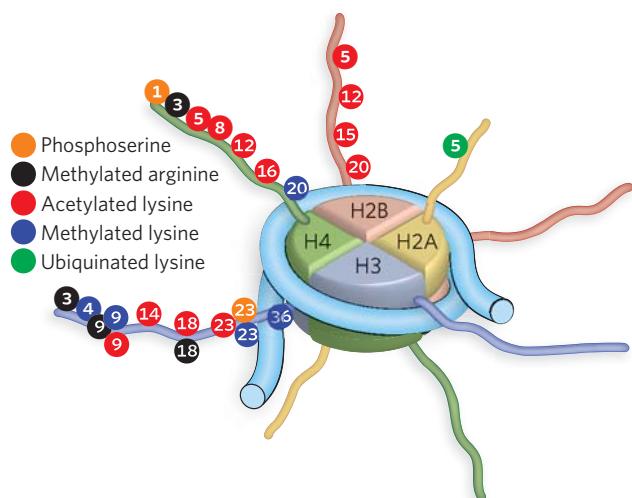


FIGURE 10-25 The modification of nucleosome N-terminal tails by small molecules. Modifications are shown on just one tail of each type of core histone. Residue numbers are indicated.

HIGHLIGHT 10-1 A CLOSER LOOK

The Use of a Histone Variant in X Chromosome Inactivation

Female mammals have two X chromosomes, and males have only one. Therefore, we might expect all the genes on the X chromosome to be expressed at twice the level in females compared with males. Most fertilized cells harboring an extra autosomal (non-sex) chromosome are lethal at the embryonic stage, due to the enhanced gene dosage from the extra chromosome. So, how do female cells, with two X chromosomes, survive with double the gene dosage? Or, how do male cells survive with half the necessary gene dosage? The answer lies in a fascinating process called X chromosome inactivation, in which

one of the two X chromosomes in every cell of the female is converted into highly condensed heterochromatin, a form of very tightly condensed DNA, silencing its genes. Inactivation of the X chromosome is also known as lyonization, after its discoverer, Mary Lyon.

In the two- or four-cell stage of development of female mammals, only



Mary Lyon [Source: Courtesy of Medical Research Council, London.]

the paternally derived X chromosome is inactivated; gene silencing of this type, specific to either paternal or maternal genes, is known as imprinting (see Chapter 21). However, both X chromosomes become active again in the early blastocyst, before one is permanently inactivated a short time later. This permanent inactivation is random; the maternal or paternal X chromosome is targeted for inactivation. Thus, the female body is a mosaic of cells, some of which have an active maternal X chromosome and others an active paternal X chromosome. In other words, all cells derived from a particular blastocyst cell contain the same inactive X chromosome, and during development the blastocyst cells give rise to an organism with a mosaic of two cell types. Because of the random nature of X chromosome inactivation, an animal that is heterozygous for an X-linked trait has a mosaic phenotype, as seen in the mottled coat color of a calico cat (**Figure 1**).

The inactive X chromosome contains an abundance of the histone variant macroH2A. This variant contains a large C-terminal region that doubles the size of the protein. The current hypothesis is that the extra protein sequence binds RNA. Essential to X chromosome inactivation is the presence of a non-coding RNA called XIST (see Figure 21-21). XIST RNA is transcribed from only one X chromosome, whereupon it acts in *cis* (see text) to coat the entire chromosome, encapsulating it. The macroH2A

DNA and consequent transcriptional activation. Histone deacetylases (HDACs) also exist; deacetylation of Lys residues generally results in transcriptional repression (Table 10-3). Initially, investigators thought that acetylation, which neutralizes the charge on lysine, simply relaxed the grip of a nucleosome on the DNA, thereby opening chromatin to regulatory proteins. More recent research indicates that acetylation has a larger effect on nucleosome-nucleosome contacts and alters higher-order chromatin structure in that way, rather than changing nucleosome-DNA affinity.

A more striking way in which histone tail modifications function is by recruiting enzymes that recognize particular modified amino acid residues. Proteins with **bromodomain** motifs recognize acetylated Lys residues (**Figure 10-26**). Bromodomain proteins are

usually contained within a larger, multiprotein complex, such as a chromatin remodeling complex (see Table 10-2) or a complex that acetylates histones (see Table 10-3). If a chromatin remodeling complex contains a subunit with a bromodomain and also a subunit containing histone acetylase activity, when it binds to an acetylated nucleosome it will acetylate histones in a neighboring nucleosome. By this means, a specific pattern of acetylation can be propagated in a targeted area of the chromosome. Propagation of the acetylation pattern typically leads to higher levels of gene expression. Creation of a specific chromatin state underlies the epigenetic inheritance of gene expression patterns not encoded by DNA.

Another common modification of histone tails is methylation of Lys and Arg residues. Histone methylation can result in either gene activation or gene



FIGURE 1 The coat color variation of a calico cat is an example of a mosaic phenotype. [Source: Agefotostock.]

histone variant binds XIST and may facilitate this process. The condensed and inactivated X chromosome, called a Barr body, is easily identified in cells stained with an antibody specific for macroH2A1. **Figure 2** shows, on the left, two blastocyst cells stained for total DNA and, on the right, the same two cells stained using an antibody to the unique C-terminal region of macroH2A1, thus revealing the X-inactivated chromosome containing macroH2A1. Other modifications associated with X chromosome inactivation are cytosine methylation at CpG residues, low levels of histone acetylation, and methylation of Lys⁹ on histone H3. These modifications are typically associated with transcriptional repression.

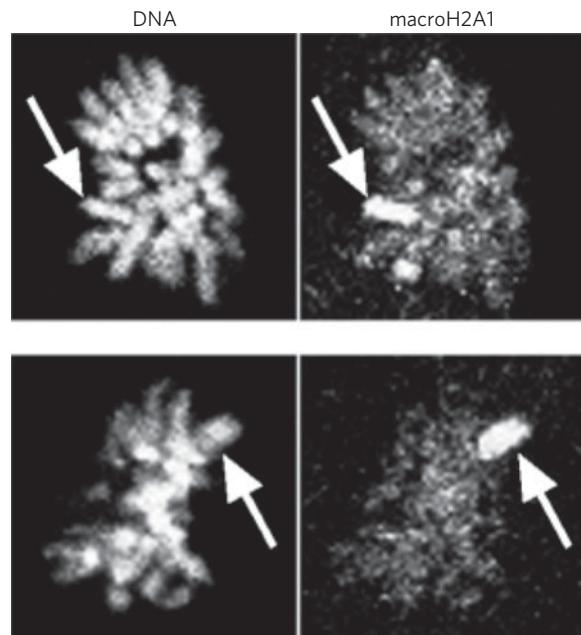


FIGURE 2 Cells of two different mouse blastocysts are stained for total DNA (left) or for macroH2A1 (right) using an antibody to macroH2A1. The arrows point to the inactive X chromosome containing macroH2A1. [Source: C. Costanzi et al., *Development* 127:2283–2289, 2000. Courtesy of J. R. Pehrson.]

repression. For example, in H3, methylation of Lys⁴ is associated with transcriptional activation, whereas methylation of Lys⁹ typically results in repression by recruiting chromatin remodeling complexes that condense chromatin. Proteins that bind to methylated Lys residues contain motifs called **chromodomains** (see Figure 10-26). Like bromodomain proteins, chromodomain proteins can be recognized by this specific sequence motif. Chromodomain proteins are found in complexes with other enzymes that further modify chromatin structure. Methylation was once thought to be irreversible, but recent experiments have identified enzymes (histone demethylases) that remove methyl groups from histone tails. Some amino acid residues, such as Lys⁹ or Lys¹⁴ of H3, can be either methylated or acetylated, and this seems to stabilize the open or closed state of the nucleosome.

Phosphorylation is another type of modification commonly found on histone tails of H3 and H4. The phosphorylation of Ser residues incorporates a negative charge into a histone tail, yet somehow results in a greater condensation of chromatin structure and repression of transcription. Histones can also be modified by ubiquitination. Recently, histone modifications by sumoylation, proline isomerization, and ADP-ribosylation have been observed (see Chapter 21). The function of many of these modifications is still unclear.

Histone Modifications and Remodeling Complexes May Read a Histone Code

Some researchers have proposed that chemical modifications of histone tails can recruit proteins in a stepwise fashion, establishing a pathway that can activate

Table 10-3 Histone Modifying Complexes**Histone Acetyltransferases (HATs)**

Group	HAT	Organism	Protein Complex	Domain That Associates with Histones	Effect on Transcription
Gcn5	Gcn5	Yeast	SAGA, ADA, SILK	Bromodomain	Coactivation
	Gcn5L	Mammal, fruit fly	STAGA, TFTC	Bromodomain	Coactivation
	PCAF	Mammal	PCAF	Bromodomain	Coactivation
MYST	ESAI	Yeast	NuA4	Bromodomain	Coactivation
	SAS2	Yeast	SAS-1	Bromodomain	Coactivation
	Tip60	Mammal	Tip 60	Bromodomain	Coactivation

Histone Deacetylases (HDACs)

Class	HDAC	Organism	Protein Complex	Effect on Transcription (or other effect)
I	HOS2	Yeast	SETSC	Repression
	HDAC1	Mammal	mSin3, Nurd, N-CoR-2	Repression
II	HDAI	Yeast	HdaI complex	Repression
	HDAC4	Mammal	Unknown	Repression
III	HST1	Yeast	Set3C	Repression
	SIR2	Yeast	Sir4, REMT	Repression
	SIRT6	Mammal	Unknown	DNA repair

the expression of a particular gene. For example, let's imagine a gene activation pathway that begins when a histone is acetylated. This modification then recruits (via a bromodomain subunit) a complex containing a HAT that propagates the modification to neighboring histones and partially opens the chromatin structure. The resulting modifications then recruit a chromatin remodeling complex (also via a bromodomain) that completes the task of opening the chromatin structure for transcription.

The basis of the hypothetical **histone code** is that successive events directed by histone modifications drive transcriptional activation. A reasonable amount of research evidence supports two-step switches, in which one histone modification leads to another type of modification. Although many researchers consider the histone code as still a working hypothesis, the numerous types of histone modifications and their resulting effects provide a rich library for combinatorial regulatory signals (Figure 10-27).

Supporting evidence for a histone code derives from studies of the expression of the human IFN- β globin gene (Figure 10-28). First, the Gcn5 coactivator binds within a complex of proteins to a specific DNA

sequence upstream of the gene. The Gcn5 subunit is an acetyltransferase (HAT) that acetylates Lys⁸ of H4 and Lys⁹ of H3. Next, a protein kinase phosphorylates Ser¹⁰ of H3. The kinase may be a subunit of a protein complex that binds to the promoter region. This modification then promotes acetylation of Lys¹⁴ of H3 by Gcn5. These modifications, in turn, recruit a SWI/SNF chromatin remodeling complex, through a bromodomain subunit, which then remodels the chromatin and exposes a DNA sequence called the TATA box. Transcription activator TFIID binds the TATA box and, through a bromodomain, also recognizes the acetylated Lys⁹ and Lys¹⁴. The specific interactions of TFIID with the DNA and modified histones promote transcription. The proteins and DNA sequences involved in the initiation of transcription are discussed in greater detail in Chapter 15.

Histone Modifying Enzymes Maintain Epigenetic States through Cell Division

Certain modifications of nucleosomes are passed down to daughter cells during cell division, in mitosis and meiosis. These modifications can activate or repress

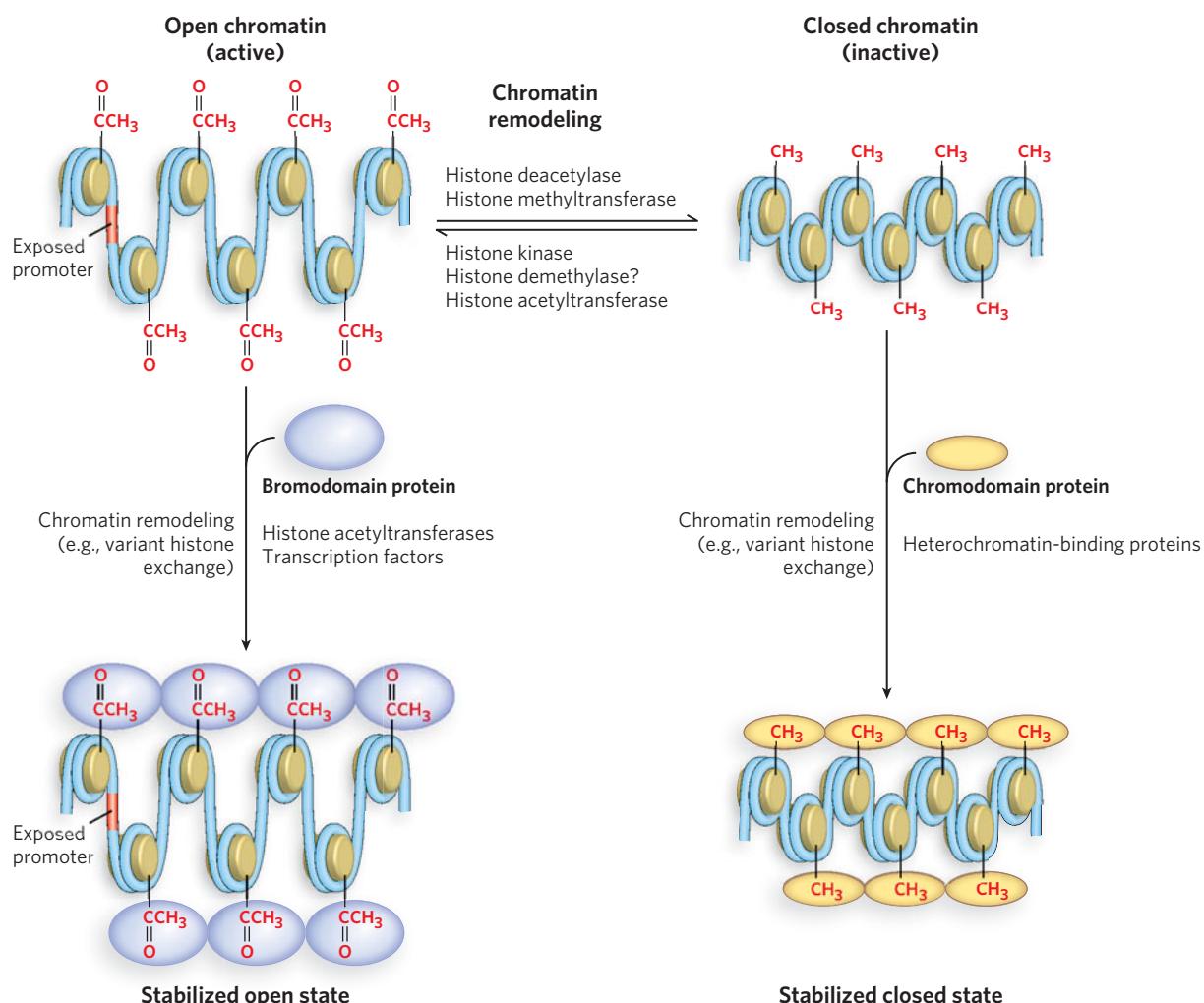


FIGURE 10-26 Bromodomains and chromodomains.

Chromatin structure is made more open or closed by various modifications performed by the different types of enzymes shown in the figure and explained in the text. Acetylated nucleosomes (top left) are recognized by bromodomain

proteins and may help stabilize the open chromatin state (bottom left). Methylated histones (top right) are recognized by chromodomain proteins that may help promote the closed state (bottom right).

certain genes. Inheriting the control of gene expression is essentially like inheriting any other trait. In other words, in addition to inheriting genes, offspring also inherit the nucleosome modifications that control expression of those genes. The underlying basis of this epigenetic inheritance is very different from classical Mendelian genetics, which is defined solely by the DNA sequence of a gene.

Histone modifications underlie the majority of epigenetic traits, although the direct methylation of cytosine bases in DNA also plays a role in epigenetic inheritance. The diversity of histone modifications provides a rich combination of modification patterns that can be coupled to epigenetic inheritance. Variant

histone subunits also play a role in epigenetic marks in chromatin, facilitating or suppressing specific functions such as chromosome segregation, transcription, and DNA repair. Histone modifications do not disappear at cell division or during meiosis, and thus they are a part of the information transmitted from one generation to the next in all eukaryotic organisms. These modifications help define which genes are expressed by daughter cells. Epigenetic modifications are important during an organism's development and in maintaining cell and tissue types. Epigenetic traits are also important in the process of imprinting in germ cells, in which one copy (allele) of a gene is silenced in the fertilized egg and remains turned off in

	N-terminal tail	Modification state	Associated protein motif	Function
H3	N	Unmodified	Sir3/Sir4/Tup1	Silencing
	N	Acetylated	Bromodomain	Transcription
	N	Acetylated	?	Histone deposition?
	N	Phosphorylated	SMC/condensins	Mitosis/meiosis
	N	Phosphorylated/acetylated	?	Transcription
	N	Methylated	?	Transcription?
	N	Higher-order combinations	?	?
H4	N	Acetylated	?	Transcription
	N	Acetylated	RCAF?	Histone deposition
CENPA	N	Phosphorylated	?	Mitosis

FIGURE 10-27 Histone modifications and their effects.

Specific combinations of modifications of histones H3, H4, and CENPA (an H3 variant) are recognized by different proteins and have diverse effects on chromatin. Acetylated

residues are lysines, phosphorylated residues are serines, and methylated residues are lysines and arginines.

[Source: Adapted from B. D. Strahl and D. Allis, *Nature* 403:41–45, 2000, Fig. 2.]

every cell of the developing organism. Humans contain at least 80 known imprinted genes that are controlled by epigenetic marks, without which the fertilized egg cannot proceed past the blastula stage (see Chapter 21). The control of epigenetic marks can also be influenced by environmental factors, and when disturbed they can lead to disease, including cancer (Highlight 10-2).

Epigenetic inheritance of a chromatin state during cell division requires the preservation of histone modification patterns during DNA replication. If the histone octamer were completely displaced during replication, all the epigenetic information encoded in the histone modifications would be lost in the daughter cells. Studies on the fate of individual histone subunits during replication demonstrate that histone octamers split apart during replication, but

the H3-H4 tetramers remain bound to the DNA (Figure 10-29). The parental (marked) H3-H4 heterotetramers are distributed randomly on the two daughter DNA strands and coat only half of the total DNA after replication. New H3-H4 heterotetramers that lack the modification pattern (i.e., are unmarked) are assembled onto replicated DNA by the CAF-1 chaperone. Current research suggests that the parental H2A-H2B dimers remain in the vicinity after being displaced by the replication fork and quickly reassemble with H3-H4 heterotetramers on the newly replicated DNA, chaperoned by NAP-1. New, unmodified H2A-H2B dimers must also be assembled on the newly replicated DNA. Hence, four types of nucleosomes form on the daughter DNA strands: nucleosomes containing old (parental) H3-H4 and new H2A-H2B, new H3-H4 and old H2A-H2B,

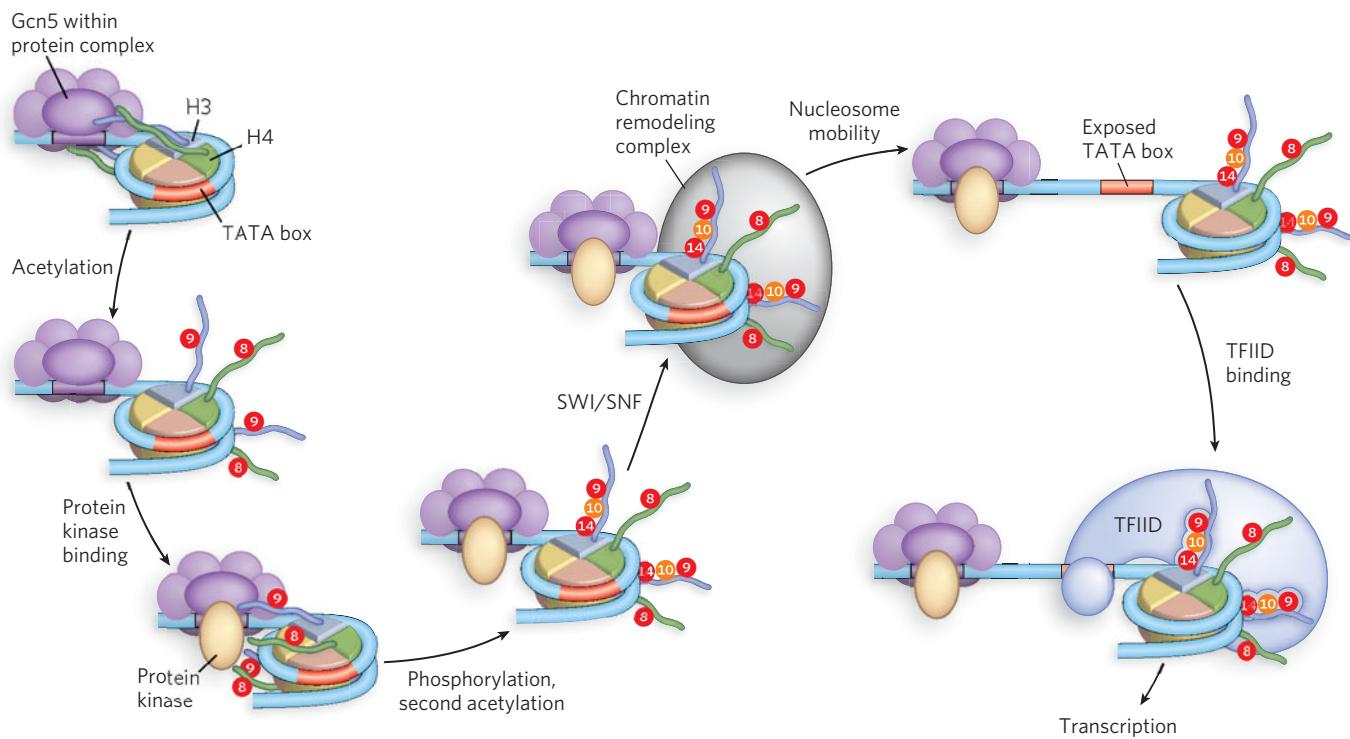


FIGURE 10-28 An example of a histone code. Activation of the IFN- β globin promoter requires an ordered succession of events driven by histone modifications that occur in a temporal sequence, as described in the text. [Source: Adapted from T. Agalioti, G. Chen, and D. Thanos, *Cell* 111:381–392, 2002, Fig. 5.]

HIGHLIGHT 10-2 MEDICINE

Defects in Epigenetic Maintenance Proteins Associated with Cancer

The development of cells into tissues, organs, and whole organisms requires individualized programs of gene expression for different cell types. Many of these programs are controlled by inheritable epigenetic mechanisms. Epigenetic controls often rely on chromatin structures based on specific histone tail modification patterns that provide or restrict the accessibility of DNA to transcription factors. Epigenetic controls are also maintained by chromatin remodeling complexes, histone chaperones, and DNA methyltransferases.

Molecular studies of cancer cells reveal that many types of cancer are associated with mutations in genes encoding proteins that act in epigenetic pathways. Presumably, the disruption of epigenetic pathways can activate genes that promote cell growth or repress genes that limit cell growth, leading to tumor formation. For example, aberrant histone modification patterns are observed in mammalian

cancers that include mutations in certain subunits of chromatin remodeling complexes. Mutations that inactivate the SNF5 subunit, common to all SWI/SNF complexes, lead to a predisposition to familial cancer and a condition known as rhabdoid predisposition syndrome. Aberrations in certain HAT genes, as well as in particular HDACs, are associated with various hematological malignancies. Abnormal histone methyltransferases have also been linked to a predisposition to cancer. For example, the mutant enzyme SUV39H1 targets a Lys residue in the N-terminal tail of H3 in mice.

Epigenetic control as a basis for cancer has a positive aspect, in that altered epigenetic states are reversible. Cancers based in epigenetic states offer the promise of strategic therapies to block the progression of cancer cells by reestablishing the correct epigenetic state. Regaining normal gene expression by resetting epigenetic states in cell lines has already been accomplished with certain inhibitors of HATs and HDACs. These findings hold promise for future diagnostics and treatments for cancer.

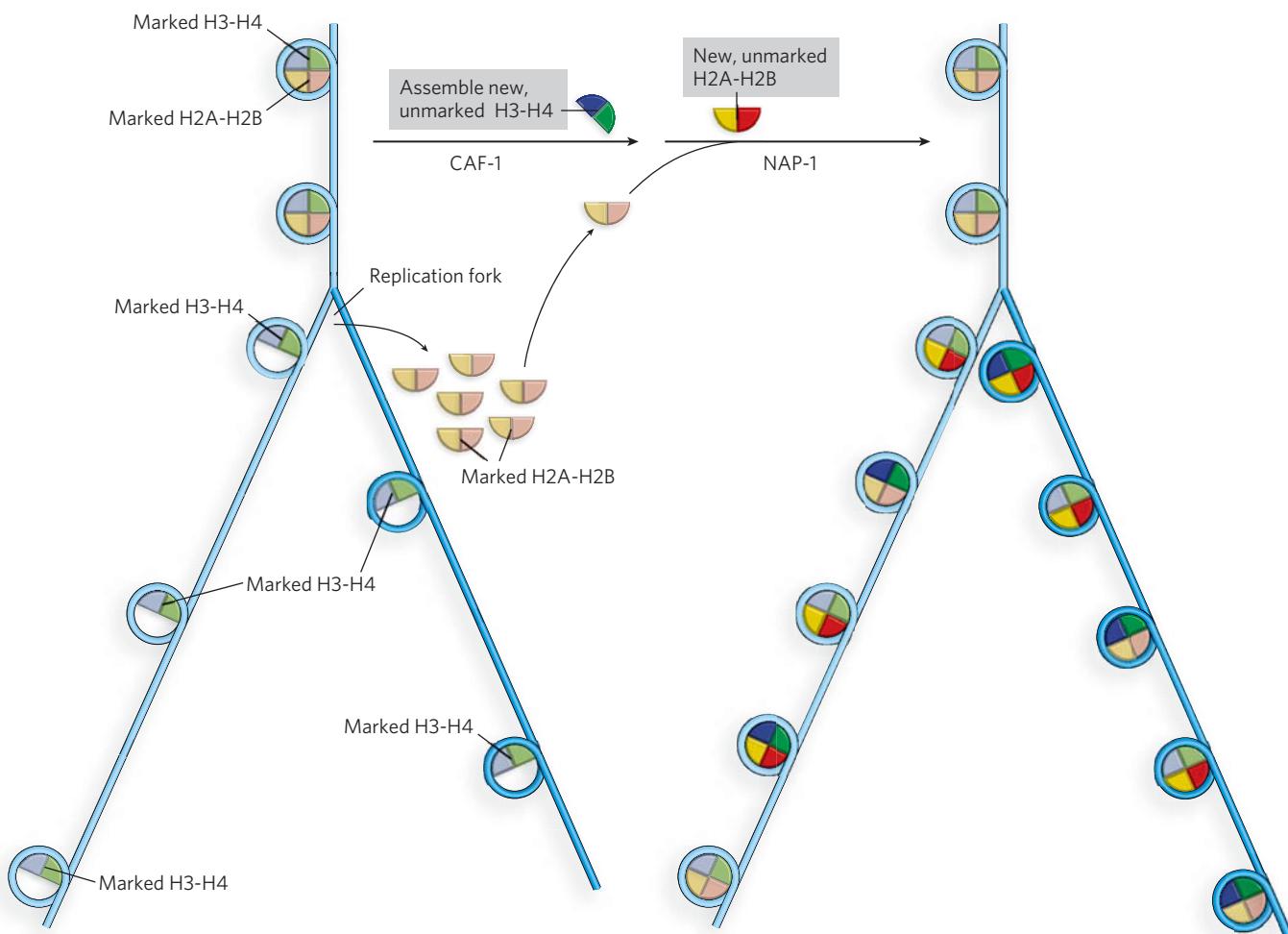


FIGURE 10-29 Preserving histone modification patterns during DNA replication. After DNA replication, the two daughter strands lack histones H2A-H2B, and the H3-H4 heterotetramers distribute among the two new strands (left). The parental (marked) H3-H4 heterotetramers bind quickly to both daughter strands, along with new (unmarked) H3-H4 heterotetramers (right). The old (marked) and new

(unmarked) H2A-H2B heterodimers reassemble randomly on daughter strands with old and new H3-H4 heterotetramers. The epigenetic marks are subsequently spread to adjacent nucleosomes on the two daughter strands, thus preserving the histone modification pattern of the parental DNA.

entirely parental H2A-H2B-H3-H4 histones, or entirely new H2A-H2B-H3-H4 histones (see Figure 10-29). Overall, the newly replicated DNA has only half of the parental epigenetic information in its histones, but the daughter DNA duplexes are “salted” with the parental histone modification pattern.

If half of the epigenetic histone modifications are lost during replication, how is the epigenetic state preserved in daughter cells? The answer lies in the ability of the cell to propagate the histone modification pattern of the parental nucleosomes to new histone subunits. Histone modifications are transmitted to nearby nucleosomes through the action of histone

modifying complexes that recognize and bind modified residues on the parental histone subunits. For example, acetylated or methylated residues are recognized by protein complexes with specific bromodomains or chromodomains. The multiprotein histone modifying complex also contains an enzyme (e.g., HAT or methyltransferase) that spreads the modification to adjacent, unmodified nucleosomes (Figure 10-30). The different epigenetic marks are presumed to recruit specific histone modifying complexes that promote the modification of other, unmodified nucleosomes. These actions transmit and propagate the histone modification pattern to new

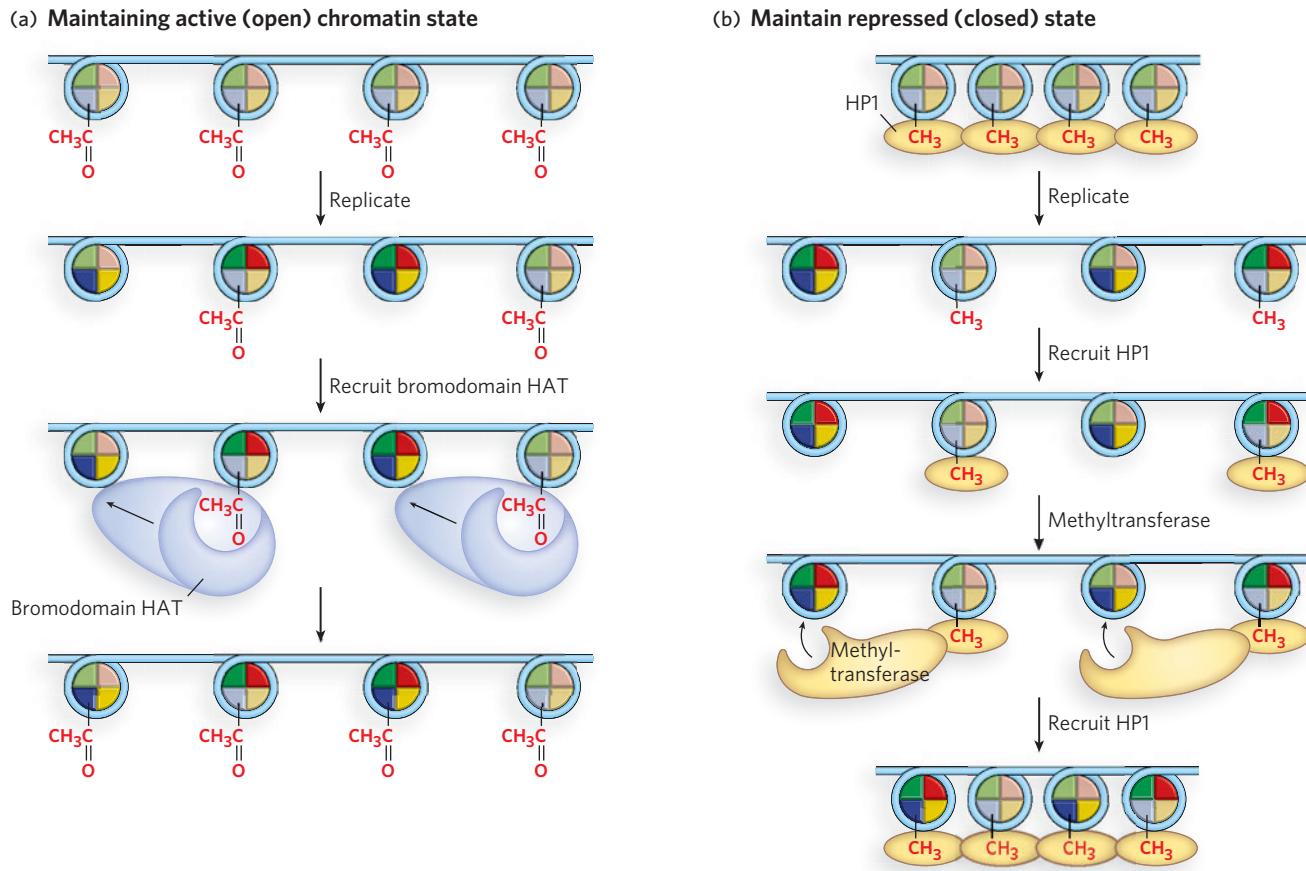


FIGURE 10-30 The spread of epigenetic states after replication. (a) The open state may be propagated through the binding of a bromodomain-containing complex that also contains a HAT subunit, which acetylates the same residues in neighboring histone octamers. (b) The closed state may be spread through binding of HP1, which reassociates with

parental histones after replication and is associated with the repressed state of tightly packed heterochromatin. HP1 contains a chromodomain that binds methylated Lys residues in histones, and it also recruits a methyltransferase to propagate the parental methylation pattern in neighboring new histones.

histone subunits in the same vicinity, thereby preserving the epigenetic state of the parental cell in the two new daughter cells.

SECTION 10.3 SUMMARY

- Nucleosomes influence gene transcription by controlling the open or closed state of chromatin. They can be positioned at specific locations on the DNA, and they can slide along the DNA to allow or block access to specific regions of the DNA.
- ATP-driven chromatin remodeling complexes can reposition, eject, or replace a nucleosome. The three classes of chromatin remodeling complexes are SWI/SNF, ISWI, and Mi2/NURD.

- Variant histones can replace individual histone subunits in the nucleosome, thus altering the chromatin's open or closed state.
- The position of every nucleosome in a genome can be determined by ChIP-Seq and ChIP-Chip techniques.
- Chemical modifications to histone tails alter the open or closed state of chromatin. Generally, acetylation is associated with enhanced accessibility of the DNA.
- Proteins with bromodomains bind to acetylated Lys residues on the histone tail, recruiting other histone modifying complexes and spreading the pattern of acetylation to nearby histones. Methylated Lys residues can be bound by proteins with chromodomains, which likewise recruit histone

- modifying complexes that spread the methylation pattern.
- The set of histone modifications in a certain region of chromatin may be read as a code that directs transcription.
 - Chaperone proteins are necessary for assembling histones into nucleosomes.
 - Epigenetic inheritance of information not encoded in the DNA relies on the transmission of histone variants and modifications through successive cell generations. Maintenance of the epigenetic state of chromatin during DNA replication requires equal distribution of H3-H4 heterotetramers on each daughter DNA. The remaining half of the parental epigenetic information is then spread to nearby newly formed histones on the two daughter strands.

Unanswered Questions

The organization of DNA in chromosomes is still largely a mystery. At the level of individual DNA segments, the details of how chromatin remodeling complexes and histone modifying enzymes control access to the DNA are still to be explored. The way in which epigenetic marks are interpreted is only now coming into focus; how epigenetic marks are read and how they relate to normal functions and disease are poorly understood. We can expect a rapid pace of new discoveries in this exciting field of inquiry.

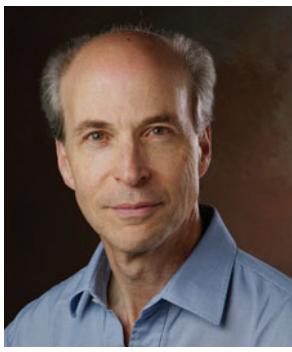
1. **How is DNA packaged in a chromosome?** We have an exceptional understanding of DNA condensation at the level of the nucleosome. But the arrangement of nucleosomes and their packing into the 30 nm filament account for only a 35- or 40-fold compaction of the DNA, far from the 10,000-fold needed to pack the genome into the cell.
2. **How do nucleosomes control individual gene expression?** The expression of genes is controlled by many factors, among which are modification of the histone tails. Do individual modifications produce a series of distinct changes in chromatin, as suggested in the histone code hypothesis? If so, what are the steps that distinguish the expression of individual genes?
3. **How are different epigenetic states specified?** The exact type and combination of histone modifications that encode various epigenetic states are poorly understood, yet they are of utmost importance to human health and disease. Are epigenetic states that are inherited through generations altered by environmental factors? Can diseases that derive from an epigenetic source be reversed by therapeutic intervention? Understanding how epigenetic states are achieved and maintained will be a most exciting avenue of future research.

How We Know

Kornberg Wrapped His Mind around the Histone Octamer

Kornberg, R.D. 1974. Chromatin structure: A repeat in unit of histones in DNA. *Science* 184:868–871.

Kornberg, R.D., and J.O. Thomas. 1974. Chromatin structure: Oligomers of the histones. *Science* 184:865–868.



Roger Kornberg [Source: Courtesy of Roger Kornberg.]

In the 1970s, scientists had already determined that the nucleosome contains four “core” histones in a 1:1:1:1 stoichiometry, and they thought that histones were arranged in some type of spiral. Kornberg’s goal was to determine the crystal structure of the histone-DNA complex. But when he purified the separate histone subunits according to established purification protocols and mixed them together, they did not assemble into an oligomer. Kornberg deduced that the pure histones failed to assemble because they were denatured by the acid and guanidine hydrochloride used in the purification. Around this time, a new histone purification protocol was developed that did not use denaturing solvents, but the procedure went largely unnoticed because it did not separate the four histones; rather, it split them into two peaks, one containing H2A and H2B, and the other containing H3 and H4. Kornberg realized that this mild purification method probably kept the histones intact and that the two peaks might be heterodimer complexes of histone subunits that lie adjacent to each other in the nucleosome.

To test this idea, Kornberg treated the fraction containing H3 and H4 with a chemical cross-linking agent—a molecule that has two reactive groups and can covalently link two proteins, provided they are close together. In SDS-polyacrylamide gel electrophoresis (SDS-PAGE), a cross-linked H3-H4 heterodimer should produce a band that migrates at a position representing the sum of the masses of H3 and H4. Cross-linking is

Roger Kornberg is best known for his discovery of the structure of eukaryotic RNA polymerase II (see Chapter 15). But well before that Nobel Prize-winning work, he performed seminal studies showing that the nucleosome consists of a histone octamer with DNA wrapped around it. By the time of this work

not 100% efficient, so the expected experimental result would be three bands, corresponding to H3, H4, and cross-linked H3-H4. Instead, Kornberg observed eight bands! The eight bands corresponded to the masses expected for an $\text{H3}_2\text{-H4}_2$ heterotetramer: H3, H4, $\text{H3}\text{-H4}$, $\text{H3}\text{-H3}$, $\text{H4}\text{-H4}$, $\text{H3}\text{-H3-H4}$, $\text{H3}\text{-H4-H4}$, and $\text{H3}\text{-H3-H4-H4}$ (Figure 1). Supporting this conclusion, sedimentation analysis yielded a molecular mass of 53.9 kDa for the largest H3-H4 complex, very close to the 53.2 kDa expected for a $\text{H3}_2\text{-H4}_2$ heterotetramer.

Combining the known 1:1:1:1 ratio of core histone subunits with his finding that H3-H4 is a heterotetramer, Kornberg hypothesized that the histone subunits assemble into an octamer containing two of each subunit. He proposed how they are arranged and surmised that about 200 bp of DNA wraps around the histone octamer, because micrococcal nuclease treatment of chromatin was known to give a repeat fragment length of 200 bp. We now know that Kornberg’s insightful conclusions were right on target!

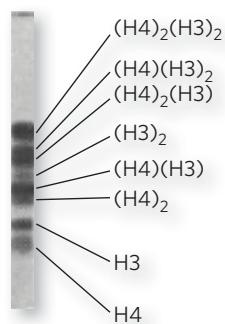


FIGURE 1 In Kornberg’s experiments, chemical cross-linking revealed that H3-H4 forms a heterotetramer. An H3-H4 complex was treated with a chemical cross-linker, followed by analysis by SDS-PAGE to separate the cross-linked proteins according to mass, which decreases from top to bottom of the gel shown here. [Source: Adapted from R. D. Kornberg and J. O. Thomas, *Science* 184:865–868, 1974, Fig. 1.]

A Transcription Factor Can Acetylate Histones

Brownell, J.E., and C.D. Allis. 1995. An activity gel assay detects a single, catalytically active histone acetyltransferase subunit in *Tetrahymena* macronuclei. *Proc. Natl. Acad. Sci. USA* 92:6364–6368.

Brownell, J.E., J. Zhou, T. Ranalli, R. Kobayashi, D.G. Edmondson, S.Y. Roth, and C.D. Allis.

1996. *Tetrahymena* histone acetyltransferase A: A homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell* 84:843–851.

For many years, histones were thought to have only a structural function—packing DNA into the confines of a nucleus. We now know that histones regulate gene expression and are involved in epigenetic inheritance. Early genetic and biochemical studies of transcription factors had identified the basal RNA polymerase machinery and several transcription coactivators, but little did anyone know that many of these factors are enzymes that covalently modify histones.

With hindsight, we can see that as far back as the 1960s, researchers had correlated histone acetylation with gene regulation, suggesting that histones do not just play a structural role. The idea that histone modification could regulate transcription heated up significantly with the discovery of a nuclear histone acetyltransferase (HAT) by David Allis's laboratory in 1995. The Allis group chose macronuclear extracts of *Tetrahymena thermophila* as an enzyme source, because this ciliate contains large amounts of acetylated histones. HAT activity was followed during purification by an activity gel assay. The assay used SDS-PAGE, starting with a gel in which histones were uniformly distributed by adding them to the polyacrylamide solution before pouring the gel and polymerizing it into a solid matrix. Column fractions of the *Tetrahymena* macronuclear extract were analyzed on this gel, then the gel was soaked in buffer lacking SDS, allowing proteins to renature. The buffer also contained ^3H -labeled acetyl-CoA, the substrate for HAT activity. Covalent attachment of [^3H]acetyl groups to the uniformly distributed histones in the gel occurred only where a HAT enzyme renatured in the gel. Unused [^3H]acetyl-CoA was removed by soaking the gel, followed by fluorography to visualize the position of the HAT activity, where the ^3H -labeled histones overlapped with the HAT (**Figure 2**). The Allis lab referred to the *Tetrahymena* HAT as p55 because it migrates as a 55 kDa protein.

Next, the researchers identified the gene encoding p55. The sequence finding was astonishing: p55 was homologous to a very well-studied transcription activator, the yeast Gcn5 protein. This result implied that Gcn5 has HAT activity and that this enzymatic activity may account for the activator's regulatory function. In fact, Allis's group used their gel assay to demonstrate that Gcn5

has HAT activity (**Figure 3**). Since this discovery, several transcription factors have been shown to contain a subunit with HAT activity.

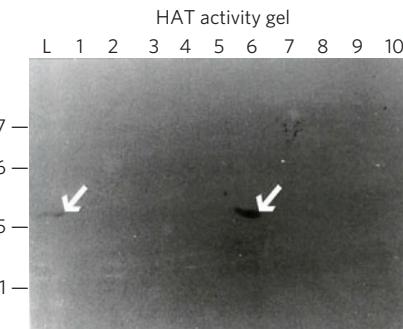


FIGURE 2 Fractions of *Tetrahymena* macronuclear proteins eluted from an ion-exchange chromatography column were analyzed by SDS-PAGE. Lane numbers (1 to 10) represent fraction numbers; L is the starting extract. Numbers on the left indicate molecular mass in kilodaltons. The p55 protein contains HAT activity, and can be visualized in the activity gel both in the initial extract (lane L) and in the column fraction in lane 6 (arrows). [Source: Adapted from J. E. Brownell and C. D. Allis, *Proc. Natl. Acad. Sci. USA* 92:6364–6368, 1995, Fig. 5.]

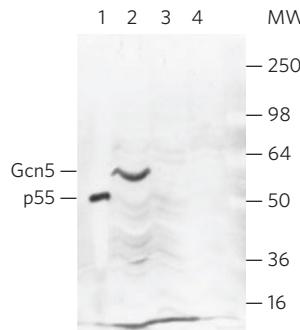


FIGURE 3 The activity gel result showing that Gcn5 has HAT activity. Macronuclear extracts of *Tetrahymena* show the expected HAT activity of p55 (lane 1). The gene for Gcn5 was cloned into an expression plasmid and then induced. The induced Gcn5 (60 kDa) protein has HAT activity (lane 2). Lane 3 shows the result for uninduced cells; lane 4, induced cells lacking the expression plasmid containing the Gcn5 gene. [Source: Adapted from J. E. Brownell et al., *Cell* 84:843–851, 1996, Fig. 4.]

Key Terms

chromatin, p. 332	linker histone, p. 338	epigenetic inheritance, p. 343
histone, p. 332	30 nm filament, p. 339	ChIP-Seq, p. 345
nucleosome, p. 332	solenoid model, p. 339	ChIP-Chip, p. 345
cross-linking, p. 334	zigzag model, p. 339	histone chaperone, p. 348
histone octamer, p. 334	chromosomal scaffold, p. 341	histone acetyltransferase (HAT), p. 348
core histone, p. 334	nucleoid, p. 341	bromodomain, p. 350
histone-fold motif, p. 335	chromatin remodeling complex, p. 343	chromodomain, p. 351
histone tail, p. 336	histone modifying enzyme, p. 343	histone code, p. 352
chromatosome, p. 338		

Problems

1. When proteins are incubated with the detergent sodium dodecyl sulfate (SDS), they take up the detergent and are partially denatured, losing much of their structure, and tend to take on a consistent mass-to-charge ratio (see Highlight 4-1). In SDS-polyacrylamide gel electrophoresis, proteins are separated almost entirely as a function of their mass. Histones are an exception. On these same gels, many histones migrate more slowly than they should, as though they were much larger than they actually are. Suggest an explanation for this behavior.
2. Which of the following protein modifications—acetylation, phosphorylation, and methylation—could change the net charge on the surface of a modified histone?
3. In bacteria, the transcription of a subset of genes is affected by DNA topology, with expression increasing or (more often) decreasing when the DNA is relaxed. When a bacterial chromosome is cleaved at a specific site by a restriction enzyme (one that cuts at a long, and thus rare, sequence), only nearby genes (within 10,000 bp) exhibit either an increase or a decrease in expression. The transcription of genes elsewhere in the chromosome is unaffected. Explain.
4. In different regions of chromatin, the ratio of histone H1 to histone H2A may vary, but the ratio of histone H2A to histone H2B is generally the same. If the amount of H1 increases in a region of chromatin, will transcription of genes in that region increase or decrease? Explain your answer.
5. In chromatin, nucleosomes are organized in higher-order structures, the 30 nm filaments. Although the detailed structure is not known, which features of the 30 nm filament have been experimentally determined?
6. In eukaryotes, chromosomes are packaged into successively higher-order structures, such as the 30 nm filaments. In bacteria, DNA is not packaged into such stable proteinaceous structures, and histonelike proteins bind to the DNA less tightly. Suggest an explanation for this difference.
7. Describe at least three differences between chromatin regions that are transcriptionally active and those in which genes are transcriptionally silent.
8. How does epigenetic inheritance differ from Mendelian inheritance?
9. During replication, nucleosomes are partially displaced and distributed on the daughter DNA strands. New histone subunits are added to bring the entire complement of nucleosomes up to the required level. Nucleosomes on the DNA to be replicated may have modified histone subunits, but the new histones that appear after replication lack the modifications (at least transiently). Which of the following statements describes how the modified and unmodified histone subunits are distributed in nucleosomes after replication?
 - (a) The modified and unmodified histones are assembled randomly into nucleosomes.
 - (b) The modified histone subunits stay together in nucleosomes, separate from unmodified nucleosomes.
 - (c) The H3-H4 modified pairs stay together, and the H2A-H2B modified pairs stay together, and nucleosomes assemble with modified and unmodified H3-H4 and H2A-H2B pairs. The various combinations occur at random on each daughter DNA molecule.
 - (d) Modified nucleosomes are segregated to one daughter chromosome, and completely unmodified nucleosomes are segregated to the other daughter chromosome.
10. The human genome contains about 3.1×10^9 bp of DNA. Assuming that the DNA is covered with nucleosomes spaced as described in this chapter, how many molecules of histone H2A are present in one somatic human cell? (Do not consider any reductions in H2A due to its replacement by H2A variants.) How would the number change after DNA replication but before cell division?
11. Roger Kornberg's histone cross-linking experiments defined an H3-H4 heterotetramer as a nucleosome substructure (see How We Know). Suppose nucleosomes actually contained two H3 subunits but only one H4 subunit, forming a stable H3-H3-H4 heterotrimer. How would the cross-linking results have been different?

Data Analysis Problem

Lowary, P.T., and J. Widom. 1998. New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning. *J. Mol. Biol.* 276:19–42.

12. In their efforts to define the rules for nucleosome positioning on DNA, Lowary and Widom obtained a library of 5×10^{12} DNA molecules, each with a different randomized sequence. All of the molecules were assembled from three synthesized segments of 72, 76, and 72 bp each, with two different 6 bp linkers that contained restriction sites. Each molecule was flanked by short segments of DNA that served as targets for PCR amplification. The total length of all the random DNA segments was 220 bp. The entire library was PCR-amplified to increase the total amount of DNA. Then, histone octamers were mixed with the DNA, in a ratio of 10 DNA molecules per histone octamer. The nucleosome-DNA complexes were isolated

after dialysis into a relatively high concentration of salt, the bound DNA was amplified by PCR, and the procedure was repeated. The same cycle was repeated 12 to 15 times, and eventually the bound DNAs that came through the selection were cloned and sequenced to determine which DNA sequences bound most tightly to nucleosomes. This led to a series of nucleosome positioning rules. For example, the dinucleotide TA tended to appear at 10- or 20-nucleotide intervals in sequences that were strongly bound by nucleosomes.

- (a) Why was the random DNA length set at 220 bp?
- (b) Why were nucleosomes added at a level that could bind only 10% of the DNA molecules present?
- (c) What was the function of the high salt concentration?
- (d) Why was the DNA amplified by PCR in each cycle?

Additional Reading

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DNA Replication



Robert Lehman [Source:

Courtesy of American Society for Biochemistry and Molecular Biology.]

measured conversion of the 5'-³²P to a form that was protected from removal by an enzyme, presumably due to incorporation into a new phosphodiester bond. This assay worked, and the DNA strands were joined after incubation in the extract! But I wanted to get definitive proof that the strands were sealed by a phosphodiester bond. Although it sounds obvious now, at the time we thought it possible that a protein linker might be the cause of the DNA strand joining.

We used two different enzyme nucleases to degrade the DNA. One enzyme digested DNA to mononucleotides leaving the ³²P at the 5' end; the other enzyme digested DNA in the opposite direction to give a mononucleotide with ³²P at the 3' end. If the linkage in the DNA were due to a phosphodiester bond, both of these products would be observed, depending on which nuclease was used. But if the linkage was due to a protein, neither of these would show up in the analysis, and we would have seen ³²P attached to a protein instead. I'll never forget the electric moment when my students and I looked at the two chromatograms used to analyze the DNA degradation products, and there they were staring right at us—the 5'-³²P-labeled mononucleotides and 3'-³²P-labeled mononucleotides, the exact products expected for a true phosphodiester bond. It was wonderfully exciting to discover a new enzyme, and DNA ligase has turned out to be one of the central players involved in DNA replication and repair in all cells.

Moment of Discovery

I started my laboratory at Stanford to study DNA recombination using the biochemical methods established by Arthur Kornberg for working with DNA polymerase. Matt Meselson's lab at Harvard had evidence that DNA recombination involved breaking and joining of DNA strands, so we set out to find an enzyme that sealed the break by forming a phosphodiester bond.

Using *E. coli* cell extracts and a substrate DNA duplex with single-stranded breaks (nicks) labeled with ³²P at their 5' termini, we

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11.4 Initiation of DNA Replication 390

11.5 Termination of DNA Replication 395

—Robert Lehman, on discovering DNA ligase

Having covered the properties of DNA, RNA, and protein—their chemical structures and functions and the methods for studying them—we now make a transition and begin to delve deeply into the heart of molecular biology. In this chapter and Chapters 12–18, we explore the mechanisms of biological information transfer. Proteins and nucleic acids enable the flow of biological information through a network of processes that collectively allow the cell to grow, reproduce, maintain its structures and organization, acquire energy, sense and respond to its environment, and, in multicellular organisms, diversify into tissues and organs. Although we know a lot about the individual molecules and reactions that conduct this information flow in the cell, we have only recently begun to understand how the pieces fit together.

This chapter focuses on the process of **DNA replication**—the duplication of the cellular genome, in which the stored genomic information is handed down to the next generation. DNA replication is central to life and to evolution; without it, there could be no transfer of information across generations. Any modifications to the genome that help or hurt the organism would be lost instead of inherited by its offspring. DNA replication is also highly regulated in response to the environment. Replication should occur only when the cell has sufficient resources to divide and form two new cells. In multicellular organisms, loss of control over this process leads to cancer—uncontrolled cell division that eventually kills the entire organism.

The structure of DNA is so elegant and simple that one might think the process of duplicating DNA would reflect this simplicity. But the replication of DNA is far from simple. Imagine the evolutionary pressure to develop a robust enzymatic machinery that duplicates this large set of instructions with the high-fidelity imperative of maintaining the species. The accuracy of replication is particularly crucial because even a seemingly low error rate of one incorrect base pair in a thousand would produce three million mutations after a single replication cycle of the DNA in a human cell! As we'll see, this evolutionary pressure has resulted in novel enzymatic architectures working in ways that are beautiful to behold. The interplay of numerous replication proteins follows a complex choreography to produce two identical DNA molecules from one.

The principles of DNA replication are surprisingly similar in bacteria, archaea, and eukaryotes alike. We begin the chapter with some classic studies that provide an overview of the replication process, and take a look at the chemistry of the reaction, the structure of DNA polymerase (the enzyme that joins nucleotides into a DNA chain), and the many other proteins needed to

replicate double-stranded DNA. We then explore how replication starts at specific origin sequences and how replication forks—the sites of DNA polymerization—are established. Initiation is a key regulatory step in replication because, once replication is initiated, it doesn't stop until the entire DNA molecule is successfully duplicated. Finally, we discuss how the terminal ends of chromosomes are replicated, a process that controls the very lifespan of individual eukaryotic cells.

11.1 DNA Transactions during Replication

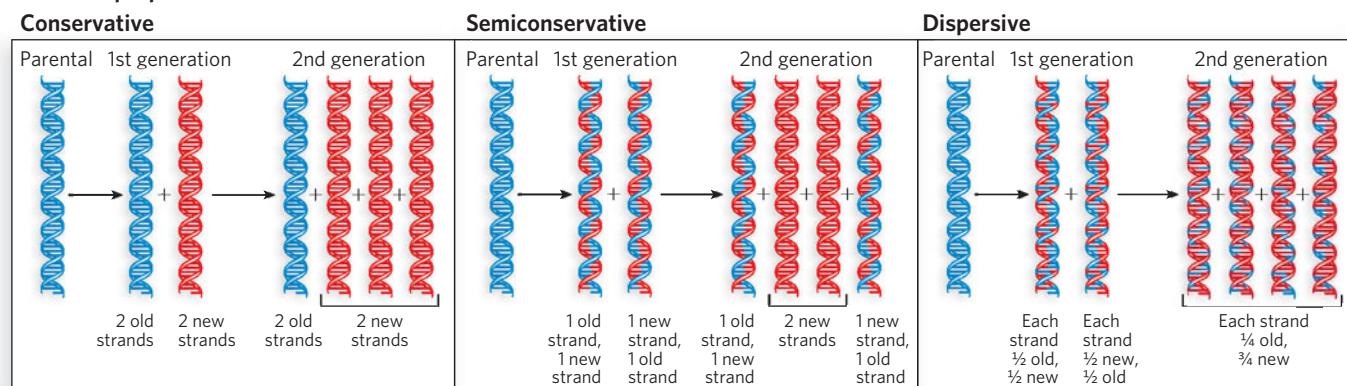
In all organisms, replication begins at specific locations on the chromosome where DNA polymerase is recruited, then travels bidirectionally along the DNA until the whole chromosome is copied. During this process, each of the two complementary strands of duplex DNA is used as a template for a newly synthesized strand. We review here a few of the exciting, now classic, discoveries about the replication process.

DNA Replication Is Semiconservative

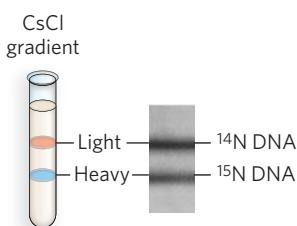
Watson and Crick hypothesized that during DNA replication, the double helix is unwound and each parental DNA strand is used as a template to generate a new daughter strand, and thus two daughter duplexes, following the A=T and G≡C base-pairing rules. This mode of replication is called **semiconservative**, because each daughter duplex conserves only one strand of parental DNA; the other strand is completely new. However, two other hypotheses were also proposed to explain how DNA could be replicated (Figure 11-1a). In one alternative hypothesis, conservative replication, the two parental strands would remain together while acting as template for production of a new DNA duplex. Another possible mechanism was dispersive replication, in which the duplex would be replicated in a random patchwork.

An elegant experiment to distinguish among these three replication mechanisms was devised by Matthew Meselson and Franklin Stahl. First, they grew *Escherichia coli* cells for multiple generations in a medium containing $^{15}\text{NH}_4$ as the sole nitrogen source to uniformly label the cellular DNA with the heavy nitrogen isotope, ^{15}N . Although DNA contains only about 17% nitrogen by mass, and the mass of ^{15}N is only 7% greater than that of ^{14}N , the resulting 1.2% increase in the mass of DNA labeled with ^{15}N is enough to cleanly resolve $[^{15}\text{N}]$ DNA from $[^{14}\text{N}]$ DNA in a density gradient. Figure 11-1b shows one of their control experiments in which an equal mixture of $[^{14}\text{N}]$ DNA and $[^{15}\text{N}]$ DNA was added to a solution

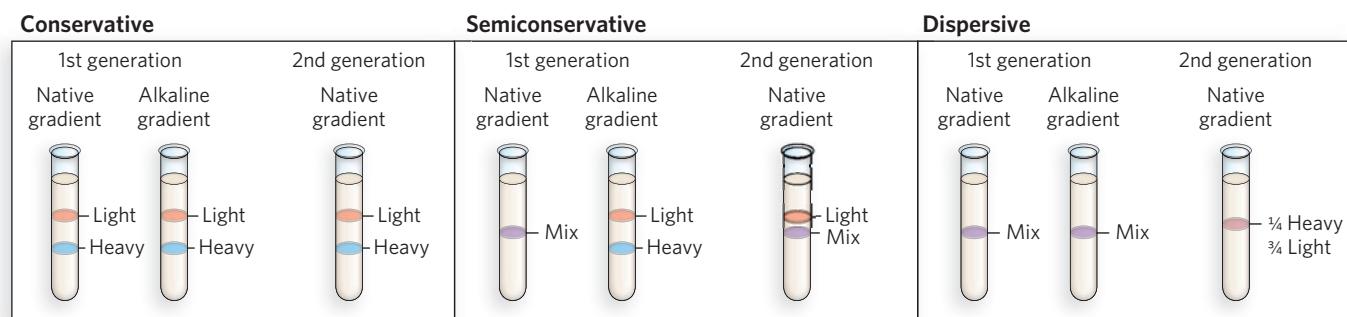
(a) Three proposed models



(b) Control experiment



(c) Predicted outcomes



(d) Observed result

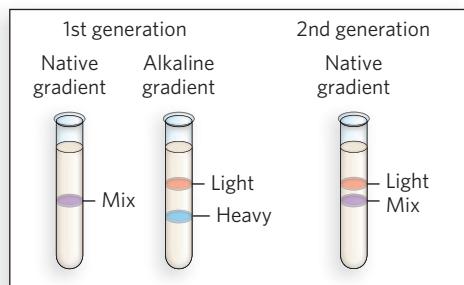


FIGURE 11-1 Semiconservative DNA replication. (a) Three possible mechanisms of replication, and the distribution of parental DNA (blue) and daughter DNA (red) expected for each, after one and two generations of growth. (b) In one of Meselson and Stahl's control experiments, equal amounts of double-stranded [^{14}N]DNA (both strands light) and [^{15}N]DNA (both strands heavy) were mixed and analyzed in a native CsCl density gradient (DNA is double-stranded) and in an alkaline CsCl density gradient (DNA strands separate) for conservative, semiconservative, and dispersive replication, following one round (first generation) or two rounds (second generation) of replication. See text for details. (d) The observed result matches the results predicted for semiconservative replication. [Source: Adapted from M. Meselson and F. W. Stahl, *Proc. Natl. Acad. Sci. USA* 44:671–682, 1958, Fig. 2.]

CsCl density gradient (DNA is double-stranded) and in an alkaline CsCl density gradient (DNA strands separate) for conservative, semiconservative, and dispersive replication, following one round (first generation) or two rounds (second generation) of replication. See text for details. (d) The observed result matches the results predicted for semiconservative replication. [Source: Adapted from M. Meselson and F. W. Stahl, *Proc. Natl. Acad. Sci. USA* 44:671–682, 1958, Fig. 2.]

of CsCl (cesium chloride) in a tube and subjected to ultracentrifugation, forming a density gradient of CsCl from the top to the bottom of the tube. As the density gradient formed, DNA molecules coalesced into sharp bands at positions corresponding to their density (and thus their mass). The “heavy” [¹⁵N]DNA formed a band below the band of “light” [¹⁴N]DNA.

To analyze DNA replication in cells, ¹⁵N-labeled cells were transferred to unlabeled (i.e., ¹⁴N) medium and allowed just one round of replication, then DNA was extracted and analyzed in a CsCl density gradient under either of two conditions: native, in which the two DNA strands of a duplex remain together, or alkaline, in which the duplex separates into two single strands of DNA. The expected results for each of the three hypotheses are shown in Figure 11-1c for both native and alkaline CsCl gradients. We’ll focus first on the expected results for the native CsCl gradient, where DNA remains double-stranded.

If replication were conservative, two bands of duplex DNA would be observed: one of lower density, containing two new strands of [¹⁴N]DNA (light/light), and one of higher density, consisting of the original parental [¹⁵N]DNA duplex (heavy/heavy) (see Figure 11-1c, left). If replication were semiconservative, only one band of duplex DNA would be observed, each duplex consisting of one parental [¹⁵N]DNA strand and one [¹⁴N]DNA daughter strand (see Figure 11-1c, middle). This heavy/light band of DNA would migrate at a position in the CsCl gradient that is intermediate between the heavy/heavy and light/light DNA bands. If replication were dispersive, the result observed in a native CsCl gradient would be the same as for the semiconservative model: one band of heavy/light DNA (see Figure 11-1c, right). The actual result of this experiment is illustrated in Figure 11-1d. The native CsCl gradient showed only a single band of heavy/light DNA, located between the positions of light/light and heavy/heavy DNA. This result ruled out the conservative model of replication, but did not distinguish between the semiconservative and dispersive models.

The alkaline CsCl gradient, in which the two strands of duplex DNA separate, distinguishes between the semiconservative and dispersive models of replication. The two strands of DNA following semiconservative replication will be very different: one is completely heavy [¹⁵N]DNA, and the other is completely light [¹⁴N]DNA, and therefore two bands should be observed (see Figure 11-1c, middle). In contrast, the two strands produced by dispersive replication would both be composed of 50% heavy and 50% light DNA, and thus only one band would be observed and it would migrate at the heavy/light position in the alkaline CsCl gradient

(see Figure 11-1c, right). The observed results supported the semiconservative mechanism and ruled out the dispersive mechanism.

To be sure of their conclusion, Meselson and Stahl performed one more experiment to distinguish between semiconservative and dispersive replication. Cells were allowed to proceed through a second round of replication in unlabeled medium, and the DNA was again analyzed in a CsCl gradient. The expected results are shown in Figure 11-1c (the second generation).

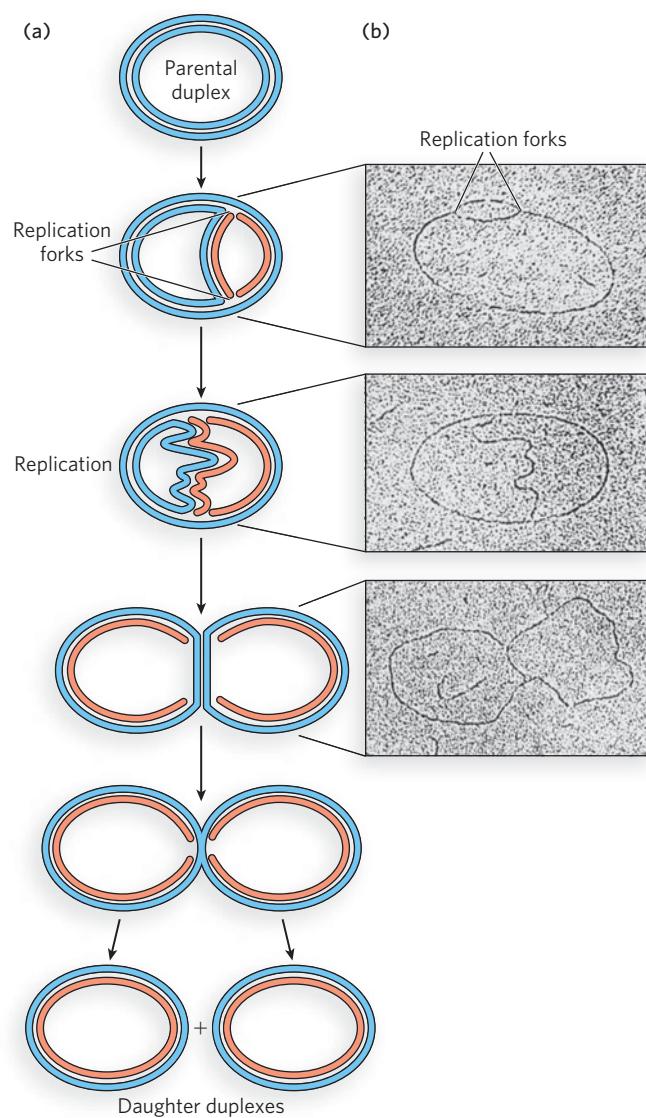


FIGURE 11-2 Simultaneous replication of both DNA strands. (a) Replication stages of a circular, double-stranded DNA, based on electron microscopic observations. Blue strands are parental DNA; red strands are new DNA. (b) Electron micrographs of plasmid DNA undergoing replication from a single origin. [Source: (b) Bernard Hirt, Institut Suisse de Recherches Experimentales sur le Cancer.]

Dispersive replication would produce just one band with a density corresponding to 25% [¹⁵N]DNA and 75% [¹⁴N]DNA. A very different result is expected for semiconservative replication: the first-generation single band of heavy/light DNA should split into two bands, one of light/light DNA and the other of heavy/light DNA. The observed result, illustrated in Figure 11-1d (second generation), showed these two bands, thus confirming the semiconservative mechanism.

Replication Is Initiated at Origins and Proceeds Bidirectionally

Experiments using density gradients could not distinguish whether synthesis initiates at one site or at multiple sites on the chromosome and whether the sites are defined or random. These questions were answered by observing replicating circular plasmid DNA in the electron microscope. The resulting images of DNA molecules undergoing replication contained a loop or bubble (Figure 11-2). This replication intermediate resembles the Greek letter theta (θ), and this mode of DNA synthesis is often called θ -form replication. The images captured in these experiments also revealed that DNA is not completely unwound before it is replicated. Instead, as the strands separate, they are simultaneously converted into two sister duplexes. This is evident from the observation that all the DNA strands appear to be the thickness of duplex DNA. The point where DNA synthesis starts is a specific DNA sequence called the **origin of replication**.

The point where the parental duplex separates and the daughter duplexes arise is the site of new DNA synthesis, referred to as a **replication fork**.

A single origin of replication is sufficient for the comparatively small bacterial chromosome, but eukaryotic cells contain much more DNA. Also, eukaryotic replication forks travel at about one-tenth the speed of bacterial replication forks, possibly due to the complex compaction of eukaryotic chromosomes (see Chapters 9 and 10). It would take more than 100 hours to duplicate the DNA in a human cell if each chromosome contained only one replication fork that started from a single origin. To duplicate larger eukaryotic genomes within a biologically relevant timeframe, numerous replication forks are generated on each chromosome (Figure 11-3a).

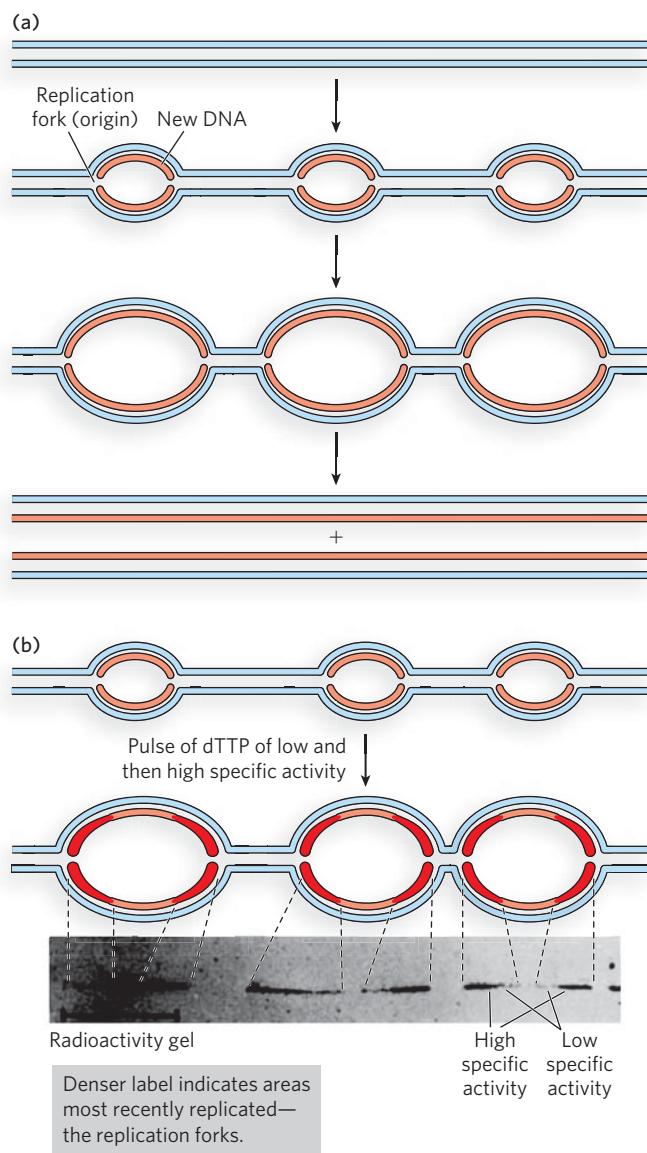


FIGURE 11-3 Multiple eukaryotic replication forks.

(a) Replication bubbles from multiple origins expand and meet head-on to produce two new linear duplex chromosomes. Parental DNA is blue; newly replicated DNA is red. (b) Chinese hamster ovary cells were labeled with a pulse of [³H]dTTP, first of low specific activity, then of high specific activity. The autoradiograph shows the newly replicated, ³H-labeled DNA initiated at multiple origins. Increased density of radioactivity at both ends of a bubble, at the replication forks, reveals that each new DNA strand is growing away from the midpoint, and thus that replication is bidirectional. Replication forks had formed prior to addition of [³H]dTTP, and thus the new DNA is not radioactive. On addition of [³H]dTTP, the new DNA becomes labeled. The label intensity increases at each forked junction because of the increase in specific activity of the label during the experiment. Therefore, the taper in the autoradiograph reveals that the direction of fork movement is bidirectional, away from the midpoint (origin) between two sets of replication forks. [Source: (b) Photo from J. Huberman and A. Tsai, *J. Mol. Biol.* 75:5-12, 1973. Courtesy of Joel Hu.]

As in bacteria, replication bubbles grow bidirectionally in eukaryotes, with moving replication forks on either side. Using a variation on the methods used in bacteria, other scientists tested this in mammalian cells. They supplied the cells with [³H]thymidine of low specific activity, followed by a short pulse of [³H]thymidine of high specific activity. The resulting autoradiograph, in Figure 11-3b, shows a gradient of radioactive density that is heaviest at forked junctions moving in opposite directions, indicating that replication forks travel bidirectionally from each origin. Only the replication bubbles, not the entire DNA, are visible in the autoradiograph because the duration of the experiment was short, far less than needed for a full round of replication. Experiments of this type have also been performed in bacteria, archaea, and other eukaryotic cells, all with similar results.

We know that specific origin sequences are present in bacteria, archaea, and the simple eukaryote *Saccharomyces cerevisiae* (baker's yeast), but the nature of the origins of replication in complex eukaryotes remains uncertain. More details on replication origins, including how they initiate replication, are discussed in Section 11.4.

Bidirectional replication is not universal. The *E. coli* Col E1 plasmid is an example of a circular plasmid with a defined origin that replicates in a single direction. The genome of some organisms, such as bacteriophage Φ29 and adenovirus, is a single, linear, double-stranded DNA with no internal origin. These genomes are replicated starting from their ends. Another form of replication, used by certain phages with a circular genome, involves a single replication fork that proceeds multiple times around the circular DNA to generate numerous copies of the genome.

Replication Is Semidiscontinuous

The Y-shaped replication fork consists of a parental duplex DNA stem and two prongs, the new daughter duplexes. The parental DNA strands are antiparallel, so the links between nucleotides in the daughter strands run in opposite directions (Figure 11-4). However, all known **DNA polymerases**, the enzymes that synthesize DNA, extend DNA in only one direction: 5'→3'. Hence, both daughter strands cannot be replicated in the same direction that the replication fork moves. As we'll discuss later in the chapter, DNA polymerase cannot initiate DNA chains; these must be initiated by short sections of RNA, referred to as primers, synthesized by an enzyme called primase.

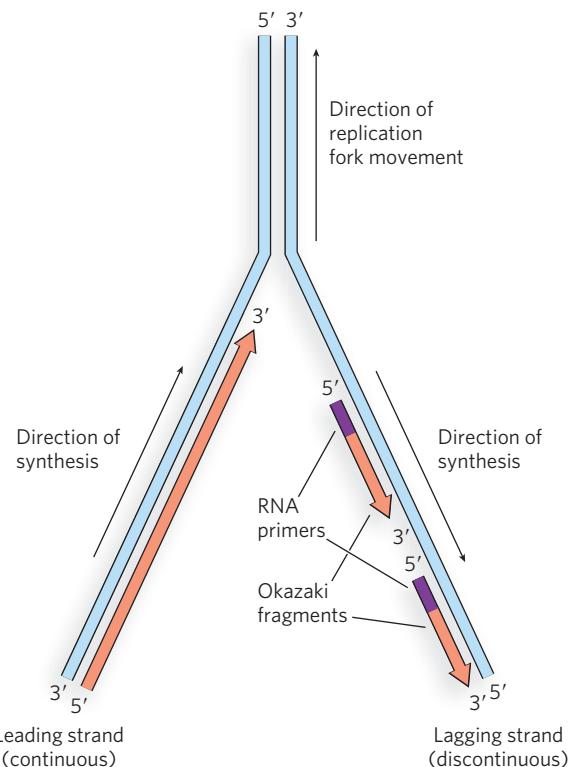


FIGURE 11-4 Structure of the replication fork. DNA polymerases can extend DNA only in the 5'→3' direction, but the two parental strands are antiparallel. Therefore, one daughter strand (the leading strand) is synthesized continuously, in the direction of fork movement, while the strand synthesized in the opposite direction (the lagging strand) must be replicated discontinuously as a series of Okazaki fragments.

KEY CONVENTION

The direction of synthesis by DNA polymerases refers to the direction in which each new nucleotide is chemically linked to the growing daughter strand. All DNA polymerases function in the 5'→3' direction, linking the α-5'-phosphate of a new dNTP to the 3' position of the nucleotide residue at the end (i.e., the 3' end) of the chain.

Reiji Okazaki and his coworkers investigated the proposal that one of the daughter strands is continuously extended in the same direction that the replication fork moves, while the other strand is synthesized in the opposite direction. The strand that is made in the direction opposite to fork movement is still synthesized in the 5'→3' direction, and thus must be reinitiated at intervals and synthesized as a series of

fragments (see Figure 11-4). This mode of replication is described as **semidiscontinuous** because only one daughter strand is synthesized continuously; the other strand is made as a series of discontinuous fragments. The continuously synthesized daughter strand is called the **leading strand**, and the discontinuous daughter strand is the **lagging strand**—the “lagging” designation is based on the fact that some unrepli-cated single-stranded DNA is generated on the lagging strand by the moving fork and therefore the conversion to duplex DNA on this strand lags behind that of the leading strand.

The model of semidiscontinuous replication predicts that during chromosome replication, short DNA fragments are produced on one strand. These fragments are expected to exist only transiently before being connected together. To look for these lagging-strand fragments, Okazaki used *E. coli* cells infected with T4 phage. Because T4 makes many copies of itself simultaneously, detecting lagging-strand fragments is made easier by their abundance. T4-infected *E. coli* cells were labeled with radioactive nucleotide precursors for brief time intervals, then analyzed in alkaline CsCl gradients to denature the new radioactive DNA strands from the unlabeled parental strands. Small fragments in the 1,000 to 2,000 nucleotide (1 to 2 kb) range were observed, as predicted, and have come to be known as **Okazaki fragments**. Lagging-strand Okazaki fragments are 1 to 2 kb long in bacteria but shorter, 100 to 200 nucleotides, in eukaryotes. Each Okazaki fragment is primed at the 5' end by a short RNA, of 10 to 13 nucleotides. The RNA primer is removed by nucle-ase action and Okazaki fragments are joined by ligase soon after the replication fork has passed.

SECTION 11.1 SUMMARY

- DNA replication is semiconservative; each daughter chromosome contains one parental strand and one newly synthesized strand.
- Replication is initiated at specific sites on the DNA, the origins of replication. Circular bacterial chromosomes can have only one origin, whereas long, linear eukaryotic chromosomes are dotted with numerous origins.
- Replication usually proceeds bidirectionally from the origin, at growing points known as replication forks.
- At a replication fork, the parental DNA strands are separated and used as templates to simultaneously form two new daughter duplexes.

- The two strands of DNA in a duplex are antiparallel, yet DNA polymerases extend DNA only in the 5'→3' direction. Therefore, only one strand, the leading strand, is extended “continuously” in the direction of replication fork movement; the other strand, the lagging strand, is synthesized “discontinuously” in the opposite direction as a series of Okazaki fragments. This is the process of semidiscontinuous replication.

11.2 The Chemistry of DNA Polymerases

The first DNA polymerase was identified in the 1950s by Arthur Kornberg and his postdoctoral fellow, Robert Lehman (see How We Know). In so doing, they initiated what would become an entire field of study on DNA replication enzymology. Initially, *E. coli* DNA polymerase I (Pol I) was simply called “DNA polymerase” as it was presumed to be the only DNA polymerase in the cell. We now know, after decades of study, that *E. coli* contains five different DNA polymerases, involved in a variety of cellular processes. In fact, Pol I mainly functions in the repair of damaged DNA rather than in chromosome replication. Nonetheless, we focus here on Pol I because the study of this enzyme taught us features of DNA synthesis that are common to all DNA polymerases, and it remains the most intensively studied and well-characterized DNA polymerase.

DNA Polymerases Elongate DNA in the 5'→3' Direction

Early work on Pol I led to the definition of two central requirements for DNA polymerization. First, all DNA polymerases require a **template strand** that guides the polymerization reaction according to the Watson-Crick base-pairing rules: where dC is present in the template, dG is added to the new strand, and so on (Figure 11-5a). Second, DNA polymerases require a **primer strand** that is complementary to the template and contains a free 3'-OH group to which a nucleotide can be added. In other words, DNA polymerases can only add nucleotides to a preexisting strand; they cannot synthesize DNA starting from only a template strand. The free 3' end of the primer, where nucleotides will be added, is called the **primer terminus**. The double-stranded DNA formed by the primer and template is the **primed template**. Most primers are oligonucleotides of RNA rather than DNA, and specialized enzymes (primases) synthesize primers when and where they are required (see Section 11.3).

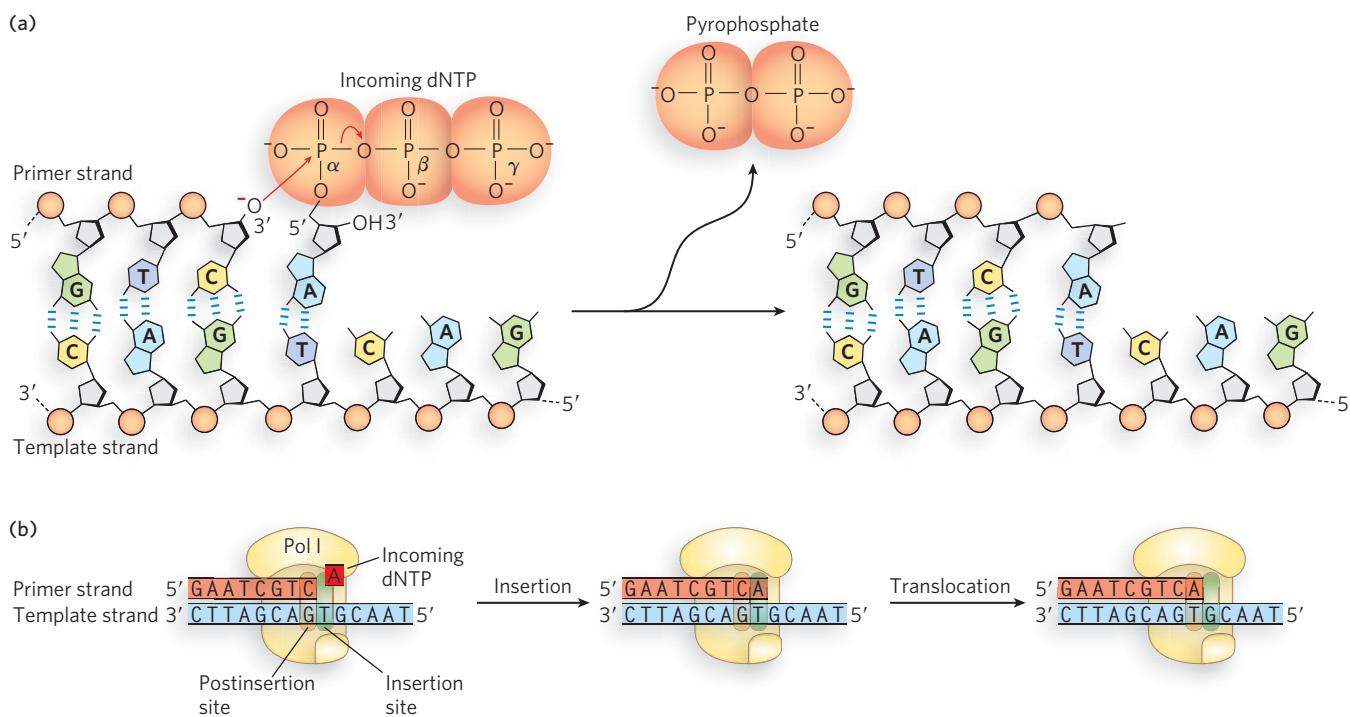


FIGURE 11-5 The DNA polymerase reaction. (a) DNA polymerases require a primer strand and a template strand (i.e., a primed template). As dNTPs are added to the 3'-OH group of the primer strand, this strand grows in the 5'→3'

Study of Pol I confirmed that the nucleotide precursors to DNA are the four deoxyribonucleoside 5'-triphosphates (dNTPs). The studies also showed that the different dNTPs bind the same active site. Pol I differentiates among dNTPs only after it undergoes a conformational change in which the bound dNTP is base-paired to the template strand. Only the correct geometry of an A=T or G≡C base pair fits into the active site. An incorrect fit results in dissociation of the dNTP and the binding of a new one. Normally, the polymerase is able to distinguish the correct nucleotide with this method, but one in every 10⁴ to 10⁵ nucleotides is added incorrectly.

The 3'-hydroxyl group of the primer strand, (dNMP)_n, is activated to attack the α phosphorus of the incoming dNTP, resulting in attachment of a dNMP to the primer 3' terminus and release of pyrophosphate (PP_i) (see Figure 11-5a). The overall reaction is:



Following incorporation of a dNMP, Pol I must slide forward on the new 3' terminus to incorporate another dNTP. Therefore, the DNA polymerase active site must have at least two sites (Figure 11-5b). The template nucleotide that pairs with the incoming dNTP is positioned in the **insertion site**. The primer 3'-terminal base pair is positioned in the **postinsertion site**. After

direction. Incoming dNTPs are complementary to the template strand. (b) The insertion and postinsertion sites in DNA polymerase properly align the primed template for sequential addition of incoming nucleotides.

incorporation of a dNMP at the primer terminus, the new terminal base pair occupies the insertion site and must be translocated to the postinsertion site, allowing the next template nucleotide and a new dNTP to occupy the insertion site. Translocation of DNA can occur by sliding of the enzyme or by its dissociation from the DNA, followed by rebinding with the terminal base pair in the postinsertion site.

DNA synthesis proceeds with only a minimal change in free energy, given that one phosphodiester bond is formed at the expense of a somewhat less stable phosphoanhydride bond. However, noncovalent base-pairing and base-stacking interactions of the newly added nucleotide residue provide additional stabilization that favors incorporation of the correct dNMP into the growing DNA molecule. Pol I can also catalyze the reverse reaction, called pyrophosphorolysis, in which PP_i and primed DNA drive Pol I to remove dNMPs from the primer strand and release them as dNTPs. In the cell, pyrophosphorolysis is largely prevented by the action of pyrophosphatase, which splits pyrophosphate into two molecules of P_i, thereby removing PP_i so that the reverse reaction cannot occur. Pyrophosphate hydrolysis is energetically favorable and goes to near completion.

At first glance, the use of a dNTP to form one dNMP link to DNA might seem a waste of energy. Why not use

dNDP as the nucleotide precursor, which would produce the same DNA product and just one inorganic phosphate (P_i) instead of PP_i , which is then split into two P_i ? The drawback would be that the reverse reaction is easily initiated with just P_i , a common molecule in living cells, as product. Using triphosphate precursors instead of diphosphates ensures that the reverse reaction will not occur, because PP_i is eliminated by pyrophosphatase—making the DNA polymerase reaction irreversible. This is probably why all known DNA polymerases use triphosphate precursors. The same strategy also operates for RNA polymerases, which use NTPs and release PP_i during RNA synthesis.

The initial studies of Pol I, performed decades ago, demonstrated its substrate requirements for a primer template and dNTP precursors. Many different DNA polymerases have been studied over the years, from sources as diverse as phages, other viruses, various types of bacteria, archaea, and eukaryotes, and mitochondria from many different species. All known DNA polymerases use the mechanism shown in Figure 11-5.

Most DNA Polymerases Contain DNA Exonuclease Activity

DNA nucleases are a class of enzymes that degrade DNA. Nucleases that shorten DNA from the ends are called **exonucleases**; **endonucleases** cut DNA at internal positions. Despite repeated attempts to separate the exonuclease activity from Pol I during enzyme purification, the two opposed activities—polymerase and nuclease—remained together, and Kornberg had no choice but to come to the paradoxical conclusion that the same enzyme that makes DNA also degrades it. In fact, Pol I has two different exonuclease activities: one starts at the 3' end and degrades DNA in the 3'→5' direction (opposite to the direction of DNA synthesis), and the other starts at the 5' end and degrades DNA in the 5'→3' direction. The active sites of the two DNA exonucleases of Pol I are distinct from each other, and from the DNA polymerase active site.

KEY CONVENTION

Exonucleases that digest a DNA strand from the 3' terminus are called 3'→5' exonucleases, because the strand shortens at the 3' end while the 5' end remains intact. In contrast, 5'→3' exonucleases digest DNA from the 5' terminus.

The 3'→5' Exonuclease DNA polymerases are typically very accurate and produce only about one error every 10^4 to 10^5 nucleotides incorporated, by incorrect base

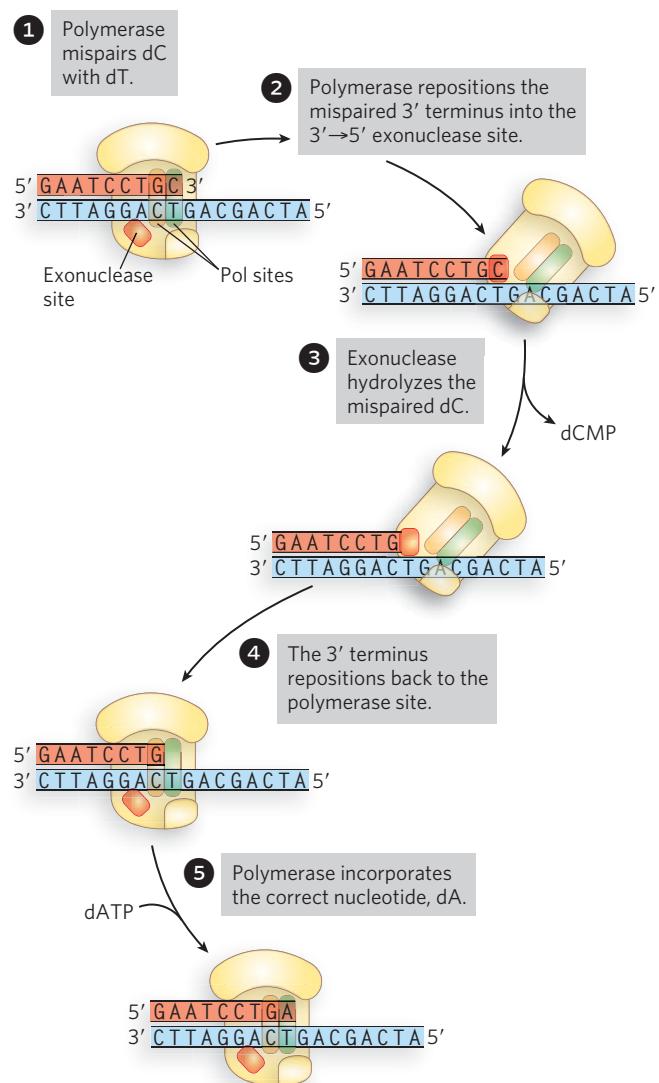


FIGURE 11-6 The 3'→5' proofreading exonuclease of DNA polymerases. The 3'→5' exonuclease active site is distinct from the polymerization site and it proofreads the DNA polymerase product.

selection. This error rate is improved 10²- to 10³-fold by 3'→5' exonuclease activity. When an incorrect dNMP is incorporated, the 3'→5' exonuclease removes the mismatched nucleotide, giving the polymerase a second chance at incorporating the correct one (Figure 11-6). This activity, known as **proofreading**, is not the reverse of the polymerization reaction—pyrophosphate is not involved, and the mismatched nucleotide is released as a dNMP, not a dNTP. Also, proofreading by the 3'→5' exonuclease occurs at a separate active site from polymerization. The mismatched primer terminus repositions from the polymerase site to the exonuclease site, where water is activated to hydrolyze the 3' nucleotide from the primer strand. Most DNA polymerases contain

this proofreading exonuclease activity, although some do not and thus become more capable of bypassing a DNA lesion.

One way in which DNA polymerase makes errors is by incorporating dNTP tautomers (see Chapter 6) at the polymerase active site. Purine and pyrimidine tautomers can form non-Watson-Crick base pairs with nucleotides in the template strand. Sometimes these incorrect base pairs are indistinguishable in shape and size from correct A=T or G≡C base pairs, thus “fooling” the enzyme into incorporating the incorrect nucleotide. Tautomers exist only transiently, and rapidly revert to their usual bonding structure. When a tautomer of an incorrect nucleotide is incorporated into the growing DNA chain and then rapidly reverts to its normal structure, the primer terminus becomes unpaired and no longer fits into the polymerase active site for addition of the next dNTP. This significantly slows further chain extension and gives time for the mispaired 3' terminus to relocate from the polymerase site to the 3'→5' exonuclease site, where the mispaired nucleotide is quickly removed. The DNA can then transit back to the polymerase site, allowing the polymerase to have another try.

When base selection and proofreading are combined, Pol I leaves behind one net error for every 10^6 to 10^8 nucleotide additions. The DNA polymerase involved in chromosome replication has a similar error rate. How accurate must a DNA polymerase be to replicate the *E. coli* genome without making a mistake? Replication of the 4.6×10^6 bp (4.6 Mbp) chromosome requires polymerization of 9.2×10^6 dNTPs. An error rate of about 1 in 10^7 would result in only one incorrect nucleotide insertion per cell division. In fact, the observed accuracy of the overall replication process in *E. coli* is one error in 10^9 to 10^{10} polymerization events. The additional accuracy derives from a repair system that recognizes and removes mismatches that escape both the polymerase and the proofreading exonuclease activities (see Chapter 12). At this level of accuracy, only a single error is incurred in every 100 to 1,000 new cells.

The 5'→3' Exonuclease Pol I also contains a second exonuclease that degrades DNA in the 5'→3' direction, the same direction as DNA synthesis. The 5'→3' exonuclease is unique to Pol I and reflects the enzyme's role in DNA repair. Pol I performs a host of clean-up functions during replication, recombination, and repair, which require the trimming of single-stranded DNA ends and the removal of RNA primers or DNA lesions. Both exonucleases of Pol I are applied to these tasks. However, only the 5'→3' exonuclease is capable of functioning at the same time as the polymerase, because the two activities act in the same direction. As the

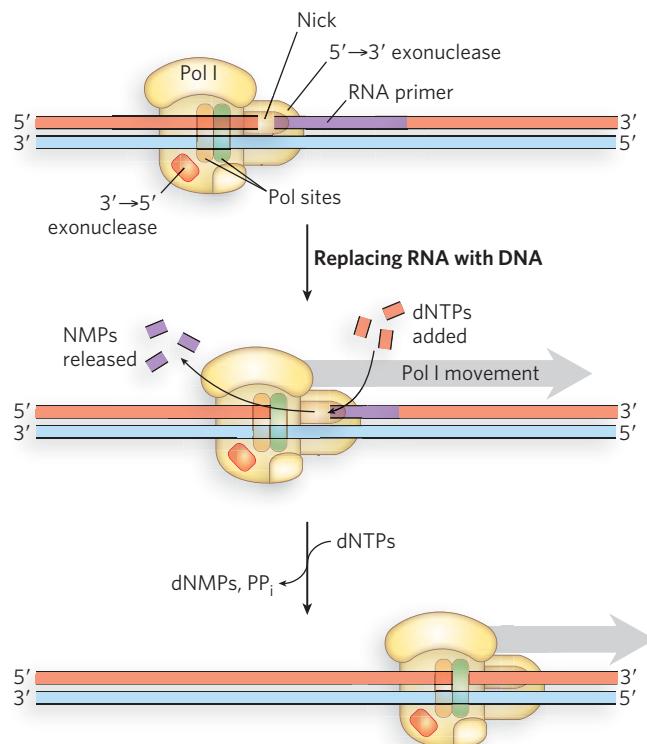


FIGURE 11-7 **Nick translation by Pol I.** DNA polymerase I (Pol I) is organized into three major domains: DNA polymerase, 3'→5' proofreading exonuclease, and 5'→3' exonuclease. At a nick—here, the gap between lagging-strand fragments—Pol I degrades the RNA primer in the 5'→3' direction, releasing NMPs, and simultaneously extends the 3' terminus with dNTPs in the same direction. The net result is movement of the nick in the 5'→3' direction along the DNA until all RNA is removed. DNA ligase can then seal the fragments (not shown here).

5'→3' exonuclease degrades a DNA or RNA strand in the duplex, the polymerase simultaneously adds dNTPs behind it. This concerted action of 5'→3' excision and DNA polymerization is called **nick translation** (shown in Figure 11-7 for removal of an RNA primer). “Nick translation” refers to the fact that a nick in the DNA gets translated along the length of the strand. The nick in DNA during nick translation is a discontinuity in the phosphodiester backbone between the 3' hydroxyl of one nucleotide and the 5' phosphate of the adjacent nucleotide. After Pol I dissociates from DNA, the nick is sealed by DNA ligase (see Section 11.3).

Five *E. coli* DNA Polymerases Function in DNA Replication and Repair

Escherichia coli contains five different DNA polymerases (Table 11-1). The large excess of intracellular Pol I delayed the discovery of the other DNA polymerases. Then, in the 1970s, DNA polymerase II (Pol II) and

Table 11-1 The Five DNA Polymerases of *E. coli*

DNA Polymerase	Number of Subunits	Mass (kDa)	Gene(s)	Function	3'→5' Exonuclease?	5'→3' Exonuclease?
Pol I	1	103	<i>polA</i>	Okazaki fragment processing and DNA repair	Yes	Yes
Pol II	1	88	<i>polB</i>	Translesion synthesis	Yes	No
Pol III	3	167	<i>dnaE, holE, dnaQ</i>	Chromosome replication	Yes	No
Pol IV	1	40	<i>dinB</i>	Translesion synthesis	No	No
Pol V	2	69	<i>umuC, umuD</i>	Translesion synthesis	No	No

DNA polymerase III (Pol III) were discovered in studies using a *polA* mutant strain, depleted of most Pol I. These studies showed that Pol III was the DNA polymerase that replicates the chromosome, sometimes referred to as a replicase, or chromosomal replicase; Pol II seems to be involved in DNA repair. Pol IV and Pol V weren't discovered until 1999.

Pol IV and Pol V are different from the other DNA polymerases in that they lack a 3'→5' proofreading exonuclease and thus often incorporate the wrong nucleotide. These low-fidelity polymerases are produced in cells when the DNA sustains damage that stalls the replication fork (see Chapter 12). The low accuracy of Pol IV and Pol V enables them to insert an incorrect nucleotide opposite a damaged template base. Although this results in an error, it gets the replication fork moving again. The ability of the replication fork to move past

a damaged site is a matter of life and death, and all cells—bacterial, archaeal, and eukaryotic alike—contain these error-prone “translesion” DNA polymerases.

DNA Polymerase Structure Reveals the Basis for Its Accuracy

The crystal structure of *E. coli* Pol I resembles a right hand, with domains referred to as the palm, thumb, and fingers. All DNA polymerases have these same structural features. The bound DNA lies on the palm domain, which contains the polymerase active site and is the most conserved feature among all DNA polymerases. The fingers domain contains the dNTP-binding site, and the thumb domain partially curves around the duplex portion of the primed template, tightening the grip on DNA (Figure 11-8).

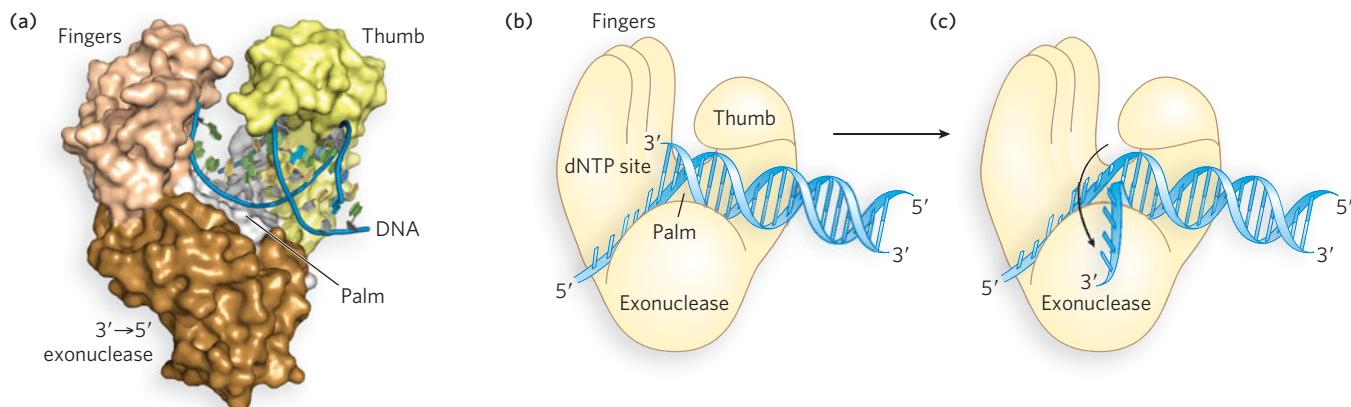
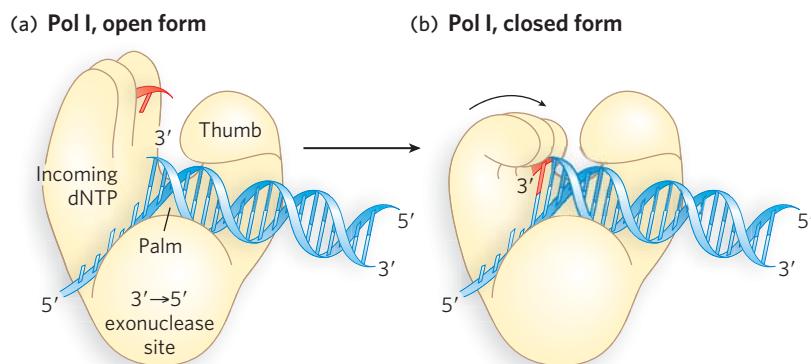


FIGURE 11-8 The structure of Pol I. (a) Crystal structure of *Thermus aquaticus* Pol I bound to DNA. All DNA polymerases are shaped like a hand and contain domains referred to as fingers, thumb, and palm. The 3'→5' exonuclease is in a separate domain from the polymerase active site. *E. coli* Pol I (not shown) also contains a 5'→3' exonuclease. (b) The 3'

terminus of the DNA binds to the palm, but neither the duplex DNA nor the template 5' single-stranded DNA enters the cleft between the fingers and thumb. The dNTP-binding site is located in the fingers. (c) A mispaired 3' terminus frays by about four nucleotides to insert into the 3'→5' exonuclease site. [Source: (a) PDB ID 4KTQ.]

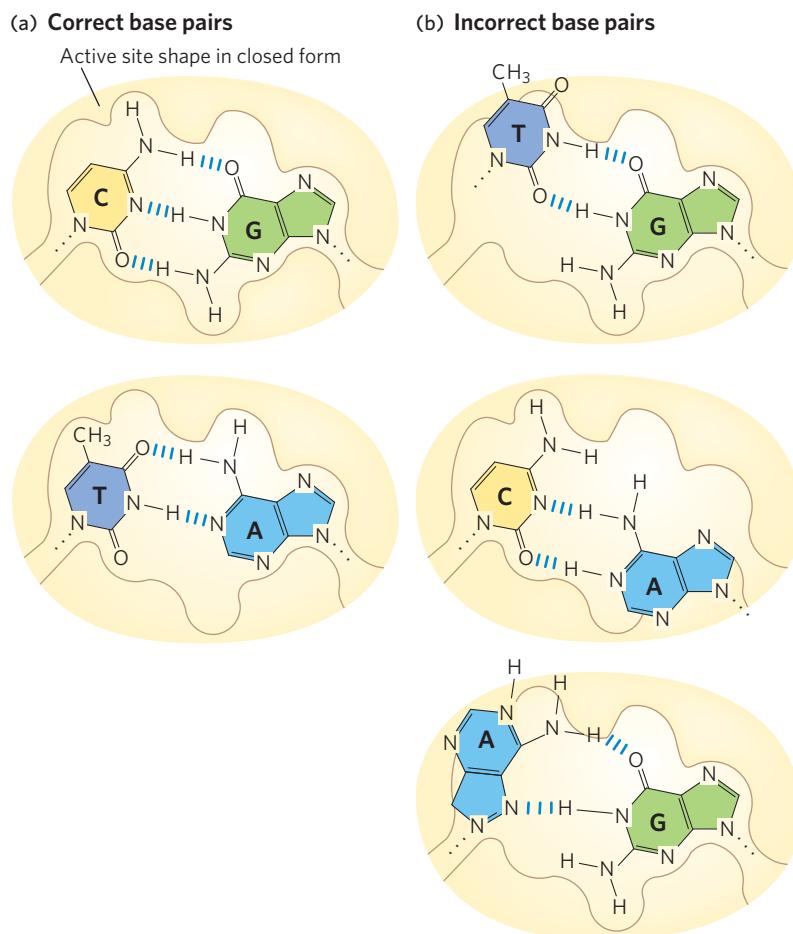
**FIGURE 11-9** Open and closed forms of Pol I.

(a) In the open form, a dNTP binds to the fingers domain. (b) In the closed form, the fingers domain undergoes a 40° rotation that moves the dNTP into base-pairing position with the template and forms an active site cavity that fits the shape of a correct Watson-Crick base pair.

A dramatic conformational change occurs on the binding of a correct dNTP to the fingers domain. The domain rotates inward about 40°, carrying the dNTP down to the template. In so doing, the enzyme forms an active site enclosure with a shape that corresponds to a correct base pair. This conformation of Pol I is often referred to as the **closed form**, to distinguish it from the **open form** prior to dNTP binding (Figure 11-9). The A=T and G≡C base pairs have similar shapes, and either pair fits into the active site cavity of the closed

form. However, incorrect base pairs cannot be accommodated and this prevents Pol I from completely closing around the DNA (Figure 11-10). Only when Pol I is fully closed do the catalytic moieties at the active site properly align for rapid catalysis. Thus, the accuracy of the polymerase functions at the level of shape recognition and provides a classic example of an induced-fit catalytic mechanism (see Chapter 5).

Incorporation of an incorrect base pair is 10- to 1,000-fold slower than incorporation of a correct base

**FIGURE 11-10** Base pairing in the Pol I active

site cavity. (a) The shape of the active site in the closed conformation. Correct G≡C and A=T base pairs fit into the active site.

(b) Incorrect base pairs do not fit.

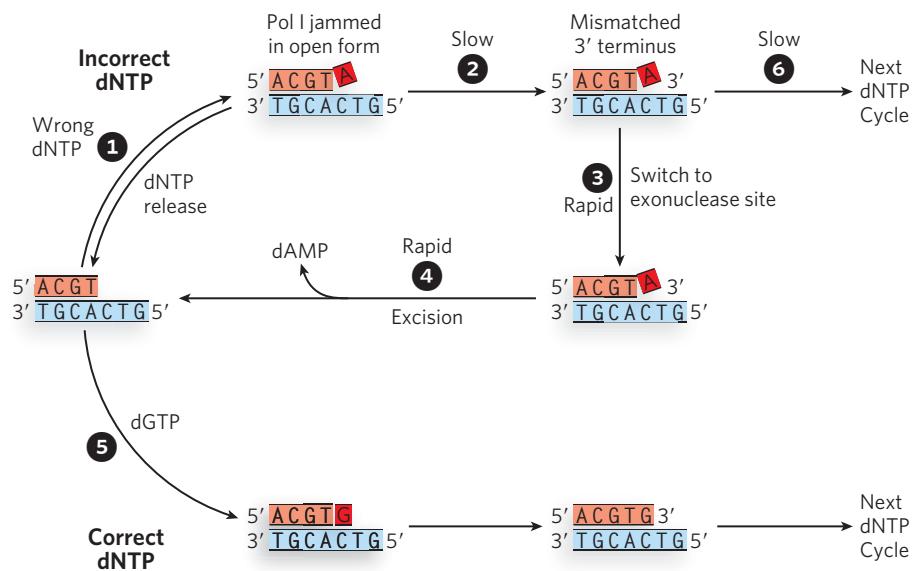


FIGURE 11-11 Favored incorporation of a correct dNTP over an incorrect dNTP. (1) When an incorrect dNTP enters the insertion site on Pol I, binding is readily reversed (back arrow). (2) In the rare instance of incorporation of the incorrect dNTP, the process is slow due to the imperfect active site fit in the closed form. (3) In the favored (rapid) route, the mispaired 3' terminus is shifted to the 3'→5'

exonuclease, and (4) the mismatched nucleotide is excised to re-form the original primed site. (5) This allows Pol I to insert the correct nucleotide on the second try. Binding and incorporation of a correct dNTP is rapid, and paves the way for the next round of incorporation. (6) If the incorrect nucleotide remains, the mismatched DNA is slow to act as substrate for the next round of dNTP incorporation.

pair, which gives the polymerase pause prior to incorporation of an incorrect nucleotide. This kinetic pause gives time for the incorrect dNTP to dissociate and for Pol I to bind another dNTP. Even when a mismatched dNTP is incorporated, the proofreading 3'→5' exonuclease usually excises it. The 3'→5' exonuclease is a separate domain located 20 to 30 Å from the polymerase active site. Stalling of catalysis by an incorrect 3' terminus buys time for the mismatched primer terminus to relocate to the 3'→5' exonuclease domain for proofreading (see Figure 11-8). The slow incorporation yet rapid removal of a mispaired dNTP underlies the inherent accuracy of DNA polymerases. Accuracy is further enhanced by a vastly diminished rate of dNTP incorporation at a mismatched 3' terminus (Figure 11-11).

The polymerase active site contains two magnesium ions that are held in place by conserved Asp residues (Figure 11-12a). One Mg^{2+} deprotonates the primer 3'-OH group to form the 3'- O^- nucleophile. The other binds the incoming dNTP and facilitates departure of the pyrophosphate leaving group. This two-metal-ion catalyzed reaction is remarkable in that no amino acid side chain plays a direct role in catalysis; the two metal ions do it all. The 3'→5' exonuclease uses a similar two-metal-ion mechanism, in which one Mg^{2+} deprotonates H_2O to form the nucleophile (HO^-) for hydrolysis of the 3' dNMP, and the other promotes

departure of the leaving group by stabilizing the charge of the dNMP product (Figure 11-12b). The exclusive use of metal ions to catalyze DNA synthesis suggests that the first polymerase may have been an RNA molecule, as RNA is highly effective in the coordination of metal ions (see Chapter 16).

Surprisingly, certain mutations result in DNA polymerases that are even more accurate than wild-type DNA polymerases. Many of these “antimutator” DNA polymerases contain a hyperactive 3'→5' exonuclease; they even excise perfectly good bases. Cells with antimutator DNA polymerases display lower levels of spontaneous mutation. Why haven’t cells with the more accurate DNA polymerases been selected by evolution? The energy cost of using only a highly accurate polymerase must outweigh the benefit; spontaneous mutation also provides variation within a population, which is a necessary component of evolution.

Processivity Increases the Efficiency of DNA Polymerase Activity

During DNA synthesis, the DNA product must be moved to the postinsertion site so that the new 3' terminus lies in the insertion site for addition of the next dNTP. To accomplish this repositioning, a polymerase can take either of two paths. It can fully dissociate from

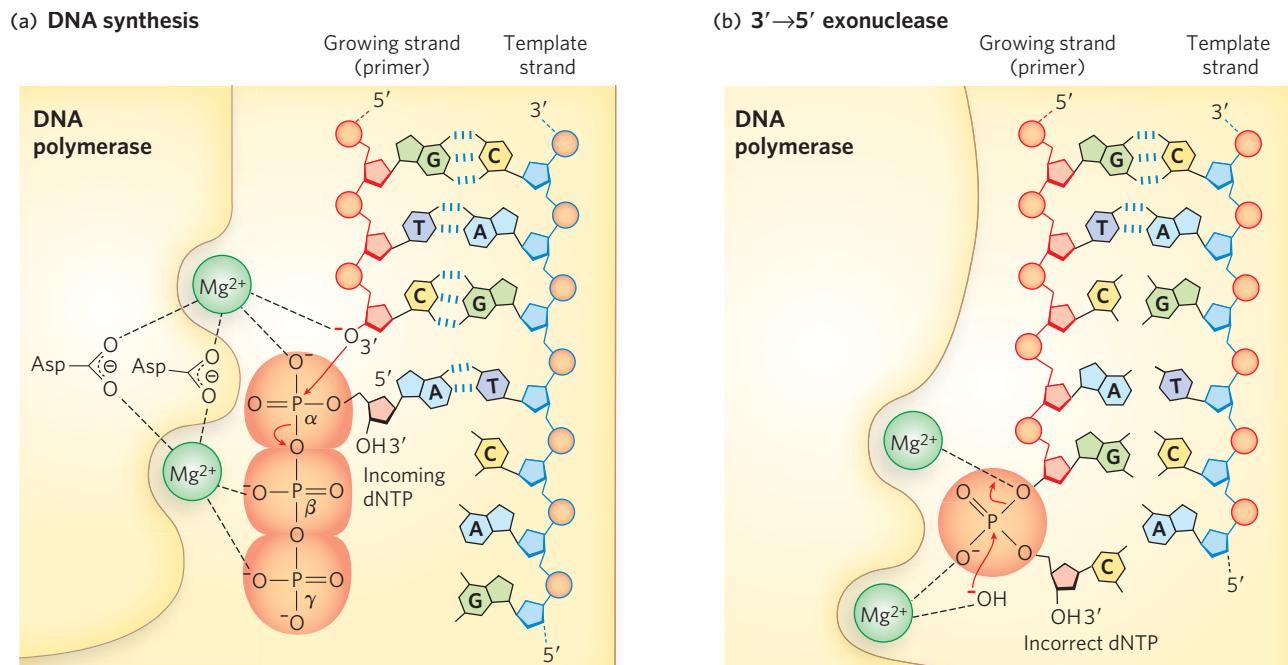


FIGURE 11-12 The role of metal ions in DNA polymerase catalysis. (a) The DNA polymerase active site contains two divalent metal ions (Mg^{2+}) held in place by residues in the palm, including conserved Asp residues. The two ions play

an essential role in catalysis, as described in the text. (b) The $3' \rightarrow 5'$ exonuclease also uses a two-metal-ion catalyzed reaction.

the DNA and rebind the primer terminus in the postinsertion site; this dissociation followed by rebinding at each nucleotide addition is referred to as **distributive synthesis**. Alternatively, the polymerase may simply slide forward one base pair along the DNA to reposition the 3' terminus, without dissociating from the DNA. When a polymerase remains attached to DNA during multiple catalytic cycles, the process is known as **processive synthesis**.

The “processivity number” is the average number of nucleotides incorporated before the enzyme dissociates from the DNA. Processivity can result in exceedingly efficient polymerization, because much time is wasted by a dissociated polymerase in locating and rebinding a 3' primer terminus. Pol I has a processivity number of 10 to 100 nucleotides, depending on conditions. In contrast, Pol III, like most DNA polymerases that replicate chromosomes (i.e., replicases), has a processivity number in the thousands, which, as we'll see, results from a protein ring that encircles the DNA.

- A dNTP that correctly base-pairs to the template strand is incorporated into the primer strand with the release of pyrophosphate. Pyrophosphate is hydrolyzed by pyrophosphatase, which reduces the concentration of pyrophosphate in the cell and makes the reverse reaction extremely unlikely.
- DNA polymerases are inherently very accurate, and are made even more accurate by a proofreading $3' \rightarrow 5'$ exonuclease.
- Pol I also contains a $5' \rightarrow 3'$ exonuclease that degrades DNA while, at the same time, the polymerase synthesizes DNA, in the process of nick translation.
- *E. coli* has five DNA polymerases. Pol III is responsible for chromosome replication, and Pol I is used to remove RNA primers and fill in the resulting gaps with DNA. The other three (II, IV, and V) are involved in DNA repair and in moving replication forks past sites of DNA damage.
- Binding of a dNTP to a DNA polymerase results in a large conformational change, yielding an active site in which only correct base pairs fit.
- DNA polymerases often have high processivity, in which many nucleotides are added to a DNA chain in one polymerase-binding event.

SECTION 11.2 SUMMARY

- DNA polymerases require a primed template and extend the 3' terminus of the primer strand by reaction with dNTPs.

11.3 Mechanics of the DNA Replication Fork

The advance of a replication fork requires the coordinated action of several different types of proteins. As we'll see, many of these proteins work together as a highly dynamic "replisome" machine. Some proteins at a replication fork dissociate from the DNA and are replaced by new ones every few seconds, whereas others are left behind at intervals to direct clean-up processes after the replication fork has passed. In addition, unwinding of the parental DNA requires it to be broken and resealed hundreds of thousands of times, to relieve torsional stress.

Replication fork mechanics are best understood in the *E. coli* system, where the process is streamlined and illustrates the fundamental mechanism of a moving replication fork. Even so, more than a dozen different

proteins are involved (Table 11-2). We describe here the individual replication proteins and how they act together at a replication fork in *E. coli*, then describe replication mechanisms in eukaryotes. Cellular processes in eukaryotes are generally more complex, and several more proteins are involved at the replication fork.

DNA Polymerase III Is the Replicative Polymerase in *E. coli*

Pol III is responsible for replicating the *E. coli* chromosome. The **Pol III core** is a heterotrimer that contains one each of α , ϵ , and θ subunits. The DNA polymerase activity is contained in the α subunit; the ϵ subunit contains the proofreading 3'→5' exonuclease. The role of the θ subunit is currently unknown. The crystal structure of the Pol III α subunit reveals the hand shape of all DNA polymerases and the presence of a PHP domain, unique to Pol III, whose function is unknown

Table 11-2 Proteins Involved in Replication of the *E. coli* Chromosome

Protein	Number of Subunits	Mass, Total (kDa)	Gene(s)	Function(s)
DnaA	1	52	<i>dnaA</i>	Initiator, binds <i>oriC</i>
HU	2	19	<i>hupA, hupB</i>	Stimulates open complex at <i>oriC</i>
DnaC	1	29	<i>dnaC</i>	Helicase loader
DnaB	6 (homohexamer)	300	<i>dnaB</i>	Helicase
Gyrase	4	400	<i>gyrA, gyrB</i>	Type II topoisomerase (A_2B_2)
SSB	4 (homotetramer)	76	<i>ssb</i>	Stabilizes and protects single-stranded DNA
Primase	1	60	<i>dnaG</i>	Synthesizes lagging-strand RNA primers
Pol III holoenzyme	17	796	See Table 11-3	Chromosomal replicase, consists of Pol III core, β clamp, and γ complex clamp loader
Pol I	1	103	<i>polA</i>	Okazaki fragment processing: removes RNA
RNaseH	1	18	<i>rnhA</i>	Okazaki fragment processing: removes RNA
Ligase	1	74	<i>lig</i>	Okazaki fragment processing: joins segments
Dam methylase	1	32	<i>dam</i>	Methylates adenines in GATC sequences
RNA polymerase	5	454	<i>rpoA, B, C, D</i>	Stimulates open complex at <i>oriC</i>
SeqA	1	20	<i>seqA</i>	Binds hemimethylated GATC, inhibits <i>oriC</i>
Hda	1	28	<i>hda</i>	Induces DnaA to hydrolyze ATP
Tus	1	36	<i>tus</i>	Participates in replication termination
Topoisomerase IV	4	308	<i>parC, parE</i>	Decatenates completed chromosomes (C_2E_2)

Table 11-3 Subunit Composition of the Three Components of the *E. coli* Pol III Holoenzyme

Holoenzyme Component(s)	Subunit	Number of Subunits	Mass per Subunit (kDa)	Gene	Function(s) of Subunit
Two Pol III cores	α	2 (1 per Pol III core)	130	<i>dnaE</i>	DNA polymerase
	ε	2 (1 per Pol III core)	28	<i>dnaQ</i>	3'→5' exonuclease
	θ	2 (1 per Pol III core)	9	<i>hole</i>	Binds ε
Two β clamps	β	4 (2 per clamp)	41	<i>dnaN</i>	Sliding clamp
One γ complex clamp loader	γ	1	48	<i>dnaX</i>	ATPase
	τ	2	71	<i>dnaX</i>	ATPase; binds Pol III and DnaB
	δ	1	39	<i>holA</i>	Opens clamp
	δ'	1	37	<i>holB</i>	Binds δ
	χ	1	17	<i>holC</i>	Binds SSB
	ψ	1	15	<i>holD</i>	Binds χ and γ
Totals		17 subunits	796 kDa		

(Figure 11-13). In *E. coli*, as in many bacteria, the α subunit recruits the ε subunit for 3'→5' proofreading activity. In some bacteria, but not *E. coli*, the PHP domain in the α subunit has a 3'→5' exonuclease activity, and these α subunits may not recruit an ε subunit.

The Pol III core is just one part of a much larger protein assembly called the **Pol III holoenzyme**, which replicates both leading and lagging strands (Table 11-3). The Pol III holoenzyme includes two Pol III cores, two ring-shaped **β sliding clamps** that improve processivity, and one **clamp loader** that assembles β clamps onto the DNA (Figure 11-14). The clamp loader includes two

τ subunits with C-terminal domains that protrude from the clamp loader and bind to the Pol III cores.

The Pol III core itself is capable of DNA synthesis at a slow rate, but DNA synthesis by the Pol III holoenzyme is exceedingly rapid, nearly 1 kb/s. The β clamps and clamp loader help maintain contact between the Pol III core and DNA, making it highly processive (~100 kb per binding event). Having two DNA polymerases in one holoenzyme assembly facilitates the coordinated synthesis of the leading and lagging strands at the replication fork. Sliding β clamps are assembled onto both DNA strands by the single clamp loader.

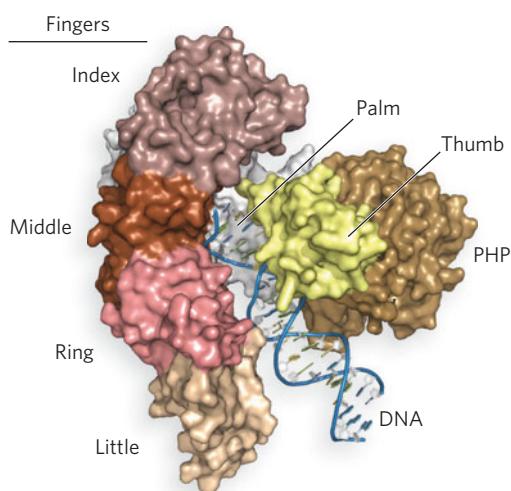


FIGURE 11-13 The *E. coli* Pol III α subunit. A ribbon diagram of the Pol III α subunit, with the palm, fingers, thumb, and PHP domains labeled. [Source: PDB ID 3E0D.]

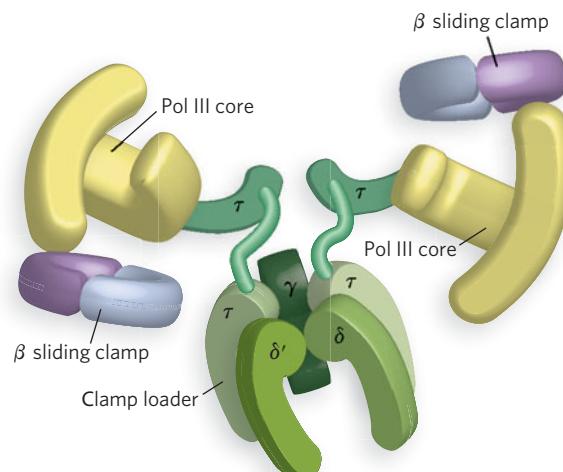


FIGURE 11-14 The architecture of *E. coli* Pol III holoenzyme. The C-termini of the τ subunits protrude from the clamp loader and bind the Pol III cores. Each Pol III core also attaches to a β sliding clamp. [Source: Adapted from N. Yao and M. O'Donnell, *Mol. Biosyst.* 4:1075–1084, 2008.]

A DNA Sliding Clamp Increases the Speed and Processivity of the Chromosomal Replicase

The processivity (1 to 10 nucleotides) and rate of synthesis (10 nucleotides per second) of the Pol III core is dramatically enhanced by the β sliding clamp. The β clamp is a homodimer, shaped like a ring that encircles the duplex DNA (Figure 11-15a). The β clamp has sixfold symmetry; it is constructed from a single domain repeated three times in each monomer. The topological binding of the β clamp to DNA allows the clamp to slide along the duplex while staying tightly attached (see How We Know). The β clamp binds to the Pol III core (Figure 11-15b) and thereby holds Pol III to the DNA while sliding along the duplex, enabling very high processivity (Figure 11-16).

An example of the remarkable speed and processivity conferred by the β clamp is shown in Figure 11-17. The DNA substrate used in this experiment is the large (5.4 kb) single-stranded, circular DNA genome of phage φ X174. With the Pol III core (within the holoenzyme) bound to the β clamp at a primed site, [32 P]dNTPs labeled on the α phosphate were added to start the polymerase reaction. At different times, reaction samples were analyzed in an agarose gel, followed by autoradiography to visualize the newly synthesized radioactive DNA. The results show that Pol III goes full circle within a few seconds. When the β clamp was omitted in a control reaction, no DNA synthesis was observed, because Pol III runs into secondary structures that block

synthesis (data not shown). However, even in the absence of these secondary structures, Pol III would require about 9 minutes to complete the 5.4 kb DNA without the help of the β clamp.

The β sliding clamp does not assemble onto DNA by itself; it requires a multiprotein clamp loader to open and close the ring around the DNA. The clamp loader, called the γ complex, consists of several subunits, γ , τ_2 , δ , and δ' , arranged in a circular pentamer (see Figure 11-14), and two small subunits, χ (chi) and ψ (psi), that connect to other proteins at the replication fork. The clamp loader uses the energy of ATP binding to open the clamp. In this operation, the clamp loader sits on top of the clamp and forces it to open (Figure 11-18a).

The clamp loader contains an inner chamber that binds primed DNA, and this positions the DNA through the sliding clamp. The clamp loading activity is specific to a primed site, because the DNA must bend out of a gap in the side of the clamp loader and only single-stranded DNA, not double-stranded DNA, has the flexibility to make this sharp bend. The γ , τ_2 , δ , and δ' subunits of the clamp loader all contain a homologous region that binds ATP and is found in all **AAA+** proteins, ATPases associated with a variety of cellular activities. The clamp loading reaction is an unusual enzyme-catalyzed process because the DNA and protein substrates are not converted to a new product—they are simply intertwined. Many of the proteins involved in DNA replication contain AAA+ domains and drive protein and DNA conformational changes (see Section 11.4).

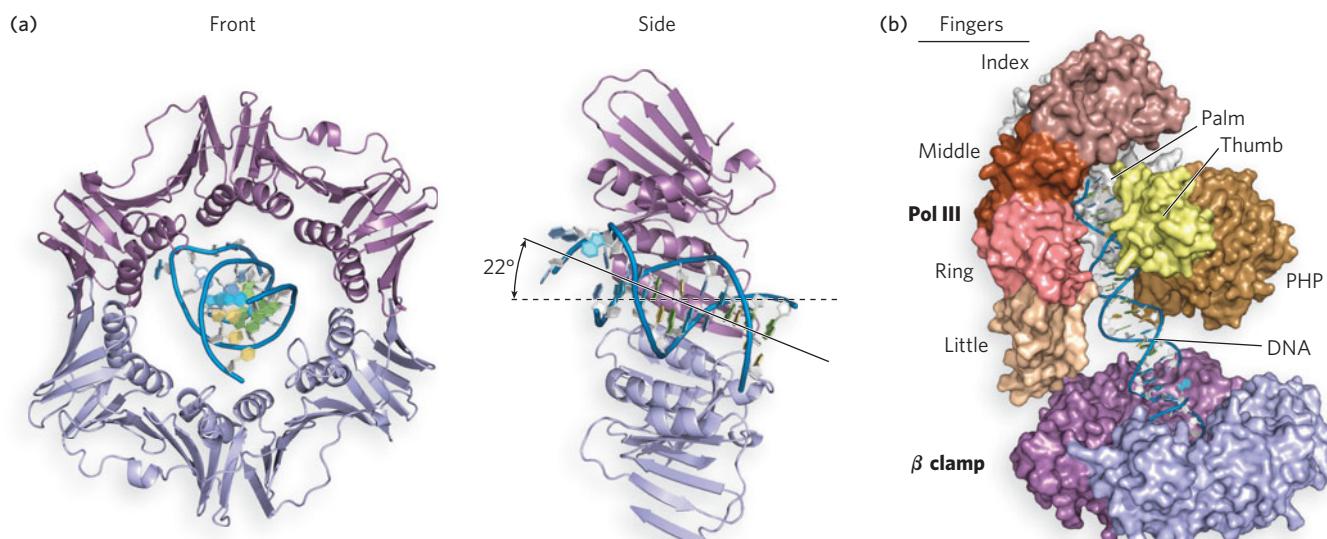


FIGURE 11-15 The *E. coli* β sliding clamp. (a) Frontal view (left) and side view (right) of the *E. coli* β clamp bound to DNA. The DNA is tilted 22° from the perpendicular. Subunits

of β are shades of purple; the DNA is blue. (b) Model of *E. coli* Pol III α bound to the β clamp. [Sources: (a) PDB ID 3BEP. (b) PDB ID 3BEP and 3EOD.]

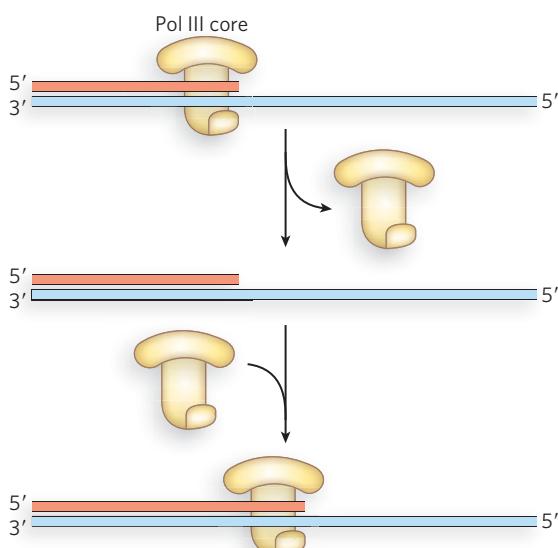
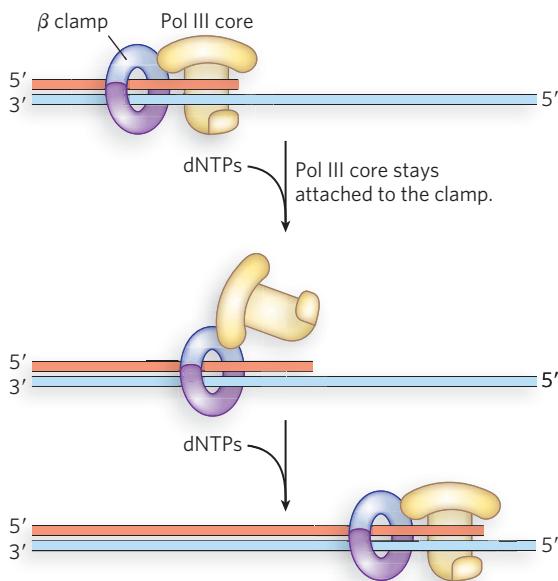
(a) Distributive Synthesis**(b) Processive Synthesis**

FIGURE 11-16 Processivity conferred by the β clamp. (a) In distributive synthesis, the Pol III core extends a primed site by a few nucleotides and then dissociates from the DNA. It must rebind the primed site to continue synthesis. (b) Binding of a Pol III core to the β clamp tethers the core to the DNA for processive synthesis. When the Pol III core detaches from the DNA, it stays attached to the β clamp and rapidly reattaches to the DNA.

In the absence of ATP the clamp loader cannot bind the β clamp, because the subunits are oriented in a way that blocks their interaction with the clamp. ATP

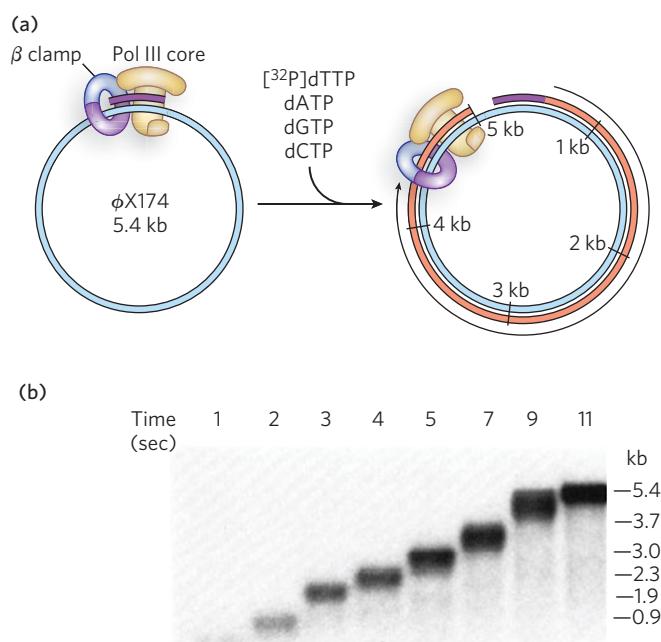


FIGURE 11-17 Rapid DNA synthesis by *E. coli* Pol III. The β sliding clamp is loaded onto a primed site by a clamp loader in the Pol III holoenzyme. Synthesis of the 5.4 kb circular DNA of bacteriophage ϕ X174 is completed within 11 seconds, as shown in the autoradiograph from the experiment described in the text. [Source: Photo from M. E. O'Donnell and A. Kornberg, *J. Biol. Chem.* 260:12,875, 1985, Fig. 2A.]

binding to the γ and τ subunits induces a conformational change that enables the γ complex to bind and open the clamp (Figure 11-18b). ATP is also needed for the γ complex to bind DNA. ATP hydrolysis causes the γ complex to revert to the form that cannot bind β and DNA, thereby ejecting the γ complex and allowing the β ring to close around DNA. It is important that the clamp loader be ejected at the end of the reaction because it binds to the same spot on β to which the Pol III core must attach.

Many Different Proteins Advance a Replication Fork

The simultaneous replication of both DNA strands at a replication fork requires the interaction of many proteins in addition to the Pol III core and β clamp (Figure 11-19a). Here we give particular attention to the *E. coli* system, but all cells contain these basic protein components for chromosome replication.

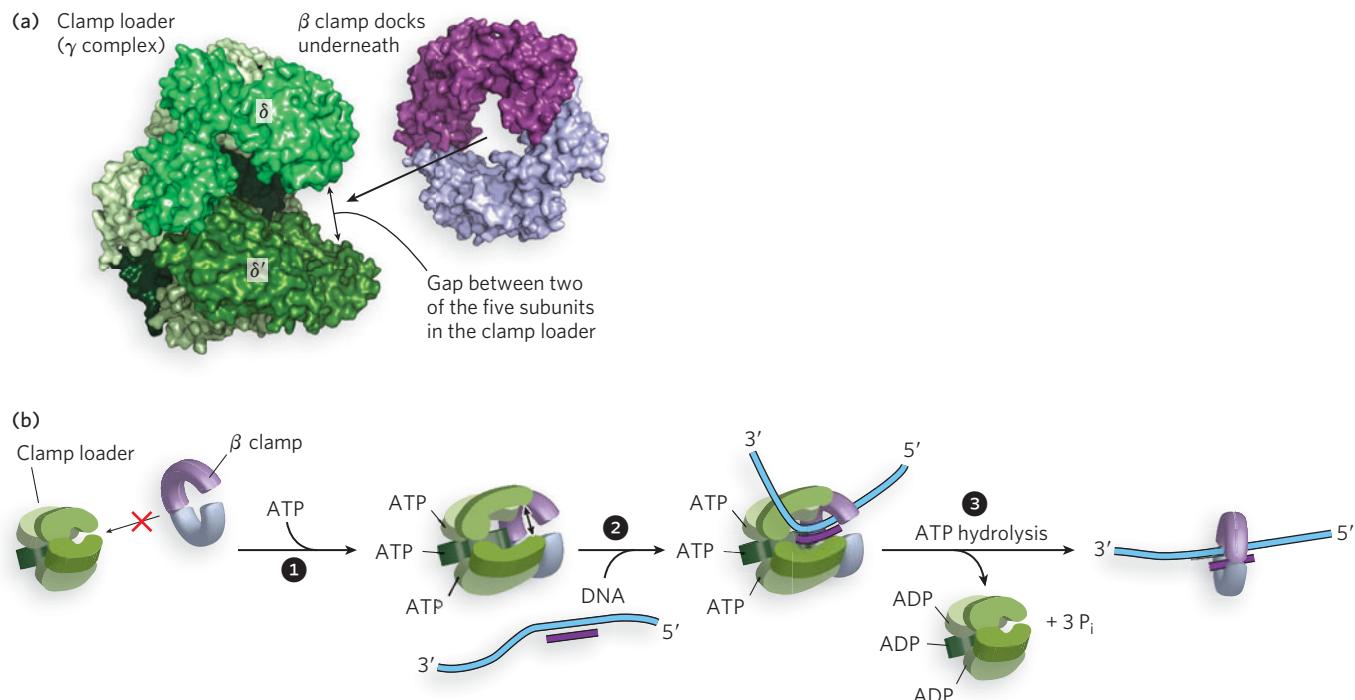


FIGURE 11-18 The *E. coli* γ complex clamp loader. (a) The five clamp-loading subunits are arranged in a circle, with a gap between the lower parts of two subunits, δ' and δ . The β clamp (purple) docks underneath the clamp loader. An α helix in the δ subunit binds to β and opens the clamp. (b) The clamp loading mechanism. (1) ATP binding to the γ subunits powers a conformational change that enables the binding and opening of the β clamp. (2) The combined

γ complex-ATP- β clamp binds primed DNA in a central chamber, and the single-stranded template DNA passes through the gap in the side of the clamp loader. (3) ATP hydrolysis ejects the clamp loader, allowing β to close again around the DNA. [Sources: (a) PDB ID 1JR3.

(b) Adapted from N. Yao and M. O'Donnell, in *Encyclopedia of Biological Chemistry*, 2nd ed. (W. J. Lennarz et al., eds.), Elsevier, 2010, in press.]

DNA Helicase The two strands of the parental DNA duplex are separated by a class of enzymes known as **DNA helicases**, which harness the energy of NTP hydrolysis (usually ATP) to drive strand separation (Figure 11-19b). Helicases are used in a wide variety of DNA and RNA transactions. DNA helicases usually load onto DNA at a single-strand gap in the duplex and move along the DNA strand in one direction (fueled by NTP hydrolysis), unwinding the duplex as they move. The direction of helicase translocation along DNA is characteristic of the particular helicase (see Chapter 5).

KEY CONVENTION

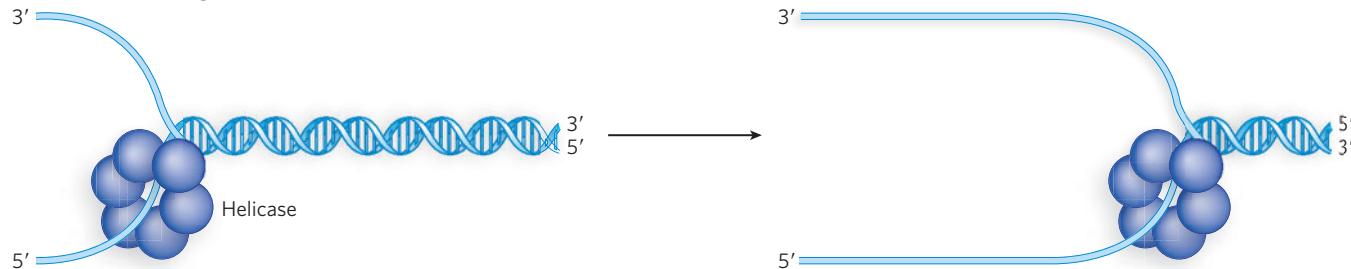
A helicase translocates along a single strand of DNA in one direction, parting the duplex as it moves. The direction of movement is specified by convention to be the direction along the strand to which the enzyme is bound. If the helicase binds to a DNA strand and progresses from the 5' end toward the 3' end, it is said to be a 5'→3' helicase.

Helicases that function at a replication fork are typically ring-shaped hexamers that encircle one DNA strand. The ring shape of replicative helicases is thought to enhance their grip on DNA for processive unwinding. The *E. coli* replicative helicase is a hexamer of DnaB protein; it encircles the lagging strand and translocates in the 5'→3' direction. An assay that demonstrates the action of DnaB is shown in Figure 11-20. The DNA substrate contains a central single-stranded DNA region for helicase assembly, flanked on both sides by duplex DNA of different lengths; one strand of each duplex is radioactively labeled. The direction of helicase translocation is determined by observing which DNA fragment is unwound from the substrate, as analyzed on a polyacrylamide gel. The result shows that DnaB translocates in the 5'→3' direction along a single strand of DNA and unwinds the duplex at only one of the two ends. Helicases require NTPs for translocation, and a control reaction conducted in the absence of ATP shows no unwinding.

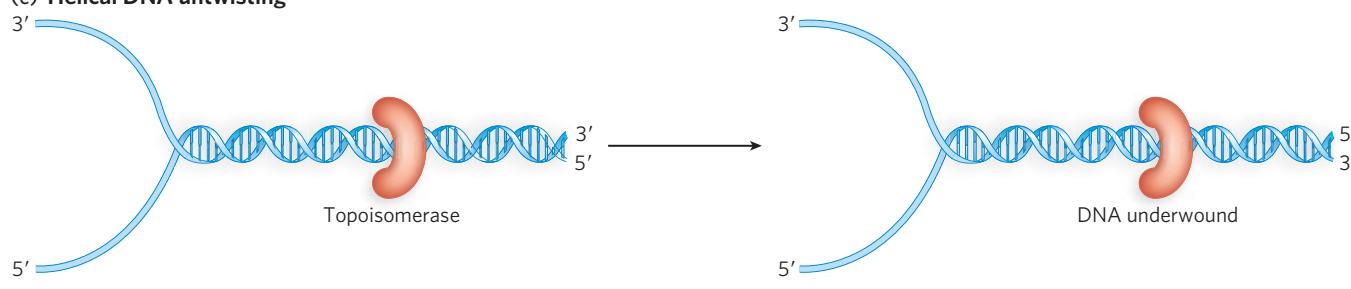
(a) Processive synthesis



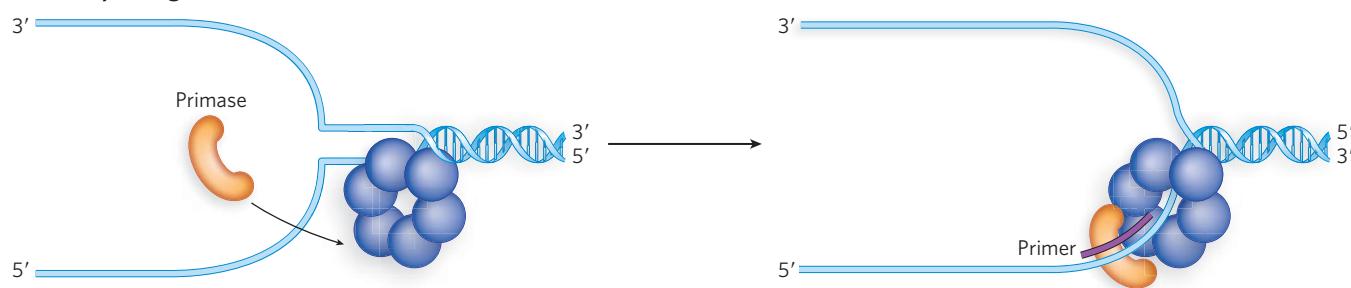
(b) DNA unwinding



(c) Helical DNA untwisting



(d) RNA priming



(e) DNA sealing



(f) ssDNA protection



FIGURE 11-19 Activities required at a DNA replication fork.

(a) DNA polymerase and the β sliding clamp are required for processive DNA synthesis. (b) Replicative helicases are homohexamers that encircle single-stranded DNA and translocate on the DNA strand to separate the strands of the parental duplex. (c) A topoisomerase removes twists in the DNA. In *E. coli*, the topoisomerase is DNA gyrase. (d) Primase (an RNA polymerase) makes short RNA primers to initiate DNA synthesis. Primases typically bind the helicase, thus localizing primers to the replication fork. (e) Ligase seals DNA nicks, joining Okazaki fragments together (after removal of the RNA primers). (f) Single-stranded DNA-binding protein (SSB) binds cooperatively to single-stranded DNA (ssDNA), removing secondary structure in the DNA strand and protecting it from the action of nucleases.

Topoisomerase As a helicase separates the parental duplex, the strands must be untwisted. But unless the DNA strands are broken, the number of turns will remain the same. For example, about half a million twists must be removed to duplicate the 4.6 Mbp *E. coli* genome. The gargantuan task of untwisting DNA is performed by **topoisomerases** that act on duplex DNA ahead of the replication fork (Figure 11-19c). In *E. coli*, gyrase, a type II topoisomerase, is the primary replicative topoisomerase (see Chapter 9).

Primase As discussed earlier, DNA polymerase requires a preformed primer from which to elongate. Cells contain specialized RNA polymerases called **primases** that synthesize short RNA primers specifically for initiating DNA polymerase action. In *E. coli*, an RNA primer of 11 to 13 nucleotides is synthesized by the DnaG primase. RNA primers are needed to initiate each of the thousands of Okazaki fragments on the lagging strand. The leading strand is also initiated by primase at a replication origin. *E. coli* DnaG primase must bind the DNA helicase for activity, and this localizes its action to the replication fork (Figure 11-19d). RNA synthesis is less accurate than DNA synthesis (see Chapter 15), and the use of RNA to prime DNA synthesis provides a way of recognizing and removing the less accurate primer before Okazaki fragments are joined together.

Pol I and Ligase RNA primers must be removed at the end of each Okazaki fragment and replaced with DNA. This is achieved through the nick translation activity of Pol I (see Figure 11-7), which removes the ribonucleotides while simultaneously replacing them with deoxyribonucleotides. A ribonuclease called **RNaseH** can also remove RNA that is base-paired to DNA. Cells often have one or more enzymes with RNaseH activity.

The nick in the phosphodiester backbone is then sealed by **DNA ligase** in a reaction that requires ATP (or NAD⁺ in *E. coli*) (Figure 11-19e; see also Figure 5-12). Ligase acts only on a 5' terminus of DNA, not on RNA. This specificity ensures that all the RNA at the end of an Okazaki fragment is removed before the nick is sealed. Both ligase and Pol I interact with the β sliding clamp.

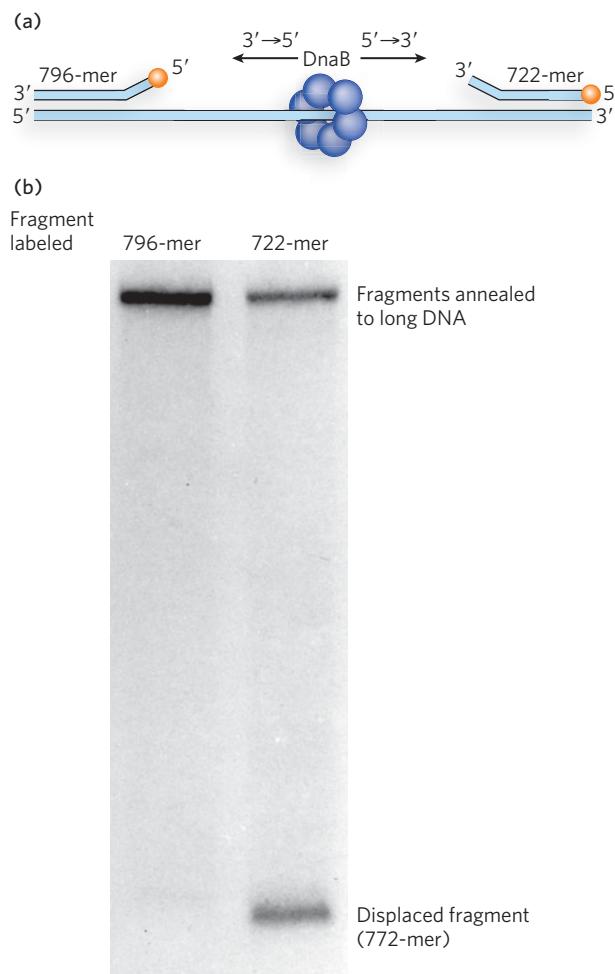


FIGURE 11-20 An assay for determining the direction of **DNA helicase activity**. (a) DnaB helicase is added to a long, single-stranded DNA that has short ³²P-labeled DNA strands of different sizes annealed to its ends (shown here are a 796-mer and 722-mer). Each annealed DNA strand has a single-stranded tail to mimic a replication fork. DnaB initially binds to the single-stranded DNA region, then translocates in one direction to unwind one of the two annealed-DNA duplexes. (b) The DNA-unwinding products are analyzed in an agarose gel. The result here shows that DnaB displaced only the 722-mer, revealing that DnaB translocates in the 5'-3' direction along single-stranded DNA. [Source: (b) J. H. LeBowitz and R. McMacken, *J. Biol. Chem.* 261:4738–4748, 1986.]

SSB Single-stranded DNA produced by helicase unwinding is quickly bound by **single-stranded DNA-binding protein (SSB)**, protecting the DNA from endonucleases (see Figure 5-3). SSB stimulates DNA polymerase activity by melting small DNA hairpin structures (i.e., separating base pairs) in the single-stranded template (Figure 11-19f). SSB is found in all cell types and binds DNA in a sequence-independent fashion. *E. coli* SSB is a homotetramer, but other SSBs range from monomers (e.g., gene 32 protein in T4 phage) to heterotrimers (e.g., RPA in eukaryotes, discussed below).

Helicase Activity Is Stimulated by Its Connection to the DNA Polymerase

The *E. coli* DnaB helicase connects to the Pol III holoenzyme through the same τ subunits that bind the DNA polymerase. Without this connection to polymerase, DnaB helicase is slow, unwinding about 35 bp/s. On connection of DnaB to the Pol III holoenzyme, unwinding proceeds at a rate of approximately 700 bp/s. The complex of Pol III holoenzyme, DnaB helicase, and primase forms a **replisome**. The two strands of DNA at a replication fork thread through the replisome (Figure 11-21). The leading-strand Pol III- β clamp complex moves continuously with DnaB helicase, while the lagging-strand Pol III- β clamp complex repeatedly moves on and off the DNA to extend multiple RNA primers made by primase.

DNA Loops Repeatedly Grow and Collapse on the Lagging Strand

The lagging-strand polymerase must repeatedly extend RNA primers into full-length, 1 to 2 kb Okazaki fragments. As we've seen, however, the direction of chain growth on the lagging strand is opposite to that on the leading strand. How can the lagging-strand Pol III synthesize DNA in the opposite direction to replication fork movement, yet remain tethered to the replisome? To accommodate these opposed directions, the lagging-strand template is pulled up through the polymerase during chain extension to form a loop (Figure 11-22). As an Okazaki fragment is extended, the double-stranded portion of the loop grows longer. As the replication fork generates more single-stranded DNA, it also adds to the growing DNA loop. When the Okazaki fragment is complete, the polymerase bumps into the fragment it made previously and lets go of the DNA loop so that it can extend a new RNA primer for the next Okazaki fragment. The process of repeated loop growth and disassembly is often referred to as the **trombone model** of replication, because it resembles movement of the slide when playing a trombone. The trombone

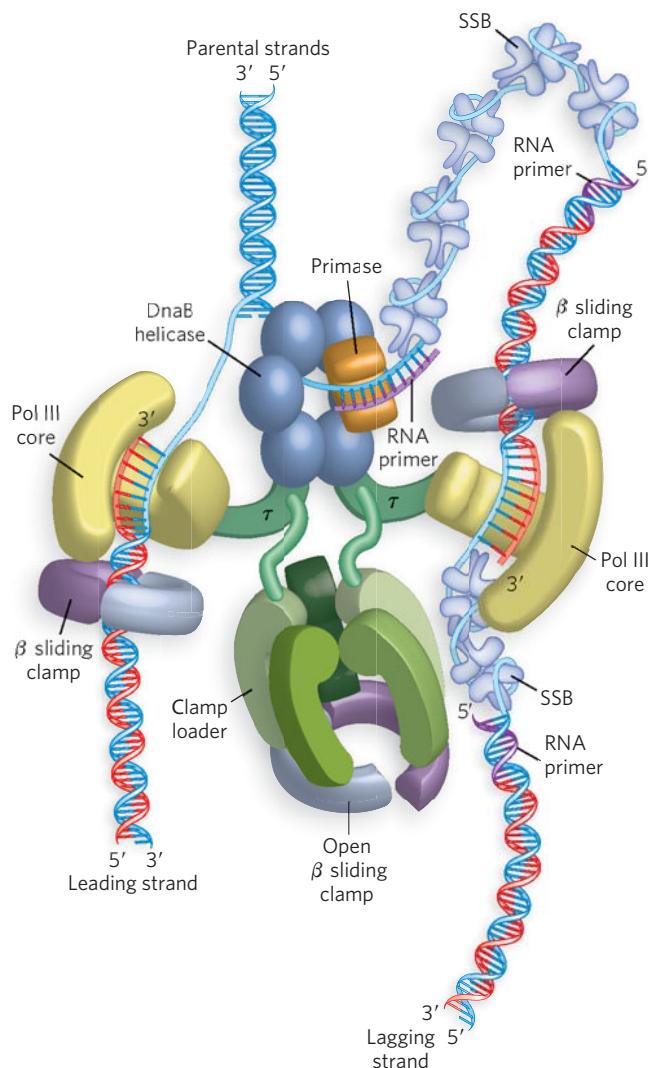


FIGURE 11-21 The architecture of the *E. coli* replisome at a replication fork. DnaB helicase encircles the lagging strand, and the Pol III holoenzyme connects to DnaB via the τ subunits of the clamp loader. Pol III cores connect to β clamps. Primase (DnaG) transiently associates with DnaB for the synthesis of an RNA primer on the lagging strand. The lagging-strand Pol III core- β clamp travels with the replisome, yet extends DNA in the 5'-3' direction, resulting in a DNA loop. The lagging strand is bound by SSB. The clamp loader is shown bound to a β clamp that it has opened in preparation for loading the clamp onto the RNA primer. [Source: Adapted from N. Y. Yao and M. O'Donnell, *Cell* 141:1088-1088e1, 2010.]

model was first proposed in 1980 by Bruce Alberts. At a replication fork speed of about 700 nucleotides per second, and with Okazaki fragments of 1 to 2 kb, a new loop is formed every 2 to 3 seconds.

When the lagging-strand polymerase finishes an Okazaki fragment, it must dissociate from the DNA in order to transfer to a new RNA primer and extend the

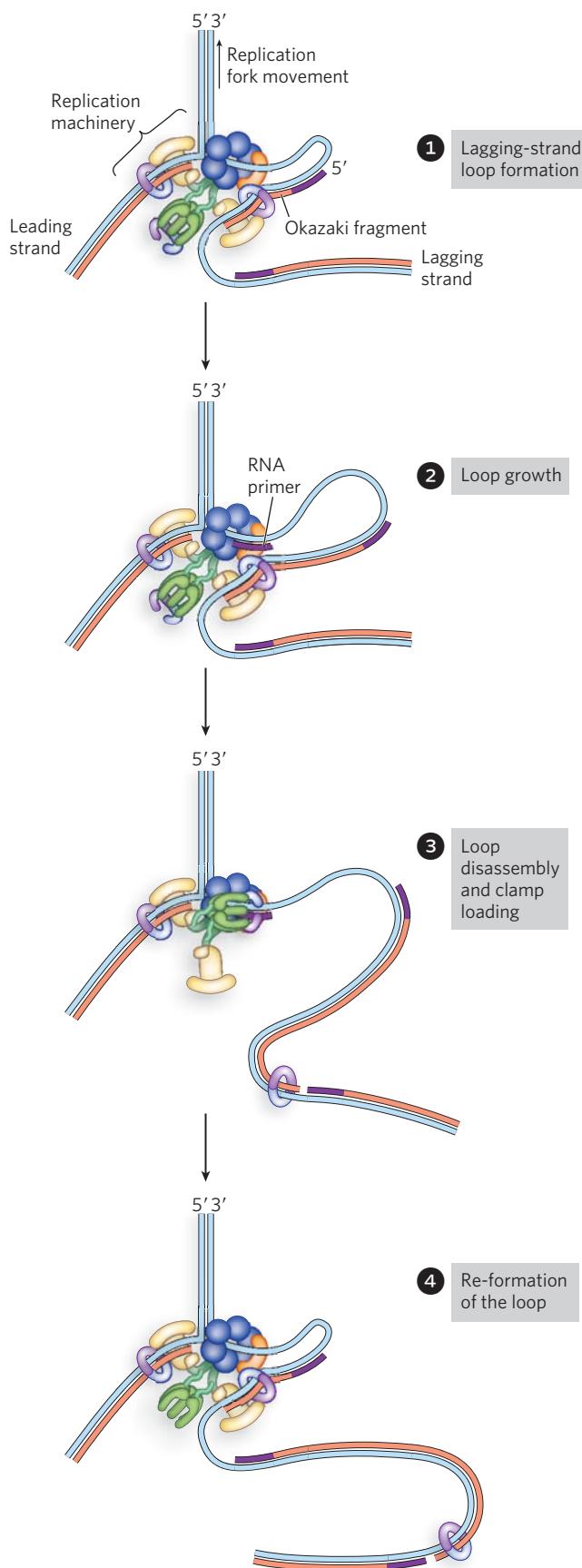


FIGURE 11-22 The trombone model of replication fork function.

function. The lagging-strand polymerase extends the 3' terminus of an Okazaki fragment in the opposite direction to fork movement, yet this polymerase is part of the replisome and thus moves with the fork. The opposed directions result in formation of a DNA loop for each Okazaki fragment. As multiple Okazaki fragments are synthesized, loops repeatedly grow and are released, similar to the movement of a trombone slide as the instrument is played.

next fragment. It does so by detaching from the β clamp, leaving it behind on the DNA. There are two ways the polymerase can detach from the clamp. It can detach after completing an Okazaki fragment, in what is known as **collision release**, because the polymerase collides with the previous Okazaki fragment when it departs from the clamp. Or, the polymerase can detach from the clamp before the Okazaki fragment is complete, which is referred to as **signaling release**, because it is thought to be signaled by priming events. Following a signaling release, the Okazaki fragment is completed by a separate polymerase. Once released, the lagging-strand Pol III core can associate with a new RNA-primed site on which a new β clamp has been assembled (Figure 11-23). This process results in a buildup of β clamps on replicated DNA. These leftover clamps perform additional functions, as we'll see shortly, but ultimately they, too, must be removed and recycled.

An experiment using a simple model system first suggested that Pol III hops from one β clamp to another to recycle among Okazaki fragments on the lagging strand (Figure 11-24). In this experiment, the Pol III holoenzyme was assembled on one DNA substrate, then mixed with two competing DNA substrates of different size, each with one site primed for DNA synthesis. Only one of the competing DNAs contained a β clamp at the primed site. Replication was initiated using [32 P]dNTPs, and timed aliquots were analyzed in an agarose gel. As the result shows, Pol III replicated the initial 5.4 kb DNA, then transferred to the DNA substrate containing the preassembled clamp. Because only the competing DNA with a preassembled β clamp was replicated, the experiment suggests that Pol III leaves the β clamp behind on the first template, then



Bruce Alberts [Source: Painting by Jon Friedman, NAS. Courtesy of Bruce Alberts.]

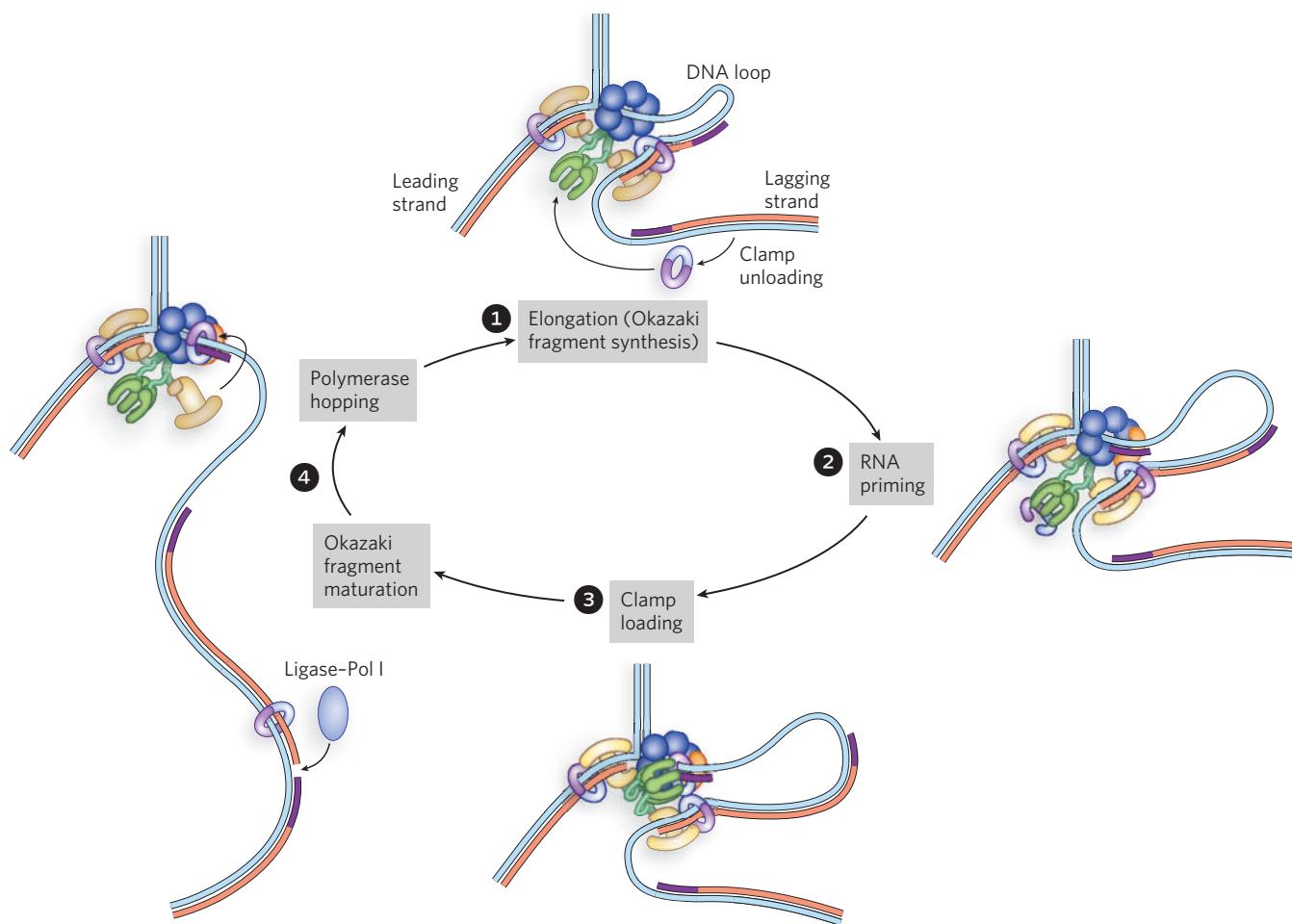


FIGURE 11-23 The Okazaki fragment cycle. ① The lagging-strand Pol III core-β clamp extends an Okazaki fragment, creating a loop. ② Primase binds to DnaB helicase and synthesizes an RNA primer (purple). ③ The clamp loader assembles a β clamp onto the new RNA primer. ④ The lagging-strand Pol III core ejects from the β clamp

on the Okazaki fragment, leaving the clamp on the DNA, and the Pol III core binds a new β clamp on the RNA primer for the next Okazaki fragment. Pol I exonuclease activity removes RNA primers, and ligase joins Okazaki fragments together. [Source: Adapted from N. Y. Yao and M. O'Donnell, *Cell* 141:1088–1088e1, 2010.]

hops to another DNA that contains a new β clamp. Further studies confirmed that Pol III indeed hops from one clamp to another, leaving clamps behind on the DNA as it does so.

Okazaki Fragments Require Removal of RNA and Ligase-Mediated Joining of DNA

The RNA at the 5' terminus of each Okazaki fragment must be removed and the gap filled in with DNA. This job is performed by the nick translation activity of Pol I (see Figure 11-7). The cell also has a backup enzyme, RNaseH, that can remove the RNA, in which case the single-strand gap must be filled in by a DNA polymerase. Processed fragments are then joined together by ligase to form a continuous duplex. Both Pol I and

ligase interact with the β clamp, and the β clamps left behind by the replisome are thought to attract Pol I and ligase for RNA removal and sealing of the fragments.

Okazaki fragments outnumber β clamps in the cell by about 10 to 1, so clamps must be recycled during chromosome replication. Clamps are removed from DNA by the δ subunit of the clamp loader, which is produced in excess in the cell (relative to the other γ complex subunits). By itself, the δ subunit can open and unload β from DNA, but it cannot assemble the β clamp onto DNA.

The β clamp binds many proteins, including all five *E. coli* DNA polymerases, ligase, the γ complex, and several proteins not described in this chapter. These proteins all bind to the same spot on β. Therefore, when the β clamp is being used by a DNA polymerase or another protein, the recycling of β is blocked. Only

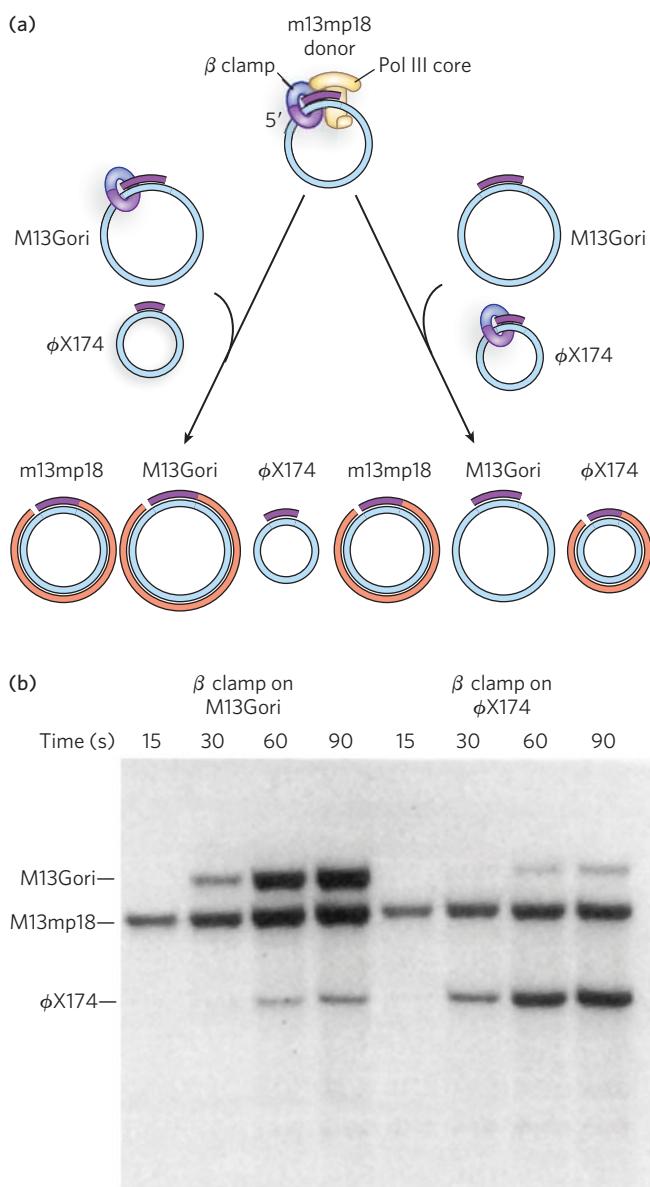


FIGURE 11-24 Transfer of Pol III from an old β clamp to a new β clamp. In these experiments, the Pol III holoenzyme is assembled onto a primed donor DNA circle (M13mp18), then the Pol III-DNA is mixed with two competing primed acceptor DNA circles of different sizes, only one of which contains a β clamp. In the experiment on the left side, the M13Gori DNA contains a β clamp and the other (ϕ X174) DNA does not. In the experiment on the right side, ϕ X174 DNA contains a β clamp and the other (M13Gori) does not. Replication is initiated and timed. Aliquots of the reaction mixtures are analyzed on an agarose gel. The acceptor DNA with a β clamp is replicated in preference to the acceptor DNA without a β clamp. [Source: Photo from P. T. Stukenberg, J. Turner, and M. O'Donnell, Cell 78:877–887, 1994. Permission of P. T. Stukenberg.]

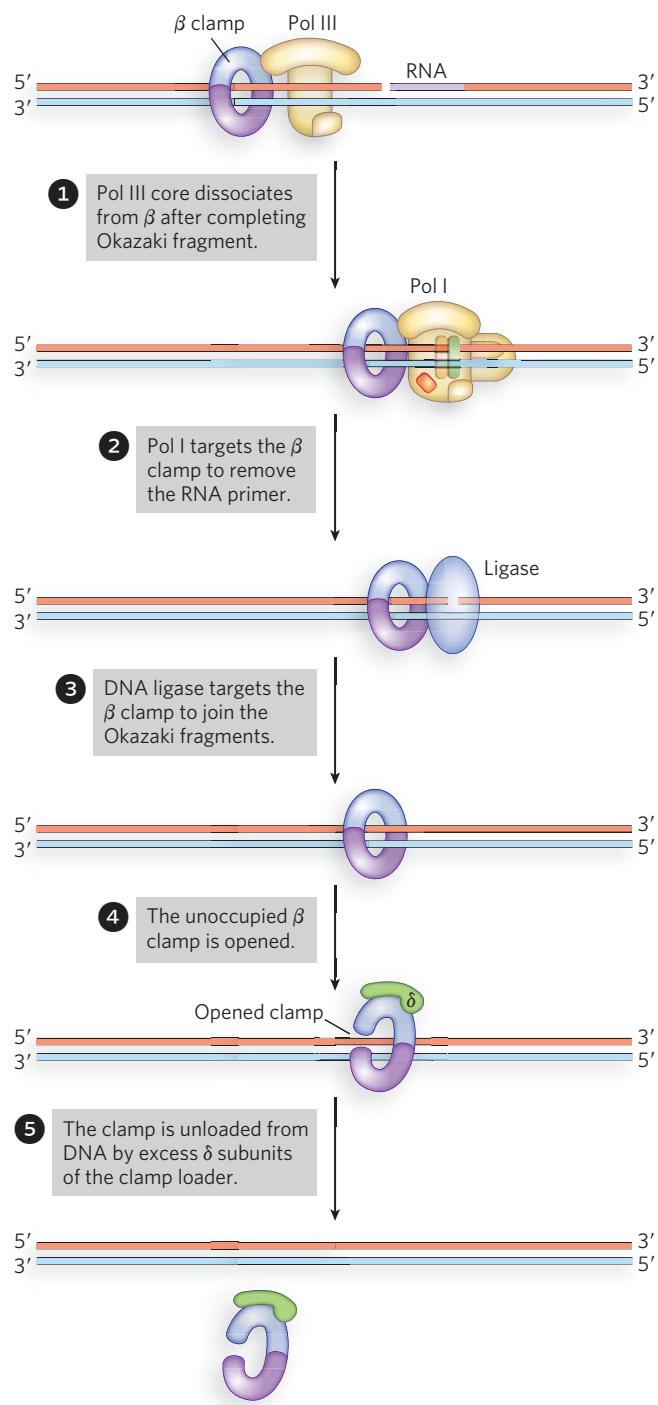
The **MCM helicase** is thought to function at the replication fork. Like *E. coli* DnaB, the MCM subunits form a ring-shaped hexamer, but each subunit is different (named Mcm2 through Mcm7, or Mcm2–7). The six subunits are homologous AAA+ proteins (each subunit $M_r \sim 100,000$). The Mcm2–7 complex associates with a heterotetramer called GINS and with the Cdc45 protein, forming a complex referred to as the **CMG complex** (for Cdc45-MCM-GINS). The roles of GINS and Cdc45 are not yet understood, and these proteins have no sequence-related homologs in bacteria. The direction of activity of the MCM helicase is 3'→5', opposite to that of *E. coli* DnaB, and therefore the MCM helicase must act on the leading strand to unwind the parental DNA. Some of the eukaryotic replication proteins, including the MCM helicase, are targets of cell cycle kinases, enzymes that phosphorylate specific proteins and are active at certain phases of the cell cycle. For example, phosphorylation of a replication protein may activate it, and this modification may occur only on entering S phase of the cell cycle. Even though most eukaryotic chromosomes are linear, they are still topologically constrained, and helicase unwinding creates torsional stress that is relieved by topoisomerases.

The eukaryotic primase is a four-subunit complex called **DNA polymerase α (Pol α)**. Priming activity is located in the smallest subunit, and it makes an RNA primer of about a dozen nucleotides. The largest subunit of Pol α is a DNA polymerase that extends the RNA primer with DNA, to a total length of 25 to 40 nucleotides. As in bacteria, the RNA is excised before Okazaki fragments are joined. The DNA made by Pol α may contain errors because the enzyme has no 3'→5' proofreading exonuclease, and we currently do not know whether the DNA made by Pol α is replaced or is corrected by repair proteins.

when β is no longer bound by other proteins is it available to be recycled (Figure 11-25).

The Replication Fork Is More Complex in Eukaryotes Than in Bacteria

Many eukaryotic replication proteins have counterparts in bacteria (e.g., the clamps and clamp loader), but the replication fork machinery of eukaryotes includes more proteins beyond those used in the comparatively simple bacterial machinery (Figure 11-26). New eukaryotic replication factors are still being identified, and details of the replication fork in eukaryotic cells are only now coming into focus.



Eukaryotes have two different chromosomal replicases: **DNA polymerase δ (Pol δ)** and **DNA polymerase ε (Pol ε)**. Both Pol δ and Pol ε are four-subunit enzymes in higher eukaryotes, and the largest subunit of each contains both a DNA polymerase and a

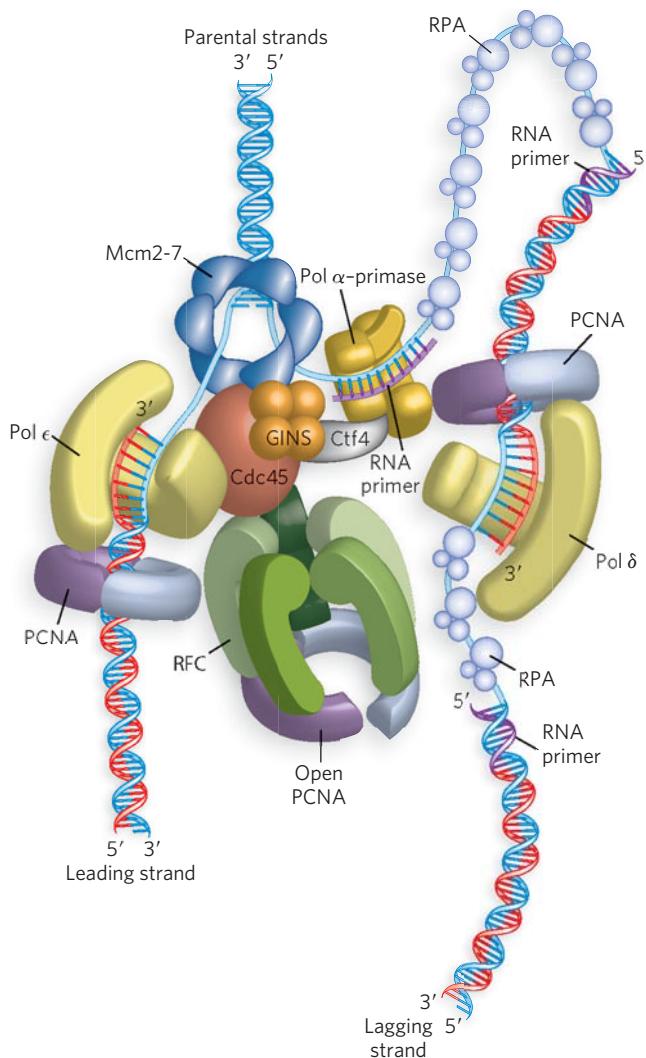


FIGURE 11-26 *A hypothetical model of the eukaryotic replication fork.* Eukaryotes contain all the proteins that function in a bacterial replisome, but most components have more subunits than the bacterial proteins, and several additional proteins function at the eukaryotic replication fork. The components and their functions are described in the text. [Source: Adapted from N. Y. Yao and M. O'Donnell, *Cell* 141:1088–1088e1, 2010.]

$3' \rightarrow 5'$ exonuclease activity (Pol δ contains three subunits in yeast). Current research suggests that Pol δ and Pol ε operate on different strands at the replication fork: Pol ε on the leading strand and Pol δ on the lagging strand.

Both Pol δ and Pol ε interact with a DNA sliding clamp called **PCNA**. This protein was named descriptively as proliferating cell nuclear antigen (PCNA) before its function as a sliding clamp was identified.

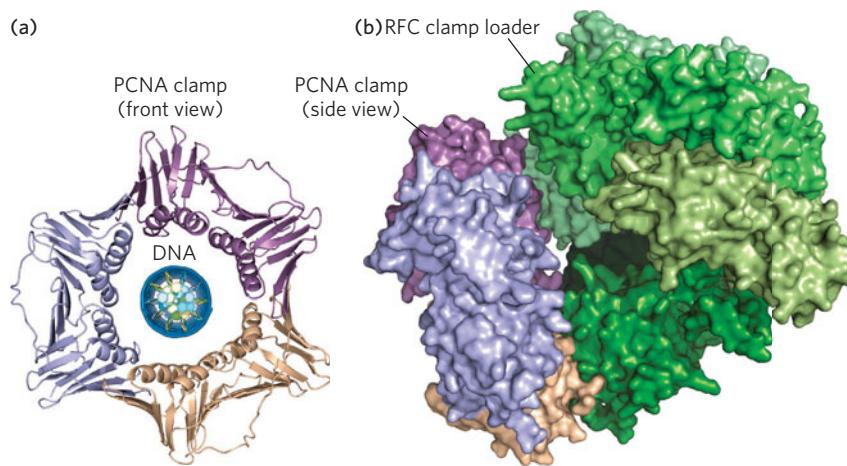


FIGURE 11-27 The eukaryotic PCNA clamp and RFC clamp loader. (a) PCNA is a homotrimer; the monomer units are shown in different colors. (b) The RFC clamp loader is homologous to the bacterial clamp loader. Compare this with Figure 11-18a. [Sources: (a) PDB ID 1AXC. (b) PDB ID 1SXJ.]

PCNA looks remarkably like the *E. coli* β clamp (Figure 11-27a). The two proteins share no sequence homology, but the three-dimensional structures are so alike that they almost certainly evolved from a common ancestor. Both proteins are constructed from a domain that is repeated six times around the ring. The three monomer units of PCNA have only two domains and trimerize to form a ring, and the two monomer units of the β clamp consist of three domains and dimerize to form the ring. The eukaryotic clamp loader, **replication factor C (RFC)**, contains five subunits similar in shape and function to the *E. coli* γ complex (Figure 11-27b). In a fascinating twist, eukaryotes contain alternative forms of RFC in which one of the subunits is replaced by another protein. These alternative clamp loaders usually function with PCNA and their intracellular role is not entirely clear. In one case, the alternative clamp loader loads an entirely different clamp onto the DNA.

Eukaryotic replication forks proceed at a rate of about 30 to 50 nucleotides per second, far slower than bacterial forks. Also, eukaryotic Okazaki fragments are considerably shorter than bacterial fragments, only 100 to 200 nucleotides long. The heterotrimeric **replication protein A (RPA)** is the functional equivalent of *E. coli* SSB. As in bacteria, the eukaryotic enzymes that remove RNA and join Okazaki fragments interact with the sliding clamp. RNA primers are removed by Fen1 nuclease, and DNA ligase I joins the fragments.

The identity and arrangement of proteins that function at the eukaryotic replication fork are still the subject of intense investigation. Several proteins with functions that, as yet, lack clear definition seem to be involved in the architecture of the eukaryotic replication fork. The numbers and types of proteins currently thought to participate in eukaryotic chromosome replication are listed in Table 11-4.

SECTION 11.3 SUMMARY

- The *E. coli* chromosomal replicase, the Pol III core, connects to the ring-shaped β clamp that encircles DNA for processive DNA synthesis. The β clamp is assembled onto DNA by a multiprotein clamp loader, the γ complex. Two Pol III cores, two β clamps, and one γ complex form the Pol III holoenzyme assembly.
- The Pol III holoenzyme, DnaB helicase, and DnaG primase form the replisome complex. The hexameric DnaB helicase encircles the lagging strand and uses ATP to unwind DNA at the replication fork. DnaG primase forms RNA primers to initiate DNA synthesis.
- Topoisomerases act ahead of the replication fork to remove superhelical tension generated by DNA unwinding. SSB binds the single-stranded DNA created by the unwinding action of helicase, preventing formation of secondary structures in the DNA and protecting it from endonucleases.
- RNA primers are removed from finished Okazaki fragments by the nick translation action of Pol I, and the processed fragments are joined by DNA ligase.
- Simultaneous replication of the two antiparallel strands of duplex DNA by two Pol III cores in the replisome requires loops to form on the lagging strand that repeatedly grow and reset for each Okazaki fragment.
- Eukaryotes have two different multiprotein DNA polymerases (Pol ϵ and Pol δ) that function on the leading and lagging strands. These DNA polymerases connect to PCNA sliding clamps loaded onto the DNA by the RFC clamp loader.
- Eukaryotes have functional counterparts for each *E. coli* replication fork protein, but the eukaryotic

Table 11-4 Proteins That Function in Eukaryotic Replication

Protein	Number of Subunits	Function(s)	Complex
ORC	6	Initiator	Prereplication complex (preRC)
Cdc	1	Helicase loader	
Cdt1	1	Helicase loader	
Mcm2-7	6	Presumed helicase	
CDK	1	S-phase cyclin kinase	Replication complex (RC)
DDK	1	S-phase cyclin kinase	
Mcm2-7	6	Presumed helicase	Replication complex (RC)
Pol α	4	Primase	
Pol δ	3-4	Replicase	
Pol ϵ	4	Replicase	
PCNA	3	Sliding clamp	
RFC	5	Clamp loader	
RPA	3	Single-stranded DNA-binding protein	
Cdc45	1	Loading of DNA polymerase onto origin; helix destabilization	
GINS	4		
Sld2	1		
Sld3	1		
Dpb11	1		
Mcm10	1		
DNA ligase I	1	Seals Okazaki fragments	Replication complex (RC)
FenI	1	Removes RNA primers	
Dna2	1	Processes Okazaki fragment	
Topoisomerase I	1	Removes supercoil stress	
Topoisomerase II	2	Removes supercoil stress	

replisome is more complex. The eukaryotic primase is a four-subunit enzyme (Pol α) that contains both DNA polymerase and primase activities. The Mcm2-7 helicase is a heterohexamer. The eukaryotic SSB homolog, RPA, has three different subunits. Eukaryotes also have proteins of undefined function, with no homologs in bacteria, that travel with the replication fork.

is more difficult in eukaryotic cells than in bacteria, because eukaryotes have numerous origins on each chromosome. The total length of DNA replicated from one origin is called a **replicon**. Many bacteria have only one origin, and the replicon is the entire chromosome. In eukaryotic chromosomes, each replicon is the section of DNA replicated from one of its many origins.

Early genetic studies by François Jacob and his co-workers showed that replication starts at a particular place on the DNA, which they termed a replicator (now known as an origin). Numerous genes encoding proteins needed for replication have now been revealed by genetic studies. Replication proteins fall into two classes: those that affect initiation and those that affect replication.

The two classes of proteins were identified by the speed at which their depletion affects DNA synthesis (Figure 11-28). Temperature-sensitive mutants for these proteins allowed incorporation of [3 H]thymidine during DNA synthesis at a permissive temperature, but no DNA

11.4 Initiation of DNA Replication

The site (or multiple sites) on a chromosome where replication is initiated is called the origin. It is the primary point at which regulatory mechanisms control DNA replication. Once replication of a chromosome has started, there is no stopping it, and the chromosome and the cell are committed to division. Control of initiation

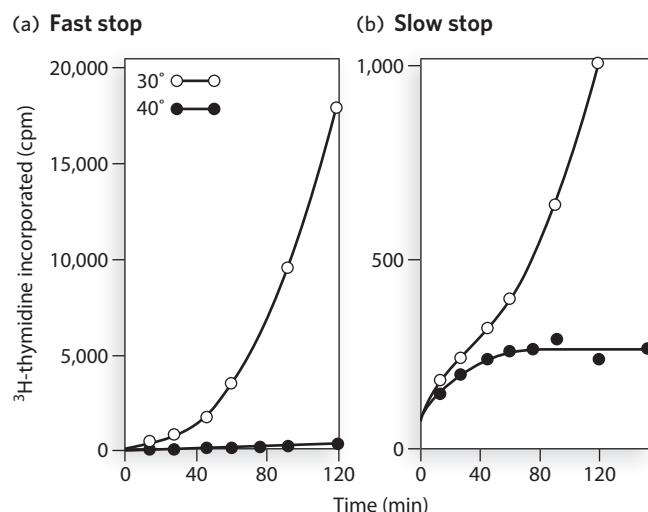


FIGURE 11-28 Two types of replication genes revealed through genetic studies. Temperature-sensitive mutants of *E. coli* were analyzed for the time needed for replication to stop after shifting cells to a nonpermissive temperature. DNA replication was observed by the uptake of [^3H] thymidine into cellular DNA (measured as counts per minute, cpm) at a permissive temperature (30°, open circles) and nonpermissive temperature (40°, solid circles). (a) A gene giving a fast-stop phenotype encodes a protein involved in progression of the replication fork. (b) A gene giving a slow-stop phenotype encodes a protein involved in the initiation of replication. [Source: Adapted from Y. Hirota, A. Ryter, and F. Jacob, *Cold Spring Harb. Symp. Quant. Biol.* 33:678, 1968, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.]

synthesis at a nonpermissive temperature. Temperature-sensitive genes encoding proteins that play a direct role in the replisome caused an abrupt end, or “fast stop,” of DNA synthesis when the cells were transferred to nonpermissive growth conditions. However, some genes displayed a “slow stop” of replication, which suggested that they encode factors needed for initiation, not for ongoing fork progression (replication). These “slow stop”

mutants allowed already-initiated DNA synthesis to continue at the nonpermissive temperature until replication of the chromosome was finished.

The **initiator protein**, which binds specific sites at the origin, is an example of a protein encoded by a slow-stop gene. Binding of the initiator protein to an origin provides a foothold for other proteins to bind, and often results in strand separation in a small region of DNA at the origin. Helicases are assembled at the unwound region, paving the way for more extensive DNA unwinding and assembly of bidirectional replication forks.

Replication from an origin is a carefully orchestrated and controlled process that involves many different proteins. We first examine initiation at the *E. coli* origin, which is understood in great detail and serves to outline the basic events involved in initiation of replication in all cells. Then we describe our current understanding of how replication from the multiple origins of eukaryotic chromosomes is initiated and controlled.

Assembly of the Replication Fork Follows an Ordered Sequence of Events

The *E. coli* origin, *oriC*, was identified by a genetic technique using a recombinant DNA plasmid (see How We Know). The minimal *E. coli* origin (*oriC*) is 245 bp long and contains four copies of a nine-nucleotide (9-mer) consensus sequence to which the initiator DnaA protein binds (Figure 11-29). To one side of the DnaA 9-mer sites are three A=T-rich direct repeats of 13 bp each. These A=T-rich repeats are the first area in *oriC* to unwind after the initiator binds. Many origins of replication contain A=T-rich repeats that probably function in a similar way.

The *E. coli* initiator protein, DnaA, is a member of the AAA+ family. Like most AAA+ proteins, it binds and hydrolyzes ATP, although turnover is very slow. DnaA oligomerizes after binding the origin and wraps the origin DNA around the oligomer (Figure 11-30, step 1). In the presence of ATP, DnaA destabilizes the

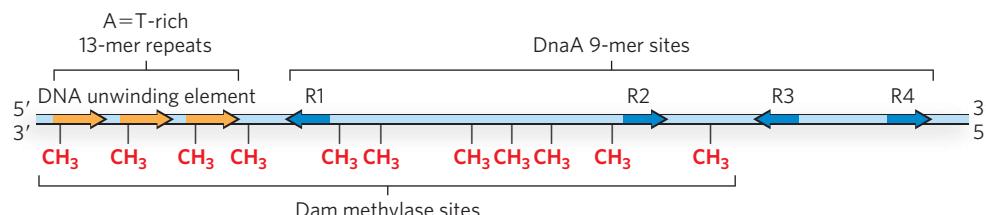


FIGURE 11-29 Structural elements of the *E. coli* origin.

The *E. coli* origin, *oriC*, contains four 9-mer DNA sites that bind the DnaA initiator protein. A possible fifth site deviates from the consensus sequence (not shown). The arrowheads indicate the relative direction of the 9-mers.

The three 13-mer direct repeats (sequences that are repeated with the same directionality) are A=T-rich and are the locus of initial DNA strand separation. The *oriC* sequence also contains 11 GATC sites that are methylated by the Dam methylase.

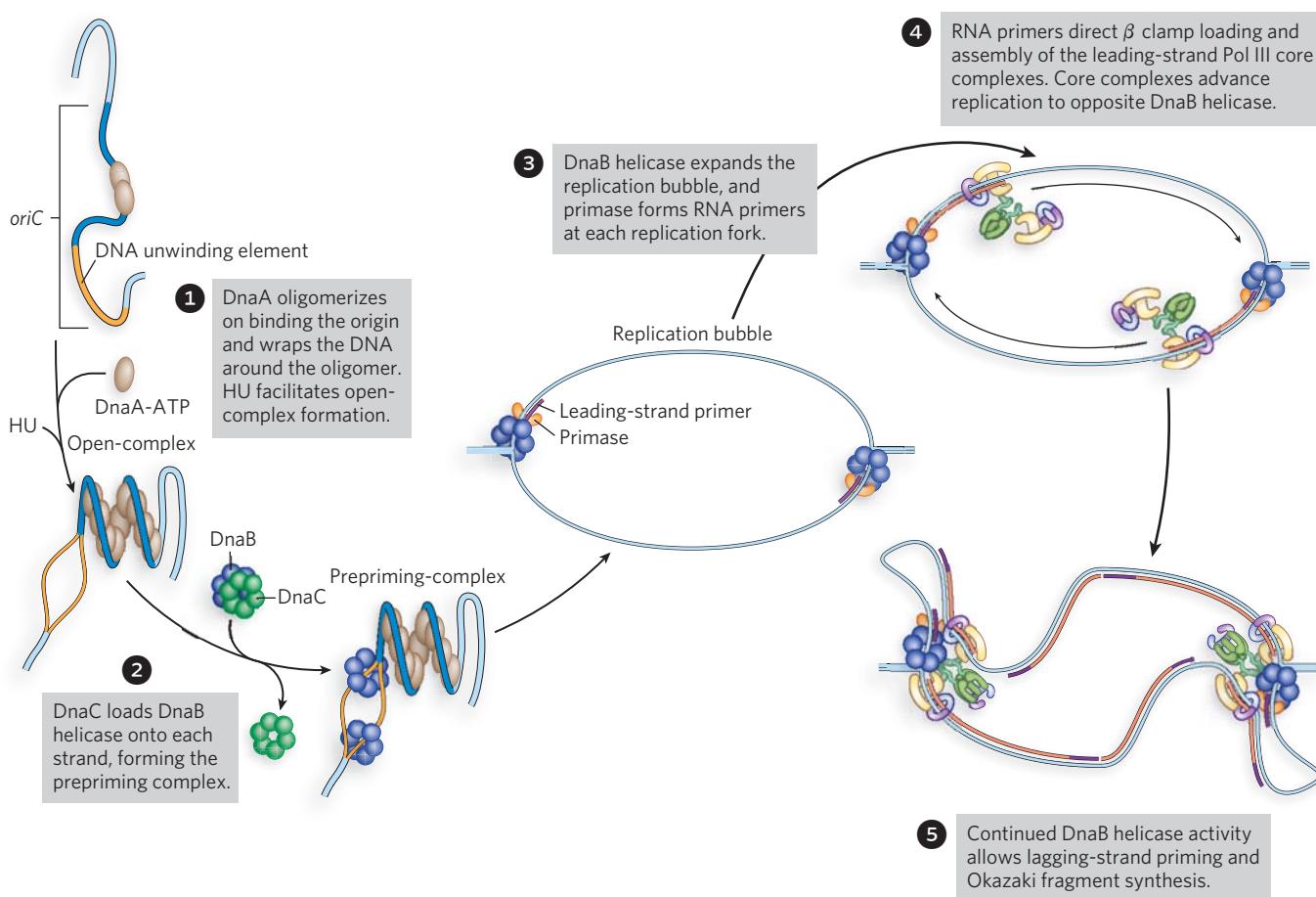


FIGURE 11-30 Activation of *oriC* and assembly of bacterial replication forks.

A=T-rich 13-mer repeats, forming a single-stranded DNA bubble. Formation of this bubble is stimulated by HU, a small, basic, histonelike protein. Because the DnaA-ATP-*oriC*-HU complex forms a bubble at the origin, it is referred to as the **open complex**.

The DNA bubble in the open complex is the nucleation point for the assembly of two hexamers of DnaB helicase (see Figure 11-30, step 2). Interaction of DnaB helicase with the DnaA initiator protein helps target DnaB to the origin, but it also requires a helicase-loading protein to assemble onto single-stranded DNA in the open complex. The helicase-loading protein is DnaC, another AAA+ protein. The helicase-loading activity of DnaC is thought to function by prying open the hexameric DnaB ring and slipping it onto the single-stranded DNA at the bubble. The ATP-bound form of DnaC binds DnaB helicase tightly and represses its helicase activity. Hydrolysis of ATP ejects DnaC from DnaB helicase, releasing DnaB for DNA unwinding. Two hexamers of DnaB helicase are assembled on the origin, one on each strand of the single-stranded

bubble. This assembled group of proteins on the DNA at *oriC* is referred to as the **prepriming complex**.

With the addition of ATP, the DnaB helicases translocate and unwind DNA (see Figure 11-30, step 3). Unwinding generates positive supercoil stress in the DNA ahead of the replication fork, and this stress must be removed by topoisomerase action (e.g., gyrase). The newly unwound DNA is coated with SSB. Primase is unable to interact with DnaB and generate an RNA primer until the bubble has been expanded to about 100 to 200 nucleotides. Once the bubble is large enough for primase, the RNA primer directs β clamp loading and assembly of the leading-strand Pol III core- β clamp complex within the holoenzyme (see Figure 11-30, step 4). This same process occurs on the other strand of the bubble and in this way forms the two leading strands for bidirectional replication forks.

Immediately after the two leading strands have formed, the bubble is completely double-stranded, and there is no single-stranded DNA for primase to initiate the lagging strand. But this is a temporary situation.

The coupled helicase-polymerase now moves rapidly, producing single-stranded DNA for primase to act upon (see Figure 11-30, step 5). The next RNA primer at each replication fork becomes the first of many lagging-strand primers. Priming is followed by clamp loading and engagement of the lagging-strand Pol III core- β clamp complex, thus completing the assembly of two bidirectional replication forks at *oriC*.

Replication Initiation in *E. coli* Is Controlled at Multiple Steps

Cell division requires sufficient nutrients and cell mass to support two new cells, so replication must be coordinated with the cell's nutritional status and growth. Regulation occurs at the initiation step, because once replication has begun, the cell is committed to division. It is also of paramount importance that the origin, once replicated, can be inactivated to prevent a second round of replication during the first round, which would commit the cell to splitting twice (resulting in four cells). However, in bacteria, when nutrients are abundant, initiation at the origin will occur a second time before the first cycle of replication is complete.

Binding of the DnaA initiator protein at *oriC* is a central point at which initiation is controlled. One mechanism for controlling initiation at *oriC* is through DNA methylation. Both strands of the palindromic sequence GATC are recognized by the enzyme **Dam methylase (DNA adenine methyltransferase)**, which methylates the N⁶ position of A residues on both strands of the GATC site. The average frequency of a GATC sequence is once every 246 bp, yet the 245 bp *oriC* contains 11 GATC sites (see Figure 11-29). Immediately after a GATC site is replicated, the new strand is not yet methylated and the GATC site is thus hemimethylated. This hemimethylated state of newly replicated DNA is only temporary, until Dam methylase acts, but the high density of GATC sites in *oriC* delays complete methylation. The SeqA protein (Seq for sequestration) binds specifically to hemimethylated GATC sites and thereby sequesters the newly replicated *oriC*, preventing DnaA from rebinding the replicated origin (Figure 11-31a). Dam methylase, working between SeqA dissociation-reassociation cycles, eventually methylates the GATC sites in *oriC*, and this blocks SeqA binding and opens up the origin to DnaA binding once more.

Initiation depends on the nucleotide-bound state of DnaA, which uses the energy of ATP binding to form the open complex at *oriC* (Figure 11-31b). When replication forks dislodge DnaA from the origin, it can rebind. But DnaA hydrolyzes the bound ATP after initiation, and even though ADP-DnaA can rebind the origin, it is inactive for open complex formation, and

reinitiation is thus prevented. Important to this regulatory step is that the exchange of free ATP for bound ADP on DnaA is slow, requiring up to half an hour—time for the cell division cycle to finish. ATP hydrolysis

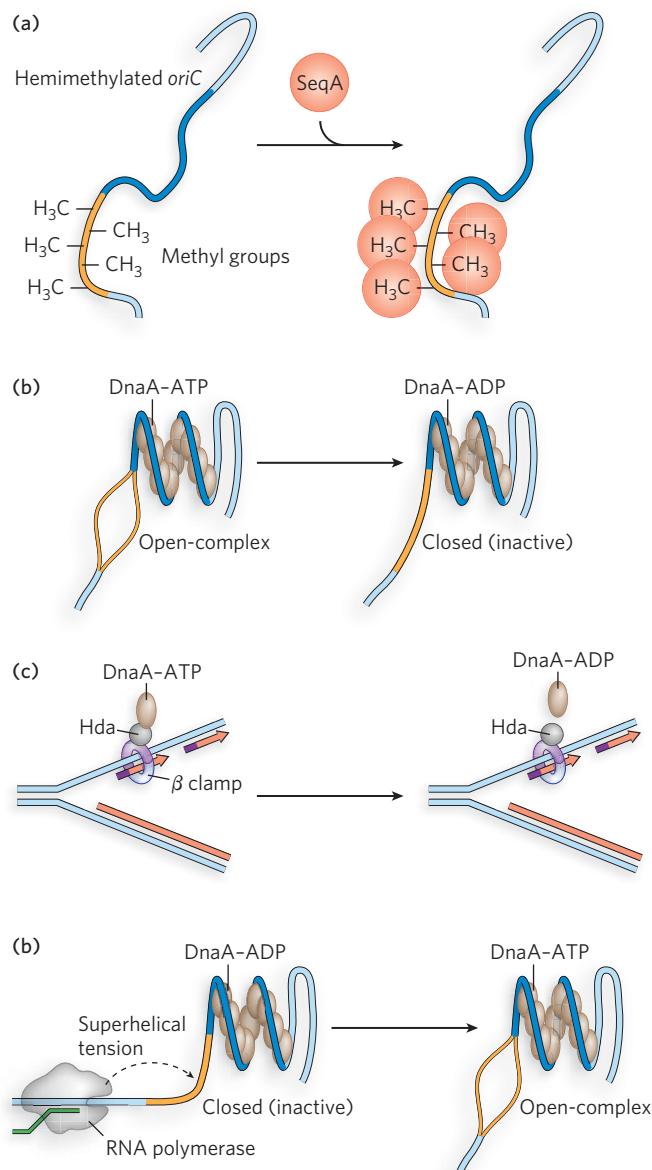


FIGURE 11-31 Regulation of the *E. coli* origin. Initiation at the *E. coli* origin, *oriC*, is regulated in several ways. (a) SeqA protein binds hemimethylated DNA and sequesters the newly replicated origin, preventing DnaA binding. (b) DnaA-ADP, formed when DnaA hydrolyzes its ATP, cannot destabilize the A=T-rich region to maintain the open complex containing a single-stranded DNA bubble, thus forming a closed complex in which the bubble has collapsed. (c) The Hda protein binds the β clamp on the DNA, causing DnaA to hydrolyze its ATP and become inactive (DnaA-ADP). (d) RNA polymerase produces superhelical tension that promotes DnaA-induced melting of the A=T-rich region.

by DnaA is ensured by the Hda protein (Figure 11-31c). After replication forks start moving, Hda binds the β sliding clamp and stimulates ATP hydrolysis by DnaA, and thus inactivation of DnaA.

The number of DnaA-binding sites in the cell may also play a role in control of reinitiation. The chromosome contains numerous DnaA-binding sites in various promoters, because DnaA is also a transcription regulator. In aggregate, these other DnaA sites far outnumber the few at the origin. Therefore, as chromosome duplication proceeds, the total number of DnaA-binding sites doubles and they may act as a sink to lower the free DnaA available for binding to *oriC*.

In lab experiments, the RNA polymerase inhibitor rifampicin blocks replication in cells, suggesting that RNA polymerase plays a role in chromosome replication. A transcribing RNA polymerase creates supercoil strain in the DNA template, and when RNA polymerase is near the origin, it stimulates activation through this supercoil strain, probably by helping DnaA destabilize the A=T-rich 13-mer repeats in forming the open complex bubble (Figure 11-31d).

Eukaryotic Origins “Fire” Only Once per Cell Cycle

The much greater DNA content of eukaryotes, coupled with their slower replication forks, necessitates multiple origins on each chromosome to allow complete replication in the 24-hour division time of, for example, a human cell. Origins are spaced 10 to 40 kbp apart along each chromosome, and multiple replication forks eventually meet to yield the two daughter chromosomes. “Firing” (activation) of an origin is under tight control, as is reinitiation at an origin that has already been duplicated.

Eukaryotes have defined cell cycle phases, with chromosome replication occurring in S phase; the duplicated chromosomes separate in M phase. (For an overview of the cell cycle, see Chapter 2.) A protein complex essential to replication is assembled on chromosome origins even before S phase. The assembly process occurs late in G₁ phase and marks origins that will be used for replication during S phase. This separation of events in the cell cycle is critical to the exquisite coordination that eukaryotes require to duplicate their long, linear chromosomes.

The simple eukaryote *S. cerevisiae* has well-defined replication origins that are 100 to 200 bp long and contain four common components: a highly conserved A sequence and the B1, B2, and B3 elements (Figure 11-32, top). Two-dimensional gel electrophoresis can be used to identify DNA segments that contain an origin (Highlight 11-1). The identification of discrete origins in yeast has made it a convenient model organism for studying

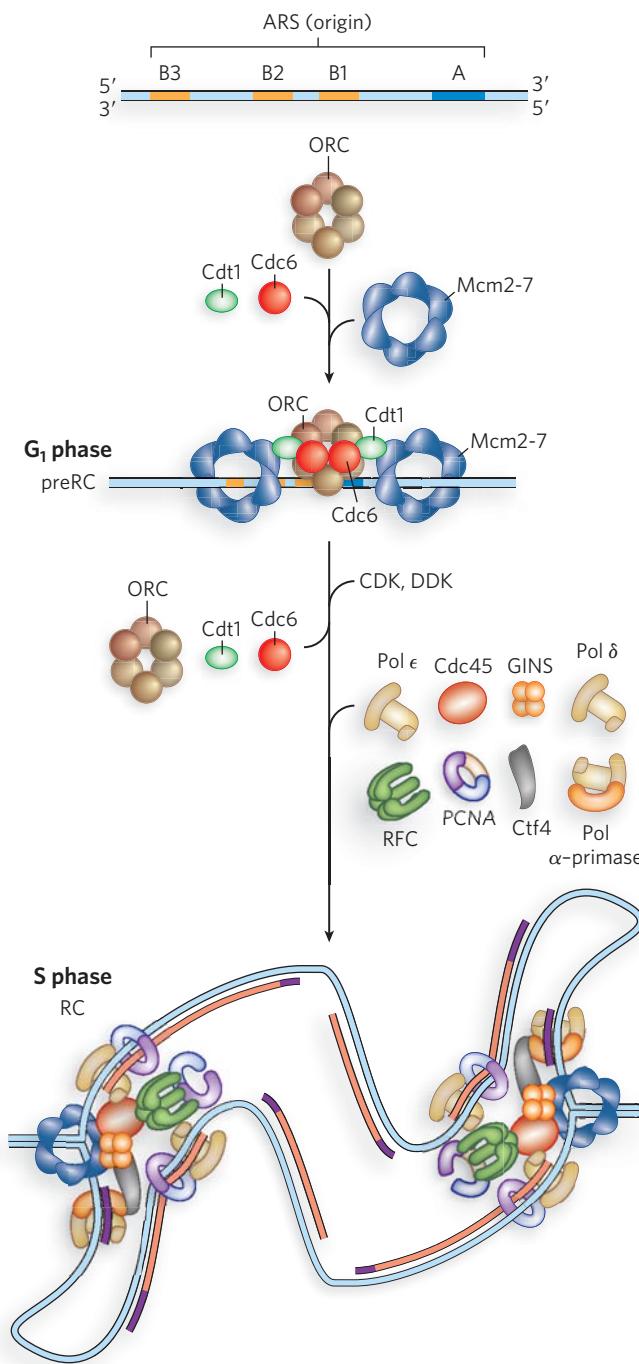


FIGURE 11-32 Assembly of eukaryotic replication forks.

The generalized structure of an origin in *S. cerevisiae* is shown at the top. The prereplication complex (preRC) assembles in G₁ phase (middle). The initiator, ORC, binds to the conserved A element and the B1 element. MCM helicases are loaded onto the DNA by Cdc6 and Cdt1. After the cell progresses to S phase (bottom), the origin forms replication forks, as cyclin-dependent protein kinases (CDK and DDK) facilitate assembly of other proteins to form the replication complex (RC) from which replication fork movement commences.

origin function in eukaryotes. Furthermore, homologs of the yeast replication proteins exist in all eukaryotes, indicating that the lessons we learn from yeast will probably generalize to more complex eukaryotic organisms.

The eukaryotic initiator is a heterohexamer called the **origin recognition complex (ORC)**. Several subunits of ORC are, like the *E. coli* DnaA initiator protein, AAA+ proteins. ATP is required for ORC binding to the origin (see Figure 11-32, middle). Cdc6 (like *E. coli* DnaC) is an AAA+ protein that is required to load the Mcm2–7 helicase onto DNA. Cdc6 is homologous to ORC subunits and is thought to bind ORC during the helicase-loading task. Cdc6 also binds a protein called Cdt1. These events occur only in G₁ phase, and the resulting complex of ORC, Cdc6, Cdt1, and Mcm2–7 is referred to as the **prereplication complex (preRC)**.

Cyclin-dependent protein kinases (sometimes referred to as cyclin kinases or cell cycle kinases) that phosphorylate certain target proteins are central to the separation of cell cycle phases. In G₁ phase there is very low kinase activity, and proteins are generally not phosphorylated. On entering S phase, *S. cerevisiae* S-phase cyclin kinases phosphorylate some of the preRC proteins. The exact protein targets are still being investigated. Phosphorylation activates the preRC, leading to dissociation of Cdc6 and Cdt1 and their degradation by proteases. The loss of Cdc6 and Cdt1 prevents further preRC assembly until the cell has divided and reenters G₁.

Several other replication factors associate with ORC early in S phase to form the **replication complex (RC)**, including most of the replication fork machinery discussed earlier. Completion of the RC is followed by DNA unwinding and full assembly of the replication forks containing Pol δ and Pol ε (Figure 11-32, bottom). Phosphorylation by S-phase cyclin kinases is necessary for replication fork assembly and confines the initiation of replication to S phase. Only after the S, G₂, and M phases are complete, and cell division is accomplished, is S-phase cyclin kinase activity reduced and Cdc6 and Cdt1 made available to direct the assembly of the preRC on the chromosomes of new cells in G₁ phase.

SECTION 11.4 SUMMARY

- The assembly of bacterial replication forks at the origin occurs in steps, starting with the binding of DnaA initiator protein, which melts an A=T-rich region. A DnaB helicase is then loaded onto each of the single strands of DNA by the DnaC helicase loader. As DNA is unwound by DnaB, DnaG primase synthesizes RNA primers; this is followed by entry of two Pol III holoenzymes to form a bidirectional replication fork.

- Origin activation in bacteria is regulated at the initiation step by various means, including DNA methylation that results in SeqA sequestering the origin, ATP turnover by DnaA, and the activity of Hda protein, which signals DnaA to hydrolyze ATP after forks are formed.
- Eukaryotes have many replication origins, and tight initiation control is achieved by dividing the activation of origins into different cell cycle phases. Some proteins can bind the origin only in G₁ phase, when cyclin kinase activity is low (preRC). Further assembly of proteins to form replication forks occurs only in S phase and is associated with phosphorylation by S-phase cyclin kinases.

11.5 Termination of DNA Replication

In both bacteria and eukaryotes, replication forks meet head-on when replication is complete. *E. coli* cells have a specialized mechanism for preventing head-on collisions between DNA polymerase and RNA polymerase. Eukaryotic cells have the additional problem of replicating the ends of linear chromosomes. Evolution has provided the solution in the form of a novel DNA polymerase—telomerase, specialized for this purpose.

E. coli Chromosome Replication Terminates Opposite the Origin

In *E. coli*, a region located halfway around the chromosome from *oriC* contains two clusters of 23 bp sequences called **Ter sites** (Figure 11-33). The two clusters of Ter sites are oriented in opposite directions. The monomeric Tus protein (termination utilization substance) binds tightly to a Ter site and blocks advance of the replication fork by stopping DnaB helicase. A fascinating property of the Tus-Ter complex is that its fork-blocking activity is polar. Replication forks are blocked when approaching a Tus-Ter complex from one direction (the nonpermissive direction), but not when approaching from the opposite (permissive) direction.

The arrangement and orientation of Ter sites is such that bidirectional replication forks from *oriC* can pass through the first set of Ter sites that they encounter, but are blocked by the second set. This arrangement localizes the replication fork collision zone to the area between the two clusters of Ter sites. Although Tus is not essential for *E. coli* growth, the Tus-Ter system presumably evolved to confer a growth advantage in the natural (nonlaboratory) setting.

Actively replicating bacteria are also growing and metabolizing and therefore actively transcribing RNA from

HIGHLIGHT 11-1 TECHNOLOGY

Two-Dimensional Gel Analysis of Replication Origins

Origins in the process of replication generate DNA molecules that contain bubbles and replication forks. These unusually shaped DNAs produce characteristic patterns in two-dimensional agarose gels (see Chapter 8). In this technique, the section of DNA to be examined for a replication origin is cut on either side of the origin with a restriction enzyme. In the first dimension of the gel, molecules are sorted mainly by size by low-voltage electrophoresis through a low-percentage agarose gel. An unreplicated 1 kbp fragment of DNA will travel farther through the gel than a replicated (2 kbp) fragment (**Figure 1a**). The second dimension is run at higher voltage to sort molecules mainly by shape. DNA fragments containing replication forks are less streamlined and will travel more slowly through the gel than unreplicated or completely replicated fragments. This two-dimensional assortment by size and shape of the same piece of DNA undergoing replication generates arc patterns. The DNA is analyzed by Southern blotting, in which DNA in the gel is transferred to nitrocellulose and probed with a radioactive DNA fragment that hybridizes to the region of interest (see Chapter 6).

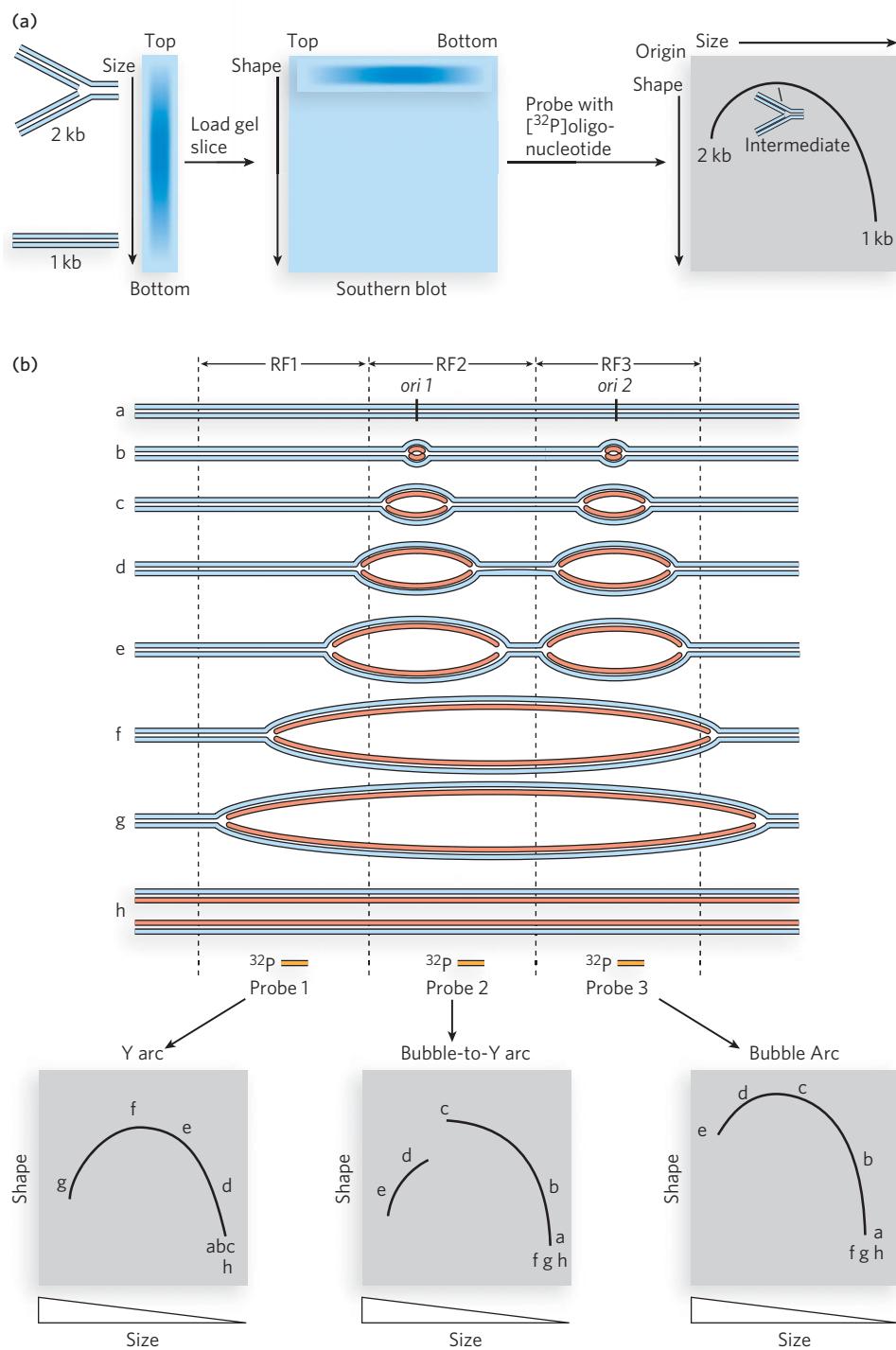
Figure 1b shows DNA structures that result from replication initiating at two different origins on one section of DNA. Vertical dashed lines represent restriction sites. After digestion by restriction enzymes, three different restriction fragments are produced, RF1, RF2, and RF3. The three panels below the DNA structures represent the results of two-dimensional gel analysis of the cut DNA using a radioactive DNA probe—probe 1, 2, or 3. These probes hybridize specifically to RF1, RF2, or RF3. The lower left panel shows the Y arc pattern, using probe 1 and RF1. This pattern is produced by DNA that contains no origin of its own. Replication forks that enter the DNA produce Y-shaped DNAs that form differently sized and shaped fragments, depending on how far the fork travels into the frag-

FIGURE 1 Replication origins can be identified based on their mobility in a two-dimensional agarose gel. (a) The steps involved in two-dimensional gel electrophoresis, showing how an arc is generated. (b) The expected patterns from analysis of linear chromosomal DNA with more than one origin—the typical case. [Source: Adapted from B. J. Brewer and W. L. Fangman, *Cell* 51:463–471, p. 464, © 1987 *Cell*.]

ment (fragments d, e, f, g are produced in succession as the fork proceeds into the fragment). The top of the arc results from DNA containing three arms of equal length (fragment f). The middle panel shows the results when probe 2 is used, which hybridizes to RF2; this produces the bubble-to-Y arc pattern. This pattern is most indicative of an origin within the restriction fragment and occurs when the origin is located to one side of the center point of the fragment. Restriction fragments that contain bubbles (fragments a, b, c) produce an arc that “breaks” when the bubble reaches the end of the fragment, to produce a Y-form DNA (fragments d and e are produced at this point). The right panel shows the bubble arc pattern generated with probe 3 and RF3. The origin in the center produces bubbles of increasing size. Due to the central location of the origin in the restriction fragment, the bubble does not produce a Y form on either end; therefore, the arc has no discontinuity and is only slightly different from the Y arc pattern.

promoters all over the chromosome. This means that collisions between RNA polymerase and replication forks are inevitable. *In vivo* studies show that codirectional collisions do not impede forks, whereas head-on collisions can

cause a fork to pause or stall. Most transcripts in bacteria are oriented in the same direction as replication, and therefore most collisions are codirectional, provided that the forks do not proceed more than halfway around the



chromosome. Perhaps the Tus-Ter system evolved to prevent replication forks from going too far around the circular chromosome, where the direction of transcription would result in head-on collisions.

Replicating the last bit of DNA between converging replication forks presents certain topological problems that must be solved to disentangle the two daughter

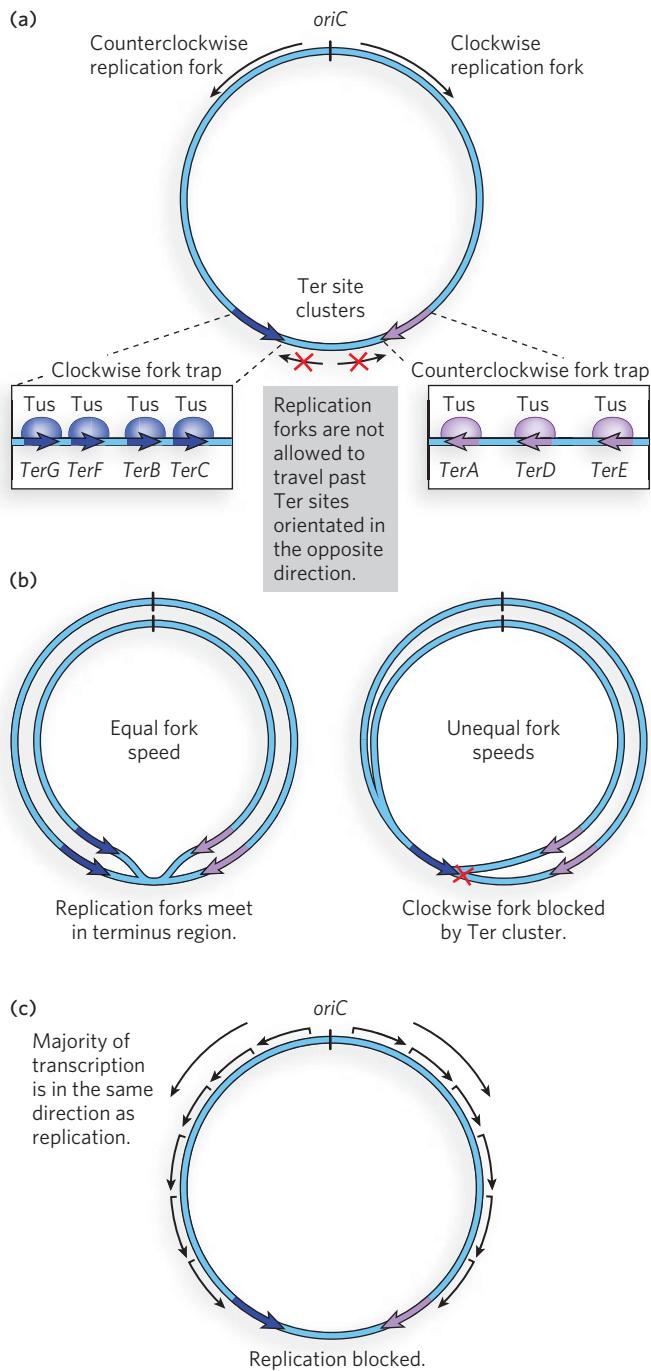


FIGURE 11-33 The role of Ter sites in control of replication termination in *E. coli*. (a) Ter sites are located in two clusters, half way around the chromosome from *oriC*. Each cluster contains multiple Ter sites oriented in the same direction, but the two clusters have opposite polarity, indicated by the arrows. Tus protein binds to a Ter site, and Tus-Ter blocks helicase approaching from one direction and not the other. Replication forks can displace Tus and pass through the first Ter cluster they encounter, but they are blocked at the second cluster, which has the opposite polarity. (b) Replication forks of equal speed meet in the terminus region (left). The Tus-Ter system ensures that even replication forks moving at unequal speed will meet in the terminus region (right). (c) The Tus-Ter system prevents a replication fork from extending much beyond the halfway point around the chromosome, and thus ensures that the fork always moves in the same direction as transcription.

gap that must be primed and filled in. The problem arises when the RNA primer at the extreme end is removed for replacement with DNA (Figure 11-34). There is no 3' terminus for DNA polymerase to extend from, so this single-strand gap cannot be converted to duplex DNA. The genetic information in the gap will be lost in the next round of replication, and repeated rounds will cause the ends to get progressively shorter until the genes near the ends are entirely lost. The resulting loss of gene function can be detrimental to a cell, disrupting cellular functions or contributing to cancer formation. This **end replication problem** does not occur in circular DNA, which has no ends. Indeed, avoidance of the end replication problem may underlie the widespread occurrence of circular DNAs in bacteria and their plasmids and phages.

The end replication problem is solved by **telomerase**, found in the Nobel Prize-winning discovery by Carol Greider and Elizabeth Blackburn. Telomerase carries its own template strand in the form of a tightly bound noncoding RNA (451 nucleotides in humans).

chromosomes. Specialized type II topoisomerases unlink the catenated daughter chromosomes (see Chapter 9).

Telomerase Solves the End Replication Problem in Eukaryotes

The replication of linear chromosome ends poses a unique problem. At the end of a chromosome, after the leading strand has been completely extended to the last nucleotide, the lagging strand has a single-strand DNA



Carol Greider

[Source: Courtesy of Carol Greider.]



Elizabeth Blackburn

[Source: Elisabeth Fall/Fallfoto.com.]

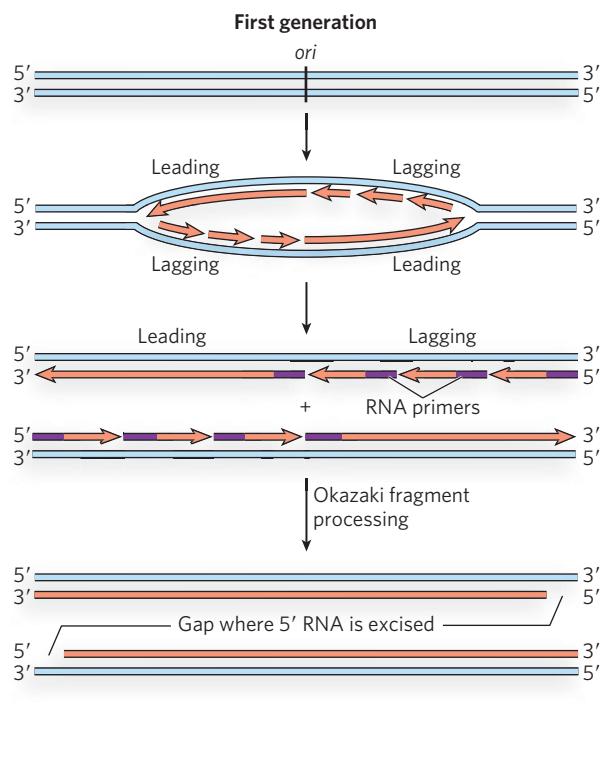


FIGURE 11-34 The end replication problem in linear chromosomes. Just two rounds of chromosome replication are shown here. In the first generation of replication (left; red indicates new DNA), lagging-strand synthesis results in an RNA primer at or near one 5' end of each new chromosome. After removal of the RNA, the 5' single-strand gap in the DNA cannot be filled, thus a 3' single-stranded DNA overhang remains. In the second generation (right; yellow indicates new DNA), each first-generation chromosome produces two new chromosomes, for a total of four new duplexes. Two of the new chromosomes have lost DNA at one end. All four chromosomes terminate with a 5' single-strand gap in the DNA after the RNA is removed. Further losses will be sustained with each new generation.

The ends of eukaryotic chromosomes are capped with telomeres, composed of hundreds of repeats of a 6 bp sequence (see Table 9-1). The RNA of the telomerase ribonucleoprotein contains a 9-mer sequence complementary to 1.5 repeat units of the telomere, and it is used as a template to extend the 3' terminus of the template strand (Figure 11-35). Three DNA nucleotides anneal to three RNA nucleotides in telomerase, which adds six dNMPs to the end of the DNA. This is followed by separation of the RNA-DNA hybrid and repositioning of the template RNA for extension of the next 6-mer repeat. Many DNA hexameric repeats are added in one telomerase-binding event. The telomerase-extended, 3' single-stranded DNA terminus is converted to duplex DNA by priming of the lagging strand and DNA polymerase activity.

Proteins Bind Telomeres to Protect the Ends of Chromosomes

The extreme 3' terminus of a new telomere still has single-stranded DNA, due to the same RNA primer-removal problem as before. But any loss of terminal nucleotides is easily tolerated because the repeating

sequence has been extended by telomerase, and the terminal repeat does not encode a biomolecule. All eukaryotic chromosomes have telomeres to begin with, but somatic cells lack telomerase activity; only germ cells contain it. In laboratory culture, the telomeres of normal somatic cells get progressively shorter with each cell division, and eventually the cells die. In contrast, telomerase is activated in cancer cells, and these cells are immortal in lab culture. These observations imply that a drug that targeted telomerase could impart mortality to cancer cells without affecting normal somatic cells. They also suggest that activating telomerase in normal somatic cells could make them immortal, although this activation could also cause cancer. These questions are currently being investigated.

The linear ends of eukaryotic chromosomes present yet another problem—they could be mistaken for sites of chromosome breakage and thereby induce the cell's recombination-mediated double-strand break repair system (see Chapter 12). In the cell, telomeric ends are sequestered by two or three specialized telomere DNA-binding proteins that cover the 3' single-stranded DNA overhang. Besides preventing recombination or end-joining, these proteins also limit the action of

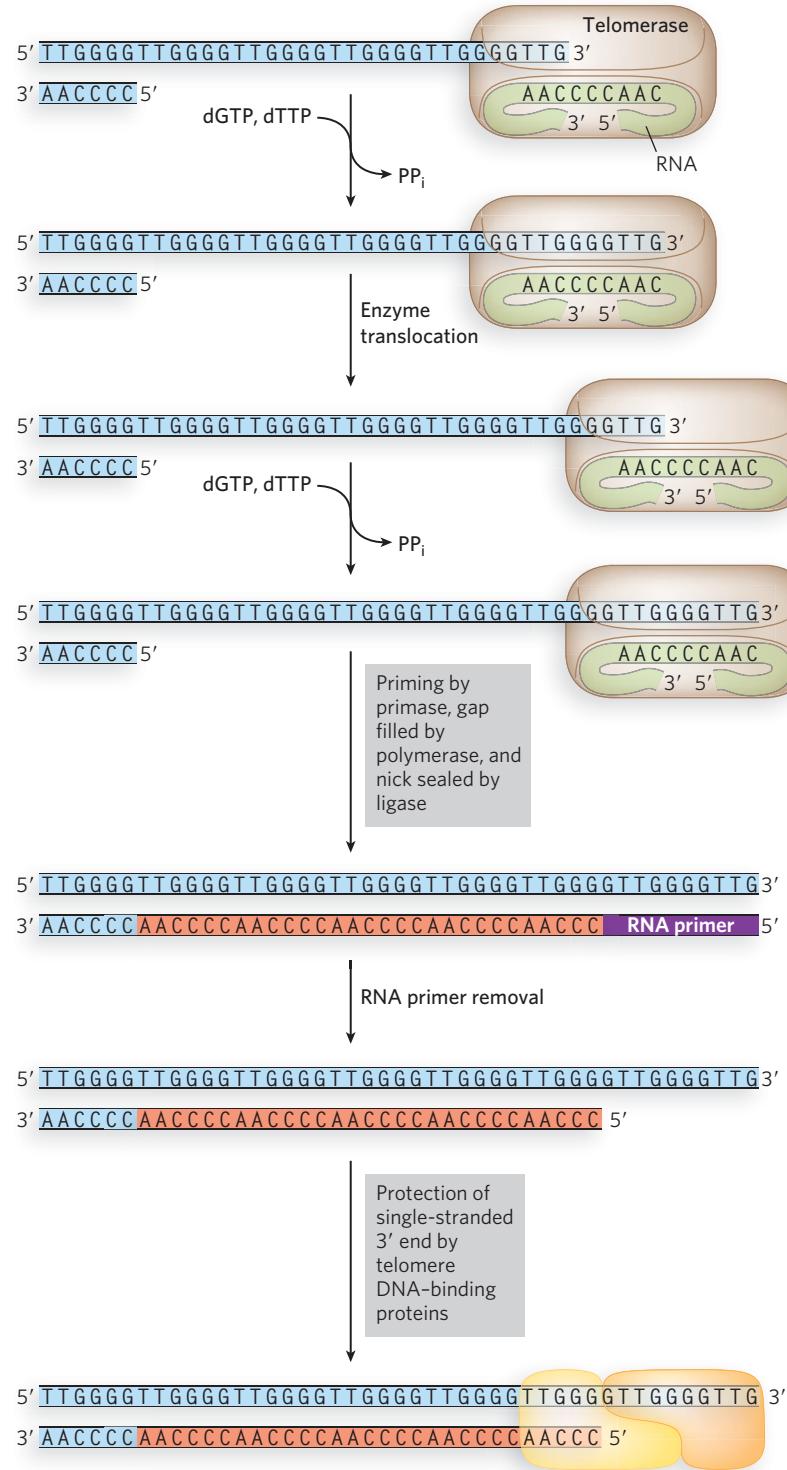


FIGURE 11-35 Extension of the ends of linear chromosomes by telomerase. Telomeres at the ends of eukaryotic chromosomes are composed of a repeating unit of a 6-mer DNA sequence. Shown here is the repeating 5'-TTGGGG-3' sequence of *Tetrahymena*. Telomerase extends the 3' single-stranded DNA end with dNTPs, using its internal RNA molecule as template. The extended 3' single

strand of DNA is filled in by RNA priming and DNA synthesis. Removal of the RNA primer for this fill-in reaction still leaves a 3' single-stranded DNA overhang; this end is sequestered by telomere DNA-binding proteins. These proteins protect the chromosome ends from becoming substrates for the cell's double-strand break repair machinery.



Titia de Lange [Source: Courtesy of Titia de Lange/de Lange & Konarska Labs.]

telomerase in cultured cells and prevent telomeres from growing abnormally long. Titia de Lange's group discovered that, in mammals, the 3'-terminal single-stranded DNA is folded back and hybridized to the duplex, to form a single-strand loop called a **t-loop** (telomere loop) (Figure 11-36). In addition, mammalian cells contain telomeric proteins that bind the duplex portion of the telomere and may assist in t-loop formation.

Telomere Length Is Associated with Immortality and Cancer

Telomeres are thought to play a central role in determining the longevity of a cell. Many key observations suggest that the regulation of telomerase activity may underpin most forms of cancer. Other observations indicate that telomerase could hold the key to immortality. These two ideas are interrelated, because cells that lack telomerase are fated to die, whereas cells expressing telomerase may live forever. Because most somatic cells lack telomerase, they have a relatively

predetermined number of cell divisions, because with each division, their telomeres shorten. When the telomeres get too short, they trigger the process of programmed cell death (i.e., apoptosis). Indeed, the cells of individuals with premature aging syndromes have shorter-than-normal telomeres. In Werner syndrome, Bloom syndrome, ataxia telangiectasia, and Fanconi anemia, for example, individuals have mutations in genes encoding proteins involved in DNA repair. The connection between repair and telomere length is uncertain at this time.

Some mutations allow cells to survive telomere loss. These mutations are typically found in genes that repress programmed cell death and promote mitotic growth. However, the cells still do not survive much longer than normal because, when a chromosome loses its telomeres, the ends are read by the cellular machinery as a double-strand break. Double-strand breaks are repaired by joining segments of DNA together, so in cells lacking telomeres, the chromosomes become joined. This preserves the chromosomes for a short while, but during cell division, the chromosomes are torn apart during anaphase, leading to further mutations and chromosomal abnormalities. The chromosomes are unstable in these cells, and the cells soon die.

Activation of telomerase prevents the loss of telomeres, often conferring immortality. Telomerase is activated in about 90% of all human cancers. HeLa cells, an immortal human cell line, have been grown in cell culture for decades in laboratories around the world. These cells are derived from Henrietta Lacks, who died of ovarian cancer in 1951.

Does the activation of telomerase hold promise as our ticket to immortality, the proverbial “fountain of youth”? Probably not. Studies in mice have shown that activation of telomerase in somatic cells leads to an increased incidence of tumors, and lifespan is shortened by cancer—which is not surprising, given that cancer cells have an activated telomerase. Several mutations are needed to form a cancer cell, not just telomerase activation. Other mutations involve suppression of the programmed cell death pathway and activation of the mitotic pathway (i.e., mutations in tumor suppressors or in oncogenes; see Chapter 12). But activation of telomerase would decrease the number of mutations needed for a cell to become cancerous. Perhaps more informative is the finding that mice without telomerase have a normal lifespan. Despite these mixed findings, research on telomerase and antiaging therapy will probably continue past the lifespan of all those who read this book.

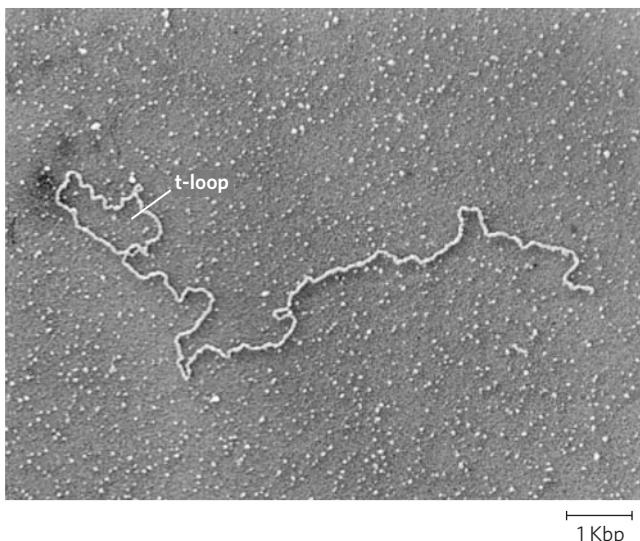


FIGURE 11-36 Mammalian t-loops. The t-loop at the end of a linear chromosome of mammalian cells is visible in this electron micrograph. The telomere has been separated from the rest of the chromosomal DNA by a restriction enzyme. [Source: J. D. Griffith et al., *Cell* 97:503–519, 1999, Fig. 3B.]

The fact stands that telomerase is associated with cancer. If telomerase could be inhibited by a drug, the telomeres in cancer cells should shorten with each cell division until the cells are induced to die. Therefore, drugs that inhibit telomerase hold promise as anticancer therapy, and this is an active area of current research.

SECTION 11.5 SUMMARY

- Termination of replication in *E. coli* occurs halfway around the circular chromosome from *oriC*. Bidirectional replication forks meet head-on within a terminus region bordered on both sides by multiple Ter sites. Tus binds to Ter and blocks replication forks in one direction but not the other, thus localizing termination to the terminus region.
- Replication of eukaryotic linear chromosomes cannot be completed at the extreme ends with the replication fork machinery. To solve this end replication problem, telomeres are synthesized at the chromosome ends by telomerase, which carries its own RNA template strand and adds multiple 6-mer repeats to the 3' terminus, extending the 3' single-stranded DNA. The single strand is then converted to duplex DNA by priming and chain extension.
- After telomere synthesis, removal of the last RNA primer still leaves a small single-strand DNA gap that cannot be filled. Because the telomeric repeats are noncoding and can be replaced by further telomerase action, their loss is of no consequence.
- Telomere DNA-binding proteins protect the ends of chromosomes from nucleases and recombination.
- Somatic cells lack telomerase, and they die when their telomeres become too short. Telomerase is activated in cancer cells, which become immortal and form tumors. Harnessing the activity of telomerase to kill cancer cells or rejuvenate normal but aging somatic cells is an important subject of medical research.

Unanswered Questions

Although we know the major actors that replicate bacterial chromosomes, the mechanics of advancing a replication fork in the highly condensed DNA of a chromosome in the cell still raises several questions. Regulation of the various steps in replication affects cell division and thus is central to preventing uncontrolled cell growth in diseases such as cancer. Control of replication will undoubtedly be an important subject of future studies.

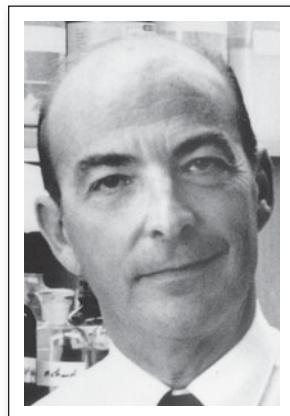
- How do replication forks respond to DNA-bound proteins?** Chromosomal DNA contains many DNA-bound proteins, including repressors, transcription activators, and nucleosomes. We know that the replisome can displace and bypass RNA polymerase in *E. coli*, but only if the direction of replication is the same as the direction of transcription. What happens when the replication and transcription machineries collide head-on? Do nucleosomes stay bound to DNA during replication, and how is the epigenetic information contained within them sustained in the daughter chromosomes?
- What protein modifications control replication?** The impact of protein modifications on replication control in eukaryotic cells is extremely important and probably involves the phosphorylation of replication proteins, because their phosphorylation state can be seen to change with phases of the cell cycle. The identity of the kinases, which proteins and amino acid residues they modify, and the change in activity these modifications bring about are nearly unexplored territory.
- What is the relationship between telomerase, aging, and cancer?** The loss of telomeres leads to chromosome instability and cell death. Most normal somatic cells lack telomerase and die when their telomeres become too short. Immortal cancer cells express telomerase and their telomeres are maintained. These observations imply that telomerase, aging, and immortality are related. The clinical ramifications of controlling telomere length in cells is a highly active area of current research.

How We Know

DNA Polymerase Uses a Template and a Proofreader: Nature's Spell Check

Bessman, M.J., I.R. Lehman, E.S. Simms, and A. Kornberg. 1958. Enzymatic synthesis of deoxyribonucleic acid. II. General properties of the reaction. *J. Biol. Chem.* 233:171-177.

Lehman, I.R., M.J. Bessman, E.S. Simms, and A. Kornberg. 1958. Enzymatic synthesis of deoxyribonucleic acid. I. Preparation of substrates and partial purification of an enzyme from *Escherichia coli*. *J. Biol. Chem.* 233:163-170.



Arthur Kornberg, 1918-

2007 [Source: AP/Wide World Photos.]

the DNA polymer was made. They developed an assay for DNA synthesis using bacterial cell extracts to which they added [^{14}C]thymidine to ensure that any radioactive polymer recovered would be DNA and not RNA. Though radioactive incorporation was feeble, it was reproducible. During fractionation of the extract, Kornberg's group discovered that several heat-stable factors (i.e., not proteins) were needed for the DNA synthesis reaction. They identified these as nucleoside triphosphates. Kornberg also found that excess unlabeled DNA had to be added to the cell extracts in order to observe DNA synthesis. These insights allowed the purification of what we now know as DNA polymerase I (Pol I).

On characterizing Pol I, the researchers were initially puzzled that it required all four dNTPs for robust DNA synthesis. If DNA were serving only as a primer, why couldn't a DNA polymer be made from just one, two, or three types of nucleotides? The finding implied that the enzyme received instructions from existing

Arthur Kornberg didn't intend to discover how DNA was made, or even to become a scientist. He was a physician on a naval ship, but soon after setting out to sea, his single publication as a medical student led to an offer to transfer to the National Institutes of Health. After jumping ship, he began an incredible scientific odyssey that founded the field of replication enzymology.

Kornberg and his group wanted to understand how

DNA acting as a template, as suggested by Watson and Crick, but at that time, the idea of an enzyme receiving direction from its substrate was preposterous. Kornberg's group conducted experiments to test whether this was in fact the case. They tested DNAs that varied in A=T versus G≡C content, and the result was astounding. Regardless of the mix of dNTPs, the ratio of A=T and G≡C in the product matched that in the template DNA. That settled it! The DNA was serving not only as primer but also as a template. To support this conclusion, they used Pol I to convert the 5.4 kb single-stranded φX174 bacteriophage genome into the duplex viral form. The double-stranded DNA product was infectious! This finding set off a flurry of newscasts: "Life created in a test tube!" More importantly, it marked the beginnings of biotechnology.

Then came John Cairns's discovery that *polA* mutant cells, with less than 1% residual Pol I activity, had no growth defects. This result, combined with genetic findings of numerous genes required for replication, revealed a process far more complex than anyone had imagined. Unsettling to Kornberg and his colleagues was the questioning of their work on DNA polymerase I in pointed editorials in *Nature New Biology*. Did the assays used to purify Pol I result in a red herring? Do "real" polymerases need a primed template? Are dNTPs the true precursors of DNA? Is a 3'→5' exonuclease proofreader needed by the "real" DNA polymerase?

Kornberg's son Tom identified both Pol II and Pol III from extracts of *polA* mutant cells. These polymerases were just like Pol I in the use of a primed template and dNTPs and the presence of a 3'→5' proofreading exonuclease. Fortunately, the controversial issues raised in *Nature New Biology* soon vanished. Coincidentally, so did the journal itself.

Polymerase Processivity Depends on a Circular Protein That Slides along DNA

Kong X.P., R. Onrust, M. O'Donnell, and J. Kuriyan. 1992. Three-dimensional structure of the β subunit of *E. coli* DNA polymerase III holoenzyme: A sliding DNA clamp. *Cell* 69:425–437.

Stukenberg, P.T., P.S.-V. Studwell, and M. O'Donnell. 1991. Mechanism of the sliding β clamp of DNA polymerase III holoenzyme. *J. Biol. Chem.* 266:11,328–11,334.

DNA polymerases that replicate chromosomes were long known to require “accessory proteins” that somehow confer rapid and processive polymerase activity. However, it seemed a contradiction that proteins that increase the affinity of polymerase for DNA also enable rapid motion along DNA. Specifically, how can a polymerase bind DNA tight and also rapidly slide along it?

Surprisingly, experiments showed that the β subunit of Pol III holoenzyme, by itself, binds to DNA. This required the γ complex and ATP. However, the β subunit only bound circular DNA and could not bind linear primed DNA. This suggested that β subunit binds DNA by encircling it, and thus slides off linear DNA. No protein was known to encircle DNA at the time, so this idea was not taken seriously. However, the test was rather simple. [^3H] β subunit was loaded onto circular primed DNA, and the reaction mixture was divided. In one tube, the DNA was linearized using BamHI, and in the other tube, the DNA was untreated and remained circular. The two reaction mixtures were then analyzed on gel filtration columns. [^3H] β bound to the large DNA molecule elutes much earlier (fractions 7 to 16) than [^3H] β not bound to DNA (fractions 20 to 40).

If the [^3H] β subunit encircles DNA like a doughnut, it should slide off linear DNA but remain on circular DNA. This was exactly the result observed. The filled circles in the upper plot in Figure 1 show the sample treated with BamHI. Most of the [^3H] β in this sample elutes late as [^3H] β not associated with DNA. In the untreated sample (unfilled circles), the early fractions show [^3H] β bound to DNA. The result is clear: β remains on circular DNA but falls off linear DNA. This behavior suggests that β is shaped like a ring that binds circular DNA but slides off the end of linear DNA.

This hypothesis was tested using DNA with two sites for a DNA-binding protein known as EBNA1 (lower plot in Figure 1). The [^3H] β was loaded onto DNA (with γ complex

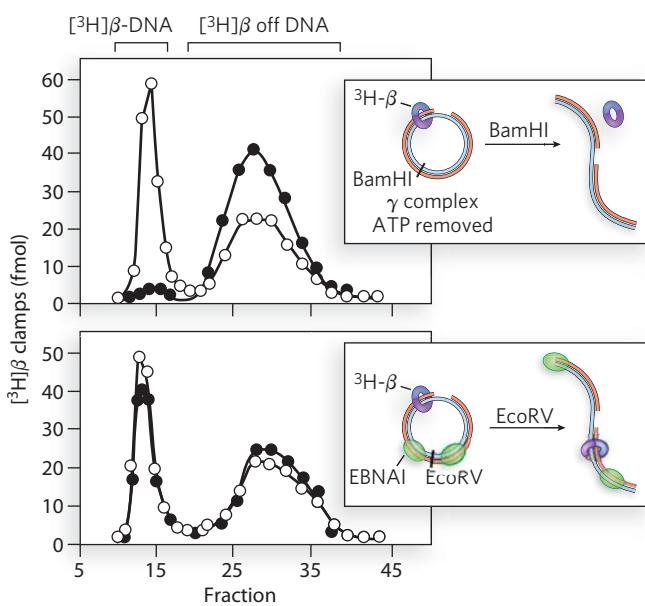


FIGURE 1 Experiments revealed that the *E. coli* Pol III β subunit binds to DNA by encircling it and slides along the duplex. [Source: Adapted from P. T. Stukenberg, P. S.-V. Studwell, and M. O'Donnell, *J. Biol. Chem.* 266:11328–11334, 1991, Fig. 3.]

and ATP), then EBNA1 was added. One half of the reaction mixture was treated with EcoRV (filled circles), which cuts the DNA between the two EBNA1 proteins. The result from gel filtration analysis (also known as gel-exclusion chromatography, see Highlight 4-1) showed that [^3H] β was retained on the linear DNA by EBNA bound to each end, supporting the idea that β DNA encircles, and slides on it. Another experiment, not shown here, demonstrated that Pol III core binds to β , implying that β acts as a clamp that encircles DNA and tethers the polymerase to the template for high processivity during synthesis. The hypothesis that β is circular was confirmed by its crystal structure (see Figure 11-15).

Replication Requires an Origin

Hiraga, S. 1976. Novel F prime factors able to replicate in *Escherichia coli* Hfr strains. *Proc. Natl. Acad. Sci. USA* 73:198-202.

Oka, A., H. Sasaki, K. Sugimoto, and M. Takanami. 1984. Sequence organization of replication origin of the *Escherichia coli* K-12 chromosome. *J. Mol. Biol.* 176:443-458.

Zyskind, J.H.W., J.M. Cleary, W.S. Brusilow, N.E. Harding, and D.W. Smith. 1983. Chromosomal replication origin from the marine bacterium *Vibrio harveyi* functions in *Escherichia coli*: *oriC* consensus sequence. *Proc. Natl. Acad. Sci. USA* 80:1164-1168.

To identify the replication origin of a host cell, plasmid, or virus, a plasmid with a known origin and a selectable marker (e.g., the gene conferring resistance to the antibiotic ampicillin) is first treated with restriction enzymes to excise the origin (Figure 2). DNA extracted from host cells is cut with a restriction enzyme to produce many fragments. Individual fragments are inserted into the cut plasmid, and DNA ligase is used to re-create plasmid DNA circles. These recombinant plasmids are transferred into *E. coli*, and the transformed cells are plated on selective media (e.g., plates containing ampicillin). To survive the antibiotic in the medium and form a colony, cells must contain the plasmid with the ampicillin-resistance gene. In turn, the plasmid must contain a functional origin of replication in order to continue duplicating itself over multiple cell generations. Surviving plasmids are isolated from the bacteria and sequenced to identify the origin required for replication.

The recombinant plasmid approach has identified numerous origins of bacterial chromosomes, plasmids, and bacteriophage. Yeast (a eukaryote) has defined origins that can be isolated in a similar way. However, eukaryotic plasmids cannot be selected using antibiotics. Instead, genes needed for the metabolism of a particular amino acid are used, and cells are plated on media lacking that amino acid. This experimental approach has not been successful in identifying replication origins in eukaryotes more complex than yeast. It is possible that higher eukaryotes do not have defined origins, and that chromatin structure defines replication start sites. Alternatively, the origins of higher eukaryotes are too large to determine by this method.

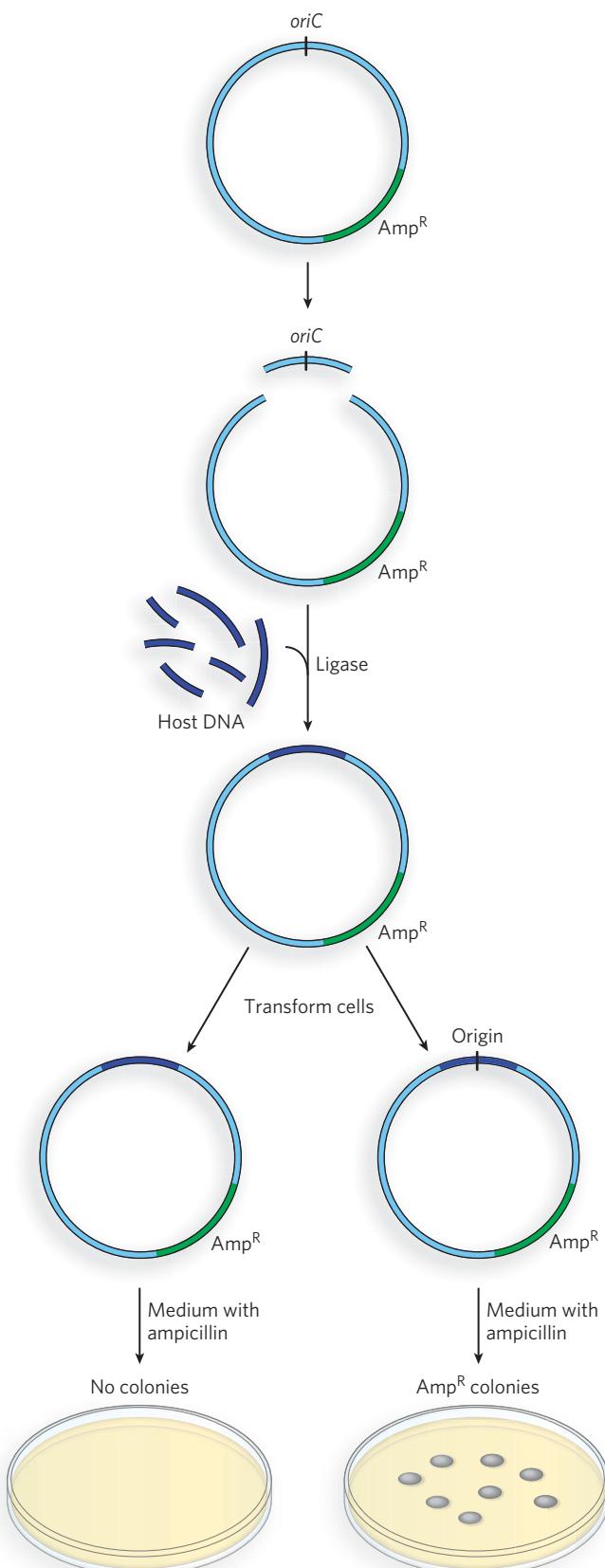


FIGURE 2 The recombinant plasmid method for identifying replication origins. *Amp^R* indicates an ampicillin-resistance gene.

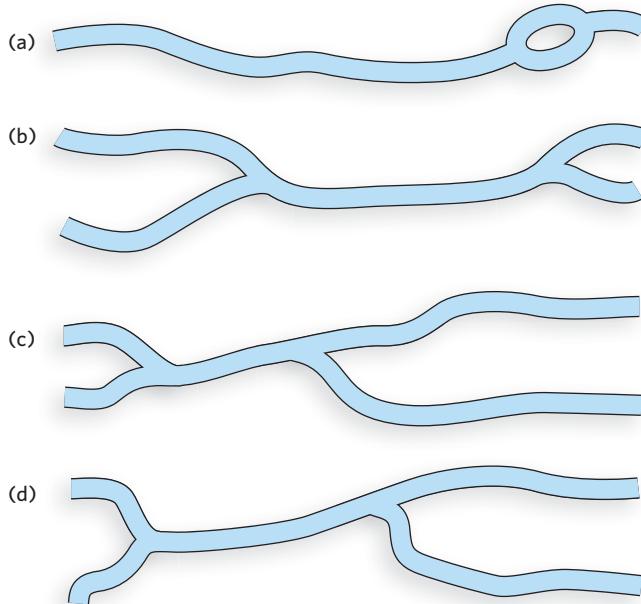
Key Terms

DNA replication, p. 364
 semiconservative, p. 364
 origin of replication, p. 367
 replication fork, p. 367
 DNA polymerase, p. 368
 semidiscontinuous, p. 369
 leading strand, p. 369
 lagging strand, p. 369
 Okazaki fragment, p. 369
 DNA nuclease, p. 371
 proofreading, p. 371
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 prereplication complex (preRC), p. 395
 replication complex (RC), p. 395
 telomerase, p. 398

Problems

1. Shown below are outlines of four linear molecules, as observed in the electron microscope, after cutting a circular plasmid once with a restriction enzyme. Does this plasmid replicate bidirectionally or unidirectionally from the origin? Explain. Order the molecules by time of replication, from the earliest to the latest.



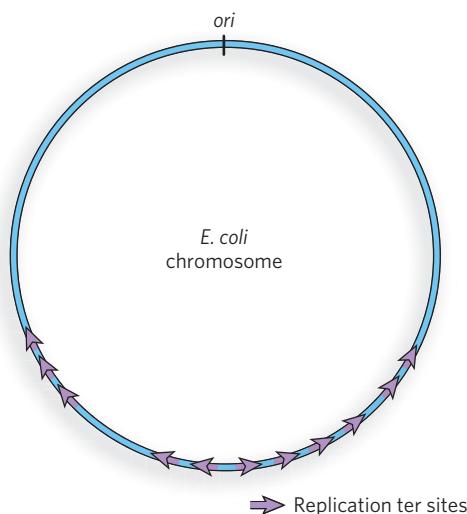
2. Arthur Kornberg and his colleagues incubated an extract of *E. coli* with a mixture of dATP, dTTP, dGTP, and dCTP. Only the dTTP was labeled with ^{32}P in the α -phosphate group. After incubation, the mixture was treated with trichloroacetic acid to precipitate DNA, but not the dNTPs. The precipitate was collected, and the extent of $[^{32}\text{P}]\text{dTTP}$

incorporated into DNA was determined. (a) If any one of the dNTPs were omitted from the incubation mixture, would radioactivity be found in the precipitate? Explain. (b) Would radioactivity be found in the precipitate if the ^{32}P labeled the β - or γ -phosphate position? Explain.

3. Provide at least two observations suggesting that Pol I is not the chromosomal replicase.
4. You are characterizing a new DNA polymerase. When the enzyme is incubated with $[^{32}\text{P}]\text{DNA}$ and no dNTPs, you observe the release of $[^{32}\text{P}]\text{dNMPs}$. This release is prevented by adding unlabeled dNTPs. Explain the reactions that most likely underlie these observations. What would you expect to observe if you added pyrophosphate instead of dNTPs?
5. Explain how the enzymes that join together Okazaki fragments ensure that all the RNA is removed from the ends of fragments before they are sealed.
6. A DNA fragment containing an origin has been identified with the recombinant plasmid approach (see How We Know). The origin contains six short palindromes: four A sites of similar sequence, and two B sites unrelated to the A sites. (a) Assuming that three of the A sites are essential, explain an approach to identify which ones are necessary for origin function. (b) Plasmids with a mutation in either of the B sites still replicate. What additional experiment would you do to determine whether B-site function is necessary at all?
7. Eukaryotic origins are tightly regulated so as not to fire more than once during S phase. Explain the key points of regulation of this process in eukaryotic cells.
8. Explain the role of the τ subunit of Pol III. (a) How many τ subunits must be present in Pol III to coordinate leading- and lagging-strand DNA synthesis at a replication

fork? (b) How many τ subunits must be present to allow Pol III to extend an oligonucleotide primer processively on a single-stranded template in vitro? Explain your answer in both cases.

9. The AAA^+ ATPases are the wrenches and crowbars of DNA metabolism. Briefly indicate what is accomplished when ATP is hydrolyzed by each of the following AAA^+ ATPases: DnaA, DnaC, and the γ and τ subunits of Pol III.
10. The replication termini (Ter sites) on the *E. coli* chromosome are oriented as shown to the right. Briefly describe what would happen in one round of replication if the orientations of all the Ter sites in the chromosome were reversed.



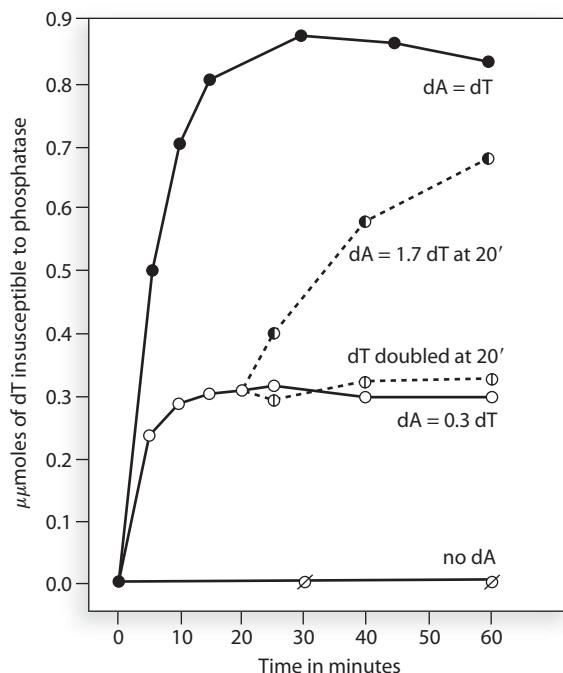
Data Analysis Problem

Olivera, B.M., and I.R. Lehman. 1967. Linkage of polynucleotides through phosphodiester bonds by an enzyme from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 57:1426-1433.

11. The discovery of DNA ligase provides some classic examples of the development of enzyme assays (see Moment of Discovery). In the paper first reporting their detection of a DNA-joining activity, Lehman and colleagues described the use of partially purified enzyme extracts from *E. coli* that had this joining activity. For a DNA substrate, they used polydeoxyadenosine, poly(dA), along with polydeoxythymidine, poly(dT), the latter digested with micrococcal nuclease (which degrades DNA strands at random locations) until the average length of the poly(dT) strands was about 250 nucleotides. These DNA strands were then labeled at their 5' ends with a ^{32}P -labeled phosphoryl group. When the labeled DNA was precipitated with HCl and filtered, the label remained with the precipitated DNA on the filter. The labeled DNA was then treated with an enzyme called alkaline phosphatase, which removes terminal phosphates from DNA; with this treatment, the label is freed from the DNA and washes through the filter.

In their experiments, Olivera and Lehman found that the poly(dT) strands were linked together, because the label became resistant to the alkaline phosphatase treatment. Some of their results are shown to the right, for a reaction containing 1 μmol (micromole) of labeled DNA ends.

- (a) Why does the linking reaction make the label resistant to alkaline phosphatase treatment?
- (b) The reaction required the addition of poly(dA) in addition to the labeled poly(dT) strands. Suggest a reason.



- (c) In one experiment, the investigators added only enough poly(dA) to allow linking of 30% of the poly(dT). After 20 minutes, they added either more poly(dA) or more unlabeled poly(dT). The reaction recommenced only in response to the poly(dA). What does this reveal about the enzyme activity?
- (d) A cofactor in the crude extract was needed for the reaction, but it could not be replaced by added ATP. Suggest a reason.

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DNA Mutation and Repair



Myron Goodman [Source: Courtesy of Myron Goodman.]

Moment of Discovery

For many years, I and others had wondered how damaged DNA is replicated in cells, thereby enabling cells to continue growing and have a chance to repair the DNA lesions. The original idea was that somehow proteins encoded by the *umu* genes could bind to DNA polymerase III—the replicative polymerase in *E. coli*—and lower its fidelity enough to allow copying of DNA containing nucleotide lesions. Our approach in the lab was

to reconstitute the whole lesion-bypass system using purified proteins.

We put purified DNA polymerase III and DNA containing a lesion together in a test tube, then began adding other proteins to activate the system. At first we added the protein RecA, an important component of DNA repair pathways, but nothing happened. Then we added a protein complex called UmuD'₂C, which was thought to activate the polymerase on damaged DNA substrates, and it worked. The damaged DNA could be copied. But the real shocker was that it worked just as well in the control experiment, when we left out the DNA polymerase III! This made us realize that the UmuD'₂C complex is itself a polymerase that has absolutely no sequences in common with any other polymerases. And we recently figured out why the RecA protein is required for the lesion-bypass reaction: long filaments of RecA in the cell are required to convert the UmuD'₂C complex—now called DNA polymerase V—to its active form by transferring a molecule of RecA from the filament to UmuD'₂C. So we now know that the active form of DNA polymerase V is two molecules of UmuD', one molecule of UmuC, a RecA molecule, and ATP.

—Myron Goodman, on his discovery of DNA polymerase V

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ADNA genome encodes the instructions for the production of every molecule in the cell and, as such, is also essential for reproduction of the cell for future generations. Because each cell contains only one or two copies of its DNA, the DNA sequence is highly protected from harm. DNA is a relatively stable molecule, but Earth's natural environment is quite toxic, and damage to DNA is inevitable. Our dependence on oxygen and water makes us vulnerable to a continuous barrage of oxidative and hydrolytic reactions, many of which strike intracellular molecules, including DNA. DNA is also subject to attack by many other reactive chemicals in the environment, both natural and synthetic, as well as various types of irradiation from the sun and radioactive decay of terrestrial elements. DNA can also be altered by mistakes made during its own replication or recombination. Damage and sequence alterations to DNA are often quickly repaired, but when they are not, the DNA becomes permanently altered and harbors a mutation.

Mutations are changes in DNA sequence, and when mutations occur in germ-line cells, or in single-celled organisms, these changes are inheritable. Indeed, some frequency of mutation is necessary to produce the variability on which natural selection acts to drive evolution. However, a mutation that confers an advantage on a cell or organism is rare. In multicellular organisms, a cell that accumulates many mutations usually dies. A cancer cell has mutations that prevent cell death, resulting in loss of cell cycle control and unregulated cell division, which leads to malignant tumors that can end the life of the entire organism.

Mutations occur through many different mechanisms, but all originate as an alteration in DNA. Only after the alteration is converted through replication into a correctly paired—but incorrect—base pair (such as an A=T pair where a G≡C pair should be) does it become a stable, inheritable mutation. Therefore the cell has a limited amount of time to fix the initial alteration and restore the DNA to its normal sequence, before replication converts the alteration into a mutation that will be passed on to the next generation. In all organisms, an army of repair enzymes has evolved that holds a constant vigil over the DNA. Indeed, the vast majority of damaged nucleotides that occur in a mammalian cell every day are repaired; fewer than one in a thousand become a mutation. DNA repair often takes advantage of the double-stranded DNA structure to restore a damaged nucleotide on one strand to the original residue, using the complementary strand as a template.

The enormous selective pressure favoring enzyme systems that repair DNA damage has led to some of the

most fascinating enzyme reactions in biology. DNA repair reactions are also among the most costly in terms of energy, testimony to the importance of their job in ensuring survival of the species. In some cases, an entire enzyme is used only once per repair event. In other words, the information contained in a cell's DNA sequence is to be preserved at all costs; the energy expended in the process is irrelevant.

We begin this chapter by defining mutations and describing how replication of a damaged nucleotide base can become an inheritable mutation. We explore different types of DNA damage that arise from agents inside the cell, as well as from those in the external environment, and then consider many of the fascinating DNA repair processes that restore the original sequence. As we'll see, certain types of DNA damage can be repaired by more than one enzyme system, whereas others are repaired by specific processes dedicated to a particular type of DNA damage. Remarkably, some repair mechanisms can recover the original, correct DNA sequence even when it cannot be obtained from a complementary DNA strand. Other types of DNA damage, however, prevent recovery of the original sequence, and repair gives rise to mutations. Although it seems counterintuitive that a repair process would produce a mutation, the alternative is cell death. For example, some DNA lesions halt the replication machinery. This is the worst thing that can happen to a cell and must be avoided at all costs. If replication can't be completed, daughter cells won't receive a full complement of DNA, and they will die. We'll explain how cells resolve these conflicts, enabling them to complete replication, even at the expense of incurring a mutation.

Most DNA repair processes evolved before the emergence of different cell types from the last common ancestor, and thus the major DNA repair pathways are similar in bacterial, archaeal, and eukaryotic cells. Because bacterial repair systems have been studied most intensively, they provide us with the highest level of mechanistic detail. However, there are several aspects of eukaryotic repair that differ from bacterial repair. We'll learn about these, and about the human diseases that arise from defective DNA repair or from DNA alterations that do not lend themselves to repair.

12.1 Types of DNA Mutations

A **mutation** is a change in a DNA sequence that is propagated through cellular generations. Mutations can be as small as a single base pair or can range from a few base pairs to thousands. Mutations of one or a few base pairs usually result from errors in replication or damaged nucleotides. Those that span large sections of

DNA are typically due to chromosomal rearrangements that arise from errant recombination.

Mutations can have different effects on gene function. A mutation in a gene product can result in a loss of function or a gain of function. For example, loss-of-function can be the result of mutations that destroy the active site of an enzyme, produce a truncated protein, or disrupt the regulation of gene expression. Gain-of-function mutations might increase the affinity of an enzyme for its substrate, remove the regulatory portion of a protein, or up-regulate gene expression. Loss-of-function mutations are generally recessive in a diploid organism, whereas gain-of-function mutations are often dominant over the wild-type allele. Examples of every type of mutation can be observed in human disease.

A Point Mutation Can Alter One Amino Acid

A change in a single base pair is often referred to as a **point mutation**. Point mutations fall into two categories (Figure 12-1). A **transition mutation** is the exchange of a purine-pyrimidine base pair for the other purine-pyrimidine base pair: C≡G becomes T=A, or T=A becomes C≡G. A **transversion mutation** is the replacement of a purine-pyrimidine base pair with a pyrimidine-purine base pair, or vice versa. For example, C≡G becomes either G≡C or A=T. Transition mutations are nearly 10 times more frequent than transversions. A point mutation in the protein-coding region of a gene can result in an altered protein with partial or complete loss of function. If the protein is central to cell viability, the cell could die.

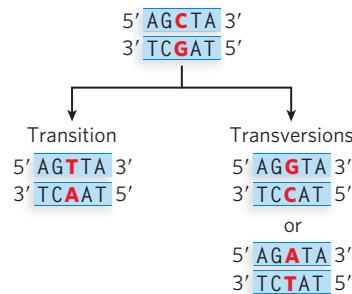


FIGURE 12-1 Transition and transversion point mutations.

The parental DNA (top) contains a C≡G base pair. There are two possible point mutations: a transition (left), in which a purine (in this case, G) is replaced with a different purine (A), producing a T=A base pair on replication; or a transversion (right), in which a pyrimidine (in this case, C) is replaced with a purine (G or A) to produce either a G≡C or an A=T base pair. (To review hydrogen bonding between base pairs, see Figure 1-3.)

Point mutations in a protein-coding region can be classified by their effect on the protein sequence. The DNA sequence encoding a protein is read in triplets, or codons. Each codon corresponds to an amino acid (see Chapter 17). A **silent mutation** is a nucleotide change that produces a codon for the same amino acid. For example, GAA and GAG both code for glutamate. A **missense mutation** is a nucleotide change that results in a different amino acid, such as a change from glutamate (GAA) to glutamine (CAA). A **nonsense mutation** changes the nucleotide sequence so that instead of encoding an amino acid, the triplet functions as a stop codon, terminating the protein.

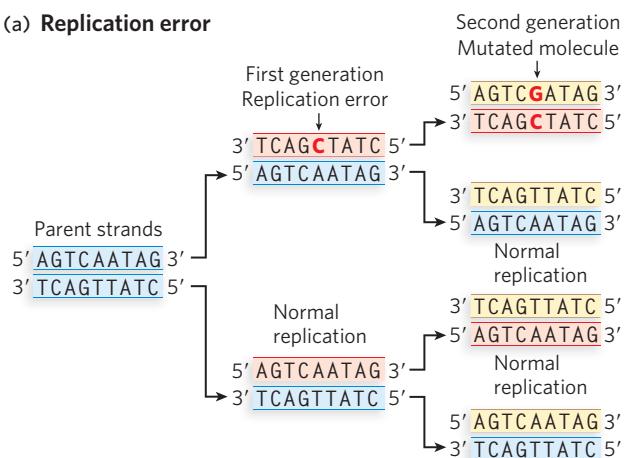
KEY CONVENTION

When a point mutation results in an altered protein sequence, the change in that protein's amino acid sequence is denoted by letters and a number—for example, E214A. The first letter is the single-letter abbreviation for the amino acid residue in the wild-type protein (E); the number is the position of the residue, numbering from the N-terminus of the protein sequence (214); and the second letter is the amino acid residue in the mutant protein (A). A nonsense mutation is identified by an X as the second letter—for example, E214X.

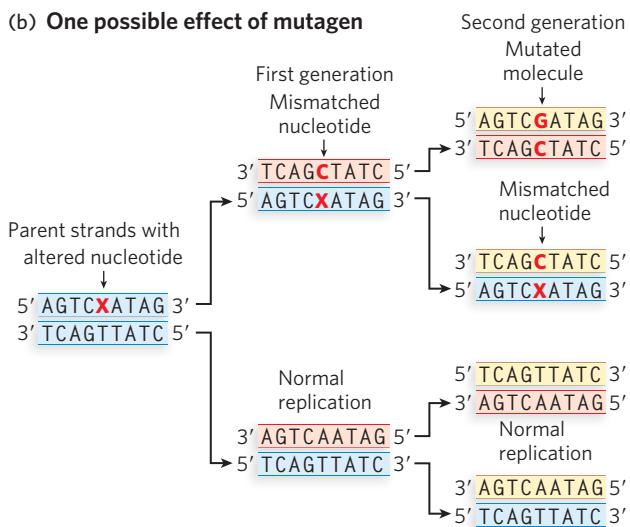
Point mutations are produced in a two-step process. In the first step, an incorrect nucleotide is incorporated by a DNA polymerase (Figure 12-2). This can happen when DNA polymerase encounters a damaged base in the template strand that no longer forms a normal base pair. It can also occur without DNA damage, because DNA polymerases, despite their high accuracy, sometimes make a mistake. For example, as discussed in Chapter 11, a DNA polymerase can incorporate a tautomeric form of a dNTP, resulting in a mismatch. Regardless of how the mismatch is formed, it is not yet a mutation, because it can be detected and repaired by processes described later in the chapter. However, if the mismatch is not repaired, it becomes a mutation in a second step during replication, which incorporates the mismatch into a fully base-paired duplex DNA. This results in a new, correctly paired base pair that can no longer be detected by repair enzymes but alters the original sequence and is therefore an inheritable mutation.

Evolution depends on mutations that can confer a selective advantage, but it is relatively rare for a mutation to have a positive outcome for the organism. Studies in *Drosophila melanogaster* suggest that mutations that alter the protein sequence are most likely to be

(a) Replication error



(b) One possible effect of mutagen

**FIGURE 12-2** The two-step process of a point mutation.

(a) DNA polymerase incorporates an incorrect nucleotide (C instead of T), leading to a mismatch in one of the first-generation cells. If the mismatch is not corrected before this cell replicates, one of the second-generation cells will incur a mutation. (b) The parental DNA contains a damaged adenine base (X). Replication over the damaged base results in a mismatched nucleotide in one of the first-generation cells. Further replication can result in a permanent mutation in a cell of the second generation.

harmful; about 70% have a negative effect, and the rest either are neutral or have a weak beneficial effect. As shown by studies in yeast, however, for mutations outside the protein-coding region, fewer than 7% are harmful.

Point mutations are known to cause a wide variety of human diseases. One example is sickle-cell anemia, resulting from a transversion that produces an amino acid change in hemoglobin: a GAG encoding glutamate

(E) at residue 6 changes to GTG, encoding valine (V) (E6V). (Sickle-cell anemia is discussed in Chapter 2, Highlight 2-1.) Another example of a point mutation in human disease is Werner syndrome, which causes premature aging due to genetic instability. The *WRN* gene encodes a helicase. Several different point mutations in the *WRN* gene lead to Werner syndrome. Some are of the missense type (e.g., K32R), although most are non-sense mutations that lead to a shorter protein product (e.g., E41X).

The most harmful mutations are those occurring in the genes involved in DNA repair, because these often result in cancer. As we'll see later in the chapter, the repair of mismatch errors requires many different proteins. A mutation in a gene encoding a mismatch repair protein can result in the production of many more mutations in the cell, as subsequent mutations are no longer corrected. Many mutations in one cell can result in cancer because, eventually, a mutation will occur in a gene (or genes) that encodes a protein needed to control cell division. In normal cells, **oncogenes** encode proteins that drive the cell division cycle forward, and **tumor suppressor genes** encode proteins that suppress cell division. Many tumor suppressors are transcription factors that regulate the expression of genes that drive the cell cycle. The transcription factor p53 and the retinoblastoma protein are examples of tumor suppressors that are mutated in many types of cancer.

Small Insertion and Deletion Mutations Change Protein Length

Another type of mutation is the gain or loss of one or more base pairs. **Insertion mutations** occur when one or more base pairs are added to the wild-type sequence; conversely, **deletion mutations** are due to the loss of one or more base pairs. Insertion and deletion mutations are collectively referred to as **indels**. Indels are caused by aberrant recombination or by template slippage by the DNA polymerase during replication. As we'll discuss in more detail in Chapter 17, proteins are encoded (via a messenger RNA intermediary) in a series of codons that starts with ATG and ends with one of three different codons that signal translation to stop (Figure 12-3a). The DNA sequence from the start codon to the stop codon is referred to as a **reading frame**. Because nucleotides are decoded in triplets, an indel mutation of only one or two base pairs in the coding sequence of a protein throws off the reading frame after the mutation, resulting in a **frameshift mutation** (Figure 12-3b). Given the frequency of stop codons in a random sequence (1 in every 20 codons), these mutations usually produce a truncated protein, especially if

(a) Reading frame of triplet codons with an ATG start codon and a TAG stop codon

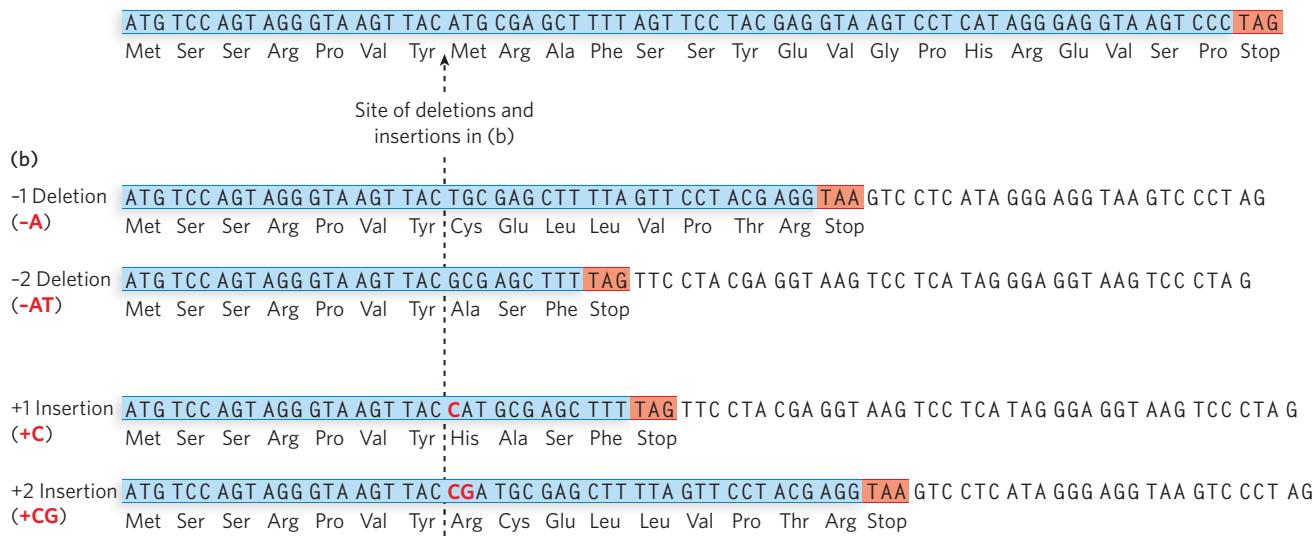


FIGURE 12-3 Insertions and deletions can lead to

frameshift mutations. (a) A reading frame in the DNA begins with the start codon, ATG, and stops with a stop codon (TAG in this case). When the coding strand of the DNA is transcribed into mRNA, these sequences are AUG

(start) and UAG (stop). (b) Insertions or deletions of one or two base pairs in a protein-coding sequence result in frameshift mutations, in which the reading frame becomes out of register relative to the wild-type sequence and typically leads to a premature stop codon.

the gene is large and the mutation occurs a few hundred base pairs prior to the wild-type stop codon. Frameshift mutations often destroy the protein's function, especially if the truncation is close to the N-terminus.

Indel mutations that occur in multiples of three base pairs preserve the reading frame of the gene. The most common such mutation is an insertion of three nucleotides. This is thought to be caused by template slippage during replication, with the inserted triplet embedded in a region of triplet repeats

(Figure 12-4). Thus, the wild-type gene has a repeating array of codons, and the protein product contains a region of repeats of the same amino acid. Although the mutant protein, with an added amino acid residue, is slightly larger than the wild-type protein, it usually retains some degree of function.

There are many examples of human disease caused by the insertion of triplet sequences, often referred to as **triplet expansion diseases** (Table 12-1). More than half of the triplet expansion diseases involve expansion of the CAG codon for glutamine (Q) and are known as

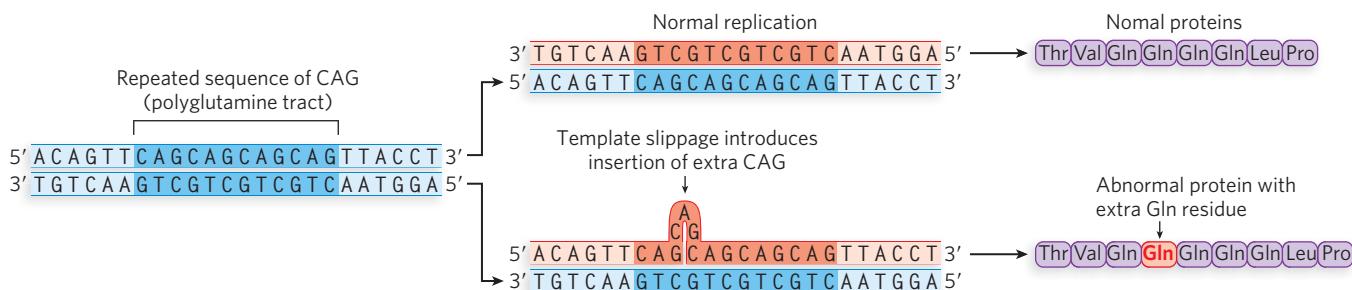


FIGURE 12-4 A three-nucleotide insertion mutation.

Template slippage on repeated sequences during replication can lead to small insertions and deletions. Shown here is a three-nucleotide (CAG) insertion in a repeated CAG region

of the coding strand of a gene sequence, resulting in an additional Gln residue in a polyglutamine tract. The protein sequence shown is the sequence encoded by the coding strand of DNA, which is transcribed into mRNA.

Table 12-1 Triplet Expansion Diseases

Disease	Repeated Codon (amino acid)	Gene	Codon Repeats	
			Normal Range	Threshold for Disease
Huntington disease	CAG (Gln)	HTT	11–35	>35
Spinocerebellar ataxia type 1	CAG (Gln)	ATXN1	6–35	>49
Machado-Joseph disease	CAG (Gln)	ATXN3	12–40	>55
Kennedy disease	CAG (Gln)	AR	9–36	>38
Fragile X syndrome	CGG (Arg)	FMR1	5–54	>230
Fragile X-E syndrome	CCG (Pro)	AFF2	6–35	>200
Myotonic dystrophy	CTG (Leu)	DMPK	5–37	>50
Friedreich's ataxia	GAA (Glu)	FXN	7–34	>100

polyglutamine (polyQ) diseases. An example is Huntington disease. When the number of CAG repeats increases to 36 copies or more, degeneration of the cerebral cortex may occur in midlife (see Figure 2-28). The number of triplet repeats correlates with the severity and time of onset of this neurological disorder.

The first triplet expansion disease to be identified was fragile X syndrome, which causes a form of mental impairment. The defect in fragile X syndrome maps to the X chromosome and involves a CGG repeat. Humans normally have 5 to 54 repeats, but some individuals have more than 230 repeats (up to 2,000), and this leads to the fragile X mental impairment. Individuals with 54 to 230 repeats show no symptoms but are carriers of the disease, because the genetic instability leading to the triplet expansion occurs in germ-line cells and therefore becomes worse with each generation—until the threshold of 230 is passed and the disease manifests. Diagnostic genetic screening for triplet expansion diseases can be performed with PCR primers that anneal to the unique sequences known to lie on either side of the repeated region (Figure 12-5).

Some Mutations Are Very Large and Form Abnormal Chromosomes

Some types of mutations involve extensive changes in the DNA sequence, most commonly caused by aberrant recombination events (Figure 12-6). Deletions of large tracts of DNA lead to a complete loss of genes and also bring genes into proximity that were once far apart. The opposite of a deletion is a **duplication mutation**,

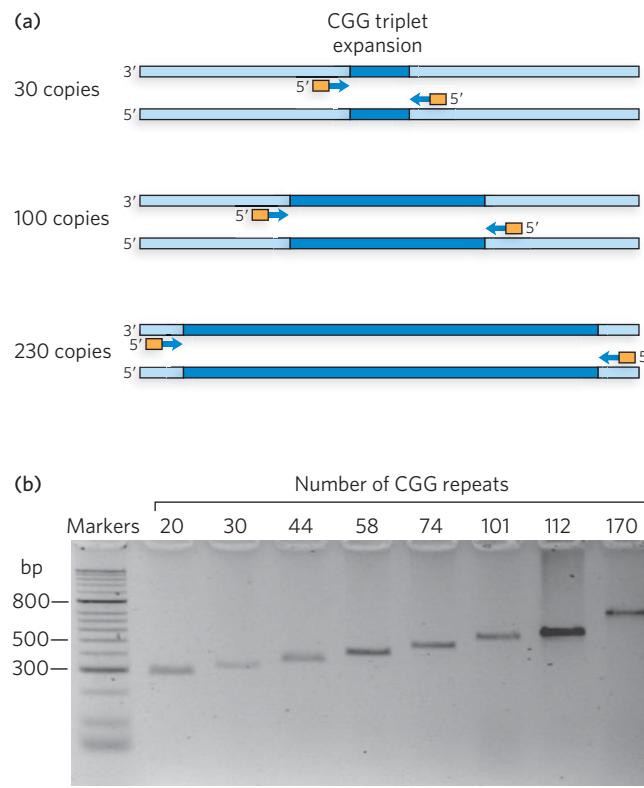


FIGURE 12-5 PCR-based genetic testing for fragile X syndrome, a triplet expansion disease. (a) The number of repeats in the fragile X locus can be determined by length analysis of PCR products. The method uses PCR primers that hybridize to unique sequences bordering the region of interest, then the PCR products are analyzed by gel electrophoresis. The size of a PCR product correlates with the size of the repeated region in the gene. (b) Size of the repeated unit in the gene for eight different patients. [Source: (b) Adapted from M. S. Khaniani et al., *Mol. Cytogenet.* 1(1):5, 2008. Courtesy of Paul Kalitsis.]

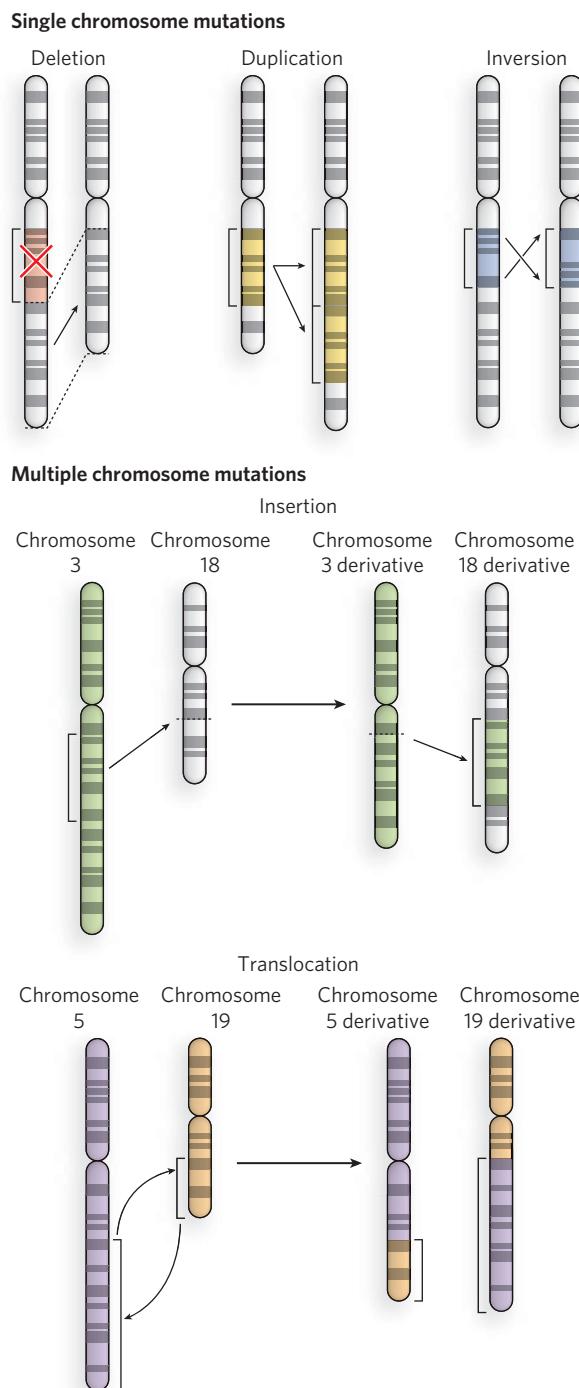


FIGURE 12-6 Large-scale mutations. Mutations that lead to alterations in a chromosome can occur internally through deletion, duplication, or inversion events (top), or they can be due to an insertion (middle) or translocation (bottom), exchanging DNA with another chromosome.

the amplification of a large tract of DNA, leading to increased gene dosage effects (i.e., increased amounts of product from the amplified gene). Chromosome **inversion mutations** result from the inversion of a

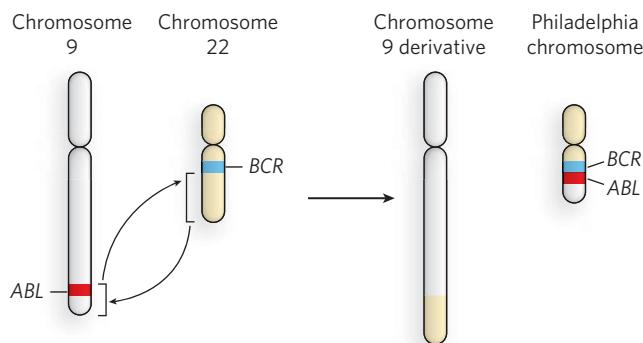


FIGURE 12-7 A chromosomal translocation resulting in a fusion gene. The chromosomal translocation that fuses the *ABL* gene with the *BCR* gene occurs when a piece of chromosome 9 (with *ABL*) is exchanged with a piece of chromosome 22. The *BCR-ABL* fusion gene causes leukemia. The small chromosome resulting from the translocation (the chromosome 22 derivative), which carries the fusion gene, is referred to as the Philadelphia chromosome, after the city where the translocation was first identified and studied.

large section of DNA in a chromosome and can have varied effects, especially on the genes at the break points. Aberrant recombination events can also occur between two different chromosomes. For example, a region of DNA from one chromosome can be transferred as an insertion to another chromosome. A chromosome **translocation mutation** occurs when two nonhomologous chromosomes exchange large regions of DNA.

Chromosomal abnormalities can result in the formation of a **fusion gene**, a hybrid of two different genes. Several types of fusion genes are associated with various forms of cancer, including lymphoma, sarcoma, and prostate cancer. An example of a chromosomal translocation that forms a carcinogenic fusion gene is the formation of *BCR-ABL*, in which one end of chromosome 9 breaks and rejoins (i.e., recombines) with part of chromosome 22, forming a very small derivative chromosome 22 (Figure 12-7). The break point in chromosome 9 occurs within the *ABL* gene, which becomes fused with the *BCR* gene in chromosome 22, forming the *BCR-ABL* fusion gene. The *ABL* protein is a cell cycle protein kinase, a tyrosine kinase that helps control the cell cycle by phosphorylating certain proteins (on specific Tyr residues) in response to cellular signals. When *ABL* becomes fused with *BCR*, the sections of the *ABL* protein that regulate the kinase activity are lost; the resulting unregulated tyrosine kinase activity leads to the uncontrolled cell division associated with lymphoblastic leukemia.

Sometimes a fusion gene retains production of the normal protein, but comes under the control of a

strong, unregulated promoter that produces too much of the protein. An example of a carcinogenic fusion gene of this type involves the *C-MYC* gene on chromosome 8, which is an oncogene. A common translocation involving this gene, found in certain types of cancer, occurs between chromosomes 8 and 14.

SECTION 12.1 SUMMARY

- A mutation is a change in DNA sequence. It can be a point mutation, affecting a single base pair, an insertion or deletion affecting more than one base pair, or a chromosomal rearrangement that affects many genes on the chromosome.
- Point mutations are classified as transitions and transversions. A transition converts a purine-pyrimidine base pair to the other purine-pyrimidine pair, or pyrimidine-purine to pyrimidine-purine. A transversion converts a purine-pyrimidine base pair to a pyrimidine-purine pair, or vice versa.
- Most mutations are produced in a two-step process. First, a nucleotide is either damaged or misincorporated during replication, then subsequent replication pairs an incorrect nucleotide with the damaged or misincorporated nucleotide.
- Most mutations are deleterious; for example, mutations in oncogenes and tumor suppressor genes that control the cell division cycle can lead to cancer.
- Insertion and deletion mutations are the addition and removal of nucleotides in a DNA sequence. Insertions and deletions that are not multiples of three nucleotides can shift a gene's reading frame, resulting in a truncated protein. Triplet expansion diseases result from three-base-pair insertion mutations caused by template slippage during replication.
- Large-scale mutations can produce abnormal chromosomes; they occur when parts of chromosomes are deleted, duplicated, inverted, or exchanged. These mutations can form fused genes, some of which cause cancer.

12.2 DNA Alterations That Lead to Mutations

DNA is subject to damage by a variety of sources. Inside the cell, reactive chemical species generated in normal metabolic processes can damage DNA. One might not immediately think of water as a DNA-damaging agent,

yet water—the universal intracellular solvent—takes its toll on DNA through spontaneous hydrolysis reactions that damage nucleotides and the DNA backbone. Also, the high degree of negative charge on DNA makes it prone to electrophilic attack by alkylating agents and by reactive oxygen species (such as hydrogen peroxide, hydroxyl radicals, and superoxide radicals), many of which are present in the normal intracellular environment. DNA is also susceptible to damage from external sources. Various types of irradiation, including x rays and UV light, can cause chemical changes in DNA. Chemicals, both natural and synthetic, can directly damage DNA or are metabolized to DNA-damaging agents. We discuss some of the most common types of DNA damage caused by these sources.

Each of these agents is capable of causing a particular type of damage to DNA. Hydrolytic damage can affect the phosphodiester backbone or a nucleotide base, whereas alkylating agents attack only bases. Irradiation can cause slightly larger lesions, such as cross-links between bases or breaks in the DNA strand. And the DNA sequence can even be altered by the very enzymes responsible for its preservation. In fact, some of the errors due to replication and recombination can be among the most difficult for the cell to detect, and sometimes escape cellular mechanisms of repair.

Spontaneous DNA Damage by Water Can Cause Point Mutations

Hydrolysis is the cleavage of a molecule by the addition of water. The reactive hydroxide ion (HO^-) is formed when a proton (H^+) dissociates from water (see Chapter 3). Hydrolysis reactions are often initiated by nucleophilic attack by a hydroxide ion; the leaving group acquires the proton, giving a net addition of water to the substrate (Figure 12-8a). At physiological pH, the concentration of hydroxide ion is low, so hydrolysis is slow—otherwise, life as we know it could not exist. However, given the large number of nucleotides in a genome, damage to nucleotide bases by even a slow rate of hydrolysis quickly adds up and becomes significant to the cell.

One result of hydrolysis is the deamination of nucleotide bases (see Figure 12-8a). **Deamination** is the removal of an amino group from a compound, and all molecules that contain an amino group are possible targets of hydrolytic attack. Three of the nucleotide bases contain an amino group—cytosine, adenine, and guanine—and thus can be deaminated by hydrolysis (Figure 12-8b). Deamination of cytosine is the most common, and it results in uracil (see Chapter 6). Adenine and guanine also undergo deamination, but at

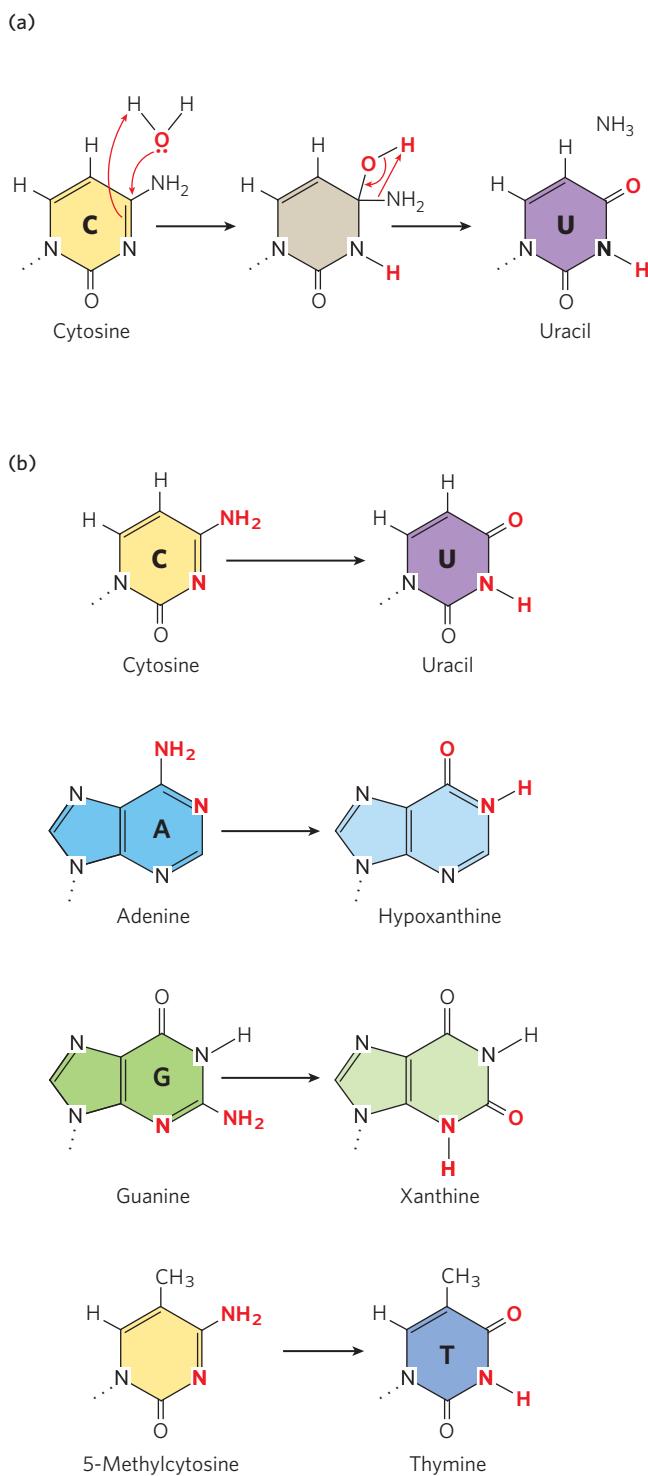


FIGURE 12-8 Deamination of nucleotide bases by spontaneous hydrolysis. In these deamination reactions, only the base is shown for each nucleotide residue. (a) A hydrolysis reaction in which water is added to cytosine, resulting in deamination to uracil and ammonia. A similar mechanism occurs in other deaminations. (b) Common deamination reactions resulting from hydrolysis of nucleotides in DNA.

only one-hundredth the rate of cytosine deamination. Deamination of adenine and guanine, unlike that of cytosine, produces bases not normally found in nucleotides: adenine yields hypoxanthine, which pairs with cytosine if left uncorrected; guanine forms xanthine, which is less deleterious because it still pairs with cytosine, through two hydrogen bonds instead of three.

Left unrepaired, a C → U change would be highly mutagenic, because uracil pairs with adenine more readily than with guanine. Hence, if the change is not repaired before replication, the cell will replicate the U to form a U=A base pair without pausing, replacing the original C≡G base pair. Repair of the uracil after replication would then insert thymine in place of uracil, completing the C≡G to T=A transition mutation. However, because DNA contains thymine rather than uracil, uracil in DNA is readily recognized as foreign and can be removed.

In eukaryotic cells, in about 5% of C residues in DNA, the cytosine is methylated to 5-methylcytosine (forming 5-methylcytidine, 5-meC), a modification that is linked to gene expression. Methylation is most common on C residues that are followed by a G, in **CpG sequences**. Methylation at CpG sequences produces 5-meCpG symmetrically on both strands of the DNA. Deamination of 5-methylcytosine produces thymine instead of uracil (see Figure 12-8b). Because thymine is a naturally occurring base in DNA, the modification is not recognized as damage and has the potential to become a transition mutation in which a G≡C base pair is changed to an A=T base pair. However, the damage does result in a mispair, from a 5-meC-G to a T-G base pair, and can be recognized by the cell's mismatch repair system (described below) before the next round of replication. Nevertheless, positions of 5-meC residues in eukaryotic DNA are associated with mutational hotspots.

Another relatively frequent hydrolytic reaction is the attack of water on the $N\text{-}\beta$ -glycosyl bond between the base and the pentose of a nucleotide residue, breaking the connection between the base and the DNA backbone. This type of hydrolytic reaction leaves an **abasic site**, a position in an intact DNA backbone that is missing the base. Hydrolysis of the $N\text{-}\beta$ -glycosyl bond occurs at a higher rate for purines—a process referred to as **depurination**—than for pyrimidines (Figure 12-9). In fact, as many as 1 in 10^5 purines (10,000 per mammalian cell) are lost from DNA every 24 hours. Depurination can lead to a mutation when the abasic site forms in single-stranded DNA, because the information in the complementary strand is not present and the DNA polymerase often inserts an incorrect nucleotide during replication to form the duplex DNA.

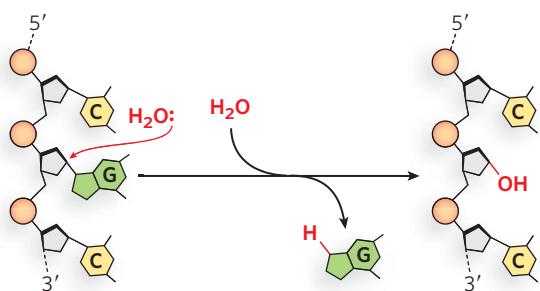


FIGURE 12-9 Depurination resulting from hydrolysis. In depurination, a purine (in this case guanine) is lost from DNA by hydrolysis of the $N\text{-}\beta\text{-glycosyl}$ bond.

Oxidative Damage and Alkylating Agents Can Create Point Mutations and Strand Breaks

The highly negatively charged DNA molecule is susceptible to electrophilic attack by alkylating agents and reactive oxygen species. Some of these DNA-damaging agents, such as cigarette smoke and industrial pollutants, come from the external environment. Often, synthetic and natural chemicals that damage DNA are not reactive per se but are transformed into DNA-damaging reagents by modification reactions. Another plentiful source of DNA-damaging reagents is the reactive oxygen species generated inside cells by aerobic metabolism (i.e., by the electron transfer chain in mitochondria) and by the detoxification system in the liver. All cells contain enzymes that convert reactive oxygen species into harmless molecules, but some of these reactive species escape the cellular cleansing systems and can damage DNA and other biomolecules.

Nitrous Acid-Induced Deamination Environmental pollutants that are metabolized to DNA-reactive forms include sodium nitrate (NaNO_3), a common food preservative, which is converted in the stomach to nitrous acid (HNO_2). Nitrosamines and nitrate salts are also converted to nitrous acid. Nitrous acid acts as a mutagen by reacting with A and C residues, altering their ability to form correct base pairs (Figure 12-10). If the modified bases are not repaired, a transition mutation will result. Bisulfite (HSO_3^-) has similar effects and is also used as a food preservative. Sodium nitrate and bisulfite do not seem to increase the risk of cancer in humans when used in this way, perhaps because, in such small amounts, they make only a minor contribution to the overall level of DNA damage.

Oxidative Damage Possibly the most important source of mutagenic alterations in DNA is oxidative damage.

The DNA of each cell in the body is subjected to thousands of damaging oxidative reactions every day. Reactive oxygen species, such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot\text{OH}^-$), and superoxide radicals ($\cdot\text{O}_2^-$), arise during irradiation or as byproducts of aerobic metabolism. Oxidative DNA damage ranges from the oxidation of base and deoxyribose moieties to the removal of bases (forming abasic sites), and it can also cause strand breaks.

The hydroxyl radical reacts with both purine and pyrimidine bases. In pyrimidines, the double bond between C-5 and C-6 is highly susceptible to attack, resulting in a variety of oxidized bases, including 5-hydroxyuracil, 5-hydroxycytosine, uracil glycol, and thymine glycol (Figure 12-11a). In purines, too, oxidation generates a spectrum of products. Among these reactions is the oxidation of guanine to 8-oxoguanine, which is extremely mutagenic. In the syn conformation (see Figure 6-16), an 8-oxoG residue base-pairs with A, and replication that occurs before 8-oxoG is repaired results in a G≡C to T=A transversion mutation (Figure 12-11b). Such G≡C to T=A transversions are among the most common mutations in human cancers.

Damage by Alkylation The addition of an alkyl group to atoms in nucleotide bases or the phosphodiester backbone is known as **alkylation**. The N-3 of adenine

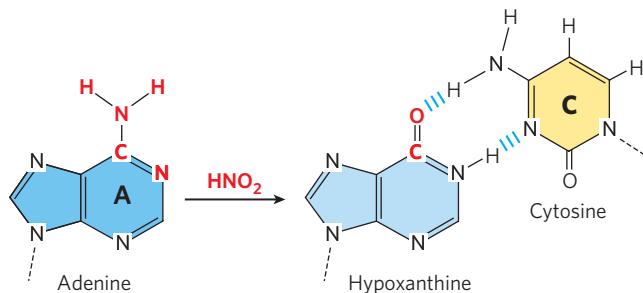
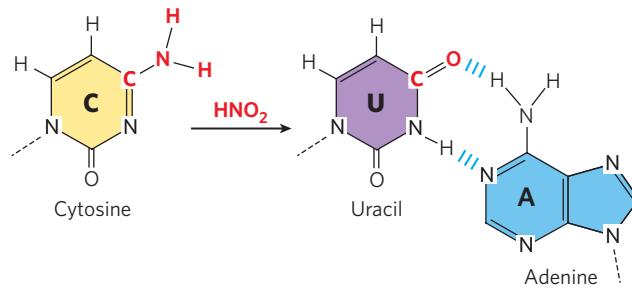


FIGURE 12-10 Deamination by nitrous acid. Nitrous acid can deaminate C and A residues, causing them to base-pair with the wrong nucleotide during replication.

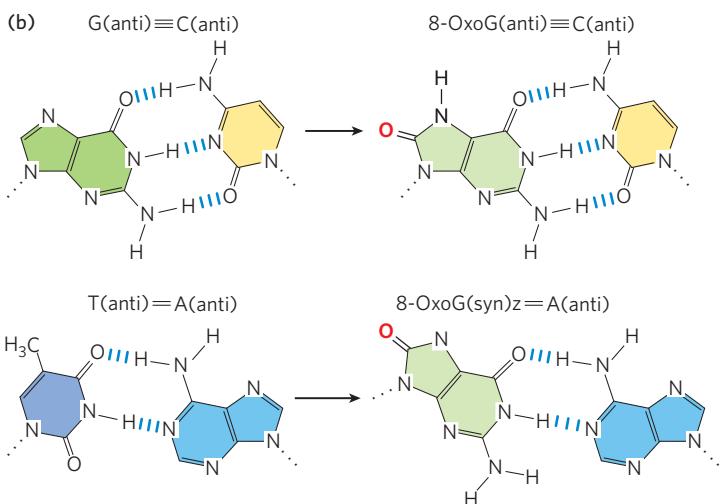
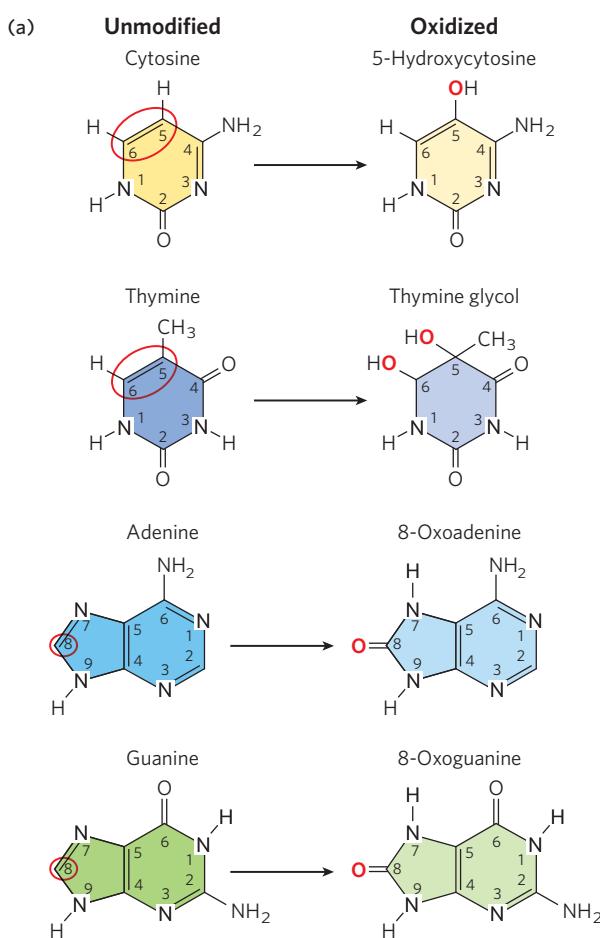


FIGURE 12-11 Oxidative damage to purine and pyrimidine bases. (a) Common positions (circled in red) of oxidative damage to nucleotide bases (left) and the products of oxidation at these positions (right). (b) Oxidation of the guanine of a G residue to 8-oxoguanine is extremely mutagenic. The 8-oxoG residue base-pairs with either A or C and thus can result in a G≡C to T=A transversion.

and O^6 of guanine are common sites of alkylation, although many other positions in all four nucleotide bases, as well as the phosphodiester backbone of DNA, can also be modified, depending on the alkylating agent (**Figure 12-12a**). Alkylation of a nucleotide base can have a range of effects on base pairing, from having no effect to completely preventing base pairing of the alkylated base with another base.

Any substance directly involved in promoting cancer is a **carcinogen**. A known carcinogen in the smoke of burning cigarettes, wood, and coal tar that reacts with DNA is benzo[*a*]pyrene. Benzo[*a*]pyrene is in a class of chemicals referred to as polycyclic aromatic hydrocarbons, which are hydroxylated in the liver as part of a detoxification process. However, hydroxylation of some polycyclic aromatic hydrocarbons results in a highly reactive epoxide. In the case of benzo[*a*]pyrene, hydroxylation forms an epoxide that reacts with purines: N^6 of adenine or N^2 of guanine (**Figure 12-12b**). Although N^2 of guanine is not a base-pairing atom, these bulky alkylated bases no longer form correct base pairs and can lead to mutations if not corrected efficiently.

Nitrogen mustard gas is an alkylating agent that was used as a weapon in World War I. Nitrogen mustards are cross-linking agents, and they react with adjacent G residues to form interbase cross-links (Figure 12-12c).

The Ames Test Identifies DNA-Damaging Chemicals

DNA-reactive compounds are referred to as **genotoxic** because they cause chemical changes in genomic DNA. Many sources of cancer in humans can be traced to DNA-damaging agents. In a seeming contradiction, not only can DNA-damaging agents cause cancer, but some

are used in chemotherapy to treat some types of cancer. Small amounts of a mutagen can do just enough damage to cause cancer. But large amounts can do more than cause mutations: they can kill the cell—they are **cytotoxic**—and can sometimes be used to kill cancer cells.

It clearly is in our interest to identify carcinogenic substances so that we can choose to avoid them and/or put them to use in treating



Bruce Ames [Source:
Courtesy of Bruce Ames.]

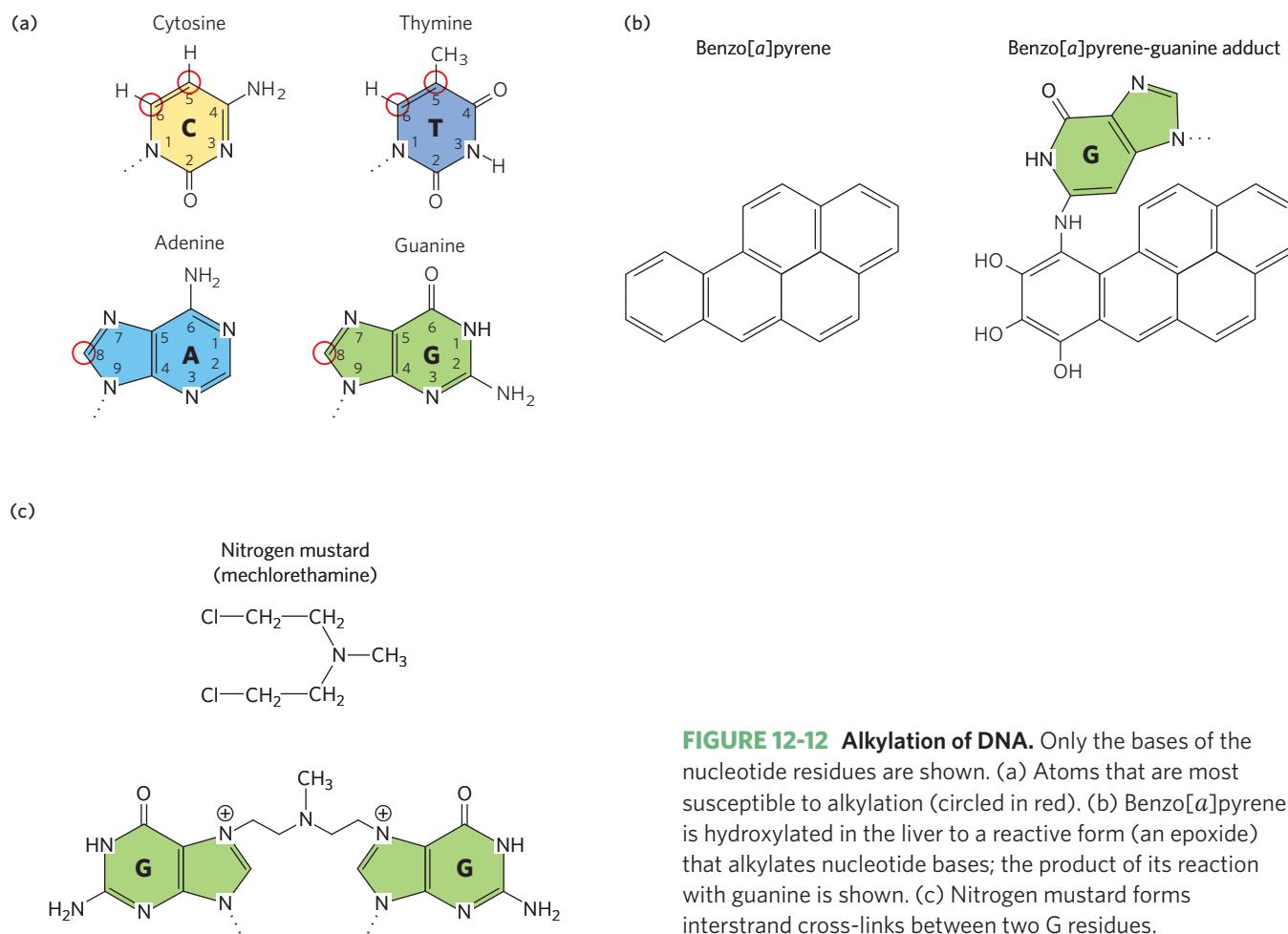


FIGURE 12-12 Alkylation of DNA. Only the bases of the nucleotide residues are shown. (a) Atoms that are most susceptible to alkylation (circled in red). (b) Benzo[a]pyrene is hydroxylated in the liver to a reactive form (an epoxide) that alkylates nucleotide bases; the product of its reaction with guanine is shown. (c) Nitrogen mustard forms interstrand cross-links between two G residues.

cancer. Many mutagens are also carcinogens. A rapid and inexpensive test for a chemical mutagen is provided by a bacterial screen invented by Bruce Ames. The **Ames test** makes use of a strain of *Salmonella typhimurium* that contains a mutation in the biosynthetic pathway for histidine and therefore requires histidine in the growth medium. These mutants are called **auxotrophs**, which are cells that have lost the capacity to synthesize various organic compounds such as amino acids. Histidine auxotrophic *S. typhimurium* cells plated on a medium lacking histidine do not survive. But a small number of the cells may acquire mutations that reverse the original mutation, and cells with a **reversion mutation** (or back mutation) of this type can synthesize histidine. These mutant cells can survive on histidine-free medium and form colonies (Figure 12-13a).

To test a potential carcinogen, the compound is absorbed on a filter-paper disk placed in the center of a plate that contains *S. typhimurium* in the growth

medium (Figure 12-13b). The chemical diffuses from the disk into the medium, creating a concentration gradient. The clear zone immediately adjacent to the disk contains the highest concentration of compound, too high to support life (Figure 12-13c–e). Beyond this lethal zone, the mutagen produces reversion mutations in some cells that enable *S. typhimurium* to form colonies. Because some compounds are mutagens only after they have been hydroxylated in the liver, the Ames test includes a step in which the compound being tested is first incubated with a liver extract. All known human carcinogens result in increased mutation in the Ames test. In fact, numerous common industrial chemicals, and even some natural products, also produce increased mutation in this test. The Ames test is therefore only the start of a process that identifies a compound as a human carcinogen. Compounds identified as mutagens in an Ames test require further testing in animals to determine whether they are likely human carcinogens.

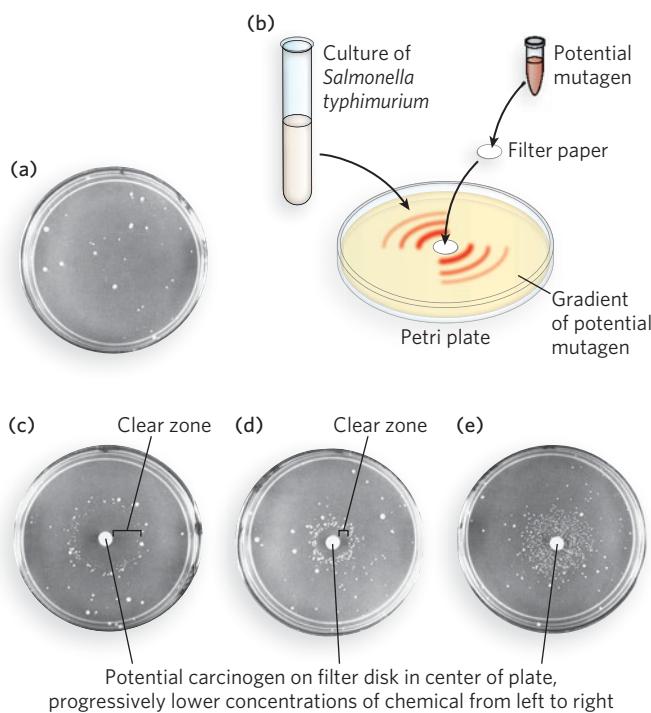


FIGURE 12-13 The Ames test for carcinogens, based on mutagenicity. The Ames test uses a strain of *Salmonella typhimurium* lacking an enzyme needed to synthesize histidine, which is plated on histidine-free medium. (a) In the absence of the chemical to be tested, a few cells develop a reversion mutation spontaneously and form colonies. (b) The chemical to be tested is soaked into a filter-paper disk, which is placed in the center of the plate, and the chemical diffuses to create a concentration gradient in the plate. (c–e) Identical nutrient plates are inoculated with an equal number of cells, but have progressively lower concentrations of the putative mutagen on the filter-paper disk. At lower concentrations, the clear zone around the disk is smaller because fewer cells are killed by the chemical. [Source: (b–e) Bruce Ames, Department of Biochemistry and Molecular Biology, University of California, Berkeley.]

DNA-Damaging Agents Are Used in Cancer Chemotherapy

DNA-reactive agents used in chemotherapy for cancer kill cells by creating broken chromosomes or stalled replication forks, either of which leads to cell death during cell division. The cytotoxic effect of DNA-damaging agents therefore requires the cell to be actively dividing. Chemotherapeutic agents are toxic to cancer cells because these cells must divide to form a tumor, but not toxic to most somatic cells that are not dividing. Nondividing or slowly dividing cells

do sustain DNA damage during chemotherapy, but the damage is often repaired before replication occurs. The adverse side effects of chemotherapeutic DNA-damaging agents (hair loss, anemia, and nausea) are due largely to their effects on the few cell types in the body that rapidly divide, such as hair follicle cells, blood cells, and the cells that line the digestive tract.

Some types of DNA-damaging agents are particularly efficient at blocking replication forks or breaking chromosomes. A common and potent chemotherapeutic agent is the cross-linking drug cisplatin. Cisplatin is an alkylating agent that forms covalent adducts with the N-7 position of the two purine residues (Figure 12-14a). The intrastrand and interstrand cross-links resulting from reaction with cisplatin can be hard to repair, and they persist until they are encountered by a replication fork, leading to replication arrest and cell death. Cisplatin is used in the treatment of bone cancer, lung cancer, certain lymphomas, ovarian cancer, and many other cancers.

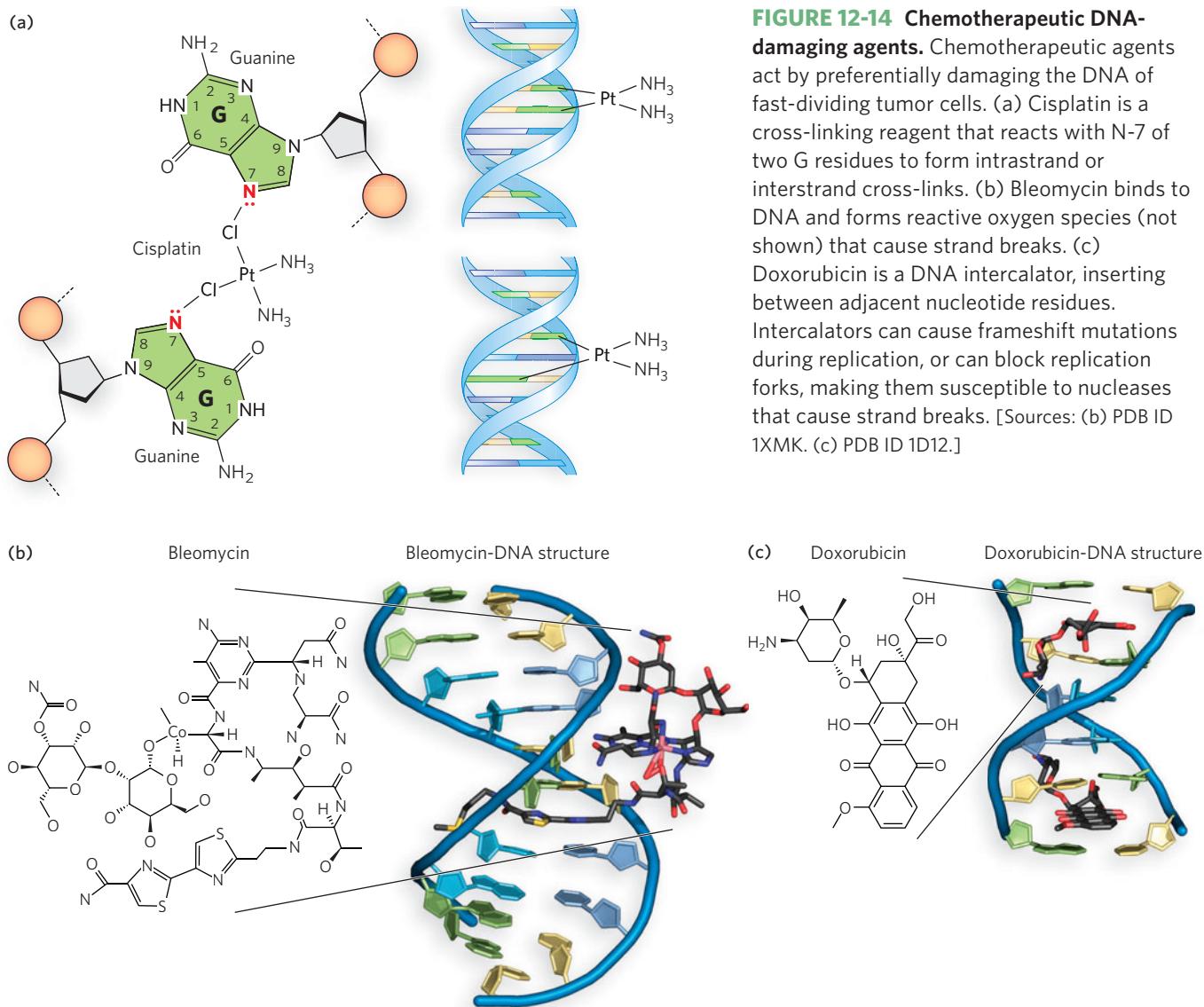
Bleomycin, a complex biomolecule isolated from a bacterium, binds an atom of iron and activates molecular oxygen to form hydroxyl radicals that damage DNA. Bleomycin also binds to DNA, and its proximity to DNA directs the hydroxyl radical-mediated damage (Figure 12-14b). Bleomycin is used to treat Hodgkin lymphoma and testicular cancer.

Anthracyclines are chemotherapeutic agents that function by intercalating into DNA, reversibly inserting between base pairs. Doxorubicin is one such agent, and intercalation leads to double-strand breaks by inhibiting the resealing step of topoisomerase II (Figure 12-14c). Doxorubicin is used to treat leukemias, Hodgkin lymphoma, and several other types of cancer, including ovarian and breast cancer and tumors of the stomach, bladder, and thyroid gland.

Chemotherapeutic DNA-damaging agents are frequently used in combination and kill cells by blocking replication or transcription, often by triggering programmed cell death. Unfortunately, the damage incurred by chemotherapy also leads to mutations in some normal cells, and people who survive the primary cancer are at increased risk of developing a secondary tumor later in life.

Solar Radiation Causes Interbase Cross-Links and Strand Breaks

Virtually all forms of life are exposed to energy-rich radiation that can cause chemical changes in DNA. We are subject to a constant field of ionizing radiation in the form of UV light (in sunlight) and cosmic rays,



which can penetrate deep into the Earth, as well as radiation emitted from radioactive elements such as radium, plutonium, uranium, radon, carbon-14, and tritium (${}^3\text{H}$). X rays used in medical and dental examinations, and in radiation therapy to treat cancer and other diseases, are other sources of exposure. UV and other ionizing radiation is estimated to be responsible for about 10% of all DNA damage caused by environmental agents.

Nucleotide bases interact very strongly with UV light (wavelengths of 200 to 400 nm), which can promote reactions that chemically change the DNA. **Pyrimidine dimers** are formed by UV-induced covalent cross-links between neighboring pyrimidines on

the same DNA strand. Pyrimidine dimers form through the condensation of two ethylene groups on adjacent pyrimidines to form a **cyclobutane ring** (Figure 12-15a, left). Adjacent T residues or adjacent C residues can react to form a **6-4 photoproduct** (see Figure 12-15a). Pyrimidine dimers cause a significant distortion in the DNA and thus can no longer base-pair with another nucleotide (Figure 12-15b). On encountering a pyrimidine dimer during replication, the DNA polymerase stalls. Repair is therefore a matter of urgency.

Gamma rays and x rays are much higher-energy radiation than UV light and generate reactive oxygen species that can break one or both strands of DNA (Figure 12-16). A break in a single strand of duplex

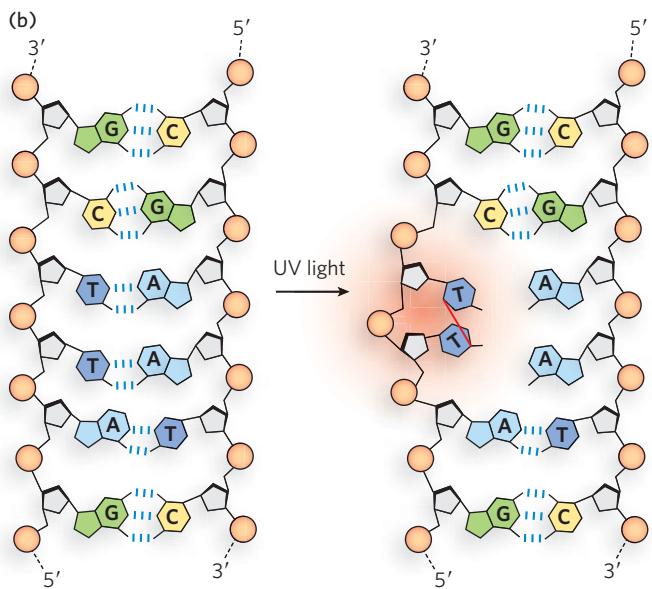
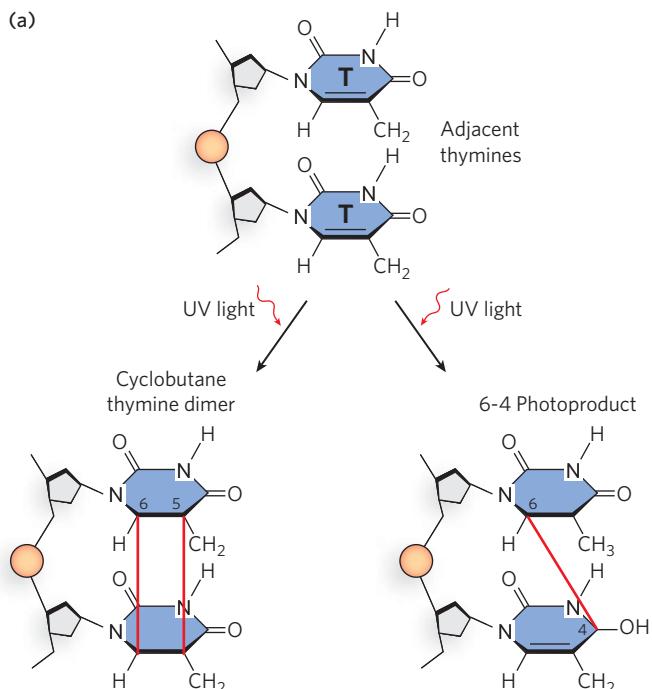
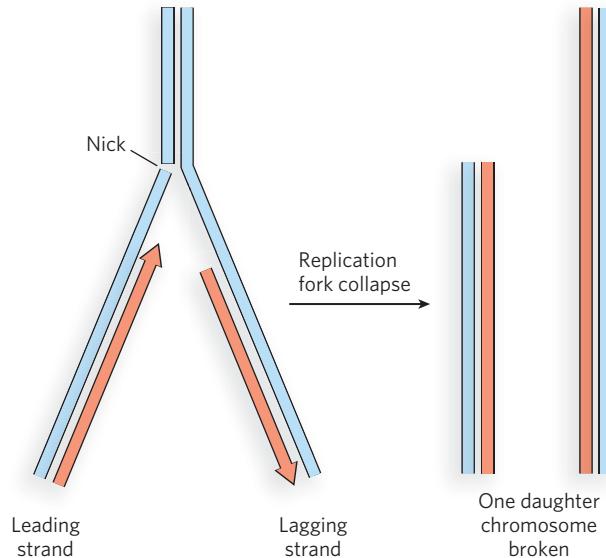


FIGURE 12-15 Pyrimidine dimers and their effect on the DNA duplex. (a) One type of reaction caused by UV light results in a cyclobutane ring, which involves atoms C-5 and C-6 of adjacent pyrimidine (in this case thymine) bases. An alternative reaction results in a 6-4 photoproduct that links atoms C-6 and C-4 of adjacent pyrimidines. (b) Formation of a pyrimidine dimer introduces a bend or kink in the DNA.

DNA is easily repaired because it can be rejoined by ligase. But when both strands are broken, the repair job is much more difficult. Rejoining of a broken chromosome is performed by either homologous

(a) A nick in one strand fragments one chromosome.



(b) Nicks in two strands fragment both daughter chromosomes.

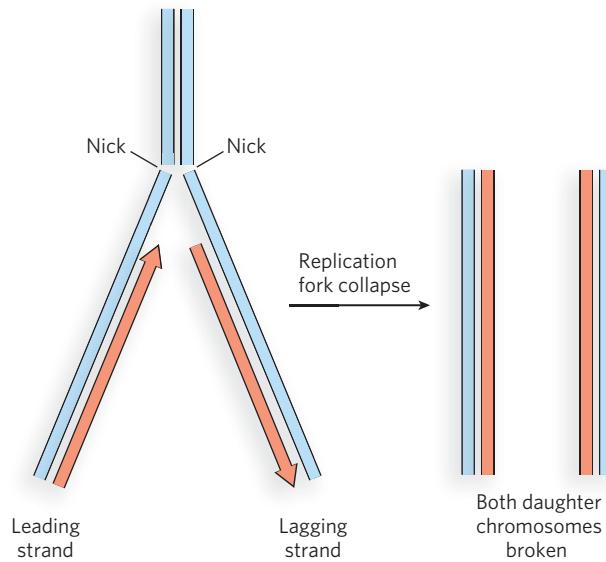


FIGURE 12-16 DNA damage caused by strand breaks during replication. (a) When a replication fork encounters a single-strand break (nick), one daughter chromosome is broken, while the other daughter chromosome remains intact. (b) When the break occurs in both strands, both daughter chromosomes are broken, and neither daughter chromosome is completed.

recombination or nonhomologous end-joining reactions, as we describe in detail in Chapter 13. These repair reactions, especially the latter, often result in mutations due to the loss of nucleotide bases.

Errant Replication and Recombination Lead to DNA Damage

Although it seems counterintuitive, many types of mutations arise from the activity of the very proteins that have evolved to maintain the integrity of genomic DNA. For example, DNA polymerases can cause damage. As we learned in Chapter 11, DNA polymerases are highly accurate and have a very low probability of making a mistake. But given the large size of genomes, even a low probability becomes a certainty. Sometimes, tautomers of nucleotide bases form correct matches with the DNA template and lead to base-pair mismatches, and sometimes the proofreading 3'→5' exonuclease misses an incorrect base and a mismatch results. Also, the polymerase can slip on repetitive sequences (template slippage), giving rise to insertion or deletion mutations. Recall, too, that not all DNA polymerases are as accurate as the primary replicase. There are rare instances in which the replication fork encounters a damaged base and low-fidelity translesion DNA polymerases are recruited that can insert an incorrect nucleotide in order to move the replication fork past the damage—allowing replication to continue, but leading to a mutation.

During cell division, homologous chromosomes are lined up in pairs so that they can be partitioned into daughter cells. The proximity of similar DNA sequences can lead to the exchange of DNA segments through homologous recombination during meiosis or mitosis. Errors in this process sometimes occur. For example, a repeated nucleotide sequence on a chromosome can lead to recombination between two regions on homologous chromosomes, or between two nonhomologous chromosomes, leading to chromosomal aberrations such as deletions, duplications, inversions, insertions, and translocations.

When chromosomal abnormalities arise during development, the anomaly is present in every cell in the body. Chromosomal abnormalities can also arise in a somatic cell of an adult, and when this happens, the abnormalities are present only in a subset of cells, such as those in a tumor formed from the mutated cell.

SECTION 12.2 SUMMARY

- Hydrolysis can deaminate nucleotide bases, altering their ability to base-pair and leading to a mismatch during replication. Deamination of cytosine to uracil is the most common type and if not repaired can lead to a C≡G to T=A transition mutation. Hydrolysis can also sever the glycosyl bond between the pentose and the base, leaving an abasic site.

- Nitrous acid, the metabolic product of a food preservative, can induce the deamination of A or C residues, resulting in a transition mutation.
- Oxidative damage is caused by reactive oxygen species that react with nucleotides at many different positions in the molecule. Oxidation can affect base pairing or cause DNA strand breaks.
- Alkylating agents attack DNA at any of several electron-rich atoms, adding bulky chemical groups to the base or the phosphodiester backbone. Alkylation can alter the base pairing of the nucleotide.
- DNA-damaging agents can lead to mutations at low concentrations and can kill the cell at high concentrations. The Ames test determines whether a compound is mutagenic in bacteria, thus identifying the compound as a potential carcinogen. Chemotherapy for cancer patients often uses DNA-damaging agents at high concentrations, thereby killing cancer cells that are replicating faster than most normal cells.
- UV light from the sun can form pyrimidine dimers that stall DNA polymerase during replication. X rays and gamma rays cause single-strand and double-strand DNA breaks.
- DNA damage can also result from errant replication, which can produce point mutations or small insertions and deletions. Errant recombination can result in large-scale chromosomal abnormalities.

12.3 Mechanisms of DNA Repair

The integrity of the information in genomic DNA is essential to cell viability. As we have seen, DNA mutations can result from a variety of causes, including replication mistakes, hydrolysis, chemical damage, and irradiation. Because the chemistry of DNA damage is diverse and complex, an elaborate set of DNA repair mechanisms is required to detect and fix damaged nucleotides before they become permanent mutations. The cellular response to DNA damage includes a wide range of enzyme systems that catalyze some of the most interesting chemical transformations in DNA metabolism. Mistakes made during replication and recombination do not involve damaged bases, yet there are enzyme systems that detect and repair these errors before they become mutations. We consider here the major repair systems in bacteria and eukaryotic cells.

Mismatch Repair Fixes Misplaced-Nucleotide Replication Errors

DNA polymerase III of *E. coli* contains a proofreading activity that confers a very low mutation rate of one error in 10^6 to 10^8 nucleotides. However, the observed accuracy of replication in *E. coli* is even higher: one error in 10^9 to 10^{10} polymerization events. The additional accuracy derives from an efficient repair process that recognizes and corrects mismatches that escape Pol III.

Mismatched nucleotides incorporated by the replication apparatus are corrected by the **mismatch repair (MMR)** system, which is conserved in all cell types from bacteria to humans. The mismatches are nearly always corrected to reflect the information in the parent strand. Given that neither strand contains a *damaged* base, the cell must discriminate between

the parental template and the newly synthesized strand, and replace only the nucleotide base in the new strand. Besides mismatches, the *E. coli* MMR system can also recognize small loops of up to 4 bp of unpaired nucleotides, formed by template slippage during replication or by recombination. Left unrepaired, these small loops of extra DNA result in deletions or insertions. Loops of more than 4 bp are not recognized by the MMR system, and there is no other mechanism to recognize these mistakes. Thus, larger indels are simply not corrected. The MMR system of *E. coli* includes at least 12 protein components that function in either the strand discrimination reaction or the repair process itself (Table 12-2).

The mechanism by which the newly synthesized strand is identified and targeted for correction has not been worked out for most bacteria or eukaryotes, but it is well understood for *E. coli* and some closely

Table 12-2 Proteins of Mismatch Repair in *E. coli* and Eukaryotes

<i>E. coli</i>		
Protein	Function	
MutS (as dimer MutS ₂)	Recognizes single base mismatches	
MutL (as dimer MuL ₂)	Binds MutS and coordinates repair	
MutH	Cleaves hemimethylated DNA	
Helicase II	Unwinds DNA	
SSB	Binds unwound ssDNA	
Exonuclease I	3'→5' exonuclease	
Exonuclease X	3'→5' exonuclease	
RecJ	5'→3' exonuclease	
Exonuclease VII	5'→3' exonuclease	
Pol III holoenzyme	Fills in gap	
DNA ligase	Seals DNA	
Eukaryotes		
Protein		
Yeast	Human	Function
MSH2/MSH6	MSH2/MSH6	Repairs single base mismatches, small loops
MSH2/MSH3	MSH2/MSH3	Repairs larger loops; functions with MSH2/MSH6 and MSH2/MSH3
MLH1/PMS1	MLH1/PMS2	Functions with MSH2/MSH6 and MSH2/MSH3
MLH1/MLH2	MLH1/MLH2	Unknown
MLH1/MLH3	MLH1/MLH3	Unknown
?		Helicase
RPA		ssDNA-binding protein
Exonuclease I		5'→3' exonuclease
RFC, PCNA, polymerase		Fill in gap
DNA ligase		Seals DNA

related bacterial species. In these bacteria, strand discrimination is based on the action of **Dam methylase**, the enzyme that methylates DNA at the N^6 position of adenine within a 5'-GATC-3' sequence (see Chapter 11). The GATC sequence on both strands of the parental DNA is methylated, but during replication, the newly synthesized strand is unmethylated for a short period (a few seconds or minutes) immediately after passage of the replication fork. During this interval of hemimethylation, proteins in the MMR complex can identify and distinguish the unmethylated new strand from the methylated parent strand. Replication mismatches in the vicinity of a hemimethylated GATC sequence are then repaired according to the information in the methylated (parental) template strand (Figure 12-17).

Studies of the MMR proteins have outlined the process by which mismatch repair occurs (see How We Know). The mismatched base pair creates a distortion in DNA that is recognized by the MutS protein. This enables MutS to bind the MutL protein, and the MutS-MutL complex, using ATP, scans bidirectionally along the DNA, forming a DNA loop. The crystal structure of MutS bound to a mismatch shows that MutS forms a homodimer (MutS_2) that binds DNA at the dimer interface (Figure 12-18). The MutS dimer also contains a hole large enough to surround the DNA, but whether MutS does encircle DNA during scanning is unknown. On arriving at a hemimethylated GATC site, the complex recruits and activates MutH, a site-specific endonuclease that cleaves unmethylated GATC sites. After strand cleavage, MutS-MutL recruits helicase II (also called UvrD), which unwinds DNA in the direction of the mismatch. During the unwinding, an exonuclease degrades the displaced DNA strand. Different exonucleases are used, depending on whether the enzyme needs to travel in the 5'→3' or 3'→5' direction along the DNA (Figure 12-19). Unwinding and DNA degradation stop shortly after the mismatch is excised, leaving a single-strand gap that extends from the mismatch to the original incision at the GATC site. The single-strand gap is coated with SSB, filled in by Pol III holoenzyme, and sealed by ligase.

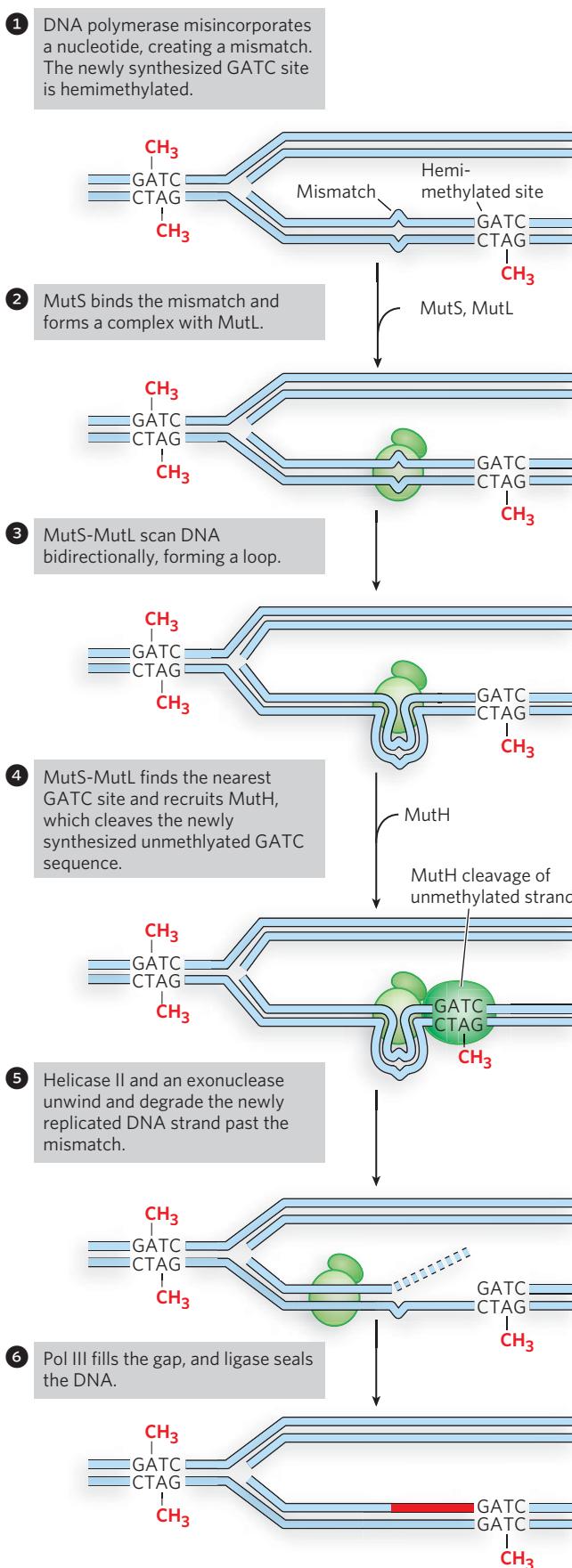


FIGURE 12-17 Mismatch repair of a nucleotide misincorporated by DNA polymerase. In *E. coli*, $\text{MutS}_2\text{MutL}_2$ binds a mismatch and scans the DNA for a GATC site. MutH nicks the DNA at the nearest unmethylated GATC site, facilitating repair of the mismatch on the newly synthesized strand by excision, filling in of the gap, and ligation.

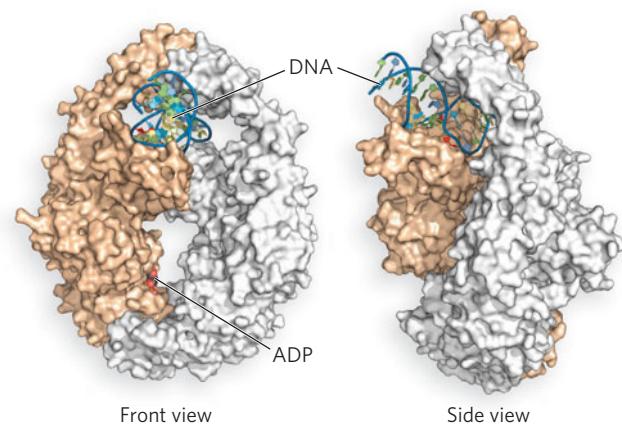


FIGURE 12-18 The structure of MutS bound to a mismatch in DNA. Front and side views of the MutS homodimer (identical subunits, orange and white) enveloping and kinking DNA that contains a mismatched base pair (red). The nucleotide is ADP; ATP is essential for MutS function. [Source: PDB ID 1E3M.]

Mismatch repair is a particularly costly process for *E. coli* in terms of energy expended. The distance between the mismatch and the GATC cleavage site can be more than 1,000 bp. The degradation and replacement

of a strand segment of this length requires an enormous investment in dNTPs to repair a single mismatched base. But this energy consumption is affordable relative to the cost of incurring a mutation. The conservation of such a high-cost repair system in all cells is a good illustration of the importance to all cells of maintaining the sequence of their genomic DNA.

Eukaryotic cells have several proteins that are structurally and functionally analogous to bacterial MutS and MutL (see Table 12-2). The MutS homologs work in heterodimers, and each has a specialized function. For instance, in yeast, heterodimers of MSH2 and MSH6 generally bind to single base-pair mismatches, and bind less well to slightly longer mispaired loops. The eukaryotic homolog of bacterial MutL is also a heterodimer that binds to the MutS homologs.

Eukaryotic cells lack homologs to bacterial MutH and Dam methylase, and they do not use methylation to distinguish between new and old strands. It is thought that, in eukaryotes, strand discrimination relies on the fact that only the newly replicated strand has nicks, and we know that these nicks are frequent on the lagging strand, given the 100 to 200 bp size of Okazaki fragments in eukaryotic cells. The nick on a newly synthesized DNA strand can be used as a starting

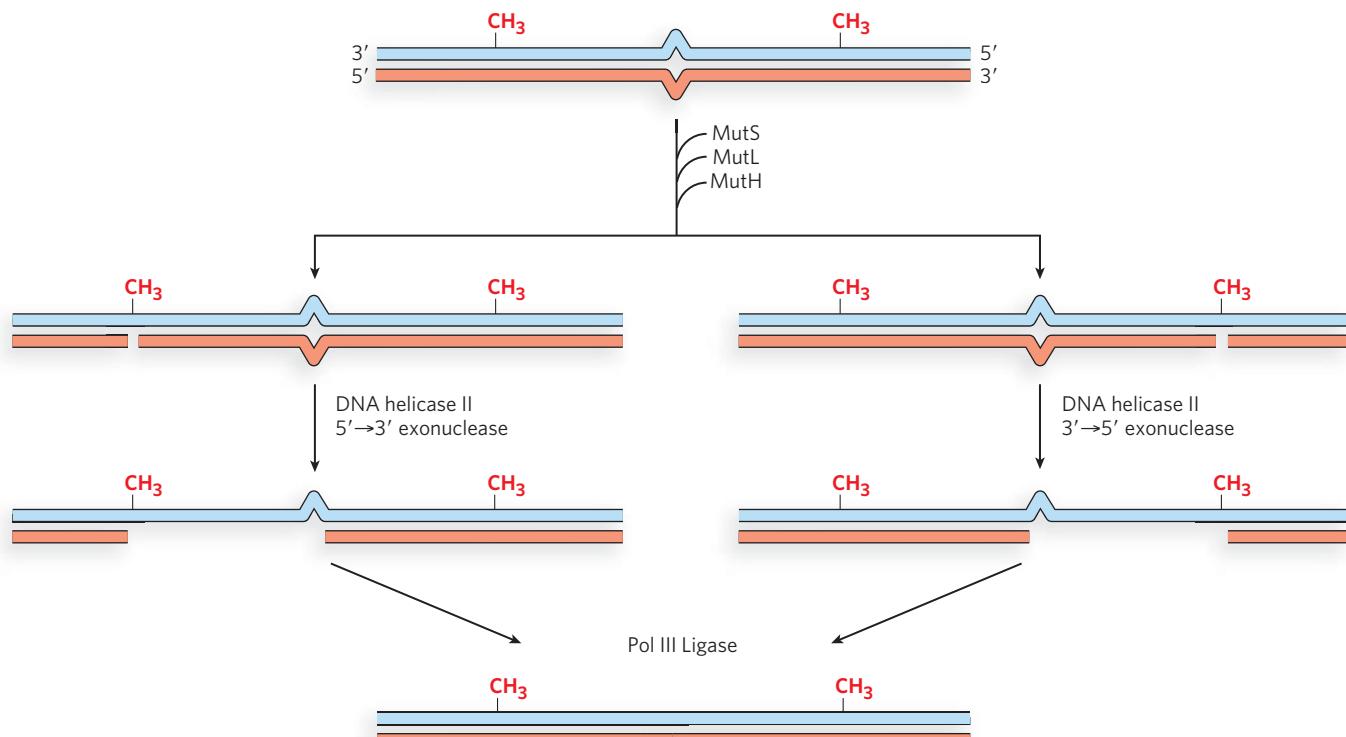


FIGURE 12-19 Multiple exonucleases involved in methyl-directed MMR. When cleavage at a hemimethylated GATC is on the 3' side of the mismatch, a 5'→3' exonuclease is

recruited, either RecJ or exonuclease VII. When cleavage is on the 5' side of the mismatch, a 3'→5' exonuclease is required, either exonuclease I or exonuclease X.

HIGHLIGHT 12-1 MEDICINE

Mismatch Repair and Colon Cancer

Most cancer cells have mutations in genes that regulate cell division (oncogenes and tumor suppressor genes). However, no single mutation is responsible for the progression from a normal cell to a malignant tumor. This progression requires an accumulation of mutations, sometimes over several decades, and is fairly well understood in the case of colon cancer (Figure 1).

Discovery of the link between MMR and hereditary nonpolyposis colorectal cancer (HNPCC) was made by the laboratories of Richard Kolodner and Bert Vogelstein, where MMR gene mutations were identified in HNPCC cells. The inherited entity is a



Richard Kolodner

[Source: Courtesy of Richard Kolodner.]

loss of function in one allele, usually of the gene encoding MLH1 or MSH2. These genes are essential to mismatch repair. Mutation of the second allele leads

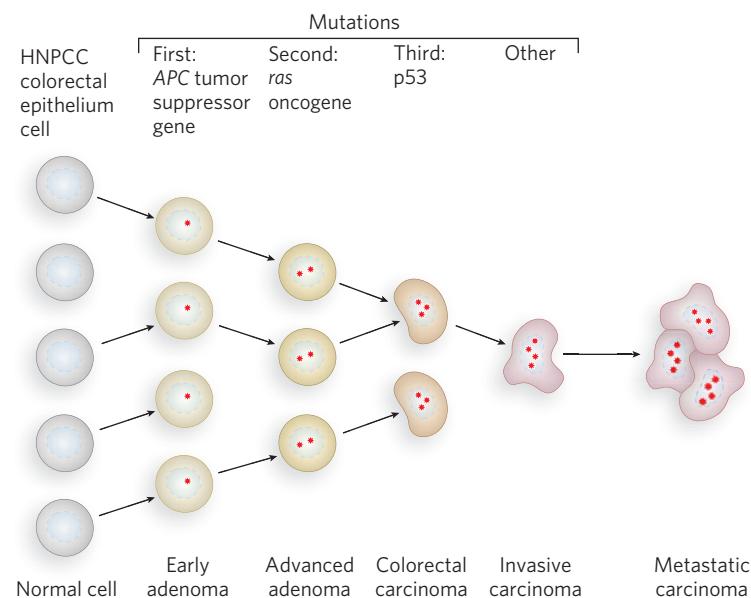


FIGURE 1 The development of colorectal cancer has several recognizable stages, each associated with a mutation. If mismatch repair becomes nonfunctional (through mutation), new mutations accrue quickly.

point to excise the DNA strand past the mismatch, and the single-strand gap is then filled in by a polymerase, much as in bacterial mismatch repair. Although this may explain how eukaryotes use nicks to distinguish new from old DNA on the lagging strand, what about the new DNA of the leading strand? The origin of strand nicks in the newly synthesized leading strand is not yet clear, but recent studies show that unlike bacterial MutL, the human MutL homolog has an endonuclease activity that depends on the PCNA clamp, suggesting that strand nicking is coordinated with DNA replication. Because PCNA has distinct “front” and “back”

sides, the orientation of a PCNA clamp used during replication may direct the endonuclease to the newly synthesized strand (see Chapter 11).

Mutations in the genes that encode mismatch repair proteins result in the accumulation of mutations throughout the human genome, because misinsertions and short indels can no longer be repaired by the MMR system. Indeed, mutations in MMR genes result in some of the most common inherited cancer-susceptibility syndromes, such as hereditary nonpolyposis colorectal cancer (Highlight 12-1). Approximately 15% of all colon cancers are of this type.



Bert Vogelstein [Source: Courtesy of Rich Riggis. Thanks to the Johns Hopkins Medical Institutions.]



Thomas Kunkel [Source: Courtesy of Thomas Kunkel.]

to the rapid accumulation of multiple new mutations that produce a malignant cell. HNPCC mutant cells have an increased frequency of small insertions and deletions in microsatellite repeats—1 to 6 bp sequences that are repeated 10 to 100 times. This is referred to as microsatellite instability.

The exact number of microsatellite repeats varies from one person to the next, but in one individual, all cells normally contain the identical number of repeats. However, in a person with HNPCC, cells contain different numbers of microsatellite repeats. Independent studies in the laboratories of Tom Kunkel and Paul Modrich showed that extracts of cells displaying microsatellite instability were defective in mismatch repair.

A test of microsatellite length is a simple indication of whether an individual has a mutation in the MMR genes of a tumor (Figure 2). PCR primers are

used to amplify specific regions containing microsatellite sequences in the genome. The tumor cell contains some microsatellite DNAs that are longer or shorter than those of a normal cell from the same individual, thus indicating a defective MMR gene. The person was born with two good alleles, but both copies for MLH1 or MSH2 became inactive during his or her lifetime. Microsatellite instability and defects in mismatch repair have now been correlated with several types of cancer other than colon cancer, including ovarian, stomach, cervical, breast, skin, lung, prostate, and bladder cancers.

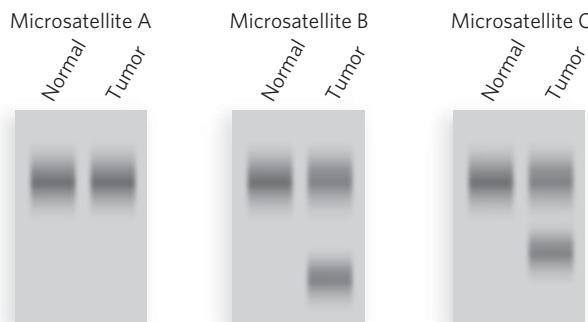


FIGURE 2 PCR primers are designed to amplify genomic DNA of three different microsatellite repeats in normal cells and tumor cells from the same individual. In this illustration of a possible result, two of the three microsatellite repeats tested have different sizes in the tumor cells—evidence that the tumor originated from a mutation in a mismatch repair gene.

Direct Repair Corrects a Damaged Nucleotide Base in One Step

Some types of DNA damage that would normally lead to a base substitution or a one-nucleotide deletion are repaired directly, without removing a base or a nucleotide. The best-characterized example of direct repair is the **photoreactivation** of cyclobutane pyrimidine dimers, first recognized in the late 1940s, before the discovery of DNA structure (see How We Know). Scientists noticed that bacterial cells and bacteriophage recovered from UV radiation damage more efficiently when exposed to

sunlight. Genetic study of this photoreactivation attributed the repair to a single gene. The gene product is an enzyme referred to as **DNA photolyase**. Photolyase uses the energy derived from absorbed visible light to reverse the damage of UV light (Figure 12-20). The energy absorbed from visible light by a first chromophore in the enzyme results in electron transfer to a second chromophore, FADH^- , to form the free radical FADH^\bullet . FADH^\bullet donates its electron to the pyrimidine dimer, reversing the cross-links and transferring the electron back to the photolyase to regenerate monomeric pyrimidines and FADH^- . Photolyases are present in almost all

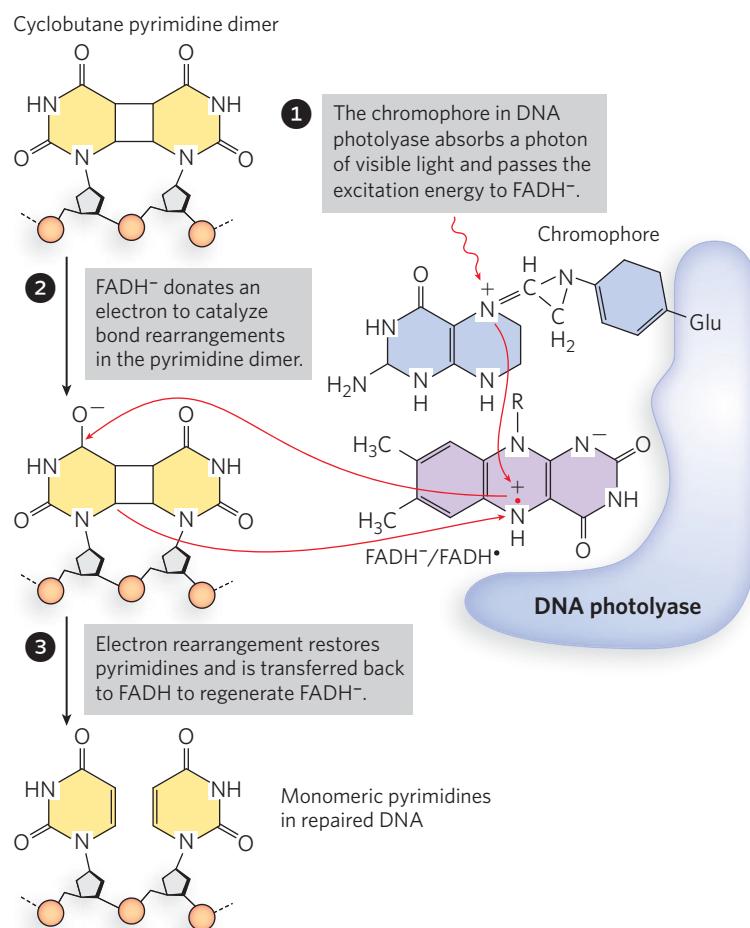


FIGURE 12-20 Photorepair of a pyrimidine dimer. *E. coli* photolyase has two chromophores (light-absorbing groups) that work in sequence to use the energy of light to repair a pyrimidine dimer.

cells—bacterial, archaeal, and eukaryotic—yet, for some reason, are not present in the cells of placental mammals (including humans).

More examples of direct repair can be seen in the repair of oxidized nucleotides. The modified base O^6 -methylguanine is a common and highly mutagenic lesion that results from alkylation (in this case, methylation) of O^6 of a G residue. It tends to pair with thymine rather than cytosine during replication, resulting in $\text{G}=\text{C}$ to $\text{A}=\text{T}$ (via $O^6\text{-meG-T}$) transition mutations (Figure 12-21a). Direct repair of O^6 -methylguanine is performed by O^6 -methylguanine-DNA methyltransferase, an enzyme that catalyzes transfer of the methyl group of O^6 -methylguanine to one of its own Cys residues (Figure 12-21b). The methyl group transfer leads to irreversible inactivation of the methyltransferase and targets it for degradation (an unusual property for an enzyme). Consumption of an entire protein to correct a single damaged base is another vivid illustration of the

priority given to maintaining the integrity of cellular DNA. Direct repair is also used to dealkylate other alkylated nucleotides.

Base Excision Repairs Subtle Alterations in Nucleotide Bases

The most prevalent means that cells use to repair damaged DNA is excision repair, of which there are two types: base excision and nucleotide excision repair. **Base excision repair (BER)** functions at the level of a single damaged nucleotide that distorts DNA very little. It is also the main pathway for the repair of single-strand DNA breaks that lack a ligatable junction and therefore require “cleaning” of the 3' or 5' terminus for ligation.

In bacterial BER, recognition of the damaged base is performed by a **DNA glycosylase**, which cleaves the nucleotide base from the pentose by hydrolyzing

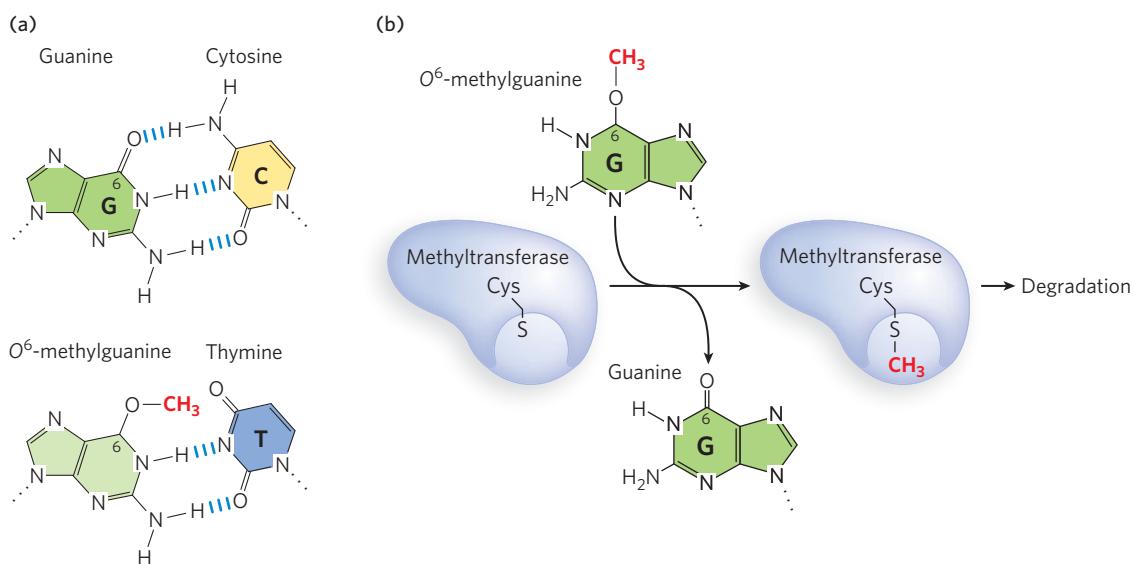


FIGURE 12-21 Direct repair of methylated nucleotide bases. (a) When the G residue of a normal G≡C base pair (top) is methylated on O⁶, the resulting O⁶-methylguanine base-pairs with thymine (bottom), and thus is highly mutagenic. (b) O⁶-Methylguanine-DNA methyltransferase transfers the methyl group from the O⁶-methylguanine onto one of its own Cys residues, and is thereby targeted for degradation.

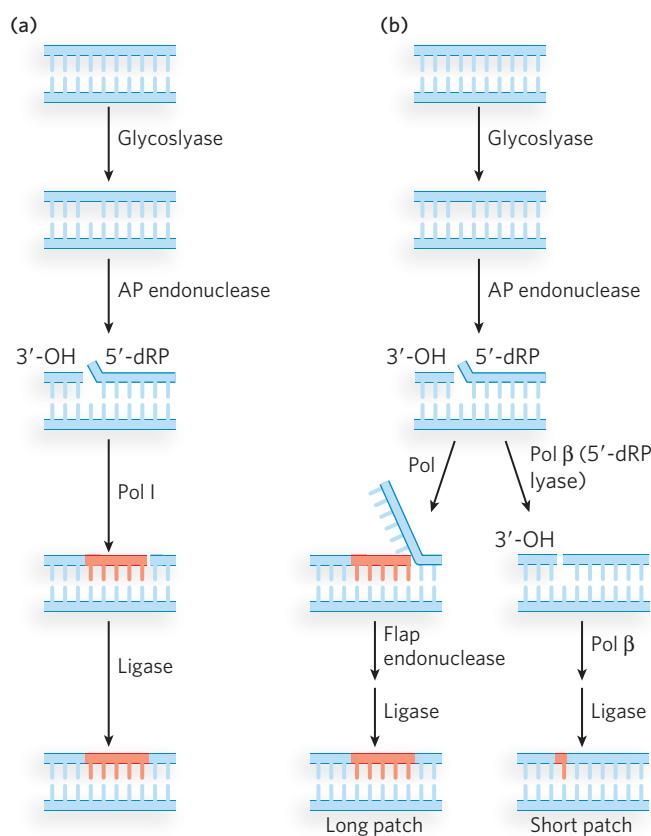


FIGURE 12-22 Base excision repair. (a) In bacteria, a glycosylase excises a damaged nucleotide base, then an AP endonuclease nicks the backbone at the abasic site. Nick translation by Pol I excises the 5' deoxyribose phosphate (5'-dRP) and some dNMPs, and synthesizes a new strand. Ligase seals the gap. (b) Eukaryotic BER, after the first two steps (similar to those in bacteria), can take either of two paths. In long patch repair, a DNA polymerase extends the DNA strand from the 3' terminus, displacing the 5' single-stranded DNA; this is followed by cleavage by a flap endonuclease and ligation. In short patch repair, only one nucleotide is inserted (by Pol β) prior to ligation.

the N-β-glycosyl bond, leaving an apurinic or apyrimidinic site (AP site). Insertion of the correct nucleotide base does not occur by re-forming the glycosyl bond with a new, correct base. Instead, the single-stranded DNA is cleaved at the abasic site by **AP endonuclease**, creating a nick with a 3' hydroxyl and a 5' deoxyribose phosphate. In *E. coli*, a segment of DNA is removed by the nick translation activity of Pol I, and DNA ligase seals the remaining nick (Figure 12-22a).

Eukaryotic BER proceeds by either of two paths; in each case, the first two steps are the same as in bacteria (Figure 12-22b). One eukaryotic BER mechanism,

similar to bacterial BER, is often referred to as long patch repair because up to 10 nucleotides are replaced. Eukaryotic DNA polymerases lack 5'→3' exonuclease activity, and therefore a special “flap endonuclease” is recruited to remove the displaced 5' terminus. The second eukaryotic BER mechanism is used the most; it replaces only the damaged nucleotide base, and thus is sometimes referred to as short patch repair. The one-nucleotide fill-in reaction is performed by Pol β , which also removes the 5' deoxyribose phosphate, leaving a 5' phosphate for ligation.

Most damaged bases repaired by the BER system remain base-paired in the helix and stack with adjacent bases. This brings up the question of how a damaged base that is buried in the DNA helix can be identified by an enzyme for repair. The crystal structure of a uracil DNA glycosylase (discussed below) reveals a fascinating recognition process in which the enzyme scans the minor groove of the helix, and damaged-base recognition is performed by kinking the DNA and “flipping” the damaged base completely out of the helix and into the enzyme’s active site (Figure 12-23a). An experiment demonstrating uracil DNA glycosylase activity is shown in Figure 12-23b. The substrate for this reaction is a synthetic 23-mer duplex in which one strand has an internal dU residue and a 5'-terminal ^{32}P label.

The uracil DNA glycosylase removes the uracil, forming an abasic site in the [^{32}P]DNA strand. Treatment with an AP endonuclease then results in cleavage of the ^{32}P -labeled strand. Analysis in a DNA sequencing gel, which separates the strands, reveals the smaller, cleaved [^{32}P]DNA strand.

All cells contain several different glycosylases that recognize different types of damaged bases (Table 12-3). There are two main types of DNA glycosylases. One type is highly specific to a particular damaged base; the other type recognizes oxidative damage, and the substrate spectrum is more diverse. As discussed earlier, spontaneous deamination of C residues to U residues in DNA is fairly frequent, and chromosomal DNA is in need of constant repair of uracil bases. A uracil DNA glycosylase is found in most cells, and it specifically removes uracil bases from DNA. This glycosylase acts only on DNA; it does not remove uracil from RNA. As may be expected, *E. coli* strains with mutations in this enzyme have a high rate of G≡C to A=T mutations.

Most bacteria have just one uracil DNA glycosylase (UDG), whereas humans and other mammals have several types (see Table 12-3), with different specificities for removing U residues. Specific UDGs remove uracils incorporated during replication or formed by deamination of cytosine in double-stranded DNA or

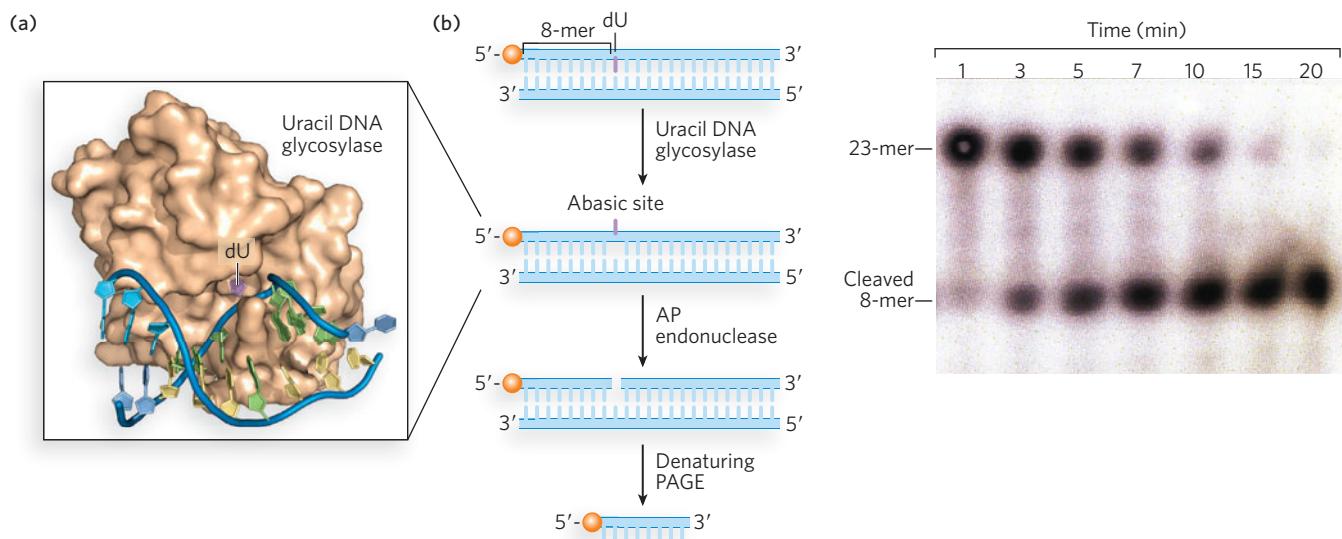


FIGURE 12-23 Uracil DNA glycosylase. (a) Human uracil DNA glycosylase (orange) bound to its DNA substrate. The uracil (purple) is flipped out of the DNA duplex and fits into the enzyme active site. (b) In this experiment, the DNA duplex is treated with *E. coli* uracil DNA glycosylase for different time intervals to produce the abasic site, then with

AP endonuclease to cleave the phosphodiester backbone. Electrophoresis in a denaturing polyacrylamide gel (right) shows results for the various time periods. [Sources: (a) PDB ID 4SKN. (b) Courtesy of Roxana Georgescu, laboratory of Mike O’Donnell, Rockefeller University.]

Table 12-3 DNA Glycosylases in Bacteria and Mammals

Lesion	Glycosylase	
	Bacteria	Mammals
Uracil	Uracil DNA glycosylase	UNG1, UNG2, MBD4, SMUG1, TDG
3-Methyladenine	AlkA	MYH
A base-paired to 8-oxoG	8-OxoguanineDNA glycosylase	8-OxoguanineDNA glycosylase
Oxidized bases	Endoglycosylase III Endoglycosylase VIII MutM Tag	NTH1 OGG1 NEIL1 NEIL2

single-stranded DNA, or formed in DNA during transcription. There is also a human DNA glycosylase that removes T residues generated by the deamination of 5-meC. Mismatch repair can also recognize T-G and U-G mismatches, and corrects them with different levels of efficiency, depending on the sequence context.

A wide variety of damaged bases can be removed by other DNA glycosylases that have evolved to recognize lesions such as formamidopyrimidine and 8-oxoguanine (both arising from purine oxidation), hypoxanthine (arising from adenine deamination), alkylated bases including 3-methyladenine and 7-methylguanine, and even some pyrimidine dimers. The BER pathway can also repair the thousands of abasic sites that arise from spontaneous hydrolysis, as well as breaks in single-stranded DNA that require processing at the 3' or 5' terminus before ligation.

Nucleotide Excision Repair Removes Bulky Damaged Bases

Nucleotide excision repair (NER) targets large, bulky lesions and removes DNA on either side of them. In contrast to base excision repair, NER does not require specific recognition of a damaged nucleotide and thus it can remove DNA lesions, even those caused by chemicals that did not exist in the environment until recently. It is the predominant repair pathway for removing pyrimidine dimers, 6-4 photoproducts, and several other bulky base adducts, including benzo[*a*]pyrene-guanine, which is formed on exposure to cigarette smoke (see Figure 12-12b). The nucleolytic activity of the NER system is novel in the sense that two incisions are made in one strand of DNA, excising the lesion; this unique enzymatic activity is called an **excinuclease**.

In *E. coli*, the NER pathway makes use of four *uvr* gene products—UvrA through UvrD—as well as several other factors (Table 12-4; Figure 12-24). First, a UvrA₂UvrB complex scans the DNA for damage. On encountering a bulky damaged base, the strands become separated to form a single-stranded DNA bubble containing the lesion and UvrA dissociates, leaving UvrB tightly bound to the damaged site. UvrB then recruits the UvrC exonuclease to make incisions in the DNA

Table 12-4 Proteins Involved in Nucleotide Excision Repair

Protein	Function
Bacteria	
UvrA	Recognizes lesion
UvrB	Unwinds DNA
UvrC	Exonuclease
UvrD	Helicase
Pol I	Fills in gap
DNA ligase	Seals DNA
Eukaryotes	
XPC	Recognizes lesion
RNA polymerase	Recognizes lesion: TCR
XPA	Verifies lesion
XPB	Unwinds DNA (TFIID subunit)
XPD	Unwinds DNA (TFIID subunit)
XPF	5' exonuclease
XPG	3' exonuclease
RPA	Stabilizes bubble
Pol δ or ε, RFC, PCNA	Fill in gap
Ligase I or IV	Seals DNA

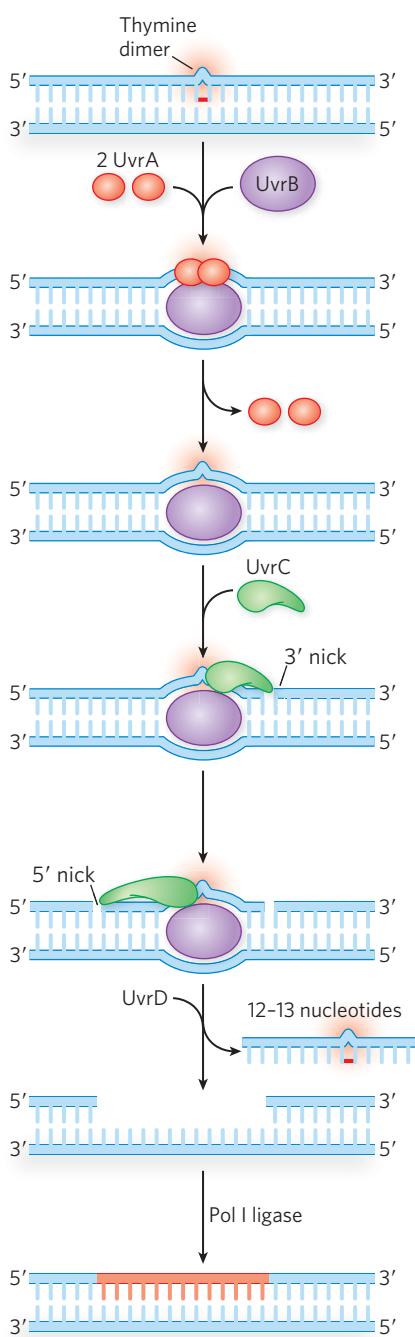


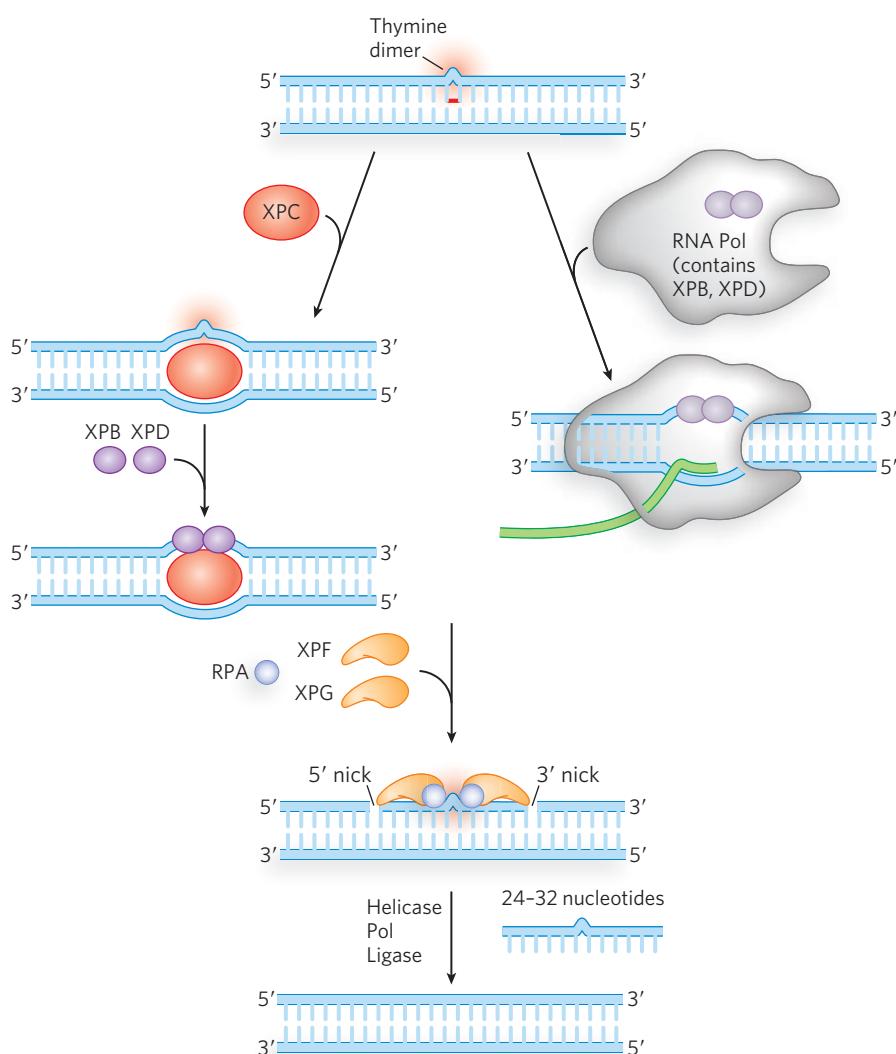
FIGURE 12-24 Nucleotide excision repair in *E. coli*. The NER pathway uses several proteins, including UvrA (red), UvrB (purple), and UvrC (green), that recognize the lesion and make incisions on either side, allowing UvrD (helicase II) to displace a section of lesion-containing DNA. The single-strand gap is filled in by Pol I, and the DNA is sealed by ligase. A transcription coupled repair (TCR) path can also be taken in which RNA polymerase stalls at the lesion on the coding strand. After the RNA polymerase is displaced, the reaction proceeds as shown here, using UvrA through UvrD, Pol I, and ligase.

backbone on the 5' and 3' sides of the damaged nucleotide(s). The incisions are precise: the fifth phosphodiester bond on the 3' side of the lesion and the eighth phosphodiester bond on the 5' side, generating a fragment of 12 to 13 nucleotides (depending on whether the lesion involves one or two bases) containing the lesion. This oligonucleotide is released through the helicase action of UvrD (also called helicase II). The small gap is then filled by Pol I, and the resulting nick is sealed by ligase.

In eukaryotes, NER follows a similar chemical path, although the enzymes are completely different in amino acid sequence and several additional factors are involved (Figure 12-25; see Table 12-4). The main actors were discovered through research on the human genetic disease xeroderma pigmentosum (XP) (Highlight 12-2). Individuals with XP are thousands of times more likely to develop skin cancer from exposure to sunlight. Studies of such patients have identified at least seven different genes that, when any one of them is defective, can contribute to XP.

Genetic studies of XP implicate the genes XPA through XPG in eukaryotic NER. Studies of the proteins encoded by these genes have revealed their roles. The XPC protein initiates the repair process by recognizing the lesion, acting like bacterial UvrA. Then XPB and XPD, which normally act as helicases in RNA transcription, are recruited to the lesion, where they separate the DNA strands to form a single-stranded DNA bubble, acting much like *E. coli* UvrB. RPA, the eukaryotic equivalent of SSB, then binds to the bubble and positions two nucleases, XPF and XPG, on either side of the lesion. XPG cleaves on the 3' side and XPF on the 5' side. The 24- to 32-nucleotide fragment containing the lesion is displaced, and the PCNA clamp recruits a DNA polymerase to fill the gap, which is then sealed by ligase.

In a fascinating reaction, eukaryotes have evolved a means to target NER to a damaged template nucleotide that has stalled RNA polymerase. This process, referred to as **transcription-coupled repair (TCR)**, differs from NER only in the way the damaged site is recognized (see Figure 12-25). In TCR, the damage is recognized by RNA polymerase, which stalls at the lesion. TCR is particularly efficient because it specifically targets repair to actively transcribed DNA that is currently yielding information needed for cell survival, rather than correcting lesions that may lie in vast untranscribed regions of the genome. Bacteria also have a type of transcription-coupled repair. When bacterial RNA polymerase stalls at a lesion, it is displaced by the Mfd helicase, which then recruits the UvrABC proteins for lesion repair.

**FIGURE 12-25** Nucleotide excision repair in eukaryotes.

NER can be initiated by two slightly different methods in eukaryotes. One pathway (left) is similar to that occurring in bacteria, except that a larger section of lesion-containing DNA is removed. The other pathway (right) is referred to as transcription-coupled repair (TCR), because the lesion is first encountered by RNA polymerase, which then stalls. Some of the NER factors are contained in RNA polymerase itself.

Recombination Repairs Lesions That Break DNA

Lesions that block the replication fork can lead to cell death if not repaired before the next round of replication. One replisome-blocking lesion in DNA is a double-strand break. The typical route by which polymerase-blocking lesions are repaired is through a high-fidelity homologous recombination pathway (see Chapter 13). Repair by homologous recombination makes use of the sister chromosome to recover the original sequence. The chromosomes are paired, and the lesion can be repaired by using the homologous strand for the correct information. The role of homologous recombination in DNA repair may even have been the main selective force that drove the evolution of recombination enzymes.

Double-strand breaks can also be repaired by nonhomologous recombination, which uses a different set of proteins, conserved from bacteria to humans. Sealing

the ends of broken DNA by this process usually incurs deletions or insertions, and therefore produces mutations. This pathway, referred to as nonhomologous end joining (NHEJ; see Chapter 13), may be particularly useful when a sister chromosome is unavailable for high-fidelity homologous recombination. Typically, only small deletions or insertions are observed as a result of NHEJ, although large deletions of more than 1 kbp can occur. The sequence at the site of the DNA religation suggests that NHEJ occurs through short, 1 to 6 bp regions of homology.

Specialized Translesion DNA Polymerases Extend DNA Past a Lesion

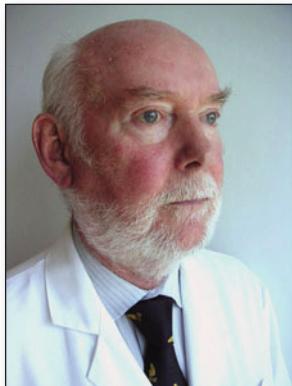
Most DNA repair occurs on double-stranded DNA, where the original sequence can be restored using information in the complementary strand. However, sometimes a lesion occurs at a replication fork after the DNA strands have

HIGHLIGHT 12-2 MEDICINE

Nucleotide Excision Repair and Xeroderma Pigmentosum

Early studies of UV-damaged *E. coli* cells showed that their survival was enhanced if the cells were incubated in growth medium before being plated. In genetic studies of this effect, researchers isolated strains with mutations in three different genes, *uvrA*, *uvrB*, and *uvrC*. Further study demonstrated that the repair of UV damage produced a patch of DNA synthesis. In the laboratory of Robert Painter at the University of California, San Francisco, a similar process was shown to occur in mammalian cells. James Cleaver, working in Painter's lab, recognized that the mammalian repair might be related to the repair of UV lesions in bacteria, but he needed similar mutants in mammalian cells to cement this connection. After reading an account of the genetics of human xeroderma pigmentosum (XP), Cleaver realized that the disease might be the sought-after connection to the UV repair pathway in bacteria. He obtained skin biopsies from patients with XP and developed cell lines that could be grown in culture.

Using the XP cell lines and methods for studying nucleotide excision repair in bacteria, Cleaver was able to identify the major protein components of NER in humans. Cell extracts from each patient were used to measure repair. No extract by itself could repair a UV lesion, but when two extracts were mixed together, repair was observed. These extracts, each missing a different



James Cleaver [Source: Courtesy of James Cleaver.]



Robert Painter [Source: Courtesy of Robert Painter.]

protein of the repair pathway, complemented each other. The researchers were eventually able to group types of XP by complementation and identify which protein was missing in each complementation group. Defects in genes encoding any of seven different proteins of NER can result in XP; the proteins are denoted XPA to XPG. Some of these—XPB, XPD, and XPG—also play roles in transcription-coupled repair. Because NER is the sole repair pathway for pyrimidine dimers in humans, people with XP are extremely sensitive to light and readily develop sunlight-induced skin cancers (Figure 1). Most people with XP also have neurological abnormalities, possibly because of an inability to repair lesions caused by the high rate of oxidative metabolism in neurons.

Various facets of NER in humans await further study. For example, the function of XPE is not yet known. Also, it seems incongruent that bacteria have a second pathway for repair of pyrimidine dimers, making use of DNA photolyase, but humans and other placental mammals do not. But mammals do have a pathway that bypasses pyrimidine dimers, involving the translesion polymerase, Pol η . This enzyme preferentially inserts two A residues opposite a T-T dimer and does not result in a mutation. Indeed, it is tempting to speculate that the appearance of Pol η replaced the need for photolyase in humans, allowing the photolyase gene to be discarded during evolution.



FIGURE 1 This boy exhibits the characteristics of xeroderma pigmentosum. [Source: cmsp.com.]

been unwound. In this case, the replicating polymerase stalls at the lesion. One mechanism that has evolved to resolve this potentially lethal situation is a pathway known as **translesion synthesis (TLS)**. Translesion synthesis

uses a bypass DNA polymerase, or TLS DNA polymerase, which usually lacks a proofreading 3' \rightarrow 5' exonuclease and is capable of extending the DNA strand across a bulky template lesion. Structural studies of a TLS



Wei Yang [Source: Courtesy of Wei Yang.]

polymerase by Wei Yang reveal a wider-than-normal active site architecture, which may explain how this class of enzymes can misincorporate nucleotides opposite noncoding DNA lesions. **Figure 12-26** shows the structure of the complex of an archaeal Pol IV (a TLS polymerase) with DNA containing a benzo[a]pyrene attached to an A residue. The TLS polymerase takes over from the high-fidelity DNA polymerase stalled at the template lesion and extends the DNA strand over the lesion. Because the lesion may be noncoding, lesion bypass by a TLS DNA polymerase often results in a mutation. The first TLS DNA polymerase was discovered in Myron Goodman's laboratory (see Moment of Discovery; How We Know).

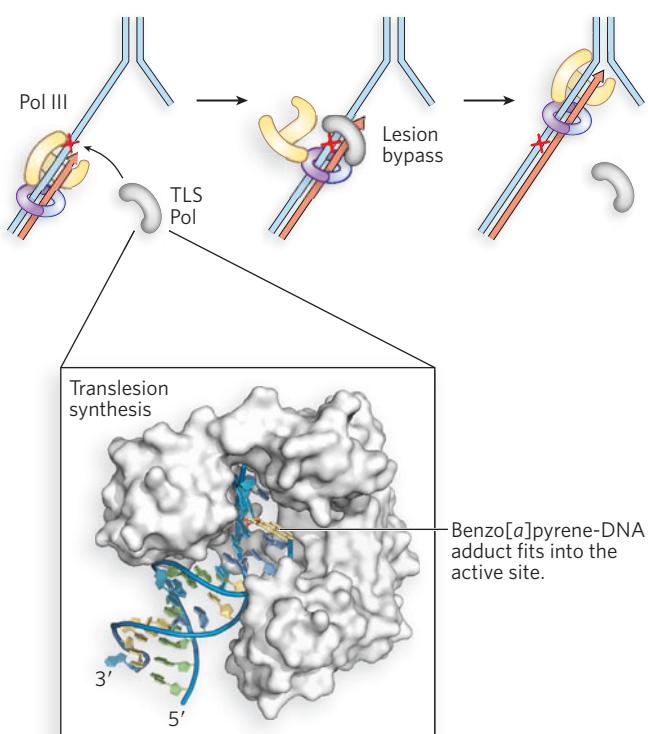


FIGURE 12-26 Translesion synthesis by TLS DNA

polymerases. When the high-fidelity *E. coli* replicase, Pol III (green), encounters a leading-strand lesion (red X), it stalls, and a TLS polymerase (TLS Pol, gray) takes over the β sliding clamp (purple) to extend the leading strand across the lesion. After lesion bypass, Pol III resumes its function with the β clamp. The structure of a TLS DNA polymerase, the archaeal Dbo4 (white), is shown binding to DNA that contains a benzo[a]pyrene adduct (yellow). [Source: (a) PDB ID 1SOM.]

Given the emphasis throughout this chapter on the importance of genomic integrity, the existence of a cellular system that increases the rate of mutation may seem incongruous. However, we can think of this as a desperation strategy. The mutations resulting from translesion synthesis are the biological price a species must pay to overcome an otherwise insurmountable barrier to replication, as it permits a few mutant cells to survive. In *E. coli*, the main TLS polymerase is Pol V, with components encoded by the *umuC* and *umuD* genes. It can extend DNA over the most common lesions, including pyrimidine dimers, 6-4 photoproducts, and abasic sites. All cells contain multiple TLS DNA polymerases (Table 12-5), each suited to bypassing particular types of lesions.

Humans have at least 10 different TLS polymerases (see Table 12-5). Pol ξ (xi) is an error-prone TLS polymerase that places nucleotides at random across noncoding lesions. In contrast, Pol η (eta) bypasses pyrimidine dimers in an error-free event, incorporating two A residues opposite the thymine dimer. Pol η goes no farther than the pyrimidine dimer; another DNA polymerase is required to extend the chain to a length that can be used by Pol δ or Pol ϵ . Pol η has very low fidelity on undamaged

Table 12-5 Specialized DNA Polymerases Involved in Translesion Synthesis

Polymerase	Family
Bacteria (<i>E. coli</i>)	
Pol IV	Y
Pol V	Y
Pol II	B
Yeast (<i>S. cerevisiae</i>)	
Rev1	Y
Pol ξ	B
Pol η	Y
Human (<i>H. sapiens</i>)	
Rev1	Y
Pol ξ	B
Pol η	Y
Pol κ	Y
Pol ι	Y
Pol λ	X
Pol μ	X
Pol β	X
Pol θ	A
Pol ν	A

DNA, and this may be why another polymerase is needed to extend DNA after Pol η has incorporated A residues opposite a thymine dimer. In other words, Pol η has evolved to be specific and accurate at a thymine dimer, not on normal, unmodified DNA, and therefore any other polymerase will do a better job than Pol η of extending DNA over an undamaged template strand.

Several other low-fidelity DNA polymerases, including Pol β , Pol ι (iota), and Pol λ , have specialized roles in eukaryotic base excision repair. Each of these enzymes has a 5' deoxyribose phosphate lyase activity in addition to its polymerase activity. After base removal by the glycosylase and backbone cleavage by the AP endonuclease, these low-fidelity polymerases remove the abasic site (a 5' deoxyribose phosphate) and fill in the gap. The frequency of mutation is minimized by the very short lengths (often just one nucleotide) of DNA synthesized.

SECTION 12.3 SUMMARY

- Cells have diverse and robust systems to repair DNA, and usually restore it to its original sequence.
- The mismatch repair system corrects nucleotide residues misincorporated during replication.
- Some types of lesions are repaired directly, such as photoreversal of pyrimidine dimers by photolyase.
- The base excision repair pathway corrects relatively small, single-base lesions and uses different DNA glycosylases to recognize particular lesions.
- The nucleotide excision repair system repairs bulky lesions by using an excinuclease that makes strand incisions on either side of the lesion. Transcription-coupled repair adapts the nucleotide excision repair system to lesions identified by a stalled RNA polymerase.
- Double-strand DNA breaks result in fragmented chromosomes and are usually repaired by homologous recombination, a high-fidelity process. Double-strand breaks can also be processed by error-prone nonhomologous end joining.
- Cells contain multiple specialized DNA polymerases that extend DNA across lesions, but translesion synthesis is usually an error-prone process that results in a mutation.

Unanswered Questions

Many mysteries about DNA repair remain to be solved. Basic research in this area currently focuses on the detailed mechanisms of the various pathways. Future studies may well discover entirely new pathways of DNA repair. We also need better ways of confirming whether particular chemicals are true human carcinogens. Only then can legal controls be put in place as safeguards against these potentially lethal agents in the environment.

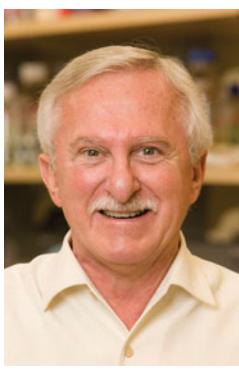
1. **Does the cell have a mechanism to avoid conversion of DNA damage into permanent mutations?** Yes, but little is known about the detailed workings of these “cell cycle checkpoint controls” that prevent the cell from dividing until all DNA damage has been repaired.
2. **How does DNA repair interweave with other DNA metabolic processes?** Repair must be coordinated with other processes of DNA metabolism. For example, enzymes of the recombination, replication, and transcription pathways may encounter lesions, and they need to couple their actions to repair enzymes. Our current understanding of these multiprotein DNA metabolic pathways—transcription, replication, and repair—suggests that they occur in a linear order of independent events. However, it seems likely that the several protein participants in any given pathway are highly coordinated, and may even function within some type of superstructure.
3. **How do translesion DNA polymerases coordinate their low-fidelity actions with the high-fidelity polymerase at a replication fork?** The primary replicase must have high fidelity, by its very nature. When encountering a template lesion, the replisome must somehow yield to low-fidelity translesion DNA polymerases to carry the DNA across the lesion, otherwise the fork may collapse and the cell could die. How do low-fidelity translesion polymerases take over at a replication fork, yet prevent fork collapse? How does the primary replicase regain access to the replication fork after the translesion polymerase has finished its work?

How We Know

Mismatch Repair in *E. coli* Requires DNA Methylation

Au, K.G., K. Welsh, and P. Modrich. 1992. Initiation of methyl-directed mismatch repair. *J. Biol. Chem.* 267:12,142-12,148.

Lahue, R.S., and P. Modrich. 1989. DNA mismatch correction in a defined system. *Science* 245:160-164.



Paul Modrich [Source:
Courtesy of Paul Modrich.]

other methylated on the C strand (**Figure 1a**).

In two sets of reactions, the two different DNAs were treated with combinations of MutS, MutL, and MutH, followed by agarose gel electrophoresis. One can make the prediction that if the mismatch repair

A landmark study by Paul Modrich and his coworkers reconstituted mismatch repair from purified proteins: MutS, MutL, MutH, SSB, helicase II (i.e., UvrD), exonuclease I, Pol III holoenzyme, and ligase. A key to success was the insightful design of a circular duplex DNA containing a single mismatch and a GATC site about 1 kbp away from the mismatch. They made two different DNAs: one methylated on the V strand and the other methylated on the C strand (**Figure 1a**).

system can distinguish new (unmethylated) from old (parental, methylated) DNA, only the unmethylated strand will be nicked. To distinguish which strand was cleaved, the agarose gel was analyzed with a ^{32}P -labeled DNA probe specific for either the C or V strand (**Figure 1b**). As a control, MboI was used to produce a marker (lane 1) because it cleaves both strands at the GATC site, the same sequence that MutH should nick (on one strand). The result shows that MutH nicks DNA only when MutS and MutL are also present: cleavage is specific to the strand that is unmethylated (Figure 1b).

Therefore, these studies demonstrate that MutS-MutL activate MutH to cleave the unmethylated strand of a hemimethylated GATC site. Further study using this system showed that the mismatched nucleotide on the nicked strand is corrected in the presence of helicase II, exonuclease I, Pol III holoenzyme, SSB, and ligase. The detailed mechanism of the mismatch repair reaction is described in the chapter text.

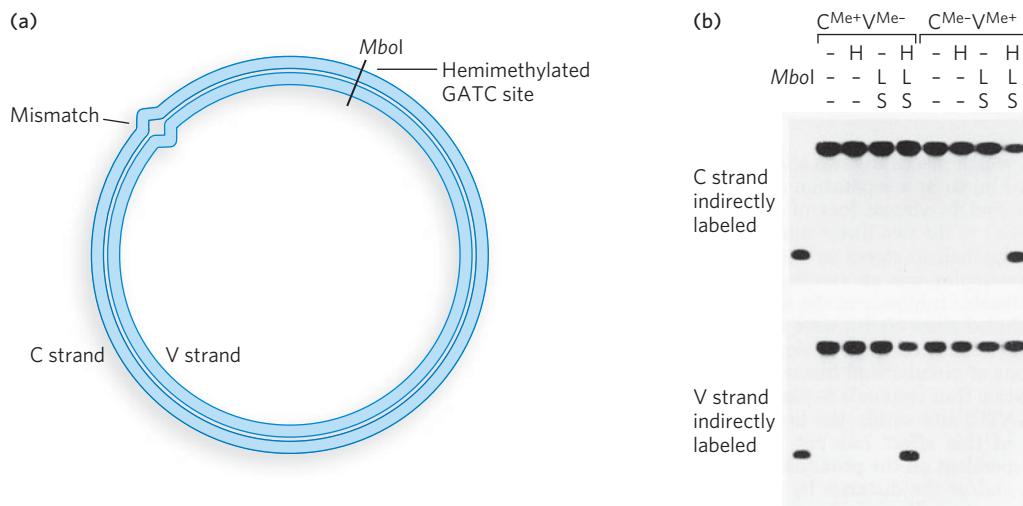


FIGURE 1 MutS and MutL are required to activate MutH, which cleaves the unmethylated strand at a GATC site. (a) The DNA substrate contained a mismatch and a GATC sequence methylated on either the V strand or the C strand.

(b) Gel analysis of reaction products: proteins added to reactions are indicated at the top of the gels. [Source: Adapted from R. S. Lahue and P. Modrich, *Science* 245(4914):160-164, 1989. Courtesy of Paul Modrich.]

UV Lights Up the Pathway to DNA Damage Repair

Delbecco, R. 1949. Reactivation of ultraviolet-inactivated bacteriophage by visible light. *Nature* 163:949–950.

Kelner, A. 1949. Effect of visible light on the recovery of *Streptomyces griseus* conidia from ultraviolet-irradiation injury. *Proc. Natl. Acad. Sci. USA* 35:73–79.

The first discovery of a DNA repair reaction was the finding that sunlight can repair UV damage. The discovery involved a serendipitous observation by Albert Kelner, an astute scientist who was studying the effects of UV light on the conidia (spores) of the mold *Streptomyces griseus*, with the hope of finding mutants that produced new varieties of antibiotics. In the course of his studies, Kelner noticed that irradiated cells sometimes had a higher rate of survival (i.e., produced more colonies) than expected. This indicated the presence of a repair pathway.

The variability in survival rate suggested that the repair pathway was inducible. During repeated attempts to identify the conditions that resulted in “induction” of the UV-damage repair pathway, Kelner obtained frustratingly irreproducible results. For example, in thinking that temperature influenced the reaction, he incubated cells that received the same dose of UV light at different temperatures. At first there seemed to be an effect, but two preparations treated in the same way gave results that differed more than 100-fold. In a keen observation, Kelner noticed that repair (i.e., increased survival) correlated with culture flasks that were closer to a window, and thus the elusive variable might be sunlight. Controlled experiments confirmed that sunlight was indeed the agent underlying the repair of UV damage. Exposure to visible light yielded more than 10^5 the number of colonies as the absence of light (**Figure 2**).

Kelner suggested that reversal of UV damage by visible light, or photoreversal, may generalize to other organisms. Having heard of Kelner’s studies, Renato Delbecco, at Indiana University looked for photoreversal in *E. coli* using a T phage. UV irradiation of T phage reduced its ability to grow in *E. coli*, as expected, but its subsequent exposure to visible light had no photoreversal effect on its viability. However, when the UV-irradiated phage were inside *E. coli* cells and then infected cells were exposed to visible light, Delbecco observed repair (increased phage viability). Hence, he concluded that visible light did not act directly to reverse UV damage, but a cellular factor was required for photorepair.

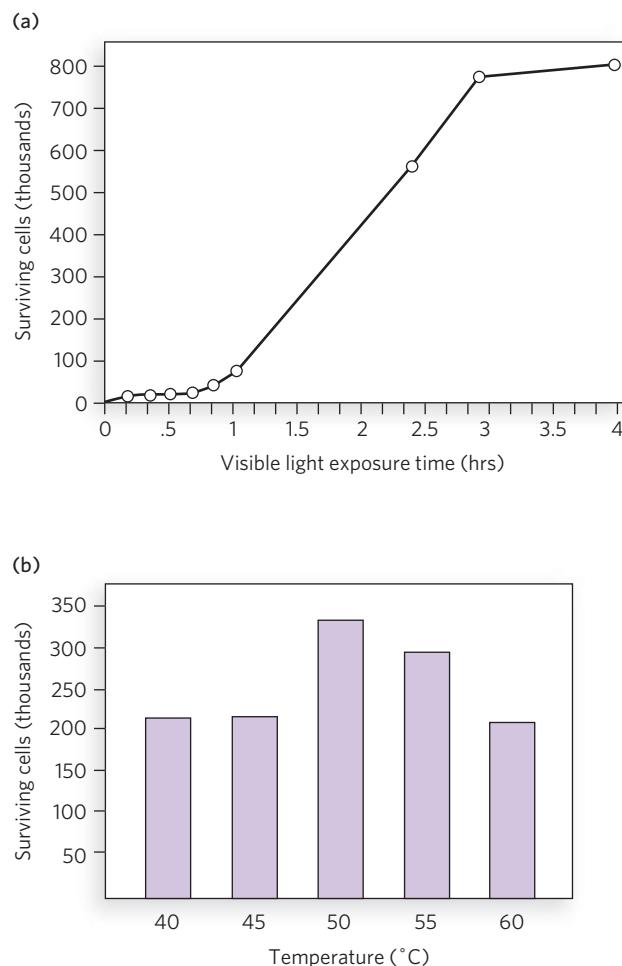
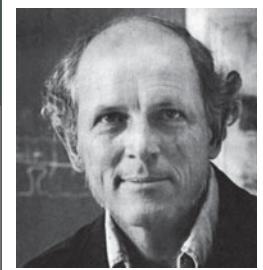


FIGURE 2 Results showing the effect on cell survival of (a) different periods of exposure to visible light and (b) different temperatures of incubation. [Source: Adapted from A. Kelner, *Proc. Natl. Acad. Sci. USA* 35:73–79, 1949.]

These studies were performed before scientists knew about DNA structure. Of course, now we know that UV light results in pyrimidine dimers and that photolyases directly reverse the pyrimidine cross-links.



Translesion DNA Polymerases Produce DNA Mutations

Rajagopalan, M., C. Lu, R. Woodgate, M. O'Donnell, M.F. Goodman, and H. Echols. 1992.

Activity of the purified mutagenesis proteins UmuC, UmuD', and RecA in replicative bypass of an abasic DNA lesion by DNA polymerase III. *Proc. Natl. Acad. Sci. USA* 89:10,777-10,781.

Harrison (Hatch) Echols

[Source: Courtesy of University of Wisconsin-Madison.]

Bacterial cells contain proteins that produce mutations in response to DNA damage. Four genes are required for this “mutagenic response” in *E. coli*: *recA*, *lexA*, *umuC*, and *umuD*. The gene product of *umuD* is first cleaved by the enzyme RecA coprotease to produce the functional form of UmuD, called UmuD', which forms a complex with UmuC (UmuC₁UmuD'₂). Harrison (Hatch) Echols and Myron Goodman intuited that UmuC₁UmuD'₂ acts during replication, and they developed an *in vitro* reaction that demonstrates lesion bypass (Figure 3a). They constructed a 5.4 kb, linear, single-stranded DNA substrate with an abasic site near one end, and placed a 5'-³²P-labeled primer (P1) just upstream of the abasic site so that they could observe polymerization by PAGE analysis in a denaturing gel. They added RecA, or UmuC, or UmuD' along with Pol III holoenzyme and SSB, and performed

each reaction in duplicate. Bypass of the abasic site is observed only in the presence of UmuC and UmuD' (Figure 3b; lanes 5 and 6 are duplicate reactions). Later studies showed that UmuC and UmuD' combine to form a distributive polymerase—one that must dissociate from and rebind to the DNA repeatedly, rather than stick to the DNA constantly as do processive polymerases (such as Pol III). The distributive nature accounts for the many bands in lanes 5 and 6 of the gel analysis. Further study also showed that UmuC is an entirely new class of DNA polymerase, with a sequence unrelated to other known DNA polymerases. The UmuC₁UmuD'₂ translesion polymerase was renamed Pol V. Soon after these studies, many other translesion DNA polymerases that are homologous to UmuC, designated Y-family polymerases, were identified from cells of all types.

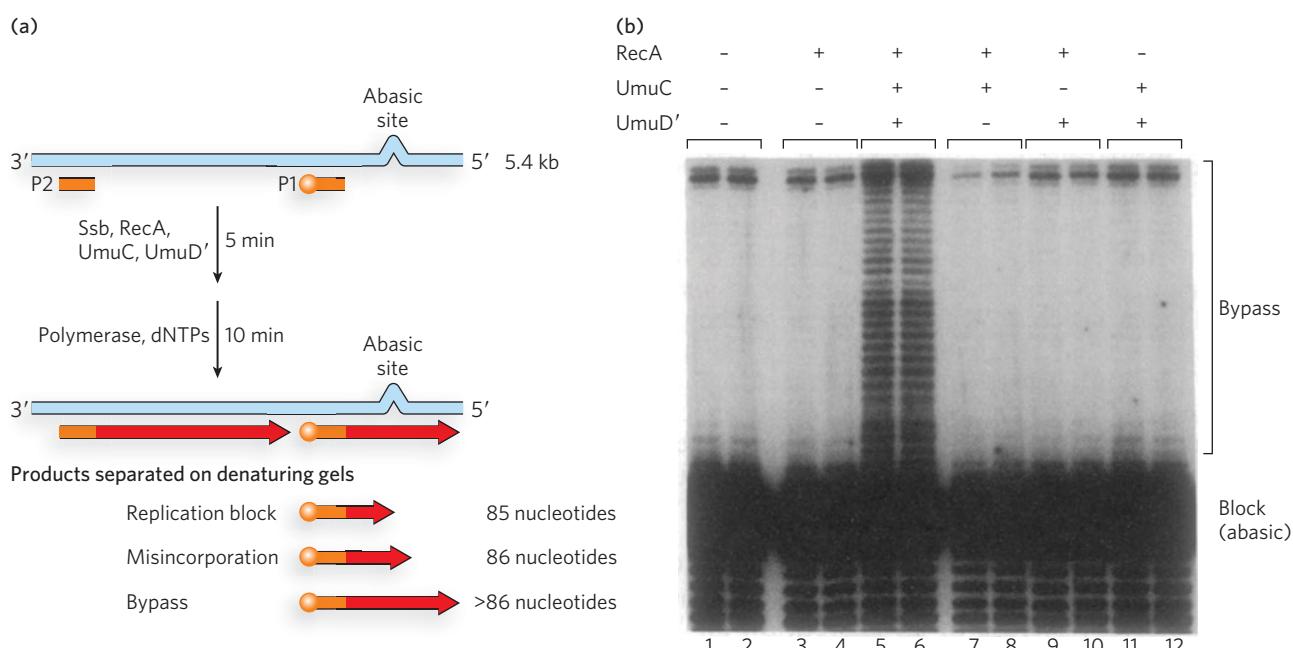


FIGURE 3 Pol V can bypass an abasic site in a DNA substrate. (a) Primer 2 (P2) is added to convert the single-stranded DNA upstream of the lesion to duplex DNA. Primer 1 (P1), labeled with ³²P, is added to observe extension over the template lesion.

(b) PAGE analysis of the reaction products. Only in the presence of RecA, UmuC, and UmuD' is extension of primer 1 observed (lanes 5 and 6). [Source: (b) Adapted from M. Rajagopalan et al., *Proc. Natl. Acad. Sci. USA* 89:10,777-10,781, 1992, Fig. 2.]

Key Terms

mutation, p. 410
 point mutation, p. 411
 transition mutation, p. 411
 transversion mutation, p. 411
 silent mutation, p. 411
 missense mutation, p. 411
 nonsense mutation, p. 411
 insertion mutation, p. 412
 deletion mutation, p. 412
 frameshift mutation, p. 412
 inversion mutation, p. 415

translocation mutation, p. 415
 abasic site, p. 417
 depurination, p. 417
 Ames test, p. 420
 reversion mutation, p. 420
 pyrimidine dimer, p. 422
 cyclobutane ring, p. 422
 6-4 photoproduct, p. 422
 mismatch repair (MMR), p. 425
 Dam methylase, p. 426
 photoreactivation, p. 429

DNA photolyase, p. 429
 base excision repair (BER), p. 430
 DNA glycosylase, p. 430
 AP endonuclease, p. 431
 nucleotide excision repair (NER),
 p. 433
 excinuclease, p. 433
 transcription-coupled repair (TCR),
 p. 434
 translesion synthesis (TLS), p. 436

Problems

- 1.** The base-pairing atoms of thymine are not directly involved in the cyclobutane ring of a pyrimidine dimer, formed by UV irradiation. Why does a pyrimidine dimer stall the replicative DNA polymerase?
- 2.** For the nucleotide sequence AAC(O^6 -meG)TGCAC, with a damaged (methylated) G residue, what would be the sequence of each strand of the double-stranded DNA in the following situations?
- Replication occurs before repair.
 - The DNA is acted upon by a glycosylase and then repaired, but only after replication has occurred.
 - Two rounds of replication occur, followed by repair.
- 3.** Name three of the common ways in which DNA lesions are incurred. What is required for these DNA lesions to result in a mutation?
- 4.** Benzo[*a*]pyrene, the cancer-causing agent in cigarette smoke, is a powerful mutagen. Benzo[*a*]pyrene itself is relatively harmless, but it is metabolized in the liver to produce active molecules that react covalently with DNA. In an experiment, benzo[*a*]pyrene is incubated with a mixture of liver enzymes to form its genotoxic metabolites. These metabolites are added to *E. coli* cells that have a mutation in a gene encoding an enzyme of the serine-synthesizing pathway (i.e., the cells are serine auxotrophs, requiring serine for growth). When the treated cells are grown on serine-containing medium, the results show that the benzo[*a*]pyrene metabolites kill cells in a dose-dependent manner. When treated and untreated serine-auxotrophic cells are plated separately on serine-free media, the cells treated with benzo[*a*]pyrene metabolites show a 10- to 100-fold increase in survivors compared with untreated cells. Explain these results.
- 5.** In the experiment described in Problem 4, some of the untreated serine auxotrophs were able to grow on a medium lacking serine. Why?
- 6.** In an experiment using *S. typhimurium* histidine auxotrophs, the cells are grown on a thin layer of agar with nutrient medium that lacks histidine. The culture ($\sim 10^9$ cells) produces ~ 13 colonies over a two-day incubation period at 37°C .
- How did these colonies arise in the absence of histidine?
 - When the experiment is repeated in the presence of 0.4 μg of 2-aminoanthracene, the number of colonies produced over two days exceeds 10,000. What does this indicate about 2-aminoanthracene?
 - What can you surmise about its carcinogenicity?
- 7.** The human disease known as xeroderma pigmentosum (XP) arises from mutations in at least seven different genes. The resulting deficiencies are generally in enzymes involved in some part of the pathway for nucleotide excision repair. The various types of XP are denoted A through G (XPA, XPB, etc.), with a few additional variants lumped together under the label XP-V. Cultures of fibroblasts from healthy individuals and from patients with XPG are irradiated with UV light. The DNA is isolated and denatured, and the resulting single-stranded DNA is examined by analytical ultracentrifugation.
- Samples from the normal fibroblasts show a significant reduction in the average molecular weight of the single-stranded DNA after irradiation, but samples from the XPG fibroblasts show no such reduction. Why might this be?
 - If you assume that an NER system is operative in fibroblasts, which step might be defective in the cells of patients with XPG? Explain.
- 8.** Describe the most critical difference between global nucleotide excision repair and transcription-coupled repair.
- 9.** What do base excision repair and repair of an abasic site have in common? How do they differ?
- 10.** Many eukaryotes have a DNA glycosylase that specifically removes T residues from DNA, but only when they are paired with G. There is no comparable enzyme that removes the G residues from G-T mismatches. Why is it

useful for a cell to always repair a G-T mismatch to G≡C rather than to A=T?

11. A gene is found that has a sequence of 11 contiguous A residues in one strand. Mutations occur at an elevated frequency in this gene, mostly in the region with the repeated A residues. Most of these mutations result in inactivation of the encoded protein, with many amino acids either missing or altered. What type of mutations would account for these observations, and how might they occur?
12. Many bacteria, including *E. coli*, are capable of growing under both anaerobic and aerobic conditions. Some mutations are introduced into an *E. coli* strain that inactivate several enzymes involved in DNA repair. The mutant strain grows normally when kept in an incubator with a 100% nitrogen gas atmosphere. However, the strain dies when exposed to a normal laboratory atmosphere. Why?

Data Analysis Problem

Tang, M., X. Shen, E.G. Frank, M. O'Donnell, R. Woodgate, and M.F. Goodman. 1999. UmuD'₂C is an error-prone DNA polymerase, *Escherichia coli* pol V. *Proc. Natl. Acad. Sci. USA* 96:8919–8924.

16. In the Moment of Discovery at the beginning of this chapter, Myron Goodman describes the translesion DNA synthesis reaction promoted by a reconstituted system including Pol III, the UmuD' and UmuC proteins, and RecA protein. A control experiment was carried out in which Pol III was left out of the reaction mixture, and translesion DNA synthesis still occurred. This was the first suggestion that UmuC and UmuD' might have DNA polymerase activity, but more work was needed to prove it.

- (a) Suggest why the control experiment might not be definitive.

Goodman and colleagues cultured *E. coli* cells that expressed Pol V, using a strain with both a mutation inactivating Pol II and a temperature-sensitive Pol III.

- (b) Suggest why the investigators did not use a strain with a completely inactivated Pol III.

The researchers partially purified Pol V and then carried out a gel filtration experiment to separate Pol III from Pol V. Their results are shown in **Figure 1**, a Western blot analysis of the fractions from the gel filtration column, using antibodies to the α subunit of Pol III and UmuC protein.

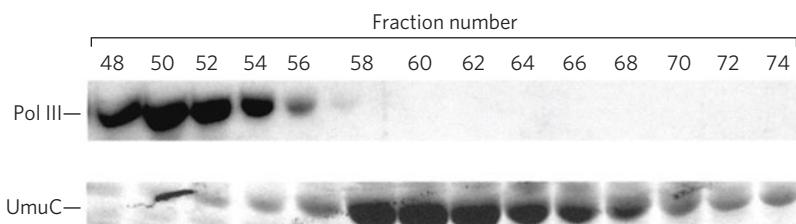


FIGURE 1

13. In an *E. coli* cell, DNA polymerase III makes a rare error and inserts a G opposite an A residue at a position 850 bp away from the nearest GATC sequence. The mismatch is accurately repaired by the mismatch repair system. How many phosphodiester bonds derived from deoxynucleotides (dNTPs) are expended in this repair process? ATPs are also used in this process. Which enzymes consume the ATP?

14. If an oxidative lesion occurs spontaneously in a single-stranded DNA fragment generated on the lagging strand during replication, it is not readily repaired by nucleotide excision repair or base excision repair. Explain why.

15. O^6 -Methylguanine lesions are repaired directly by transfer of the methyl group to O^6 -methylguanine methyltransferase. A very high level of metabolic energy is invested in this simple methyl transfer reaction. Describe this energy investment.

Three fractions—50, 56, and 64—were chosen for further analysis, as shown in **Figure 2**. DNA polymerization activity was examined on a primer template (labeled P in the gel) in which the second position in the single-stranded template had an abasic site (X). The work was done at two temperatures, 37°C and 47°C.

- (c) Suggest why DNA polymerization is greater for fraction 56 than for fraction 50.
 (d) Why does the DNA polymerization activity decline in fractions 50 and 56 at 47°C relative to 37°C?
 (e) The activity of fraction 64 does not decline with increased temperature. Suggest why.

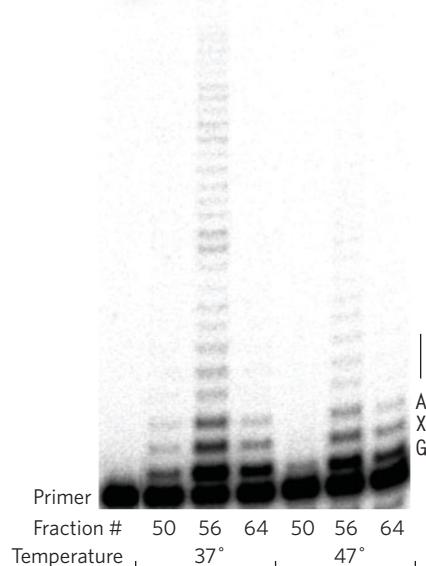


FIGURE 2

Additional Reading

General

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Recombinational DNA Repair and Homologous Recombination



Lorraine Symington [Source: Courtesy of Lorraine Symington.]

We recently figured out how double-strand DNA breaks (DSBs) are processed in eukaryotic cells, as a first step in generating meiotic crossovers. This story started some years ago when Jack Szostak showed that DSBs stimulate homologous recombination of DNA and function as natural initiators for meiotic recombination. Researchers also found that DNA ends produced by DSBs undergo degradation to produce 3' single-stranded DNA tails, which are required to initiate homologous recombination. Around the same time, Jim Haber found the same thing to be true for mating-type switching in yeast. *But how are the 3' single-stranded tails made?*

We had been using genetic and biochemical approaches over a number of years with the expectation that a single mutant in yeast would completely block 3'-tail formation, but we were unsuccessful. Then a new student arrived in my lab, Eleni Mimitou, and she hypothesized that helicases might play an important role. The first helicase-encoding gene she deleted from yeast, *SGS1*, produced a profound defect in double-strand break processing! The role of *Sgs1* at such an early stage of recombination was quite surprising because prior studies had shown a role for it in a late stage of recombination.

Eleni went on to show that the function of *Sgs1* is partially redundant with that of a nuclease called *Exo1*. When she deleted both *SGS1* and *EXO1* from yeast, the resulting strain produced only partially processed DNA ends during recombination, a result that was obvious from the very first experiment that Eleni looked at. She also found that another protein, *Sae2*, was required to complete the initial processing of DSBs. These results and other data led us to propose a two-step mechanism for the production of 3' single-stranded tails. After 15 years of working on recombination, all of these results came together in about six months! These “moments” of discovery in science make all of the struggles in between worthwhile.

—Lorraine Symington, *on discovering how DNA ends are processed to initiate DNA recombination*

13.1 Recombination as a DNA Repair Process 447

13.2 Enzymatic Machines in Bacterial Recombinational DNA Repair 453

13.3 Homologous Recombination in Eukaryotes 464

13.4 Nonhomologous End Joining 472

Genetic recombination is the exchange of genetic information between chromosomes or between different chromosomal segments in a single chromosome. Such exchanges occur by several mechanisms. Homologous genetic recombination, often simply termed **homologous recombination**, encompasses genetic exchanges at sequences that are identical, or nearly so, in both DNA segments involved in the recombination. Any sequence will do, as long as it is shared by the DNAs undergoing an exchange. Chapter 14 explores other forms of recombination, including exchanges that require a specific sequence (site-specific recombination and some forms of transposition) and some that can occur almost randomly (other forms of transposition). The idea of processes with the potential to scramble genetic information may seem incompatible with the DNA replication and repair processes that so thoroughly maintain genomic integrity. For the most part, the disconnect is illusory. First and foremost, homologous recombination is a DNA repair process, and recombinational DNA repair is where we begin our discussion.

Recombinational DNA repair is, to a large extent, directed at the repair of the most dangerous of all DNA lesions: the **double-strand break (DSB)**. Double-strand breaks usually arise during DNA replication, when replication forks encounter a single-strand break in a template strand. In DNA metabolism, this is a true show-stopper. Broken DNA ends make it impossible for DNA replication to continue. Double-strand breaks can also arise during exposure to UV light or γ radiation. The effect goes beyond a mutation that might or might not affect a cellular function; DSBs, if not repaired, typically lead to cell death.

Partial deficiencies in DSB repair systems have been connected to a genetic predisposition to many forms of cancer, including an inherited predisposition to breast cancer. A wide range of human genetic diseases that are characterized by genomic instability, developmental abnormalities, light sensitivity, as well as cancer predisposition, have been traced to defects in particular genes involved in homologous recombination. If a mammalian embryo completely lacks the capacity for DSB repair, that embryo is never born. Its cells divide a few times and then die, the genome in fragments arising from countless failed attempts to repair stalled replication forks. The capacity for the enzymatic repair of DSBs is inherent to every free-living organism.

The repair of DSBs by homologous recombination, in any context, requires the use of a homologous chromosome. The broken DNA ends are processed to produce single-stranded tails with 3' ends. Each 3' single-stranded

end is aligned with an identical sequence in a homologous chromosome or DNA segment. Strand invasion, catalyzed by specialized recombinase enzymes to be described in Section 13.2, is a process in which one strand of the target DNA is displaced and the other is paired with the invading strand. The DNAs thus joined are further processed by DNA polymerases that use the invading 3' end as a primer for DNA synthesis, then other enzymes process the branched DNA intermediate to produce two complete, repaired DNAs. All cellular chromosomes encounter many situations in which this kind of DNA repair process is needed.

The need to repair replication forks probably fueled the evolution of recombination systems. DNA damage is common. Oxygen first appeared in the atmosphere 2.3 billion years ago as photosynthesis evolved. However, the advantages of aerobic metabolism could not be fully realized until cells also developed the means to deal with oxidative DNA damage. A bacterial cell grown in an oxidative environment will suffer more than 1,000 DNA lesions per cell per generation, and a typical mammalian cell, more than 100,000 DNA lesions every 24 hours. This omnipresent spontaneous DNA damage may have limited the size of a genome that could be replicated successfully in an aerobic organism, until the advent of systems for reconstituting and restarting collapsed replication forks.

Homologous recombination systems now have a broader range of functions in diploid organisms. Eukaryotes have repurposed the homologous recombination machinery to facilitate the accurate transmission of large chromosomes from one generation to the next. DSBs are introduced in every chromosome during meiosis. The resulting recombination provides a link between replicated sister chromosomes (chromatids) and ensures their accurate segregation at cell division. This same recombination also produces chromosomal crossovers as a byproduct, exchanging large segments of genetic material between homologous chromosomes—a process that makes a significant contribution to the genetic diversity that fuels evolution. The study of homologous recombination was originally inspired by its effect on inheritance. To a great extent, the genetic recombination systems in every organism made the development of the entire science of genetics possible.

Cellular recombination systems are co-opted in additional processes that trigger changes in fungal mating types, allow pathogenic bacteria to evade host immune systems, and sometimes consummate horizontal gene transfer through genetic exchanges between cellular chromosomes and foreign DNA (see Figure 1-11). In other words, although homologous recombination began as a process of DNA repair, it has evolved into a broader

mechanism that allows populations of organisms to genetically adapt more quickly to their environment.

The recombinational DNA repair of damaged replication forks is the centerpiece of our discussion. The resurrection of collapsed replication forks represents a fascinating intersection of every aspect of DNA metabolism—replication, repair, and recombination. Our examination of recombination thus begins with replication. We then expand the discussion to include the recombination processes of bacteria and eukaryotes in a variety of contexts, as well as some alternative paths to the repair of double-strand breaks.

13.1 Recombination as a DNA Repair Process

As discussed in Chapter 12, DNA damage is common and highly deleterious. The most important consequences of the damage do not become apparent until the DNA is replicated. When a lesion exists in the DNA template, several things can happen. In some cases, DNA synthesis continues over the lesion, by translesion synthesis (TLS) (Figure 13-1a; see also Figure 12-26). Normally, this occurs only with the aid of specialized DNA polymerases capable of TLS. Translesion DNA polymerases are found in all cells and are often adapted to particular repair scenarios. More rarely, the normal cellular DNA polymerase may replicate over lesions that do not cause significant DNA distortion. For example, an O⁶-meG in the template strand pairs with T rather than C, and replication of the lesion will result in a C → T transition mutation.

Most lesions, if encountered before any repair process is initiated, cause the replication fork to stall (Figure 13-1b). The replisome cannot insert a nucleotide opposite the lesion, and replication ceases until the lesion is repaired. If the replisome encounters a lesion that is already undergoing nucleotide excision repair (NER) or base excision repair (BER), the template strand may contain a temporary break. When the replication fork arrives, one branch of the fork becomes disconnected and the replication fork collapses (Figure 13-1c)—a particularly catastrophic outcome that creates a DSB. In these last two cases, recombinational DNA repair restores an undamaged fork structure, allowing replication to restart.

In still other cases, the replication machinery is blocked by the lesion but resumes replication further downstream (Figure 13-1d). The lesion is left behind in a single-strand gap, with no undamaged complementary strand present to guide the most common DNA repair pathways. This kind of bypass-plus-restart

outcome occurs most readily when the lesion is in the lagging strand, because the inherent mechanism used to initiate the synthesis of new Okazaki fragments can simply continue downstream.

Recombinational DNA repair resolves each of the situations illustrated in Figure 13-1b-d, although the pathways differ in ways large and small, and in some cases even use somewhat different sets of enzymes. As described more fully below, a stalled replication fork is repaired by fork regression, a collapsed fork with a DSB is resolved through DSB repair, and the gap resulting from lesion bypass is filled through DNA gap repair.

Double-Strand Breaks Are Repaired by Recombination

Double-strand breaks can result from oxidative DNA damage. This is a relatively rare occurrence, but can be a byproduct of respiration in organisms growing in an oxygen-rich environment or a consequence of exposure to ionizing radiation. These lesions destroy the continuity of both template strands, and they are generally lethal if not repaired. Often, DSBs result when the replication fork encounters template strand discontinuities (see Figure 13-1c). We first consider a generalized pathway for the repair of DSBs, which will reappear in slightly altered forms as we discuss specific repair pathways. The enzymes we'll encounter are described in more detail in Section 13.2.

The repair of double-strand breaks by recombination requires the presence of another, homologous, undamaged double-stranded DNA. In a diploid cell, that double-stranded DNA is either the second copy of each chromosome or the sister chromatid present immediately after DNA replication. This second DNA molecule guides the repair process by providing a template for the restoration of genetic information that might otherwise be lost as nucleotides missing at the site of the break. The broken DNA ends are processed, with the 5'-ending strands selectively degraded to create 3' single-stranded extensions, or overhangs (Figure 13-2, step 1). Next, the 3' single-stranded extensions invade the homologous chromosome, in a process catalyzed by a ubiquitous class of enzymes called **recombinases**. In this **DNA strand invasion**, the quintessential step of homologous recombination, the invading strand displaces one strand of the homologous chromosome and base-pairs with the other (steps 2 and 3). The use of 3' ends for the invasion step has the important consequence that these ends can also act as primers for DNA synthesis. DNA polymerase-mediated extension of the invading strands (step 4) lengthens them in a manner that faithfully restores any information lost at the site of the break, using the

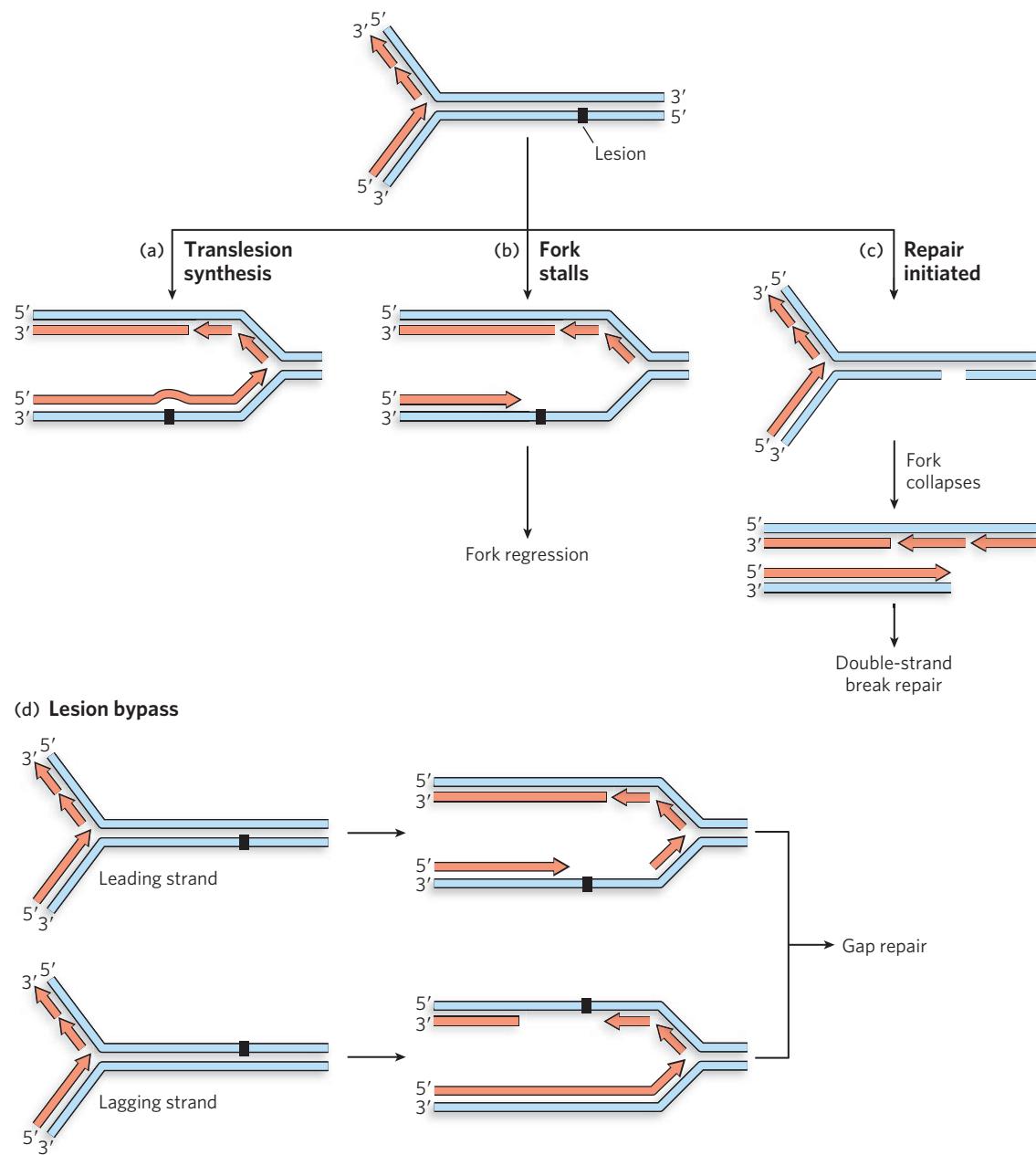


FIGURE 13-1 Possible effects of a damaged template on a replication fork. (a) Translesion synthesis occurs when the replisome encounters a lesion and keeps going. (b) Some lesions prevent progress of the replisome, resulting in a stalled replication fork. (c) If the replisome encounters a

lesion that is undergoing repair, a single-strand break can cause the replication fork to collapse, creating a double-strand break. (d) In some cases, the lesion is bypassed, leaving a single-strand gap, and replication continues downstream.

invaded chromosome as the template. At this point, the two DNA molecules are linked at two places where the invading strands cross from one template to the other, forming a branched intermediate.

This complex intermediate can be resolved in at least two ways. First, the now-lengthened invading strands can simply be displaced by the action of helicases, then can anneal to each other (Figure 13-3a). Any

remaining gaps can be filled by DNA polymerases and the ends ligated by DNA ligases to complete the repair. This type of pathway, which is quite common, is known as **synthesis-dependent strand annealing (SDSA)**.

In the second pathway (Figure 13-3b), often referred to as **double-strand break repair (DSBR)**, replication is completed by ligating the strands while they are still linked. The two four-armed junctions in

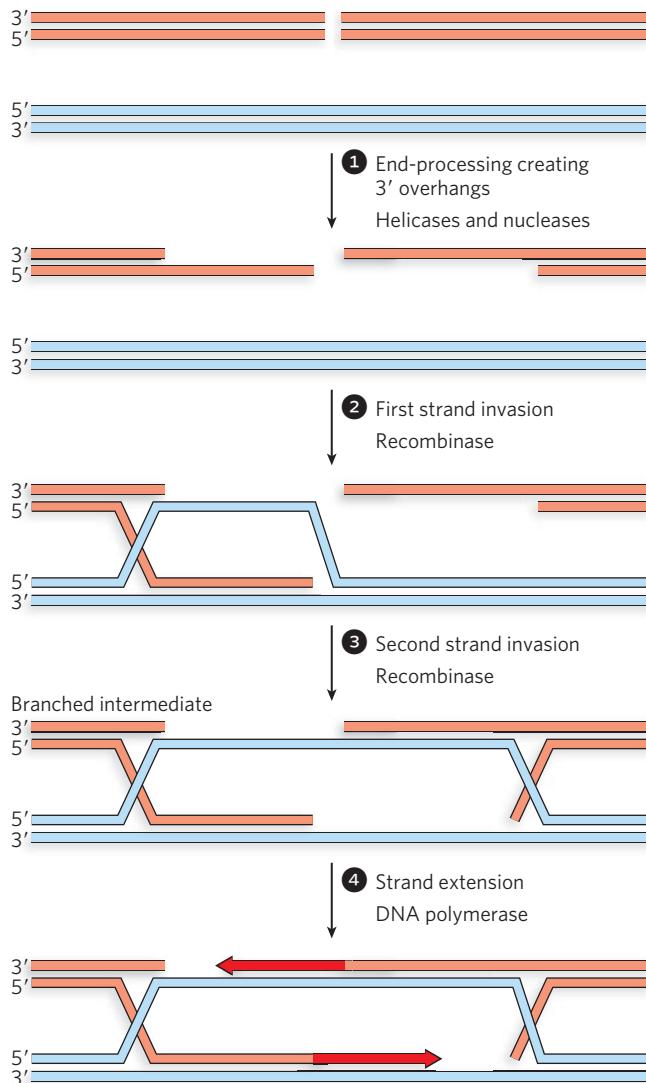


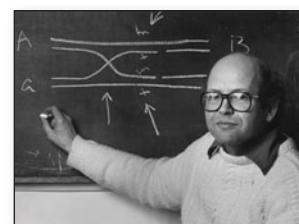
FIGURE 13-2 The repair of chromosomal double-strand breaks. Accurate repair of a DSB requires an undamaged source of duplicate genetic information—that is, homologous double-stranded DNA. The broken ends are first processed to generate 3' single-stranded extensions, and both ends are used for strand invasion of the homologous double-stranded DNA. The invading 3' ends are extended by DNA polymerases. Crossovers called Holliday intermediates are often formed where four DNA strands come together (as described later in the text).

this structure, with all DNA strands intact, are called **Holliday intermediates** (or Holliday junctions)—named for the geneticist Robin Holliday, who proposed the first recombination model that included them. Specialized nucleases recognize and cleave the Holliday intermediates in one of two ways, such that the products are viable chromosomes with a complete set of genes (see Figure 13-3b). The remaining breaks are

sealed by DNA ligase. The cleavage and rejoicing may result in either the retention of the chromosomal DNA segments originally linked on either side of the repair site or an exchange of that information in a **crossover**. If both Holliday intermediates are cleaved at the sites labeled X or both are cleaved at the sites labeled Y in Figure 13-3b, no crossover results. If one is cleaved at the X sites and the other at the Y sites, a crossover occurs, and the genetic material extending from the site of repair to the telomere is transferred between chromosomes.

The elaborate repair process in this DSBR pathway requires the careful coordination of many different enzymes. Because, in principle, recombination can occur between any two DNA segments that share sequence similarity, deleterious events may occur. For example, recombination of two repeated sequences on the same chromosome could lead to complete deletion of all the genetic information between these sequences. Such deleterious events are rare, given the very tight regulation imposed on homologous recombination systems in all cells.

We'll now look at how the various steps of SDSA and DSBR are applied to the repair of replication forks. When replication forks stall or collapse, recombinational DNA repair has a major advantage over translesion DNA synthesis: it does not cause mutations.



Robin Holliday [Source: Courtesy of Robin Holliday.]

Collapsed Replication Forks Are Reconstructed by Double-Strand Break Repair

When a replication fork encounters a break in a template strand, one arm becomes detached. The resulting double-strand break and collapse of the fork triggers a recombinational repair process. Here, and in some other pathways we'll soon encounter, the steps in Figure 13-2 will become quite familiar. In brief, repair requires the reattachment of the broken arm to recreate the fork. The reattachment involves a DNA strand invasion reaction, mediated by a recombinase.

The reconstruction of a replication fork by recombinational DSBR is shown in Figure 13-4. The broken DNA end is processed by nucleases to remove a segment of the 5'-ending strand (step 1). This creates a 3' single-stranded DNA extension, which is then bound by recombinase and used for strand invasion of the intact portion of the chromosome, such that it is paired

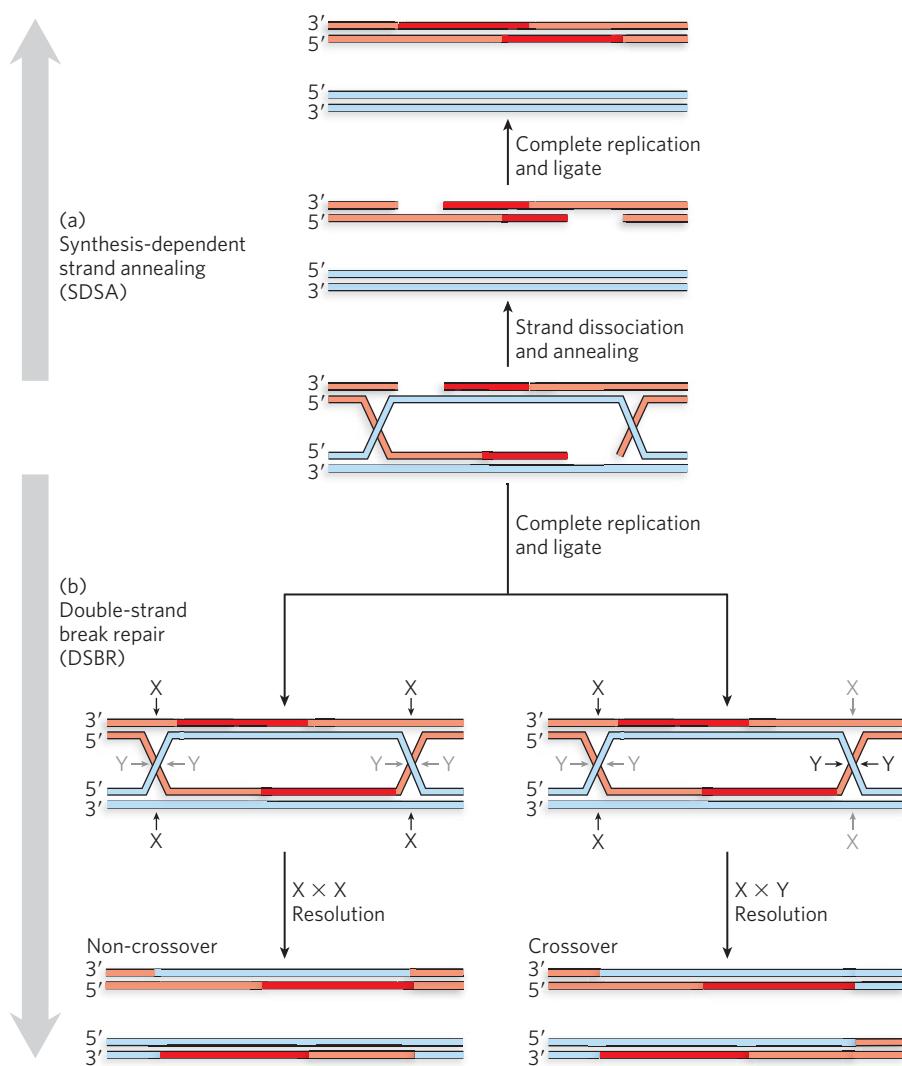


FIGURE 13-3 Two paths for completing double-strand break repair. (a) In the SDSA pathway, the invading strands dissociate and anneal to each other. Further replication and ligation complete the process. (b) In the DSBR pathway, the two

Holliday intermediates are cleaved by Holliday intermediate resolvases (see Section 13.2). Resolution of the Holliday intermediate can yield two different results with respect to the DNA flanking the repair site, as described in the text.

with its complementary strand (step 2). At the point of insertion, the other strand of the invaded duplex DNA is displaced. Once the strand invasion is complete, reconstruction of the replication fork requires the action of enzymes that promote **branch migration** (step 3). In this process, DNA branches are moved along the DNA, with some base pairs forming and others being disrupted, but with no net increase or decrease in number of paired nucleotides (see Figure 13-4, inset). Branch migration creates a Holliday intermediate, which is resolved by the successive actions of a specialized nuclease called a Holliday intermediate resolvase, along with DNA ligase (step 4). The reconstructed fork is then ready for renewed replication, and no mutations

have been added in the process. Replication is restarted with the aid of a dedicated replication restart complex that reloads the DnaB helicase onto the DNA. The other replisome components load spontaneously onto DnaB, and replication begins anew.

A Stalled Replication Fork Requires Fork Regression

When the replication fork encounters a template strand lesion that cannot be surmounted, the fork stalls at the lesion. In some cases, replication restarts downstream; in others, the replication simply halts until the lesion is repaired. In either case, the lesion is left

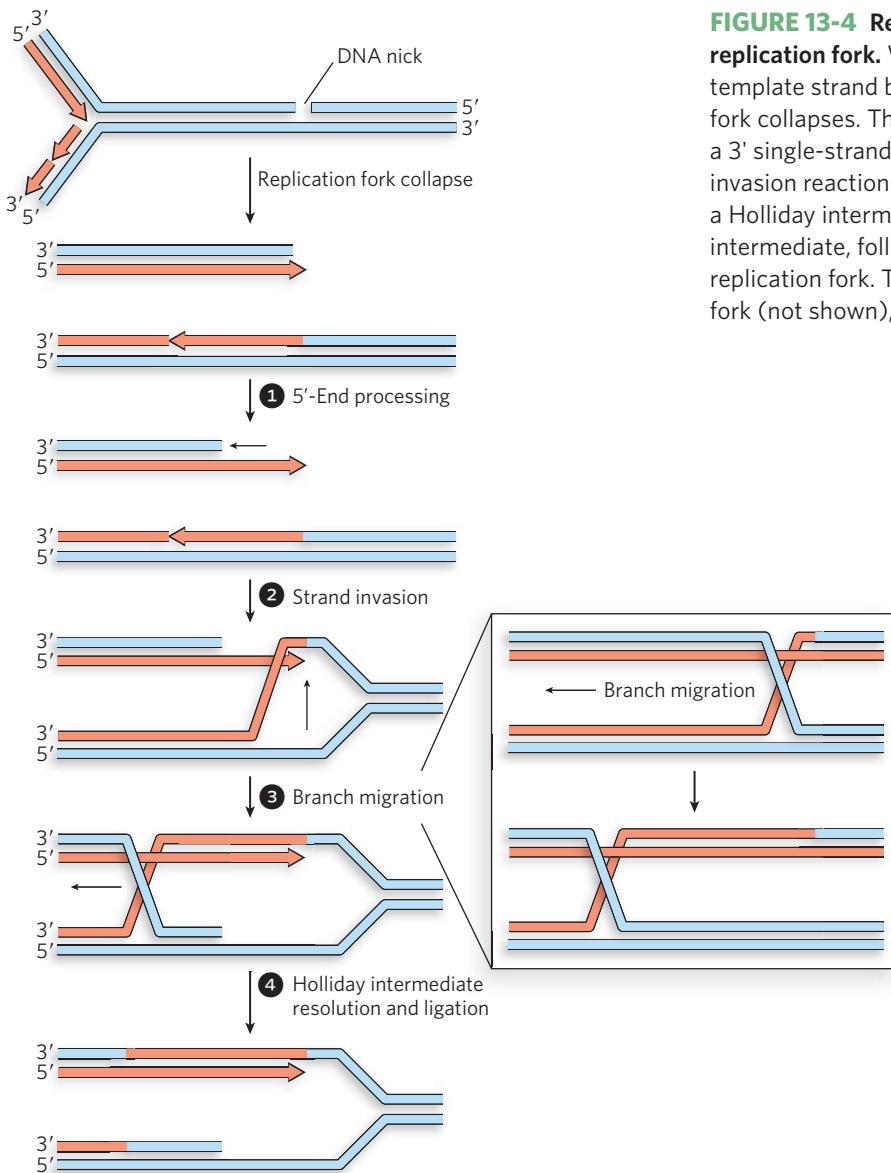


FIGURE 13-4 Recombinational DNA repair at a collapsed replication fork. When a replication fork encounters a template strand break, one arm of the fork is lost and the fork collapses. The 5' end at the break is processed to create a 3' single-stranded extension, which is used in a strand invasion reaction. Migration of the branch (inset) can create a Holliday intermediate. Resolution of the Holliday intermediate, followed by ligation, restores a viable replication fork. The replisome is reloaded onto this restored fork (not shown), and replication continues.

without a complementary strand. Most of the DNA repair processes described in Chapter 12—NER, BER, and mismatch repair—require that the lesion occur in only one strand of a duplex DNA. The lesion is simply removed from the damaged strand, and the undamaged complementary strand guides the replication and ligation needed to fill in the missing nucleotide(s). For a double-strand lesion, the reactions shown in Figure 13-5 are directed at providing a complementary strand to allow repair of the lesion and to complete the process without creating a mutation.

In many cases, branch migration forces the fork backward, a process known as **fork regression** (see Figure 13-5, step 1). As the fork migrates in reverse, the lesion-containing strand is reunited with its original complementary strand, and the newly synthesized

DNA strands are paired with each other. This results in a Holliday intermediate at the point where the four strands intersect. The regression leaves the lesion paired with its undamaged complementary strand, allowing its repair by processes such as NER. If the lesion is repaired, the Holliday intermediate can be resolved by exonucleolytic degradation of the short DNA arm (steps 2 and 3, left). A second method of resolving the Holliday intermediate can occur whether or not the lesion has been repaired. This method entails replication of the short DNA arm, followed by branch migration in the opposite direction (steps 2 and 3, right). Even if the lesion has not been repaired at this point, it is now paired with a complementary DNA strand that can be used as a template for later repair. In either case, replication must be restarted.

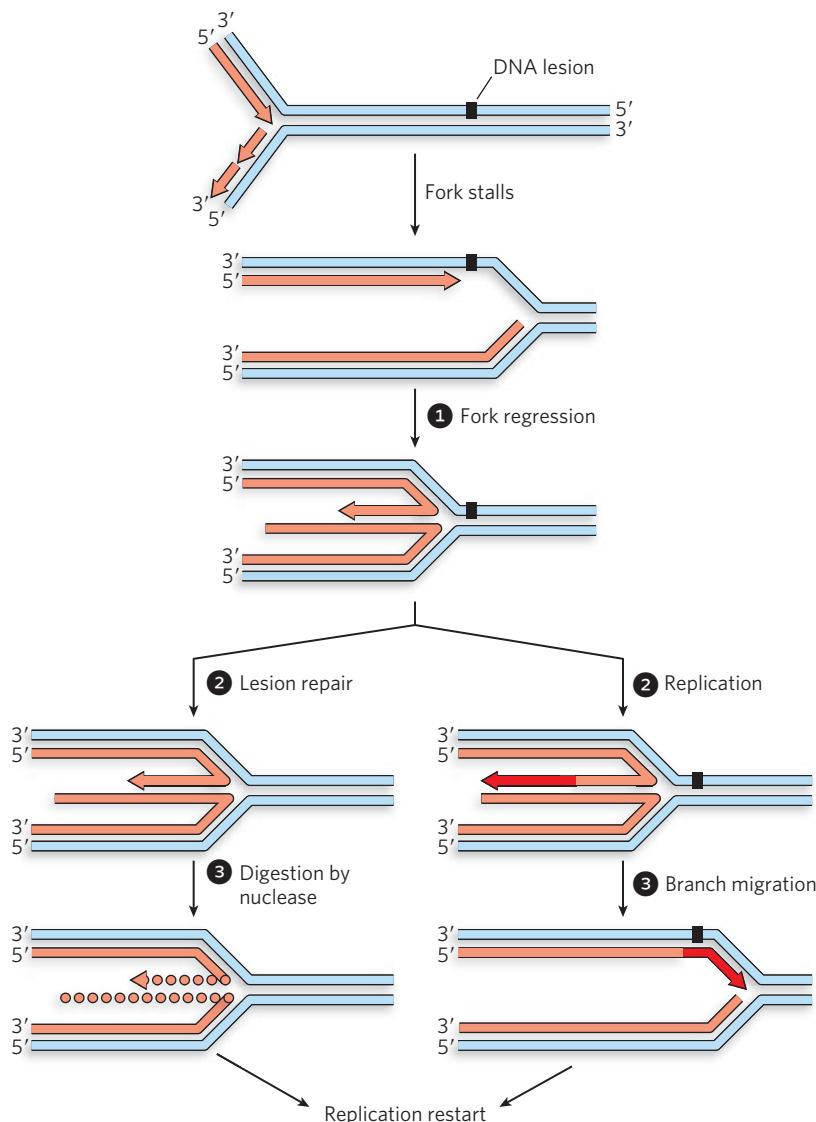


FIGURE 13-5 Repair of a replication fork stalled at a DNA lesion. When a replication fork encounters a blocking lesion in the leading strand, the fork stalls. Fork regression creates a Holliday intermediate and reunites the lesion-containing strand with the complementary template strand with which it was originally paired. Lesion repair can then proceed by the usual pathways. Replication can be restarted by

digestion of the short arm and reloading of the replisome (left). Alternatively, the 3' end of the short arm can be extended to the end of the available template (right). If the lesion is not repaired, branch migration in the direction opposite to fork regression pairs the newly synthesized strand with the lesion. Replication is restarted, and repair can occur later.

Single-Stranded DNA Regions Are Filled In by Gap Repair

In some cases, a DNA lesion is bypassed by the DNA polymerase and left within a single-strand gap, where it is not readily repaired. The repair pathway must then generate an undamaged complementary strand. The gap can be filled in by translesion synthesis (see Figure 12-26). TLS can be mutagenic, however, and is usually not the pathway of first choice. Alternatively,

recombinational DNA repair can again be used, in a variant called **gap repair** (Figure 13-6). In this mechanism, the complementary strand needed for repair of the lesion is found in the undamaged arm of the replication fork.

The steps shown in Figure 13-6 follow a pattern that should now be familiar, with some subtle variations. A recombinase binds to the DNA in the single-strand gap. The recombinase promotes a strand invasion, using the bound single-stranded DNA to

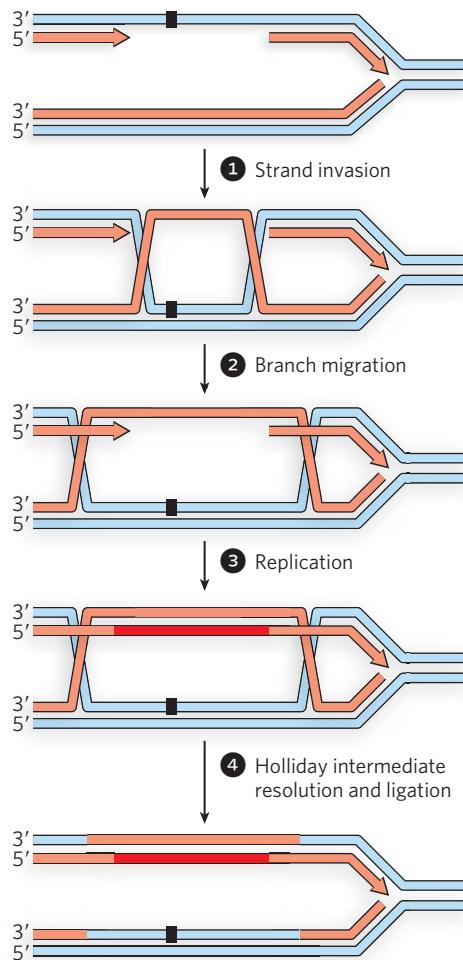


FIGURE 13-6 Repair of a DNA gap after the replication fork bypasses a lesion. If a single-strand gap is left behind by the replication fork, the information for its repair must come from the other side of the fork. A recombinase-mediated strand switch provides an undamaged template strand, which is subsequently used to extend the discontinuous strand in the gap. The process is completed by resolution of the Holliday intermediates and ligation.

invade the undamaged double-stranded DNA on the other side of the replication fork (step 1). In this instance, the invading DNA has no free end. Branch migration (step 2) generates two Holliday intermediates. The displaced strand serves as an undamaged template for replication (step 3). Both Holliday intermediates are then cleaved by Holliday intermediate resolvases, and the nicks are sealed by DNA ligase (step 4). The resolution can result in a crossover; however, many cells have enzymatic systems that direct most resolution events into a non-crossover path. In the end, the strand that had the DNA lesion now has an undamaged complement, and nonmutagenic repair by NER or BER can begin.

The pathways described in Figures 13-4, 13-5, and 13-6 provide responses to almost any deleterious event that can befall a replication fork. Recombinases, acting in concert with DNA polymerases, ligases, Holliday intermediate resolvases, and proteins that promote branch migration, provide a repair system that readily adapts to the variety of stalled or collapsed fork structures that may present themselves. In the next section, we consider these enzymes in more detail.

SECTION 13.1 SUMMARY

- Recombination is a pathway for the repair of single- and double-strand breaks, both of which can arise during replication.
- Double-strand break repair entails the generation of 3' single-stranded extensions at the broken DNA ends, which are then paired with a homologous duplex region in a strand invasion reaction catalyzed by recombinases.
- Many recombination reactions produce a four-armed DNA intermediate called a Holliday intermediate.
- Recombinational DNA repair restores DNA segments with double-strand breaks to their original genomic condition, causing no mutations.
- When a replication fork encounters a single-strand break in the template DNA, the break is converted to a double-strand break, and the replication fork collapses. The fork is then repaired by DSBR, and replication is restarted.
- When a replication fork encounters a nucleotide lesion, it may bypass the lesion through translesion synthesis or it may stall. Stalled forks may be repaired by a pathway involving fork regression.
- When lesions are left in single-strand gaps, recombination provides a nonmutagenic pathway for gap repair.

13.2 Enzymatic Machines in Bacterial Recombinational DNA Repair

The complex pathways shown in Figures 13-2 through 13-6 have been elucidated by genetic studies carried out in bacteria and eukaryotes. Many of the enzymes that promote the various steps have been characterized *in vitro*, and multiple steps of some pathways have been reconstituted with purified enzymes (Table 13-1). Complete reconstitution of a DSB repair or a replication fork repair

Table 13-1 Enzymes/Proteins Involved in Bacterial Recombinational DNA Repair

Enzyme/Protein	Size of Monomer (kDa)	Functional Form	Function
RecA	38	Filament on DNA	Bacterial recombinase
RecB	134	Part of RecBCD heterotrimer	$3' \rightarrow 5'$ helicase, forms 3' strand extensions
RecC	129	Part of RecBCD heterotrimer	Binds chi, forms 3' strand extensions
RecD	67	Part of RecBCD heterotrimer	$5' \rightarrow 3'$ helicase, forms 3' strand extensions
RecF	41	Part of RecFOR mediator complex	Bind DNA, load RecA onto ssDNA gap
RecO	27	Part of RecFOR mediator complex	
RecR	22	Part of RecFOR mediator complex	
RuvA	22	Tetramer	Binds DNA, promotes branch migration
RuvB	37	Hexamer	DNA translocase, promotes branch migration
RuvC	19	Dimer	Resolvase, resolves Holliday intermediate
RecG	76	Monomer	Helicase, promotes fork regression
SSB	19	Tetramer	Binds ssDNA
Pol I	109	Monomer	Fills in gaps
DNA ligase	75	Monomer	Seals nicks

event remains a key goal of the field. In this section we describe the major enzyme systems found in bacteria, before moving on to eukaryotic recombination systems.

RecBCD and RecFOR Initiate Recombinational Repair

In all cells, the process of recombinational DNA repair revolves around a recombinase enzyme. In bacteria, that recombinase is the RecA protein. Before RecA can act, the stage must be set by other enzymes that degrade one strand of DNA, where necessary, and load RecA onto the DNA. The RecBCD and RecFOR complexes, with three subunits each, are the initiators of recombinational DNA repair. RecBCD loads RecA protein onto DNA to repair double-strand breaks, and RecFOR does so to repair DNA gaps.

In *E. coli*, the *recB*, *recC*, and *recD* genes encode the heterotrimeric **RecBCD** enzyme, which has both helicase and nuclelease activities. This enzyme has two jobs: (1) it prepares the 3'-ending single strand by degrading the 5'-ending strand at the site of a DSB, and (2) it

directly loads the RecA protein onto the prepared single-stranded DNA tail. The RecBCD enzyme binds to linear DNA at a free (broken) end and moves inward along the double helix, unwinding and degrading the DNA in a reaction coupled to ATP hydrolysis. The structure of RecBCD reveals a great deal about how the complex works ([Figure 13-7a](#)). The RecB and RecD subunits are helicase motors, with RecB moving in the $3' \rightarrow 5'$ direction along one strand and RecD in the $5' \rightarrow 3'$ direction along the other strand. One domain of the RecB subunit is also a nuclease that initially degrades both DNA strands as they are unwound. The activity of the enzyme is altered when it interacts with a sequence referred to as **chi**, 5'-GCTGGTGG-3', on the 3'-ending strand, which binds tightly to a site on the RecC sub-unit. From that point, degradation of the strand with a 3' terminus is greatly reduced, but degradation of the 5'-ending strand is increased ([Figure 13-7b](#)). This process creates the single-stranded 3' overhang that is required for subsequent steps in recombination. The 1,009 chi sequences scattered throughout the *E. coli* genome enhance the frequency of recombination about

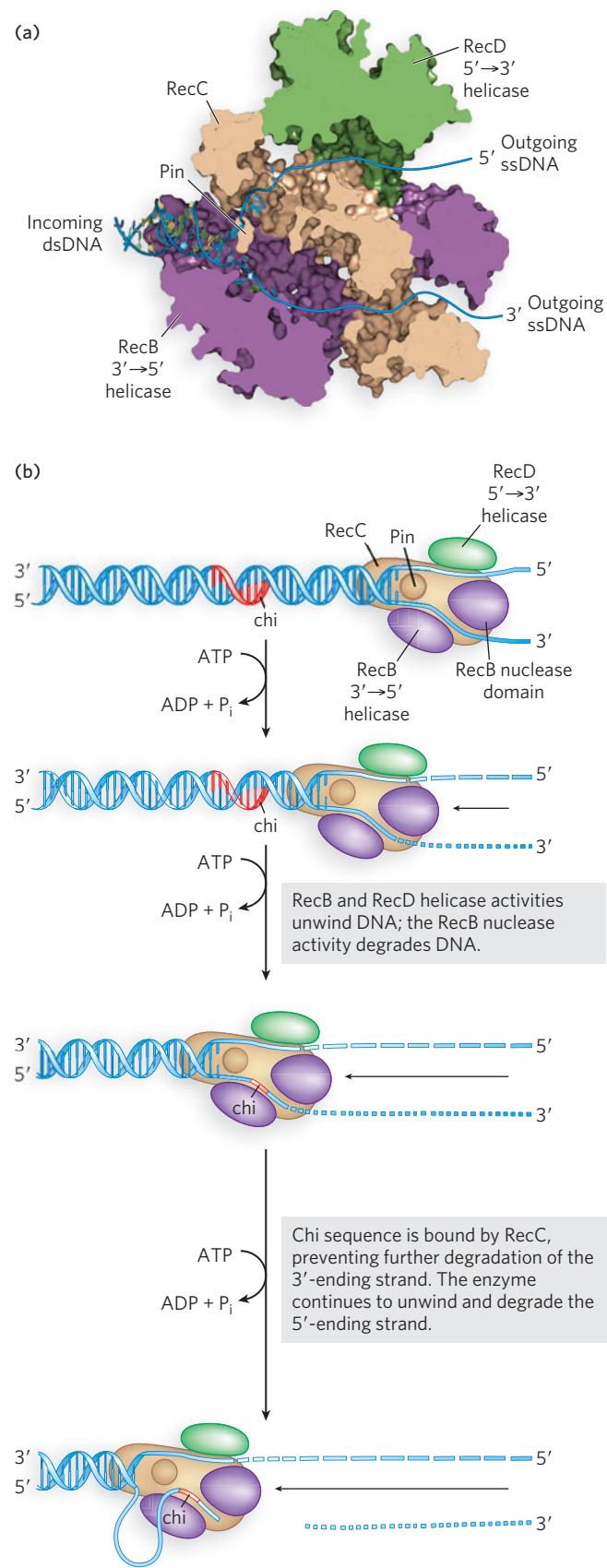


FIGURE 13-7 The RecBCD helicase/nuclease. (a) The structure of the RecBCD enzyme. (b) Activities of the RecBCD enzyme at a DNA end. The RecB and RecD subunits travel along the DNA, a process that requires ATP; RecB degrades both strands as the complex travels, cleaving the 3'-ending strand more often than the 5'-ending strand. The RecC subunit has a protrusion called a pin that facilitates separation of the DNA strands. When a chi site is encountered on the 3'-ending strand, the RecC subunit binds to it, halting the advance of this strand through the complex. Degradation of the 5'-ending strand continues as the 3'-ending strand is looped out, eventually creating a 3' single-stranded extension. RecA protein (not shown) is then loaded onto the processed DNA by the RecBCD enzyme. [Source: (a) PDB ID 1W36. (b) Adapted from M. R. Singleton et al., *Nature* 432:187, 2004.]

fivefold to tenfold within 1,000 bp of the chi site. The chi octamer sequence is greatly overrepresented in the *E. coli* chromosome (relative to a random occurrence of an eight-nucleotide sequence), and the chi sites are not randomly oriented in the DNA. Almost all have the orientation needed to facilitate the repair of DSBs that occur during replication.

As the 3'-ending strand is turned into a single strand, it is rapidly bound by the single-stranded DNA-binding protein (SSB) (see Figure 5-3). This protein directs traffic on segments of single-stranded DNA, blocking the access of some proteins and enzymes and facilitating the access of others. RecA protein is one of the proteins that is blocked, and SSB helps prevent the binding of RecA to single-strand gaps where recombination is not required (such as the transient gaps on the lagging strand during DNA synthesis). When recombinational DNA repair becomes necessary, RecA loading is mainly a process of overcoming this SSB barrier.

The second function of the RecBCD enzyme is as a loading factor for the recombinase, RecA. As the RecBCD enzyme unwinds the DNA and degrades the 5'-ending strand, RecA protein is recruited to the 3'-ending strand by a domain within the RecB subunit. This loading function serves to nucleate the RecA binding that is needed to promote strand invasion.

RecA protein must also be loaded onto single-strand DNA gaps, such as the one shown in Figure 13-6 (top). These gaps are also bound by SSB, blocking access by RecA. In this case, the key loading factors are the RecF, RecO, and RecR proteins, collectively called **RecFOR**. The RecF protein is targeted by an unknown mechanism to the double-stranded DNA immediately adjacent to the gap, on the free 5' end in the discontinuous strand. With the aid of the RecO and RecR proteins,

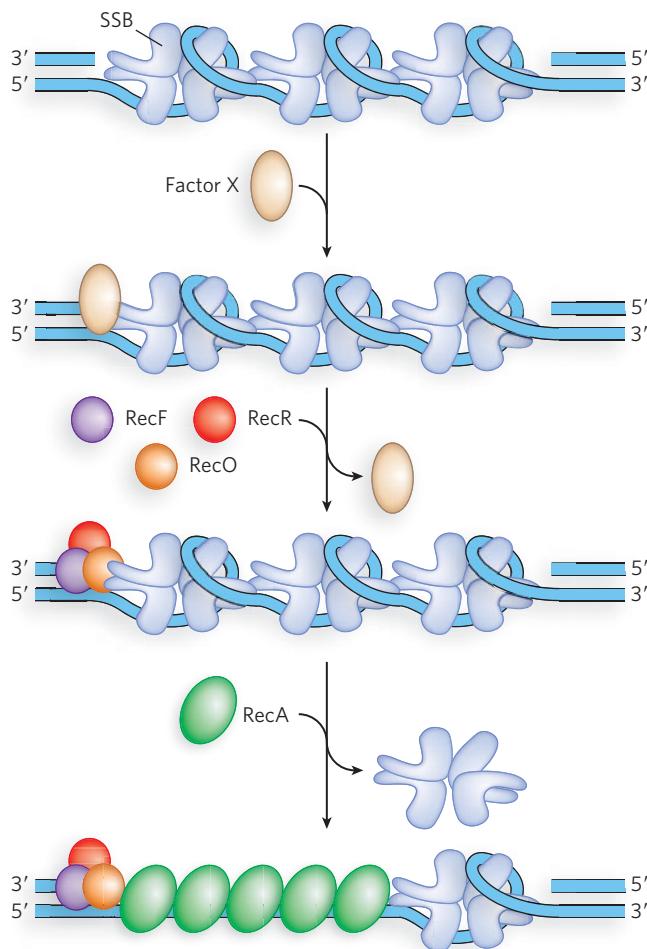


FIGURE 13-8 The role of RecFOR in loading RecA protein onto SSB-coated single-stranded DNA. An unknown targeting factor (X) is postulated to facilitate RecFOR function at gap ends. SSB is displaced, and RecA protein is loaded.

RecA protein is activated to displace SSB and initiate formation of the RecA filament needed for the subsequent phases of repair (Figure 13-8). Proteins that have the unique function of loading other proteins onto DNA are called mediator proteins. The RecFOR proteins are recombination mediators in most bacteria.

RecA Is the Bacterial Recombinase

The bacterial **RecA protein** is the prototype of a class of proteins found in all organisms, from bacteria to humans (see How We Know). The RecA-class recombinases promote the central steps in recombination reactions. They align a single-stranded DNA with a homologous double-stranded DNA and promote a strand switch that pairs the single strand with its complement in the duplex, displacing the other duplex strand. This is the reaction that results in strand invasion.

RecA-class recombinases are unusual among the proteins of DNA metabolism in that their active forms are ordered, helical filaments of up to several thousand subunits that assemble cooperatively (Figure 13-9). RecA binds most readily to single-stranded DNA. The filament forms in two steps, first nucleating on the single-stranded DNA, then growing by the addition of subunits in the 5' → 3' direction. A polar filament is created, with growth occurring at one end and most dissociation occurring at the other, trailing end. It is the filament nucleation step that is blocked by SSB. RecA is a DNA-dependent ATPase. ATP is hydrolyzed by subunits throughout the RecA filament; when hydrolysis occurs in the subunit at the trailing end, it often results in dissociation of that subunit. Once formed, the RecA nucleoprotein filament is ready to promote the DNA strand exchanges at the heart of recombination.

The RecA filament can facilitate strand exchange with a variety of substrates in vitro. The single-stranded DNA may be linear or circular, or a single-strand gap within double-stranded DNA (Figure 13-10a–c). When the exchange is initiated in a DNA gap, branch migration may move the process into the adjacent duplex, where the exchange then involves four strands (Figure 13-10d). The reactions promoted with linear single strands best mimic the strand invasion observed during DSBR, especially when the double-strand breaks occur during replication. The circular single strand, easily purified from certain bacterial viruses, has been particularly convenient for monitoring RecA activity in vitro. The single-strand gap provides a model for reactions occurring during gap repair.

In each scenario, the single strand of DNA is first bound by RecA to establish the nucleoprotein filament. The RecA filament then takes up a homologous double-stranded DNA and aligns it with the bound single strand. After homologous alignment and strand exchange, over a region that can involve hundreds of base pairs, the exchanged region can be further extended in a process that requires RecA-mediated ATP hydrolysis. The exchange occurs at a rate of 6 bp/s at 37°C and progresses in the 5' → 3' direction relative to the single-stranded DNA in the RecA filament. The initial DNA-DNA alignment involves a single strand (in a gap or a single-stranded tail) and a homologous duplex, thus three strands in all. The extension reaction is a kind of facilitated branch migration.

When purified RecA protein promotes DNA strand exchange in vitro between a circular single strand and a linear duplex, the substrates and products have distinctive structures that can be readily separated and visualized by agarose gel electrophoresis (Figure 13-11). The initial pairing of the RecA-bound single-stranded

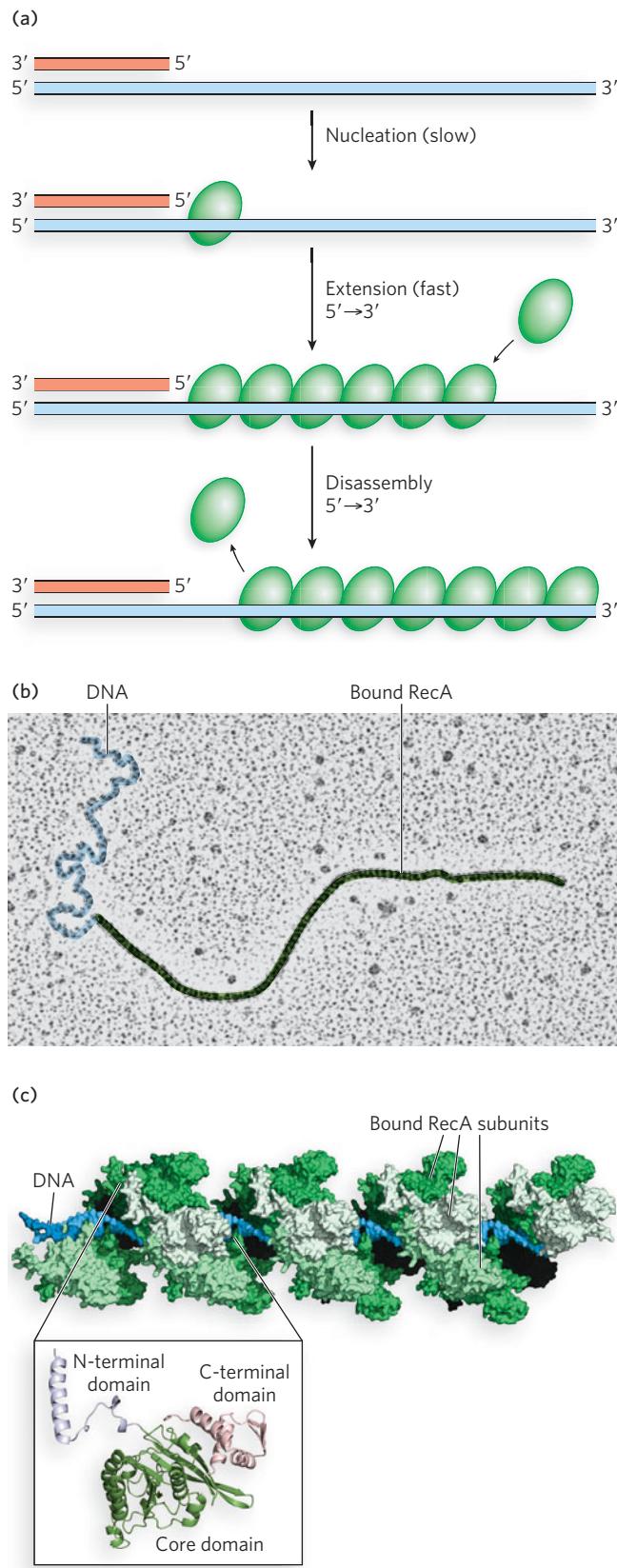


FIGURE 13-9 RecA protein filaments. RecA and other recombinases in this class function as filaments of nucleoprotein. (a) Filament formation proceeds in discrete nucleation and extension steps. Extension occurs by adding RecA subunits so that the filament grows in the 5' → 3' direction. When disassembly occurs, subunits are subtracted from the trailing end. (b) Electron micrograph of a RecA filament bound to DNA. (c) Segment of a RecA filament with four helical turns (24 RecA subunits). Notice the bound double-stranded DNA in the center. The core domain of RecA is structurally related to the domains in helicases. [Source: (b) By permission of the Estate of Ross Inman. Special thanks to Kim Voss. (c) Adapted from PDB ID 3CMX.]

circle and the linear duplex creates a branched DNA intermediate. After a period of facilitated branch migration around the circle, a nicked circular duplex and a displaced linear single strand are formed as reaction products. The initial DNA alignment requires ATP, but not its hydrolysis. The facilitated branch migration necessary to complete exchange is coupled to ATP hydrolysis.

If the DNA circle is gapped (i.e., one strand is discontinuous), the DNA strand exchange is initiated in the single-strand gap, and facilitated branch migration extends it into the double-stranded region (Figure 13-12). Because DNA is a helical structure, continued strand exchange requires an ordered rotation of the two aligned DNAs. The mechanism by which ATP hydrolysis is coupled to facilitated branch migration is not yet understood, but it probably entails the dissociation of RecA subunits at the trailing end of the RecA filament. When bound at a stalled replication fork, RecA protein can promote fork regression, using its capacity to promote branch migration.

RecA Protein Is Subject to Regulation

In principle, RecA-mediated recombination can occur between any two homologous DNA sequences. In every bacterial chromosome, some sequences, such as those that encode ribosomal RNAs, are repeated. Recombination between them could have catastrophic consequences, leading to the deletion or rearrangement of large segments of the chromosome. For this reason, recombination in general and RecA protein activity in particular are highly regulated.

Regulation occurs at three levels: transcription of the *recA* gene, autoregulation, and regulation by other proteins. Transcriptional regulation occurs within the

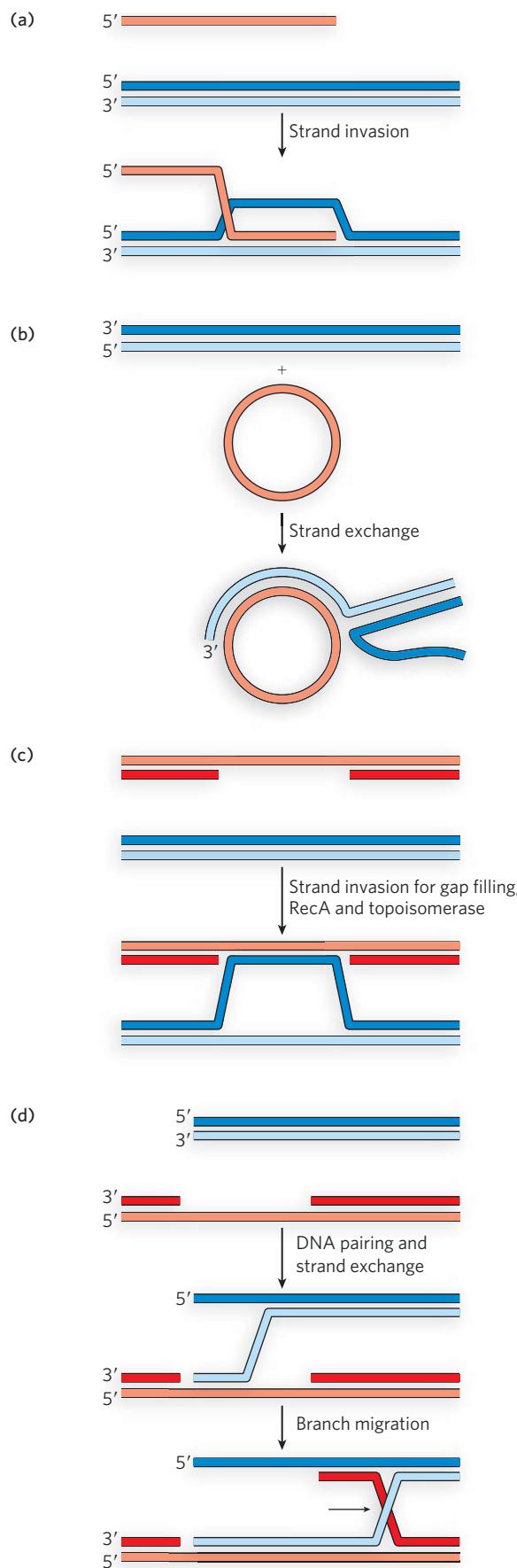


FIGURE 13-10 RecA-mediated DNA strand exchange reactions. Reactions promoted by the RecA protein of *E. coli*.

(a) Strand invasion. The product of this reaction is sometimes called a D-loop. (b) A DNA strand exchange involving three DNA strands. The substrates shown here are often used in research because the products are readily separated and visualized by agarose gel electrophoresis. (c) DNA strand exchange involving a single-strand gap. Reactions of this sort are often facilitated by topoisomerases. (d) Strand exchange involving a single-strand gap and four DNA strands. The reaction begins in the single-strand gap of one strand, and facilitated branch migration carries the reaction into the adjacent duplex.

context of the bacterial SOS response (see Chapter 20). Most regulation at other levels is directed at the formation, disassembly, and function of RecA protein filaments.

Autoregulation is “self” regulation. The RecA protein suppresses its own activities by means of a highly charged C-terminal peptide flap (see Figure 5-20). Removal of just 17 amino acid residues from the RecA C-terminus creates a RecA species for which almost all activities are enhanced. For example, whereas filament nucleation of native RecA protein is blocked by SSB, a C-terminal deletion mutant of RecA protein readily displaces SSB without the aid of RecBCD or RecFOR. In the mutant cell, this can result in elevated levels of recombination.

Many other proteins play a role in the regulation of RecA protein. As we’ve already seen, the RecBCD and RecFOR complexes facilitate the RecA filament nucleation process. Reliance on these loading functions helps direct RecA filament formation to DNA regions where it is needed. Another regulatory protein, RecX, binds to the growing RecA filament end and halts filament extension. A protein called DinI binds along the RecA filament and stabilizes it, while at the same time limiting the DNA strand exchange process. The helicase UvrD actively removes RecA filaments from the DNA when they are no longer needed. These and other proteins, working as an integrated system, help limit RecA function and direct it toward particular repair requirements.

Multiple Enzymes Process DNA Intermediates Created by RecA

RecA is not the only protein in a bacterial cell that can promote branch migration; other enzyme systems are specialized for that task. As one example, the processing of Holliday intermediates is facilitated by a complex called RuvAB (repair of UV damage). Up to two RuvA protein tetramers bind to a Holliday intermediate and form a complex with two RuvB hexamers

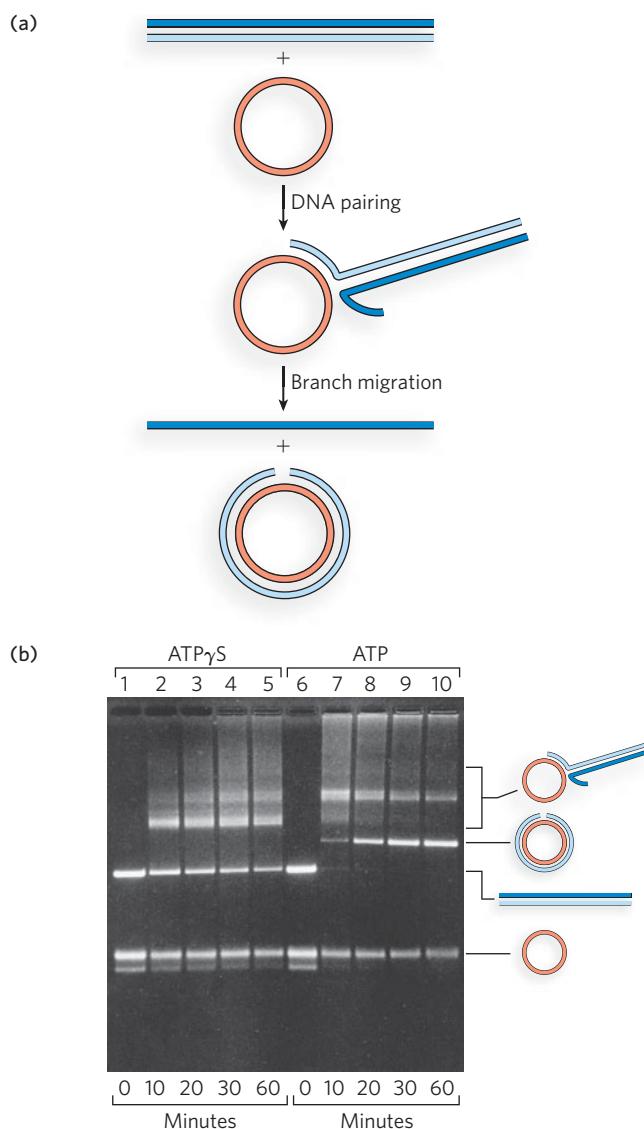


FIGURE 13-11 ATP-dependent RecA-mediated strand exchange. (a) The DNA substrates, intermediates, and products of the DNA strand exchange reaction. The circular DNAs, derived from bacteriophages, have a total length of 5,000 to 8,000 nucleotides or base pairs. With DNAs of this size, the reaction time is measured in tens of minutes at 37°C. (b) Intermediates (as diagrammed to the right of the agarose gel) generated in the reaction. No branch migration occurs when ATP γ S, a nonhydrolyzable ATP analog, replaces ATP (lanes 1 to 5). A complete reaction requires ATP hydrolysis (lanes 6 to 10). [Source: (b) Courtesy of Mike Cox.]

(Figure 13-13). The donut-shaped RuvB hexamers surround two of the four arms of the Holliday intermediate. RuvB is a DNA translocase, related in structure and function to hexameric DNA helicases. The DNA is propelled outward through the hole in the donut, away from the junction, in a reaction coupled to ATP

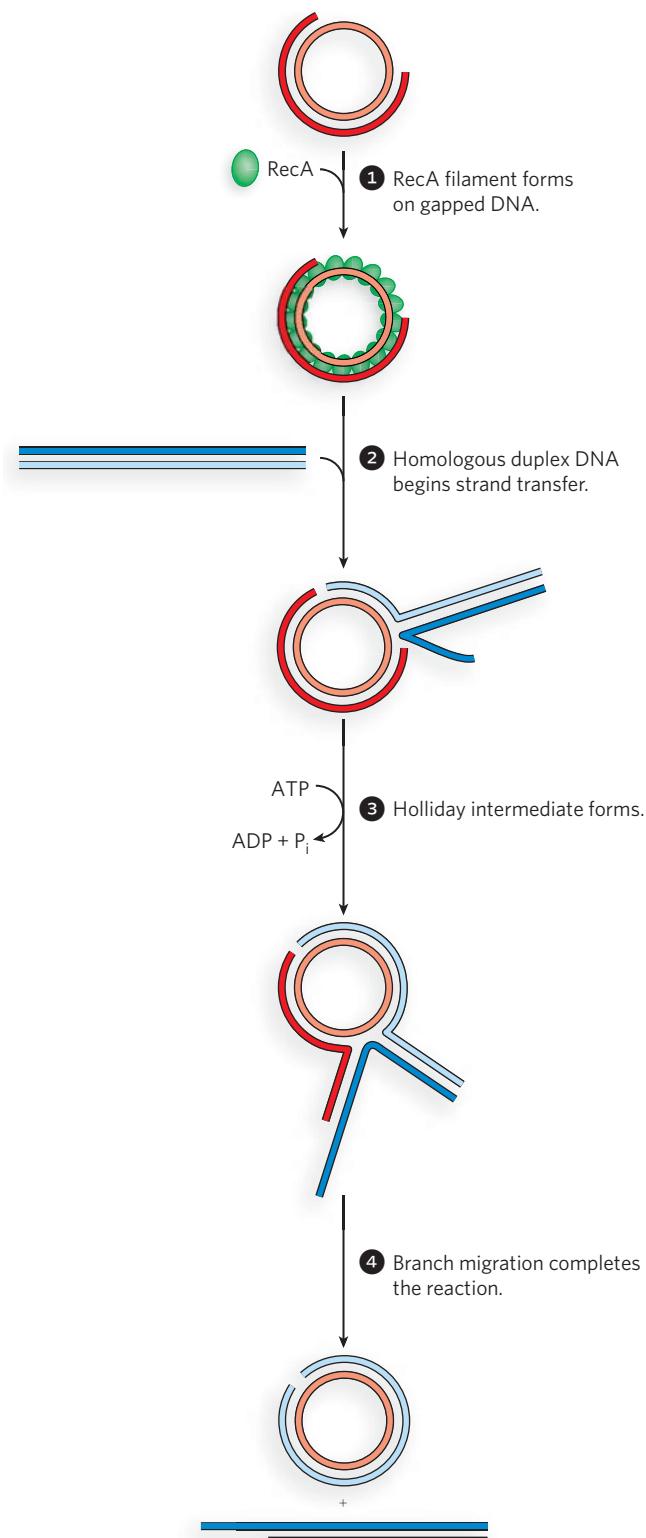


FIGURE 13-12 Steps in a RecA-mediated DNA strand exchange reaction.

hydrolysis. The result is a very rapid branch migration that can move the position of the Holliday intermediate by thousands of base pairs in a few seconds. The RuvAB complex moves the Holliday intermediate away from

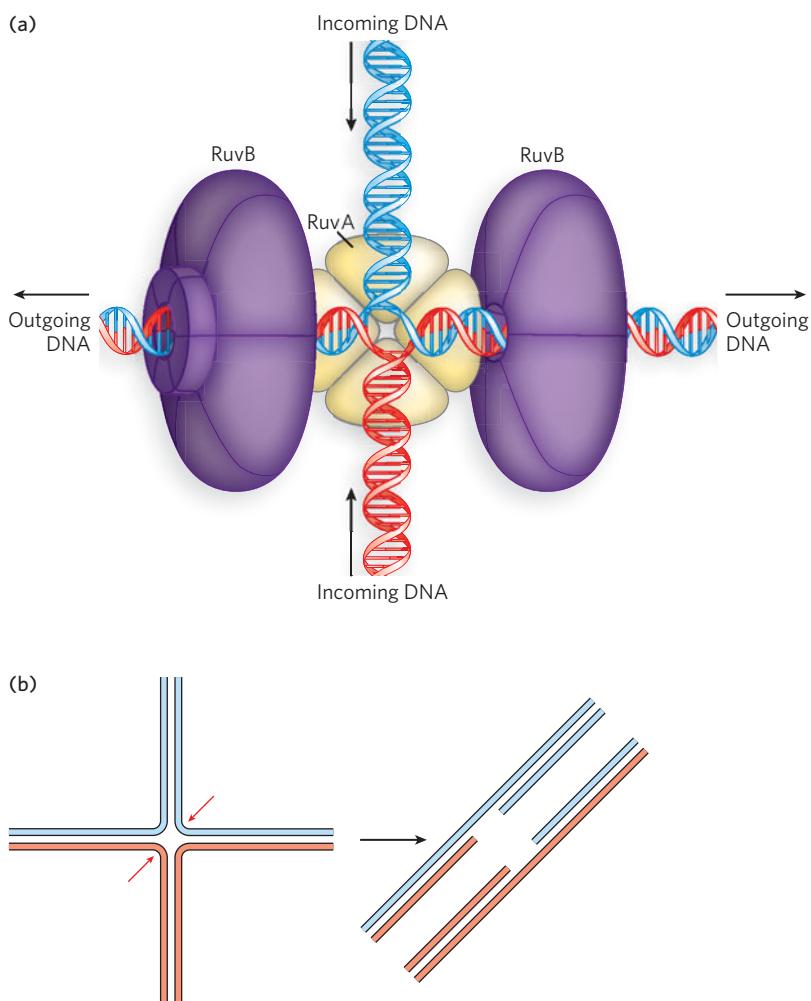


FIGURE 13-13 Catalysis of DNA branch migration and Holliday intermediate resolution by the RuvA, RuvB, and RuvC proteins. (a) RuvA binds to a four-armed junction

(Holliday intermediate) and forms a complex with RuvB, a hexameric DNA translocase, on two sides. (b) RuvC cleaves Holliday intermediates (red arrows).

the region of damaged DNA and recruits RuvC, a Holliday intermediate resolvase. RuvC replaces one of the RuvA tetramers at the junction and cleaves strands in opposing arms of the Holliday intermediate to resolve it into viable chromosomal products. The nicks in the DNA products are sealed by DNA ligase.

The bacterial recombination systems that repair replication forks can also repair DSBs created by ionizing radiation, sometimes with startling proficiency. The bacterium *Deinococcus radiodurans* can survive and prosper after absorbing doses of ionizing radiation sufficient to generate thousands of DSBs (Highlight 13-1).

The repair of stalled or collapsed replication forks is generally followed by a restart of replication. A five-protein complex called the restart primosome loads the replicative helicase, DnaB, onto the DNA at the reconstituted replication fork. The rest of the replisome assembles around DnaB, and replication starts anew.

Repair of the Replication Fork in Bacteria Can Lead to Dimeric Chromosomes

Some pathways of replication fork repair lead to the creation of a Holliday intermediate behind the reconstituted fork (Figure 13-14). This Holliday intermediate can be resolved by RuvC in either of two ways: by cleaving the crossover strands (shown as path X in Figure 13-14) or by cleaving the template strands (path Y). One resolution, cleaving the crossover strands, simply leads to the completion of replication and the segregation of two monomeric chromosomes into daughter cells. The other resolution is to cleave the template strands. This has a special consequence in the circular bacterial chromosome: it ultimately creates a single dimeric chromosome that cannot be segregated at cell division. Under normal growth conditions, this outcome is observed in about 15% of cells in an *E. coli* culture.

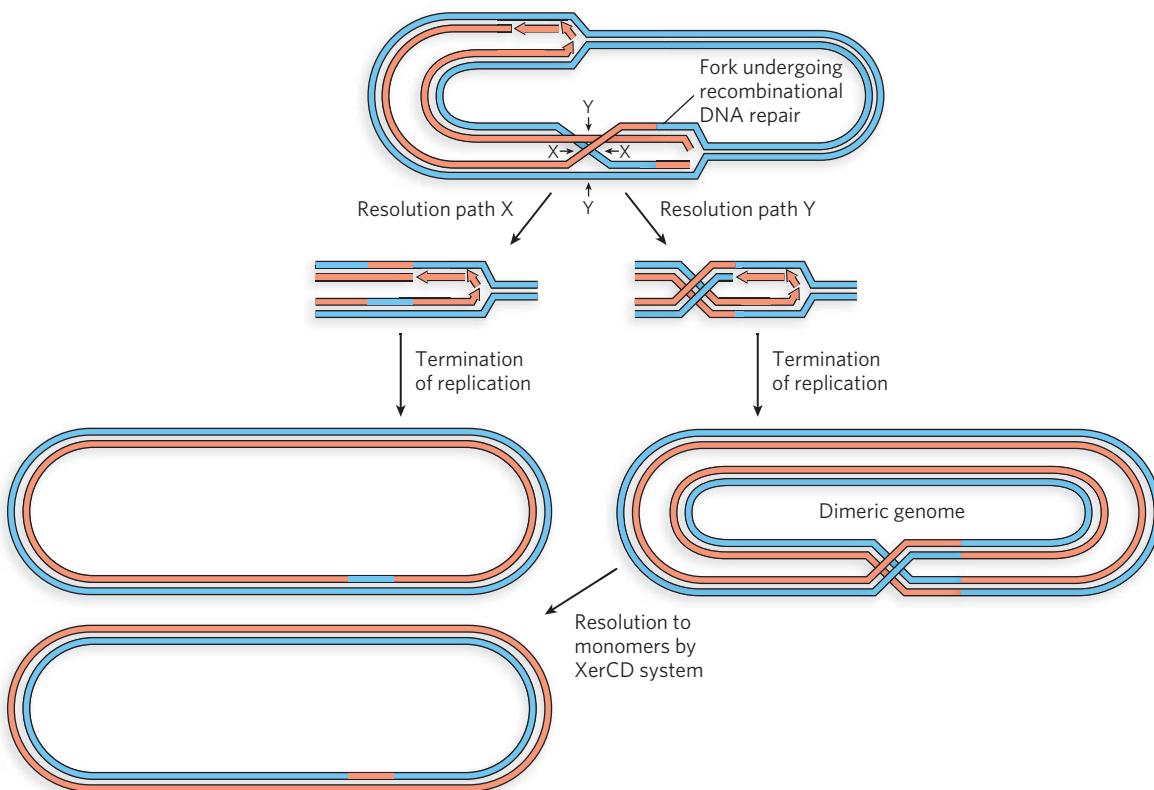


FIGURE 13-14 The generation and resolution of dimeric chromosomes formed during bacterial replication. There are two ways to resolve any Holliday intermediate. In path X, the crossover strands are cut and ligated to form two separate

chromosomes. In path Y, the crossover strands are cut and ligated to form a contiguous, dimeric form of the circular chromosome, when replication is complete.

Cells harboring dimeric chromosomes do not die. Instead, the stalled chromosomal segregation is detected, triggering the activity of a specialized site-specific recombination system, called XerCD, that converts the dimer back into monomeric circles. Site-specific recombination is a class of reaction we'll discuss

in Chapter 14. Once the monomeric chromosomes are generated, cell division completes normally. If the cells have a mutation that inactivates the site-specific recombination system, cells with dimeric chromosomes become “stuck,” unable to divide (Figure 13-15). For these cells, the mutation is lethal.

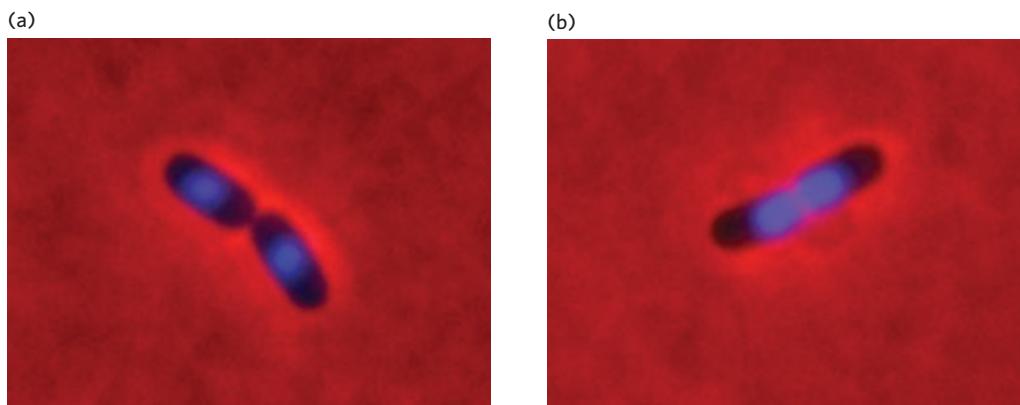


FIGURE 13-15 Cell division is hindered in bacterial cells lacking the capacity to resolve dimeric chromosomes.

(a) Wild-type *E. coli* cells immediately after cell division.
 (b) Mutant *E. coli* cells with a mutation that inactivates the dimeric chromosome resolution system, after chromosomal

division. In both photos, the chromosomes were condensed by treatment with chloramphenicol and stained with a blue fluorescent dye. [Source: Courtesy of David Sherratt, University of Oxford.]

HIGHLIGHT 13-1 EVOLUTION

A Tough Organism in a Tough Environment: *Deinococcus radiodurans*

Some radioactive isotopes, such as ^{60}Co and ^{137}Cs , emit a type of ionizing radiation called γ rays. Gamma rays are photons; they transmit energy to atoms in solution, generating ions that include the highly reactive hydroxyl radical. In a living cell exposed to γ rays, any molecule can be damaged, including proteins and DNA. Double-strand breaks are included in the carnage.

The energy deposited by electromagnetic radiation is measured in rads or gray (1 Gy = 100 rads). For a human cell, a dose of 2 Gy is lethal. In the 1950s, it became clear that some organisms are surprisingly resistant to radiation. For example, in efforts to use radioactive sources to sterilize food, some sealed food samples were spoiled by bacterial action even after exposure to γ radiation at levels up to 4,000 Gy. The culprit was a pink, non-spore-forming, nonmotile bacterium eventually named *Deinococcus radiodurans*. *D. radiodurans* can absorb the damage inflicted by γ irradiation at 5,000 Gy with no lethality. A dose of this kind causes substantial damage even to a Pyrex beaker. More relevant to the cell, a 5,000 Gy dose produces many hundreds of DSBs, in addition to thousands of single-strand breaks and other lesions.

The *Deinococcus* genome consists of four circular DNA molecules, all generally present in multiple copies. After γ irradiation, the cells stop growing and DNA repair begins. Overlapping DNA fragments are spliced together, and the entire genome is accurately reconstituted within a few hours. The cells begin to grow and divide again as if nothing had happened. It is perhaps the most remarkable feat of DNA repair we know of so far.

This process is demonstrated in the gel shown in Figure 1. Following various treatments, *D. radiodurans* genomic DNA was isolated, treated with a restriction enzyme, and subjected to pulsed field gel electrophoresis (see Chapter 7). In cells grown under normal conditions, this procedure yields the series of large DNA fragments shown in the second lane of the gel (the first lane consists of markers of known molecular weight). Immediately after γ irradiation

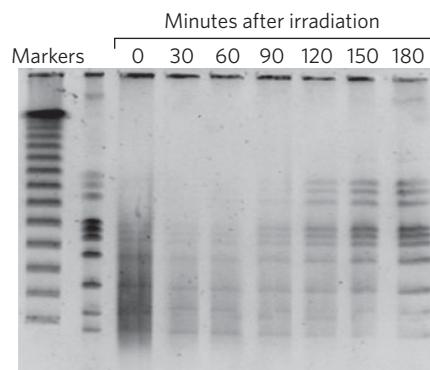


FIGURE 1 DNA from irradiated *D. radiodurans* is initially fragmented, but after several hours of repair regains its normal banding pattern. [Courtesy of John R. Battista, Louisiana State University, Baton Rouge.]

at 5,000 Gy, this banding pattern disappears, replaced by a smear of randomly sized, smaller DNA fragments. Over the next 3 to 4 hours, the normal band pattern reappears as the genome is accurately reconstituted.

Genome reconstitution in *D. radiodurans* is recombinational DNA repair on a massive scale. The *Deinococcus* RecA protein (DrRecA) plays a key role, and most of the radiation resistance of this organism disappears if DrRecA is inactivated. There are two stages of repair, each requiring about 90 to 120 minutes under optimal conditions. The first stage uses a process similar to SDSA, but with an extended phase of replication (extended synthesis-dependent single-strand annealing, ESDSA); the ends of the broken DNA fragments are processed, generating 3' extensions (Figure 2a). These are then used in strand invasion reactions. The 3' ends act as primers for extended DNA synthesis, using a homologous chromosome strand as the template. After dissociation, the long 3' extensions are annealed to each other where complementarity exists. The process is completed by nuclease treatment and ligation, as needed. The second stage uses RecA protein to carry out a larger-scale splicing of chromosomal segments (Figure 2b).

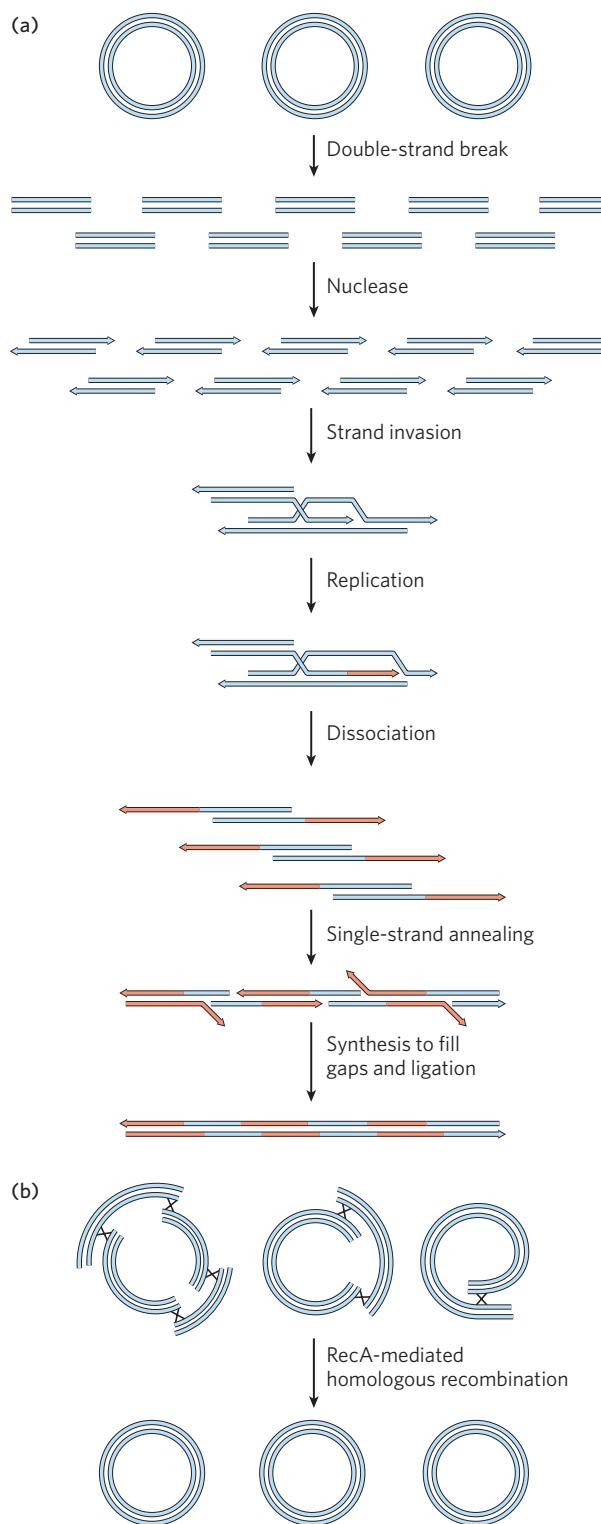


FIGURE 2 (a) The first stage of double-strand break repair in *D. radiodurans* closely resembles synthesis-dependent strand annealing (SDSA). (b) In the second stage of genomic reconstitution, large chromosomal segments are spliced together by DrRecA protein. [Source: Adapted from M. Radman et al., *Nature* 443:569–573, 2006.]

For several decades, *D. radiodurans* was considered the most radiation-resistant organism known. Recent research, however, has revealed many microbial species that are highly resistant to radiation, some of them more so than *D. radiodurans*. In addition, these highly resistant species are found in various unrelated genera, indicating that this phenotype has evolved independently many times. There are no environments on Earth that are subject to ionizing radiation at levels of thousands of gray, but these bacteria are not aliens from outer space. A few species have been found in hot springs with high radon backgrounds, where exposure to chronic low levels of radiation has forced adaptations to permit more efficient DNA repair. However, the most reliable source of bacteria with extreme radiation resistance is a desert environment, where the major selective pressure is not radiation but desiccation. When water disappears for an extended period of time, most metabolic processes, including generation of the ATP needed for DNA repair, cease. However, spontaneous DNA damage continues, and DSBs are among the accumulating lesions. The extreme radiation resistance of *D. radiodurans* and many other bacteria reflects their extraordinary capacity for rapid genomic reconstitution when desiccation gives way to conditions favorable for growth.

SECTION 13.2 SUMMARY

- The bacterial RecBCD and RecFOR complexes provide pathways for loading RecA protein onto single-stranded extensions (at double-strand breaks) or onto single-strand gaps, respectively.
- The bacterial RecA protein is the prototypical recombinase, forming a filament on single-stranded DNA and promoting strand invasion reactions.
- Recombination is a highly regulated process, and the RecA protein is the major target for regulation. Regulation occurs through transcriptional regulation, autoregulation, and regulation by other proteins.
- Recombination intermediates generated by RecA protein are processed by enzymes such as the RuvA, RuvB, and RuvC proteins.
- In the circular bacterial chromosome, resolution of Holliday intermediates associated with replication fork repair can lead to the formation of dimeric chromosomes.

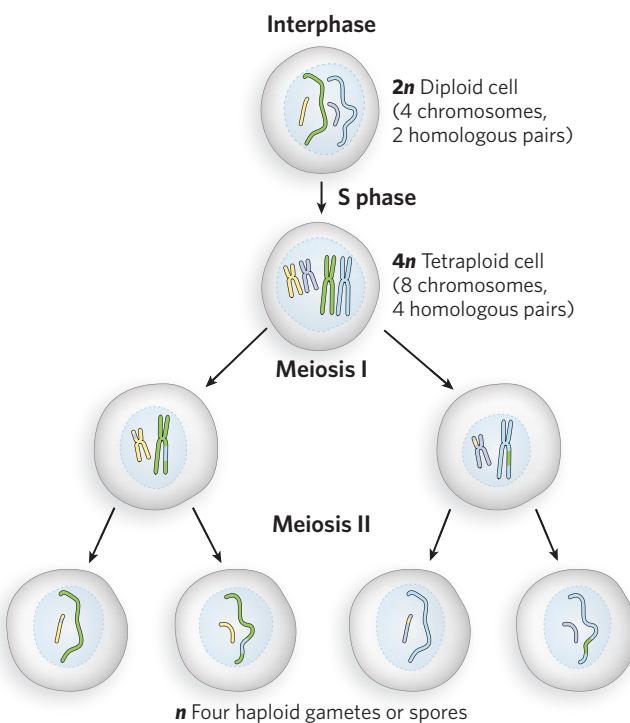


FIGURE 13-16 An outline of eukaryotic meiosis. DNA is replicated in S phase. For the two pairs of chromosomes shown here, this generates four sets of sister chromatids. The homologs are aligned in meiosis I, joined by recombination (crossovers), then segregated. In meiosis II, the sister chromatids segregate into the haploid cell products. (See also Figure 2-12, right.)

13.3 Homologous Recombination in Eukaryotes

With the evolution of more complex cells and organisms, recombination systems underwent adaptation to new functions. One well-studied example is meiotic recombination. The diploid germ-line precursor cell has two complete sets of chromosomes, or two genome equivalents. The two copies of each eukaryotic chromosome—the homologous chromosomes, or **homologs**—generally have the same genes distributed along their lengths, in the same order. However, the genes of the two homologs are not identical. The alleles inherited from each parent are often slightly different, distinguished by mutations and even by small insertions and/or deletions.

During meiosis, each pair of homologs is replicated to create four chromosome copies (two copies, or chromatids, per homolog). (See Chapter 2 for a review of meiosis and the stages of the cell cycle.) Homologous sister chromatid pairs are aligned during the first meiotic prophase (prophase I). The four sets of chromosomes are then segregated through two cell divisions (meiosis I and meiosis II) to create four haploid gametes (or spores in lower eukaryotes such as fungi), each with a complete (haploid) chromosomal complement (Figure 13-16). Homologs segregate in the first cell division, and sister chromatids segregate in the second.

The proper segregation of chromosomes when eukaryotic cells divide requires the attachment of spindle fibers to the centromeres. Chromosomes are then drawn

toward opposite poles into what will eventually become two new cells. If replicated chromosomes were scattered randomly about, and spindle fibers were attached randomly to the chromosomes, segregation of the chromosomes to daughter cells would be equally random. A given cell might receive two copies of some chromosomes and no copy of others. To segregate a complete set of chromosomes to each cell, some organizational accounting is required. Chromosome pairs are lined up and linked together in meiosis I. When spindles at opposite poles attach to the centromeres of a linked pair of chromosomes and start to pull, tension is created. This tension, sensed by a mechanism not yet understood, indicates that this pair of chromosomes is properly aligned for segregation. Once the tension is sensed, the links between the chromosomes are gradually removed so that segregation can proceed. If an improper attachment of spindle fibers occurs (e.g., if the two centromeres in the chromosome pair are linked to spindle fibers emanating from the same pole), a cellular kinase senses the lack of tension and activates a system that removes the spindle attachments, allowing the cell to try again.

A physical link between chromosomes destined to be segregated is a requirement for any orderly cell division. During mitosis, and in meiosis II, the sister chromatids produced by replication are segregated. The physical link is provided by cohesins (see Figure 9-25). Cohesins are deposited along the chromosomes as replication proceeds, ensuring that only homologous sister chromatids are linked together. These cohesin-based links ensure accurate chromosomal segregation by the spindle fibers. However, the segregation of homologs during meiosis I is an event unique to meiosis. The homologs to be segregated are not related by a recent replication event in which cohesins were deposited, and thus some other molecular device is needed to ensure that only homologous chromosomes are linked. The eukaryotic answer to this problem is homologous recombination, a process that relies on closely related sequences in the two chromosomes. Recombinational crossovers provide for the accurate alignment of homologs at the metaphase plate during meiosis I (Highlight 13-2). Many of the same enzymes are involved in both recombinational DNA repair during replication and meiotic recombination. The process again begins with DSBs, but in meiosis I, the breaks are programmed events.

Eukaryotic recombination is not restricted to meiosis. Less frequent recombination events occur during mitosis, at least some of which are associated with the

repair of stalled or collapsed replication forks. In addition, recombination systems have been appropriated to carry out some highly directed exchanges of genetic information between different segments of chromosomes, as we'll discuss later.

Meiotic Recombination Is Initiated at Double-Strand Breaks

Following the premeiotic S-phase replication cycle, homologous chromosomes are brought together. As the cell enters meiosis, in early prophase I (Figure 13-17), DSBs are introduced at multiple locations along one chromatid of each chromatid pair. The breaks are not random, and yet are not entirely predictable. Certain chromosomal sites, often referred to as hot spots, are much more likely to undergo a break than others. The hot spots are defined by features of chromosome structure that have not been entirely characterized. An open chromatin configuration, active transcription, and G≡C-rich sequences can all affect the process.

A protein called **Spo11** (so named because inactivation of this protein causes defects in yeast sporulation), closely related to eukaryotic type II topoisomerases, catalyzes formation of DSBs (Figure 13-18a; see How We Know). Spo11 is found in all eukaryotes. Acting as a dimer, it uses an active-site Tyr residue as a nucleophile in a transesterification reaction (Figure 13-18b).

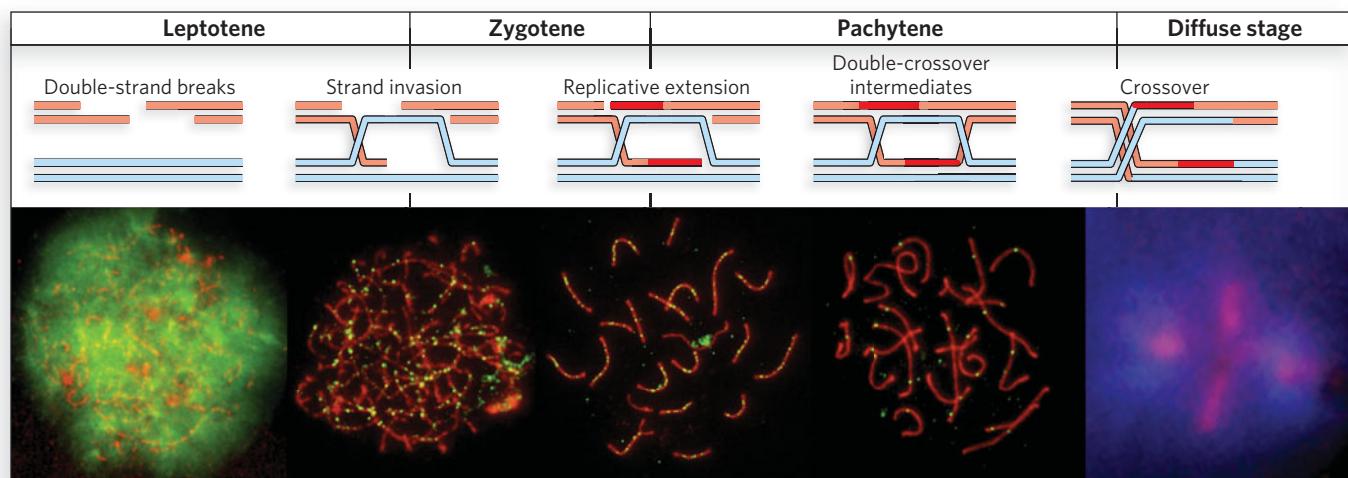


FIGURE 13-17 Homologous genetic recombination during meiotic prophase I. Prophase I includes a directed recombination process. The double-strand breaks are created and processed early. Strand invasions and replicative extension produce double-crossover intermediates, and some of these mature into crossovers. Shown here are the early stages of meiosis in mouse spermatocytes. Chromosomes are stained red, and a few recombination proteins, fused to

green fluorescent protein, appear as green foci. As prophase progresses, the homologous chromosome pairs, at first diffuse, become tightly aligned, producing rodlike structures. The chromosomes again become diffuse as prophase ends and the cells begin the first cell division. Leptotene, zygote, and pachytene are terms used to describe the subphases of meiosis prophase I. [Source: Adapted from A. McDougall, D. J. Elliott, and N. Hunter, EMBO meeting report, Jan. 14, 2005, Fig. 2.]

HIGHLIGHT 13-2 MEDICINE

Why Proper Chromosomal Segregation Matters

When chromosomal alignment and recombination are not correct and complete in meiosis I, segregation of chromosomes can go awry. Aneuploidy, a condition in which a cell has the wrong number of chromosomes, can result. The haploid products of meiosis (gametes or spores) may have no copies or two copies of a chromosome. When a gamete with two copies of a chromosome undergoes fertilization, cells in the resulting embryo are trisomic for (have three copies of) that chromosome.

In *S. cerevisiae*, aneuploidy resulting from errors in meiosis occurs at a rate of about 1 in 10,000 meiotic events. In fruit flies, the rate is about 1 in a few thousand (see Figure 2-15). Rates of aneuploidy in mammals are considerably higher. In mice, the rate is 1 in 100, and it is even higher in other mammals. The rate of aneuploidy in fertilized human eggs has been estimated as 10% to 30%, mostly with monosomies or trisomies. This is almost certainly an underestimate. Most trisomies are lethal, and many result in abortive miscarriage long before the pregnancy is detected. This is the leading cause of pregnancy loss. The few trisomic fetuses that survive to birth generally have three copies of chromosomes 13, 18, or 21 (trisomy 21 is Down syndrome). Abnormal complements of the sex chromosomes are also found in the human population. Almost all monosomies are fatal in the early stages of fetal development. The societal consequences of aneuploidy in humans are considerable. Aneuploidy is the leading genetic cause of developmental and mental disabilities. At the heart of these high rates is a feature of meiosis in female mammals that has special significance for the human species.

In a human male, germ-line cells begin to undergo meiosis at puberty, and each meiotic event requires a relatively short period of time. In contrast, meiosis in the germ-line cells of human females is a highly protracted process. The production of an egg begins with the onset of meiosis in the fetus, at 12 to 13 weeks of gestation. This initiation of meiosis occurs in all the developing fetal germ-line cells over a period of a few weeks. The cells proceed through much of meiosis I. Chromosomes line up and generate crossovers, continuing just beyond the pachytene phase (see Figure 13-17)—and then

the process stops. The chromosomes enter an arrested phase called the dictyate stage, with the crossovers in place, a kind of suspended animation where they remain as the female matures—for anywhere between 13 and 50 years. It is not until sexual maturity that individual germ-line cells continue through the two meiotic cell divisions to produce egg cells.

Between the onset of the dictyate stage and the final completion of meiosis, something can happen that disrupts or damages the crossovers linking homologous chromosomes in the germ-line cells. As a woman ages, the rate of trisomy in the egg cells she produces increases, dramatically so as she approaches menopause (Figure 1). There are many hypotheses on why this occurs, and several different factors may play a role. However, most of the hypotheses are centered on recombination crossovers in meiosis I and their stability over the protracted dictyate stage.

It is not yet clear what medical steps could be taken to reduce the incidence of aneuploidy in females of child-bearing age. What is revealed is the inherent importance of recombination and crossover generation in human meiosis.

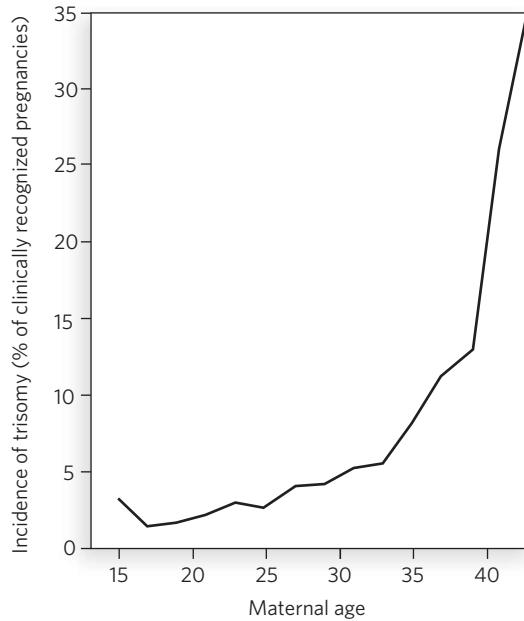


FIGURE 1 The increasing incidence of trisomy with increasing age of the mother. [Source: Adapted from T. Hassold and P. Hunt, *Nat. Rev. Genet.* 2:280–291, 2001.]

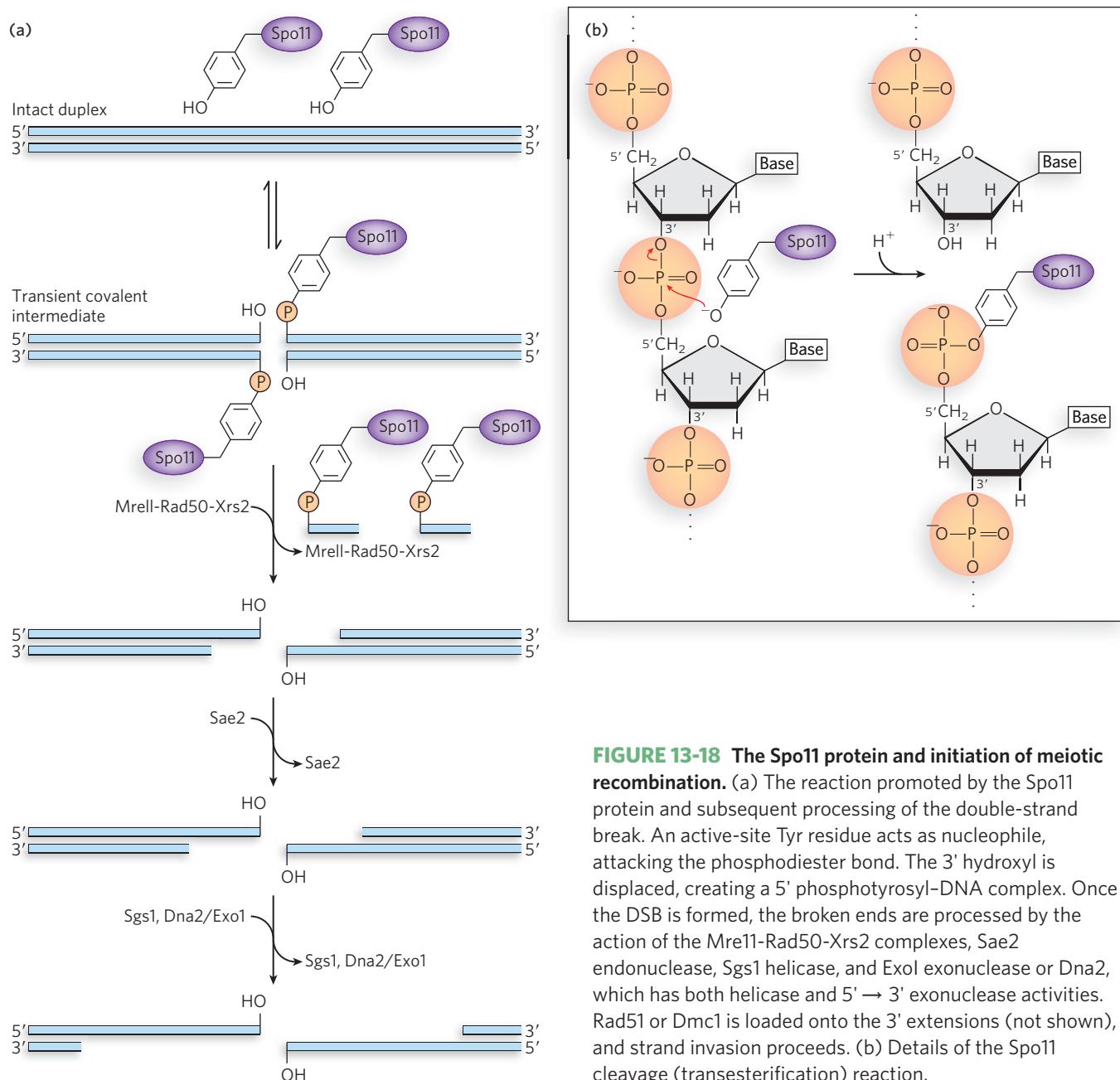


FIGURE 13-18 The Spo11 protein and initiation of meiotic recombination. (a) The reaction promoted by the Spo11 protein and subsequent processing of the double-strand break. An active-site Tyr residue acts as nucleophile, attacking the phosphodiester bond. The 3' hydroxyl is displaced, creating a 5' phosphotyrosyl-DNA complex. Once the DSB is formed, the broken ends are processed by the action of the Mre11-Rad50-Xrs2 complexes, Sae2 endonuclease, Sgs1 helicase, and Exo1 exonuclease or Dna2, which has both helicase and 5' → 3' exonuclease activities. Rad51 or Dmc1 is loaded onto the 3' extensions (not shown), and strand invasion proceeds. (b) Details of the Spo11 cleavage (transesterification) reaction.

Each subunit cleaves one DNA strand, with the phosphodiester bond replaced by a 5'-phosphotyrosyl linkage. The reaction halts at this point, and Spo11 does not carry out the additional steps of a topoisomerase reaction. A dozen or more additional proteins may cooperate in the formation of an active Spo11 complex on the DNA, and in processing the DNA after it is cleaved.

To remove Spo11 from the DNA and initiate nucleolytic degradation of the 5'-ending strand, a complex of proteins consisting of Mre11 (*meiotic recombination*), Rad50 (*radiation sensitive*), and Xrs2 (*x-ray sensitive*) binds to each Spo11 complex and cleaves the DNA

on the 3' side of Spo11, liberating the linked protein along with a short segment of the attached DNA strand. The nuclease Sae2 degrades the DNA a bit more. Three other enzymes, the helicase Sgs1 and the nucleases Dna2 or Exo1, have been implicated in the more extended degradation of the 5' ends to create long 3' single-stranded regions (see Moment of Discovery for a description of experiments leading to the discovery of the role of Sgs1 in this process). The single-stranded regions are bound by RPA, the eukaryotic single-stranded DNA-binding protein. Aided by mediator proteins, two RecA-class recombinases called **Dmc1**

(disrupted meiotic cDNA) and **Rad51** are loaded, perhaps asymmetrically, onto the 3' extensions on either side of the double-strand break. Dmc1 and Rad51 are the eukaryotic counterparts to RecA. They exhibit sequence and structural homology to RecA and, like RecA, they form extended nucleoprotein filaments on the DNA. The site is now set up for recombination.

Meiotic Recombination Is Completed by a Classic DSBR Pathway

Meiotic recombination is a directed process, which is slowly yielding its secrets to intensive research. The primary goal of recombination is to create physical links, or crossovers, between chromosomes. The formation of crossovers is very tightly regulated so that at least one crossover is created in every pair of homologs. At the same time, a process of interference, not yet understood, ensures that the total number of crossovers for each pair is limited. Where several crossovers occur between the same homologs, they are spaced far apart on the chromosomes. Once the DSBs have formed, subsequent recombination is regulated to occur almost exclusively between homologs rather than between sister chromatids.

The DSBs and Holliday intermediates have two possible fates, only one of which leads to a crossover. In one pathway, the invading strand dissociates and pairs with its complement on the other side of the break. Further extension and ligation complete the process. This pathway is equivalent to SDSA. No link between the two chromosomes is created and no crossover occurs. However, even this process is not genetically neutral. Homologous regions may have different alleles of the

same gene, with small base-pair differences. If the region where the two single strands are paired contains one of these base-pair differences, the resulting duplex will have a mismatch. This is typically repaired by the cellular mismatch repair system to create a normal base pair. The base on one strand or the other must be changed, and genetic information is lost on the changed strand. This type of outcome is referred to as **gene conversion**. It represents one byproduct of recombination not only during meiosis but also during the recombinational repair of double-strand breaks and single-strand gaps in other contexts.

In the alternative pathway, strand extension leads to displacement of one strand of the invaded duplex, and this strand eventually pairs with the other side of the DSB (Figure 13-19). DNA ligation creates a double Holliday intermediate, and thus a link between the homologs. As meiosis proceeds, the double Holliday intermediate is cleaved by resolvases (similar to the bacterial RuvC) with the potential to create a crossover. The crossover is embedded in a proteinaceous structure called a chiasma (see Figure 2-18). The overall pathway is closely related to that in a breakthrough model that first described meiotic recombination as a process of DSBR, proposed by Jack Szostak, Terry Orr-Weaver, Rodney Rothstein, and Frank Stahl in 1983.

Crossovers have two roles in meiosis. The first is to create the physical link essential for proper chromosomal segregation. The second role is a genetic one. Following segregation, the sister chromatids (now daughter chromosomes) are no longer identical. One end of at least one of each set of paired chromatids has been exchanged with the homolog, generating genetic diversity.

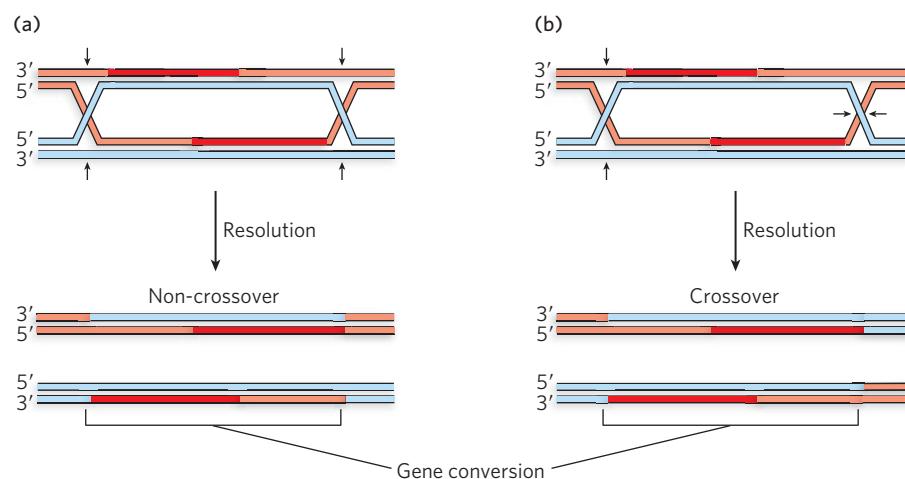


FIGURE 13-19 The two possible fates of double-strand breaks in meiosis. (a) Recombination with no crossover but some potential for gene conversion. (b) Recombination

leading to a crossover. Gene conversion can occur in some regions between the two Holliday intermediates.

Meiotic Recombination Contributes to Genetic Diversity

Meiosis, then, is not simply a mechanism for reducing a diploid genome to a haploid genome in the eukaryotic gamete (or spore). Meiosis increases the genetic diversity in a population by shuffling the genome in two ways. The first is independent assortment of unlinked genes (**Figure 13-20**). As a cell goes through meiosis I, homologous chromosomes are segregated to the daughter cells. The chromosomes assort independently. If a cell inherits chromosome 1 from a given parent, that has no bearing on which version of chromosome 2 is inherited. Genes on a given chromosome are linked and are likely to be inherited together, but genes on different chromosomes are unlinked.

Recombination during meiosis I makes an additional contribution to the genetic diversity of the gametes. Crossovers between homologs in prophase I shuffle the alleles on individual chromosomes. Each gamete ends up with one complete genomic complement, with all genes present. However, given the variation of crossover locations and the unpredictability of independent assortment, the resulting gamete population includes individual cells that may contain virtually any combination of alleles derived from each parent.

Recombination during Mitosis Is Also Initiated at Double-Strand Breaks

Double-strand breaks occur more rarely during mitosis, and recombination is correspondingly less frequent. The breaks can result from endonucleolytic action or exposure to ionizing radiation. However, the most common source of DSBs in mitosis is the encounter of a replication fork with a template strand break (see Figure 13-1). When this occurs, a cellular checkpoint is activated (a signaling pathway) that halts the progression of the cell cycle, and recombinational DNA repair processes are initiated. Mitotic recombination is generally limited to the S and G₂ phases of the cell cycle, when sister chromatids are present. Recombination between homologous chromosomes in diploid cells is much less frequent—probably a simple matter of the physical distance between homologs.

The pathways in mitotic recombination are similar to those in meiotic recombination (**Figure 13-21**). When a DSB is generated by ionizing radiation, it can be repaired by two main pathways. The break is processed by nucleases to generate 3' single-stranded tails, which are coated with RPA. With the aid of recombination mediator proteins, the recombinase is loaded onto the single-stranded DNA to promote strand invasion of a double-stranded homologous chromosome, usually

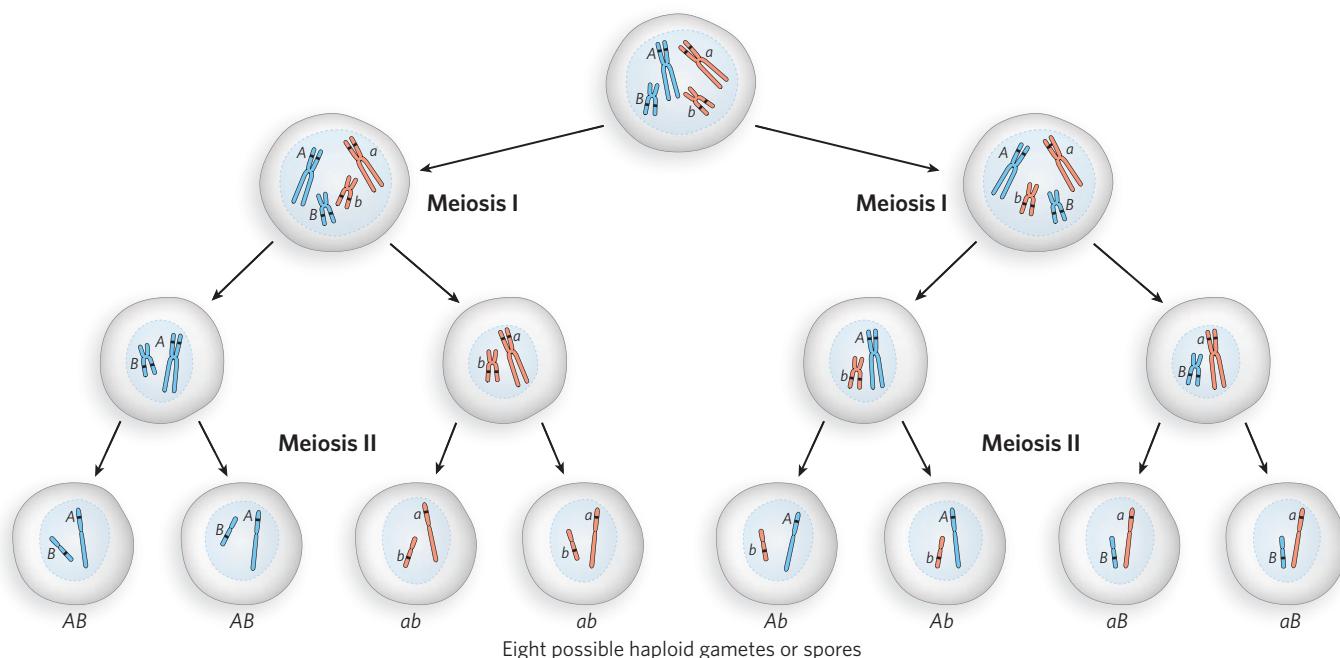


FIGURE 13-20 The contribution of independent assortment to genetic diversity. Much genetic diversity comes from the independent assortment of chromosomes during meiosis. Blue and red distinguish the chromosomes

inherited from each parent. One gene on each chromosome is highlighted, with different alleles (A or a; B or b) in the homologs. Independent assortment can lead to gametes with any combination of alleles on each chromosome.

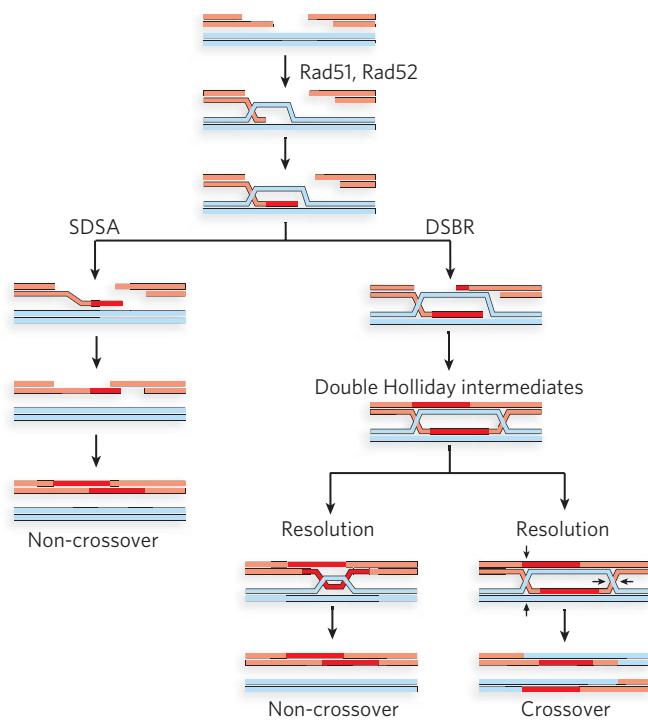


FIGURE 13-21 Mitotic recombination. The most common path of mitotic recombination is SDSA (left). After strand invasion and strand extension, the invading strand is displaced. It can then anneal with its complement on the other side of the original double-strand break. Replication, nucleolytic trimming as needed, and ligation complete the process. Crossovers occur in recombination events in the DSBR pathway (right). Rad52 protein may be involved in initiation of the second strand invasion that leads to the double Holliday intermediate. [Source: Adapted from Y. Liu and S. C. West, *Nat. Rev. Mol. Cell Biol.* 5:937, 2004.]

the sister chromatid (less frequently the homolog). The Dmc1 recombinase is specific to meiosis, so mitotic recombination relies entirely on Rad51. Recombination mediators include the Rad52 protein in all eukaryotes, and the **BRCA2** protein (*breast and ovarian cancer type 2 susceptibility protein*) in vertebrates. These proteins load Rad51 onto RPA-coated single-stranded DNA and may have additional functions. Humans with a defect in the *BRCA2* gene or certain defects in the gene for Rad52 have a predisposition to breast cancer and several other cancers. This provides a vivid illustration of the importance of mitotic recombination to maintenance of the genome.

Programmed Gene Conversion Events Can Affect Gene Function and Regulation

Saccharomyces cerevisiae is a single-celled eukaryote, familiar to bakers and brewers and the thousands of scientists who have adopted this yeast as a model

organism (see the Model Organisms Appendix). It can live with a haploid genome complement or as a diploid. Both forms are stable and both reproduce by mitosis, with daughter cells budding off mother cells. However, the haploid and diploid forms can also be interconverted under the right conditions. Haploid cells exist in two forms called **mating types**, designated **a** and **α**. A haploid cell can mate with another haploid cell of the opposite mating type (**a** with **α**, or **α** with **a**), creating a stable diploid. When conditions are unfavorable for growth, diploid cells can undergo meiosis, resulting in four haploid spores (two **a** spores and two **α** spores). The spores represent a dormant state that can survive stressful environmental conditions. When conditions improve, the spores can begin growing as haploid cells.

The mating type of a haploid cell is determined by two genes expressed at a single locus called **MAT** (Figure 13-22). If the **MAT** locus is **MAT^a**, the cell will have the **a** mating type. If the **MAT** locus is **MAT^α**, the cell will have the **α** mating type. An unusual mechanism of gene regulation governs this system, with recombination at its core.

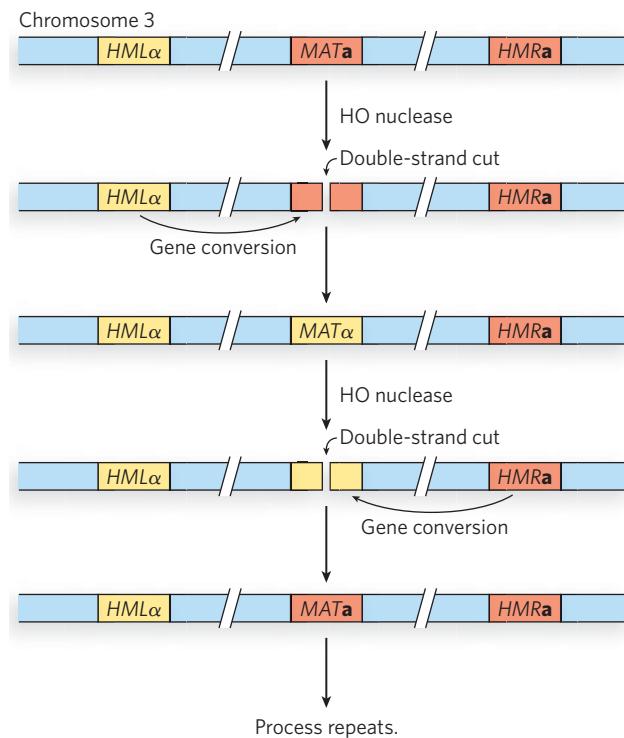


FIGURE 13-22 The mating-type loci in *Saccharomyces cerevisiae*. The genetic information expressed at the **MAT** locus on chromosome 3 determines mating type (**a** or **α**) in yeast. That information can be changed by recombination-mediated gene conversion, moving information (normally silent) that is stored at the **HML^a** and **HMR^a** loci.

Haploid cells can switch mating types, and they do so as often as every cell generation. The switch requires a directed recombination reaction initiated by a double-strand break. The *MAT* locus is cleaved by a nuclease called HO, and recombinational repair of the resulting DSB is directed by recombination with one of two mating-type donor sites (see Figure 13-22). The *HML α* locus has 700 bp of α -specific genetic information, and the *HMR α* locus has 650 bp of α -specific genetic information. (The locus acronyms are derived from *homothallic locus left* and *homothallic locus right*.) The genes at *HML α* and *HMR α* are not expressed; these loci serve only as a silent reservoir of genetic information used to change the information at the *MAT* locus by gene conversion.

The mating-type switch is a classic example of recombinational DNA repair of a double-strand break by the SDSA pathway (Figure 13-23). If the cell is initially the \mathbf{a} mating type, *MAT \mathbf{a}* is cleaved by HO, the free DNA ends are processed to generate 3' single-stranded overhangs, and the Rad51 protein binds to the overhangs and directs DNA strand invasion at a homologous part of the *HML α* locus. As the invading 3' end is extended by a DNA polymerase, the α mating-type information in *HML α* is copied. The \mathbf{a} mating-type information at the *MAT* locus is removed by nuclease digestion. Once the extending strand is long enough, it dissociates, the end is paired with a homologous gene segment in the original *MAT* locus, and the DNA gap is filled in and ligated. *MAT \mathbf{a}* is thus switched to *MAT α* . Switching from *MAT α* to *MAT \mathbf{a}* is essentially the same reaction, except that the strand invasion occurs at the *HMR α* locus.

Similar gene conversion mechanisms are surprisingly common, found in many bacteria and single-celled eukaryotes. Examples important to medicine can often be found in pathogens that are able to evade the human immune system. For example, the bacterium *Neisseria gonorrhoeae* is the agent that causes gonorrhea. The immune response to the pathogen is largely directed at antigens in the bacterial pili, cellular projections involved in adhesion to host cells and other bacteria. The bacterium can evade the host's immune system by alternately expressing different pilin genes, through antigenic variation. Switching from the expression of one pilin gene to another proceeds by a process very similar to the mating-type switch in yeast.

The use of recombination to switch expression between different sets of genes has a major advantage over the more common types of gene regulation described in Chapters 19–22. It is absolute. The mating-type locus of yeast is either \mathbf{a} or α , never an intermediate. The template donor genes are not expressed at all because they are not present in the gene expression

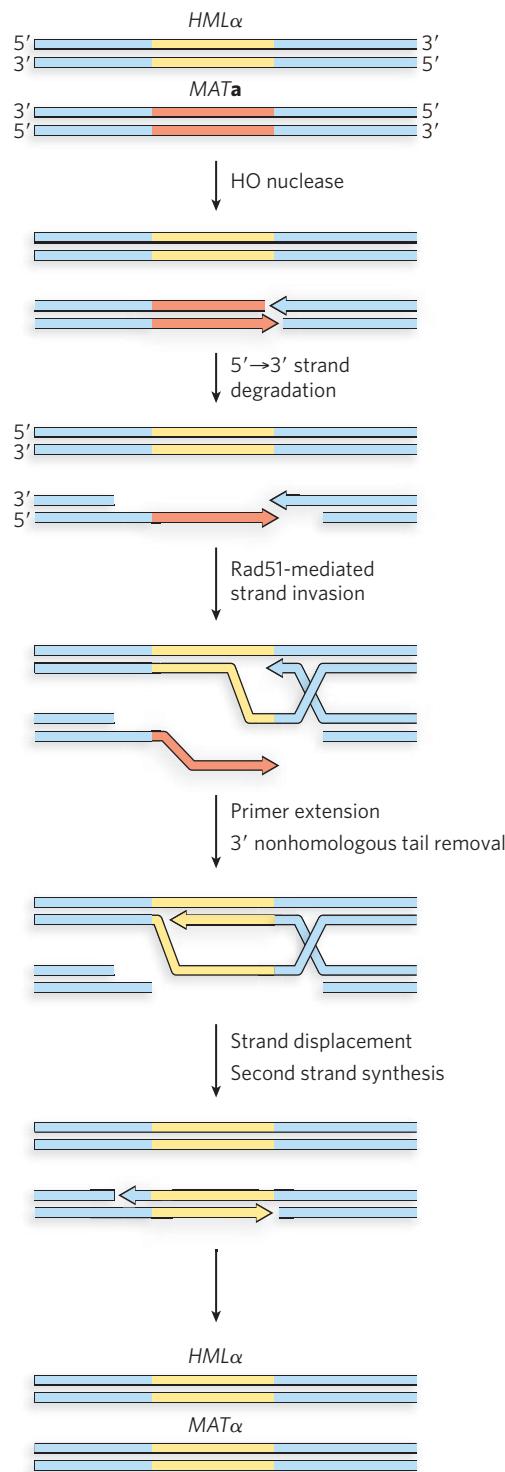


FIGURE 13-23 Pathway of the mating-type switch in yeast.

The double-strand break is introduced by the nuclease HO. The subsequent strand invasion and other steps closely resemble the SDSA pathway. The *MAT \mathbf{a}* information is converted to *MAT α* information by the resulting gene conversion.

locus. For a pathogen, even low levels of leaked expression of the wrong genes could undermine its strategy for circumventing the host's immune system.

Some Introns Move via Homologous Recombination

Some of the introns that are found in many eukaryotic (and a few bacterial) genes have the interesting property that they can move—from one gene to another copy of the same gene that lacks that particular intron. In this way, an intron that becomes associated with a specific gene can rapidly spread through a population. The movements occur whenever genetic transfers bring chromosomes together from two different sources, such as during fusion of gametes at fertilization.

The movement can occur in several ways, but the mobile introns of the group I class (see Chapter 16) use recombinational DNA repair of a targeted double-strand break (Figure 13-24). In brief, the introns

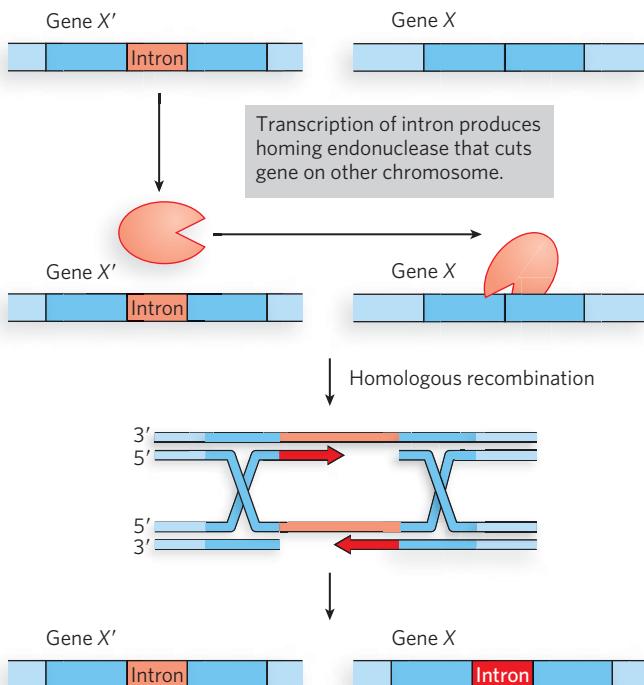


FIGURE 13-24 The homing pathway of some group I introns. In this pathway, the gene with the intron (X') and the same gene without the intron (X) are present in the cell, and an intron-encoded endonuclease creates a double-strand break in the uninterrupted gene (X). The break triggers recombinational DSBR similar to that outlined in Figure 13-2, using the intron-containing gene (X') as a template for repair. When the repair is complete, both genes have a copy of the intron. [Source: Adapted from B. S. Chevalier and B. L. Stoddard, *Nucleic Acids Res.* 29:3757–3774, 2001.]

encode a **homing endonuclease**, an enzyme that cleaves a specific sequence in any copy of the host gene that lacks the intron. The resulting DSB is repaired by recombination with the gene that *does* have a copy of the intron.

Homing endonucleases have been widely used in biotechnology. They function much like restriction enzymes (see Chapter 7), but they recognize and cleave sequences that are much larger, 12 to 40 bp, and asymmetric. These sites occur very rarely in genomes. If a site recognized by one of these enzymes is engineered into a chromosome or viral DNA, it can be reproducibly cleaved without affecting other genomic sequences.

SECTION 13.3 SUMMARY

- During meiosis, recombination generates crossovers that create a physical link between homologous chromosomes just before the first meiotic cell division.
- Meiotic recombination events are initiated at programmed double-strand breaks, and most proceed by synthesis-dependent strand annealing.
- Meiotic recombination in eukaryotes makes an important contribution to the generation of genetic diversity in a population.
- Some meiotic recombination events do not generate crossovers, but result in a more subtle exchange of genetic information known as gene conversion.
- Mitotic recombination is rarer and is also initiated at DSBs.
- Directed homologous recombination promotes a mating-type switch in yeast and can promote antigenic variation in some pathogens.
- Some group I introns migrate by means of recombinational DSBR.

13.4 Nonhomologous End Joining

Recombination provides for an accurate restoration of broken chromosomes, a considerable virtue given the importance of maintaining genomic integrity. However, recombination is complicated and requires the action of dozens of proteins. Sometimes DSBs occur when recombinational DNA repair is not feasible, such as during phases of the cell cycle when no sister chromatids are present. Another path is needed at these times to avoid the cell death that would result from a broken chromosome. That alternative is provided by

nonhomologous end joining (NHEJ). The broken chromosome ends are simply processed and ligated back together.

Nonhomologous End Joining Repairs Double-Strand Breaks

Nonhomologous end joining is an important pathway for double-strand break repair in all eukaryotes, and it has also been detected in some bacteria. In general, the importance of NHEJ increases with genomic complexity. Only a few bacteria seem to have NHEJ systems. In yeast, most DSBs are repaired by recombination, and only a few by NHEJ. In mammals, many DSBs occurring outside meiosis are repaired by NHEJ. These patterns reflect differences in cellular lifestyles. In all eukaryotic cells, recombinational DNA repair is the preferred DSBR pathway during the S and G₂ phases of the cell cycle, when chromosomes are being replicated and paired prior to cell division. Finding a homolog to direct the repair process by recombination is readily accomplished at these times. NHEJ is critical to the repair of DSBs that arise during the G₁ and the static G₀ phases of the cell cycle, when homologous chromosomes are not readily aligned. Differentiated mammalian cells may divide rarely, if at all, and typically spend much more time in the G₁ and G₀ phases than do yeast cells, which may divide every few hours. When DSBs occur during these phases, the enzymes that promote NHEJ are rapidly activated.

Unlike homologous recombinational repair, NHEJ does not conserve the original DNA sequence. When a DSB occurs during G₁ or G₀, a protein complex forms at each broken end of the chromosome, and the two DNA-protein complexes associate to form a DNA synapse. Synapsis activates a protein kinase and helicase activity within the protein complex. The subsequent DNA unwinding may produce a short, 1 to 6 bp region of microhomology that is presumably needed for end joining. Any flaps of single-stranded DNA can be trimmed by nucleases, gaps filled by DNA polymerase, and nicks sealed by DNA ligase.

NHEJ is a mutagenic process, and a smaller genome, such as that of yeast, has relatively little tolerance for the loss of information. The small genomic alterations may be tolerable in mammalian somatic cells, however, because they are not in the germ line and the mutations will not be inherited, and they are balanced by the undamaged information on the homolog in each diploid cell. Indeed, the propensity of NHEJ to create mutations has led to its being recruited in somatic cells as a source of variation in the production of genes encoding antibodies (see Chapter 14).

Table 13-2 Enzymes Involved in Nonhomologous End Joining

Enzyme	Function
Ku70	Binds to DNA ends
Ku80	Binds to DNA ends
DNA-PKcs	Protein kinase catalytic subunit
Artemis	Nuclease
Pol μ	Fills in gaps
Pol λ	Fills in gaps
XRCC4	Seals nicks
XLF	Seals nicks
DNA ligase IV	Seals nicks

Nonhomologous End Joining Is Promoted by a Set of Conserved Enzymes

In eukaryotes, at least nine proteins are used in the multiple steps of NHEJ (Table 13-2). The reaction is initiated at a DSB by the binding of a heterodimer consisting of the proteins Ku70 and Ku80 ("KU" are the initials of the individual with scleroderma whose serum autoantibodies were used to identify this protein complex; the numbers refer to the approximate molecular weights of the subunits). The Ku proteins are conserved in almost all eukaryotes. Both subunits of the Ku70-Ku80 complex have three domains, with the central domain forming a double ring (Figure 13-25). The complex binds readily to double-stranded DNA blunt ends or ends with 3' or 5' extensions. Multiple copies of the complex

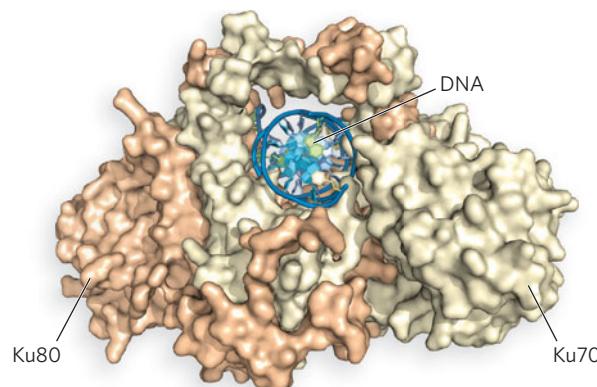


FIGURE 13-25 The Ku70-Ku80 complex. The central domains of Ku70 (yellow) and Ku80 (orange) provide an opening through which DNA can pass. The proteins slide over the DNA at a broken end. Additional domains in Ku70-Ku80 (not shown) provide interaction targets for some of the other NHEJ proteins. [Source: PDB ID 1JEY.]

may bind, sliding inward on the DNA. The Ku70-Ku80 complex binds all of the other complexes that play key roles in the subsequent steps: nuclease, polymerases, and ligase. Ku70-Ku80 thus acts as a kind of molecular scaffold. In the eukaryotic nucleus, this complex has additional roles in DNA replication, telomere maintenance, and transcriptional regulation to complement its role in NHEJ. A loss of the genes encoding NHEJ function can produce a predisposition to cancer.

NHEJ proceeds in three major stages (Figure 13-26). In the first stage, Ku70-Ku80 interacts with another protein complex containing DNA-PKcs (the 470 kDa DNA-

dependent protein kinase catalytic subunit) and a nuclease known as Artemis. Once the complex is assembled, the protein kinase activity of DNA-PK is activated. DNA-PK autophosphorylates in several locations, and also phosphorylates Artemis. Artemis is generally active as a 5' → 3' exonuclease, but when phosphorylated it acquires an endonuclease function. This endonuclease can remove 5' or 3' single-stranded extensions as well as hairpins, resecting the excess DNA in overhangs at the ends. In the second stage, DNA ends anneal and Artemis cleaves any unpaired DNA segments that are created. Small DNA gaps are filled in by the eukaryotic Pol μ or Pol λ. Finally, the nicks are sealed by a protein complex consisting of XRCC4 (*x*-ray cross complementation group), XLF (XRCC4-like factor), and eukaryotic DNA ligase IV.

DNA ends are usually not joined randomly by NHEJ. Instead, when a DSB occurs, the ends are generally constrained by the structure of chromatin and thus remain close together; they are rarely linked to the ends of other chromosomes, because all eukaryotic chromosome ends are protected by telomeres. Very rare events linking end sequences that are normally far apart in the chromosome, or on different chromosomes, may be responsible for occasional dramatic and usually deleterious genomic rearrangements.

SECTION 13.4 SUMMARY

- Nonhomologous end joining is critical to the repair of double-strand breaks that arise during the G₁ and static G₀ phases of the eukaryotic cell cycle. NHEJ is more important in cells that spend a greater amount of time in G₁ and G₀, such as the somatic cells of more complex eukaryotes.
- NHEJ is promoted by the well-conserved Ku70-Ku80 protein complex, along with additional complexes with nuclease, polymerase, and ligation activities.

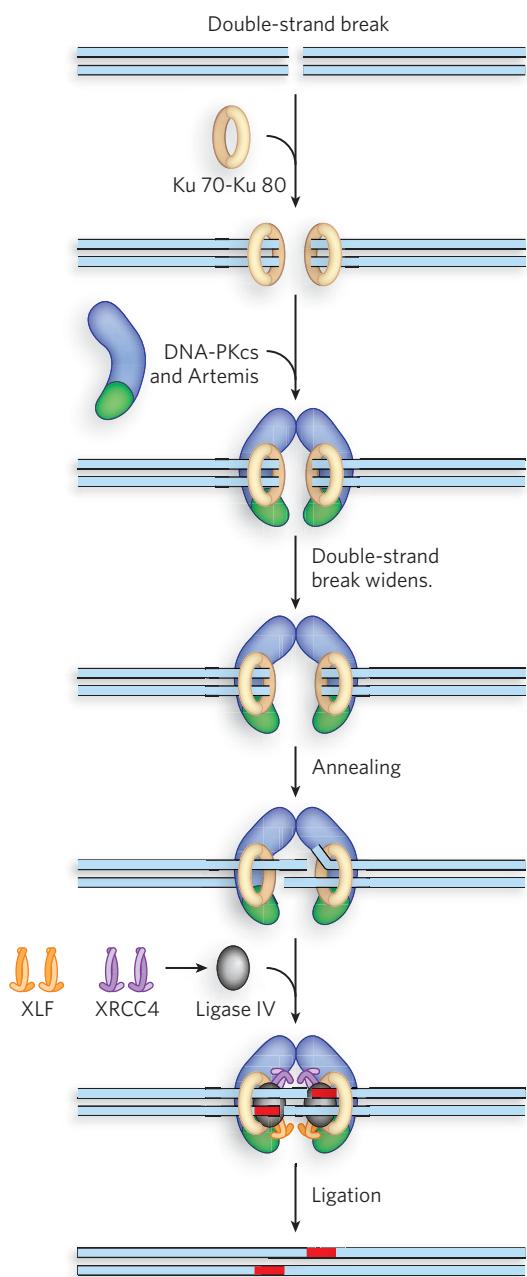


FIGURE 13-26 Nonhomologous end joining in eukaryotes. The Ku70-Ku80 complex is the first to bind the DNA ends, followed by a complex including DNA-PKcs and the nuclease Artemis. These proteins then recruit a complex of XRCC4, XLF, and DNA ligase IV. Either of two DNA polymerases, Pol μ or Pol λ (not shown), subsequently extends the annealed DNA strands, as needed, before ligation. [Source: Adapted from J. M. Sekiguchi and D. O. Ferguson, *Cell*, 124(2):260–262, 2006.]

Unanswered Questions

In every area of molecular biology there is a need to reconcile biochemistry with the observations made in living cells, using genetics and functional genomics. Recombination systems and processes are complex, and accurately reconstituting them *in vitro* with purified components remains a major challenge. Here are some of the significant questions in the field.

- 1. How often do replication forks collapse?** It is not yet clear how often DNA template lesions halt replication forks in a manner requiring replication restart, and how often lesions are simply bypassed by the replication machinery. Some *in vitro* studies show a potential for bypass even for lesions in the leading strand. However, *in vivo* studies show that replication ceases for a period when sufficient DNA damage is introduced into the genome. Although we understand the outlines of the major recovery pathways, there are doubtless many variants yet to be elucidated—along with some undiscovered enzymes—that respond to different classes of lesions and the many different DNA structures found at stalled forks.
- 2. What factors remain to be discovered in double-strand break repair, and how do they work?** In studies of recombinational DNA repair, many new proteins are still being discovered. Some of the newer discoveries involve protein factors that regulate recombination, or link it to replication checkpoints or other aspects of chromosome structure or cell division. The complexities are illustrated by proteins such as Spo11. The double-strand cleavage reaction of Spo11 has not yet been replicated *in vitro*, perhaps due to a requirement for other protein factors not yet purified (or not yet discovered). After Spo11 has cleaved a chromosome, additional enzymes must degrade the 5'-ending strand to create the 3' single-stranded extensions needed for strand invasion. The identity of the nuclease that processes these DNA ends is not yet clear. A complete DSBR reaction has yet to be reconstituted *in vitro*.
- 3. How is recombination coordinated with other aspects of DNA metabolism?** Regulation is an increasingly visible theme in this field of research. Recombination must be directed at locations where it is needed, and prevented elsewhere. When a replication fork stalls, recombinational repair systems must arrive quickly and address the situation at hand. The intricate coordination required to keep these processes on track is critical to genomic integrity, and even survival, and understanding it will keep many molecular biology laboratories engaged for decades to come.
- 4. What is the tension-sensing mechanism that facilitates proper chromosomal segregation in eukaryotic cell division?** Some of the participating proteins have been discovered, but much remains to be done in this area of molecular biology.

How We Know

A Motivated Graduate Student Inspires the Discovery of Recombination Genes in Bacteria

Clark, A.J. 1996. RecA mutants of *E. coli* K12: A personal turning point. *Bioessays* 18:767-772.

Clark, A.J., and A.D. Margulies. 1965. Isolation and characterization of recombination deficient mutants of *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA* 53:451-459.



Ann Dee Margulies, 1940/41-1980 [Source: Courtesy of Werner Maas and Renata Maas. Thanks to Alvin J. Clark.]

Sometimes it is the student who challenges the professor. This is what happened in 1962 at the University of California, Berkeley, when first-year graduate student Ann Dee Margulies came to the office of a new assistant professor, A. John Clark. Clark later related the encounter as a career-changing moment. At a time when many molecular biologists considered the problem of recombination too com-

plicated to address in any productive way, Margulies and Clark embarked on a project to find the genes that control recombination in bacteria.

The two researchers decided to use bacterial conjugation as a way to measure recombination events. As Joshua Lederberg and E. L. Tatum had demonstrated in 1946, some bacteria harbor plasmids that can be transferred between cells. These F plasmids sometimes integrate themselves into the bacterial chromosome, creating strains (Hfr strains) that can convey parts of their chromosome to other cells at high frequency. When DNA is transferred, alleles from the donor DNA can be transmitted to the recipient's chromosome by recombination. Margulies and Clark used replica plating, a technique devised by Esther Lederberg and Joshua Lederberg in 1952, to search for mutants.

They used two strains of *E. coli*. The chosen Hfr donor strain could not grow unless leucine was included in the growth medium (denoted leu^-). The recipient strain, lacking an F plasmid, had a mutation leading to a requirement for adenine (ade^-).

Conjugational crosses between the two strains produced recombinants that could grow in the absence of both leucine and adenine (leu^+ade^+).

The recipient strain was treated with the mutagen 1-methyl-3-nitro-1-nitosoguanidine (MNNG) to introduce mutations at random locations in the chromosome. They then had to search for those very rare mutations that affected recombination genes. The mutagenized cells were spread on agar plates containing leucine, where cells not killed by the mutagen grew into colonies. Strains were transferred one by one onto a second master plate that also contained leucine, creating a pattern of 50 to 100 colonies. On a third plate that lacked both leucine and adenine, a culture of the Hfr donor strain was spread uniformly, creating a thin "lawn" of bacteria that was alive but unable to grow, given the lack of adenine.

Using a piece of sterile velvet, Margulies replicated the pattern of colonies on the master plate onto the third plate. The transferred cells underwent conjugational mating with cells in the lawn of donor Hfr bacteria. Successful conjugation and recombination produced ade^+leu^+ recombinants at high frequencies that could grow into colonies on the plates lacking leucine and adenine. Occasionally, no recombinant cells would arise where a colony was expected. If the mutagenized recipient strain continued to yield no recombinants on repeated trials, it was set aside as a candidate for a strain containing a mutation in a gene required for recombination.

The procedure was laborious, but Margulies, working under Clark's guidance, persevered. After months of careful controls and screening more than 2,000 mutagenized recipient strains, Margulies found two strains that had a recombination defect. Later work established that these strains had mutations in what became known as the *recA* gene. Clark and Margulies published their results in the *Proceedings of the National Academy of Sciences* in 1965; their paper has been cited countless times. The work launched John Clark into a productive career in elucidating recombination mechanisms. Sadly, Ann Dee Margulies, the intrepid graduate student, died of cancer in 1980, at the age of 40.



A. John Clark [Source: Courtesy of Alvin J. Clark.]

A Biochemical Masterpiece Catches a Recombination Protein in the Act

Keeney, S., C.N. Giroux, and N. Kleckner. 1997. Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* 88:375–384.

Following the proposal of the double-strand break repair model for meiotic genetic recombination in 1983, evidence for the accuracy of major parts of the model accumulated quickly. In particular, it became clear that the process was initiated by double-strand breaks. The DSBs could be detected early in meiosis, especially in regions with recombination hot spots. But what protein did this? For Nancy Kleckner, a Harvard biochemist who had become intrigued with genetic material as a high school student in the 1960s, this was an obvious challenge to take up. By 1995, Kleckner's postdoctoral associate, Scott Keeney, had discovered that a protein was linked to the 5' termini at the break sites. Now, the two researchers had to identify that protein.

The answer was delivered in a biochemical exercise marked by both determination and elegance. The trick was to isolate the protein bound to the cleaved 5' ends of the DSB, but this was no simple task. Every meiotic cell has scores of such cleavage events, and they are spaced along chromosomes containing millions of base pairs, bound by hundreds of different proteins.

The researchers' first step was one that biochemists often use: amplification of the signal. Keeney, Kleckner, and others had found that when steps subsequent to formation of the DSB, such as the rapid degradation of the 5'-ending strands, were blocked, covalent protein-DNA intermediates accumulated. A mutation in the gene encoding Rad50 (rad50S) served this blocking purpose well.

Using rad50S cells as an enriched source of the protein-DNA complexes, Keeney and Kleckner, working with collaborator Craig Giroux of Wayne State University, developed a two-step purification procedure. The first step was to eliminate bulk proteins. The researchers isolated the nuclei from the cells to remove cytoplasmic proteins and extracted the nuclear DNA with guanidinium chloride and detergent at 65°C, a treatment harsh enough to strip all but covalently linked protein from the DNA. Bulk protein was separated from the DNA in a CsCl gradient.

In the second step, the researchers separated protein-DNA complexes from bulk DNA by passing the CsCl-purified material through a glass-fiber filter, to which proteins adhere. The adhered complexes were

eluted from the filter with a detergent, then treated with nucleases to remove most of the DNA. The remaining proteins were separated on a polyacrylamide gel.

The procedure was carried out in parallel on rad50S cells and on cells with a mutation that prevents DSB formation (as control). Doing so on a large, preparative scale yielded the results shown in **Figure 2**. Two bands, with apparent molecular weights of 34,000 and 45,000, were seen in the rad50S samples but not in the control samples. The two proteins were excised from the gel and identified by tandem mass spectrometry as a contaminant and Spo11, respectively. More controls were carried out to solidify the case that Spo11 is the protein bound to the break sites. In one particularly compelling experiment, Spo11 was immunoprecipitated from rad50S cells with covalently linked DNA fragments from a known recombination hot spot.

The Spo11-mediated cleavage of DNA is the first step of the elaborate process of meiotic recombination, and its mechanism still presents a biochemical challenge. Although Spo11 is clearly the protein linked to the break sites, the actual cleavage reaction has not been observed *in vitro* with purified DNA and protein. The cleavage events must be regulated, and Spo11 may act only in concert with other—perhaps many other—as yet unknown proteins.

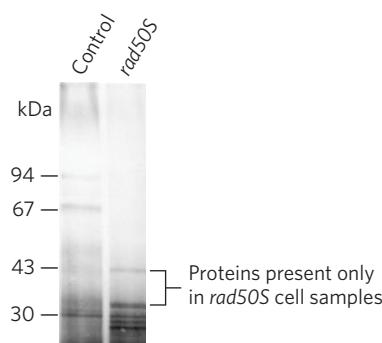


FIGURE 2 Proteins detected in the two-step purification procedure. [Source: S. Keeney, C. N. Giroux, and N. Kleckner, *Cell* 88:375–384.]

Key Terms

homologous recombination, p. 446	crossover, p. 449	Dmc1, p. 467
recombinational DNA repair, p. 446	branch migration, p. 450	Rad51, p. 468
double-strand break (DSB), p. 446	fork regression, p. 451	gene conversion, p. 468
recombinase, p. 447	gap repair, p. 452	BRCA2, p. 470
DNA strand invasion, p. 447	RecBCD, p. 454	mating type, p. 470
synthesis-dependent strand annealing (SDSA), p. 448	chi, p. 454	homing endonuclease, p. 472
double-strand break repair (DSBR), p. 448	RecFOR, p. 455	nonhomologous end joining (NHEJ), p. 473
Holliday intermediate, p. 449	RecA protein, p. 456	
	homolog, p. 464	
	Spo11, p. 465	

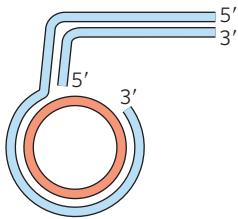
Problems

1. What are the four possible fates of a replication fork that encounters a template strand with a break or some other type of unrepaired DNA lesion?

2. A branched, circular DNA substrate is constructed to mimic one possible structure of a stalled replication fork, as shown below. An enzyme is added that promotes regression of the fork structure.

(a) Draw the structure of the product obtained if regression proceeds halfway around the circle.

(b) Draw the structure of the product if regression proceeds all the way around the circle. Assume the arm is the same length as the circle and has the same sequence.



3. Draw a Holliday intermediate and label the ends of each DNA strand so that the strand polarity is evident.

4. The RecBCD enzyme acts as a nuclease and a helicase in preparing DNA ends for RecA binding and strand invasion. RecBCD has several functions built into its three subunits. Indicate the subunit (RecB, RecC, or RecD) responsible for each of the following functions.

(a) 3' → 5' helicase motor

(b) Nuclease

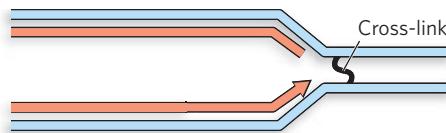
(c) 5' → 3' helicase

(d) Having a "pin" structure that helps separate DNA strands

(e) Binding to chi sites

5. Replication forks of a bacterial species are found to stall at double-strand cross-links, yielding a stalled fork with the structure shown below. The pathway for repair of these stalled forks involves the formation of a Holliday intermediate. Draw one step that will convert the fork into a structure with a Holliday intermediate. Place

an arrow on all 3' ends (only one end is so labeled below). Note that the Holliday intermediate is formed without cleaving any covalent bonds in the DNA.



6. In *E. coli* cells with mutations that eliminate the RecBCD enzyme, about 20% of the cells have linearized chromosomes when grown under normal aerobic conditions. Under similar growth conditions, fewer than 3% of the chromosomes are linearized in wild-type cells. Suggest, in two or three sentences, why this difference is observed.

7. During meiosis in yeast, if the diploid cell has alleles *a* and *A* of a particular gene, it normally forms two spores with *A* and two spores with *a*. Rarely, meiosis yields one spore with *A* and three with *a*, or three with *A* and one with *a*. How could this happen?

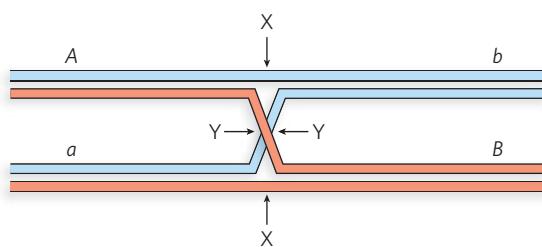
8. Unlike recombination, the repair of double-strand breaks by nonhomologous end joining creates mutations. Explain why.

9. At the yeast mating-type locus, the mating-type switch is initiated by introducing a double-strand break at the *MAT* locus. What would happen if the DSB were introduced at the *HMLα* locus instead?

10. In the study by Keeney, Giroux, and Kleckner (see How We Know), Spo11 was identified as the protein that introduces double-strand breaks to initiate meiotic recombination. To identify candidate proteins, the DNA was first extracted to remove most noncovalently bound proteins, then filtered to isolate remaining protein-DNA complexes. The samples were then extensively treated with nucleases before the samples were loaded onto a polyacrylamide gel. Why was the nuclease treatment necessary?

11. A Holliday intermediate is formed between two chromosomes at a point between two genes, *A* and *B*, as shown below. The two chromosomes have different alleles of the

two genes (*A* and *a*; *B* and *b*). Where would the Holliday intermediate have to be cleaved (points X and/or Y) to generate a chromosome with (a) an *Ab* genotype or (b) an *ab* genotype?



- 12.** The bacterium *Deinococcus radiodurans* is highly resistant to the DSB-generating effects of ionizing radiation.

Data Analysis Problem

Cox, M.M., and I.R. Lehman. 1981. Directionality and polarity in RecA protein-promoted branch migration. *Proc. Natl. Acad. Sci. USA* 78:6018–6022.

- 13.** The RecA protein promotes DNA strand exchange, as shown in Figure 13-11, with a unidirectional branch migration proceeding around the DNA circle. The direction of the branch migration was established by Cox and Lehman. A circular chromosome from bacteriophage ϕ X174 can be isolated as either a single- or a double-stranded circle. The circular map of the ϕ X174 genome is shown in Figure 1 (adapted from Cox and Lehman's paper); the single-stranded circle proceeds 5' → 3' in the clockwise direction. In the experiment, the single-stranded ϕ X174 circle was radioactively labeled uniformly along its length. The double-stranded circular DNA was not labeled, but was cleaved at a unique site (labeled A) by a restriction enzyme to generate the substrates shown in Figure 13-11. Sites for cleavage by a second restriction enzyme (labeled B) are also noted.

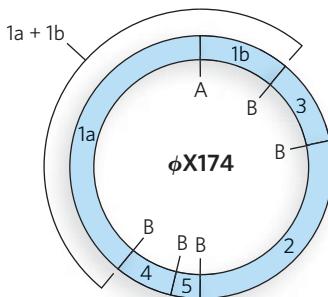


FIGURE 1

DSBs also occur (slowly) during prolonged cell desiccation, and desiccation is thought to be the selective pressure in the evolution of the extraordinary capacity of *D. radiodurans* for DNA repair. After heavy irradiation, the bacterium produces several novel proteins at high levels. One of these, called DdrA (DNA damage repair protein A), binds tightly to the 3' ends of broken DNA strands and prevents their degradation by nucleases. A mutation that eliminates DdrA function has little effect on survival after irradiation, but a large effect on survival after desiccation. Suggest an explanation for the role of DdrA during desiccation. In your answer, consider the requirements of DNA repair versus DNA degradation.

A DNA strand exchange reaction was initiated with RecA protein and ATP. At various times, aliquots were removed and treated with restriction enzyme B and with a nuclease that selectively digested all single-stranded DNA, and the DNA was subjected to agarose gel electrophoresis to separate the restriction fragments. The incorporation of radioactivity (as percentage of maximum) into the various restriction fragments was measured at various time intervals; the results are shown in Figure 2.

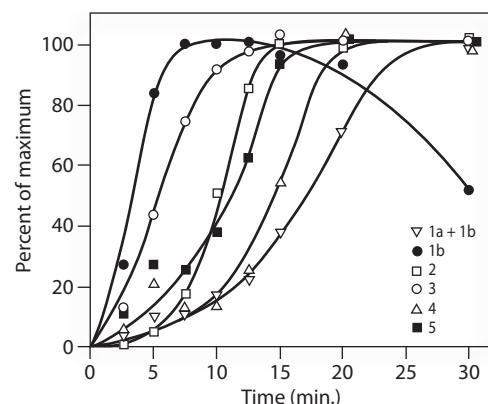


FIGURE 2

- Radioactivity is present throughout the single-stranded DNA circle, so why is there no radioactivity in any of the restriction fragments at the beginning of the experiment?
- In what direction does RecA protein-mediated branch migration proceed?
- At the end of the experiment, the amount of fragment 1b begins to decline. Suggest an explanation.

Additional Reading

General

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Site-Specific Recombination and Transposition



Wei Yang [Source: Courtesy of Wei Yang.]

take place, making the complex very difficult to crystallize.

In the early 1990s, we had to use a “divide and conquer” strategy. We started by co-crystallizing DNA containing only one res cleavage site with a dimeric $\gamma\delta$ resolvase. It was terribly exciting for me to find the initial crystals while peering into a microscope, but the experienced postdocs and students in Steitz’s lab just smiled and politely wished me luck. I understood their kindness only after finding that many protein-DNA co-crystals don’t diffract x rays well or at all.

After overcoming the diffraction problem, the phase problem, and the asymmetry of the $\gamma\delta$ resolvase dimer, the moment of visualizing the electron density of the $\gamma\delta$ resolvase-DNA complex on a computer screen for the first time was sheer joy. At that moment I was the only one in the whole world who knew how $\gamma\delta$ resolvase bound (and bent!) its recognition site. However, obtaining the crystal structure of a true recombination intermediate took another 10 years.

Today, 15 years after I solved the $\gamma\delta$ resolvase-DNA complex structure, the mechanism of how two DNA duplexes exchange partners remains a hypothesis. To fully understand a biological process, it often takes generations of scientists with each generation making additional steps forward. I was pleased to contribute my step and eagerly look forward to following the next chapters in this story.

—Wei Yang, on researching the structure and molecular mechanisms of $\gamma\delta$ resolvase

- 14.1 Mechanisms of Site-Specific Recombination 482**
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- 14.3 The Evolutionary Interplay of Transposons and Their Hosts 503**

Life might have made its first appearance on this planet in the form of a self-replicating polymer made of RNA, or something similar. The existence of this successfully self-replicating RNA would immediately have given rise to a biosphere with two possible survival strategies. First, the polymer itself could continue to use the strategy of self-replication, drawing on resources in the environment to increase its own population. Second, a smaller RNA—a primitive RNA transposable element—could insert itself into a vulnerable site in a self-replicating RNA. With this strategy, the transposable element would reproduce passively as long as the function of the self-replicator was unaffected by the addition to its mass. Thus, transposable elements may be among the most ancient of genetic entities. Always present and often deceptively simple in structure, transposons long ago perfected the art of mostly benign coexistence. Some bear a close evolutionary relationship to viruses.

Transposable elements, also called **transposons**, are genomic freeloaders, nucleic acid sequences that insert themselves into the genome of another organism and are replicated passively every time a cell divides. They can move from one location on a chromosome to another, often with no apparent regard for the surrounding DNA sequence; this process is known as **transposition**. The ability of transposons to move within a genome, defying Mendelian genetics, startled and then intrigued geneticists when these elements were discovered by Barbara McClintock in maize plants in the 1940s. Careful work by McClintock, and the eventual discovery of closely related genetic elements in other organisms by other workers, gradually overcame the initial skepticism of colleagues. Since then, studies of transposons have yielded invaluable genetic techniques for constructing transgenic model organisms and for exploring gene function.



Barbara McClintock,
1902–1992 [Source: National Institutes of Health.]

In a typical human cell, several million transposable elements account for nearly half the genomic DNA. They make up the largest portion of the genomic material that has sometimes been referred to as “selfish DNA” or “junk DNA,” perceived to be of little or no use to the host organism. However, the movement of transposable elements within a genome, involving the activity of enzymes called transposases, helps shape evolution, sometimes

providing the raw genetic material with which to craft new processes that enhance the fitness of their host.

In contrast to transposition, **site-specific recombination** is a precise and predictable process in which DNA is rearranged between two specific sequences. This can result in the insertion, deletion, or inversion—depending on the arrangement of the recombination sites—of a particular DNA segment. In a sense, recombinases, the enzymes that carry out the site-specific recombination reactions, are restriction endonucleases and DNA ligases combined into a single efficient package. However, unlike DNA ligation, the reactions do not require ATP or a similar cofactor; they are typically isoenergetic ($\Delta G^\circ = 0$), or nearly so. The reactions are often tied to genomic replication, but they have been recruited for other purposes as well. They typically provide an elegant biochemical solution to awkward topological problems that occur in plasmids and chromosomes. Site-specific recombinases are increasingly well understood. Biotechnologists love them for the precise DNA rearrangements they catalyze.

Site-specific recombination and transposition are specialized types of recombination that share two key properties. First, both generally involve the bringing together of DNA sites without extensive homology. Second, the key enzymes in both processes bear a notable evolutionary relationship to DNA topoisomerases. Site-specific recombinases and transposases inhabit an enzymatic world of thermodynamic equanimity. Like topoisomerases, they promote phosphoryl group transfers in which the net change in free energy is usually close to zero.

In this chapter, we discuss site-specific recombination and transposition in succession. We begin with site-specific recombination because it is more predictable and provides a useful introduction to chemistry that is applicable to all the processes considered here. Each process makes an important contribution to the maintenance and sometimes dynamic nature of the genome, and each one can be harnessed by the molecular biologist to study and alter those same genomes.

14.1 Mechanisms of Site-Specific Recombination

Recombination between specific sequences can result in insertion, deletion, or inversion of the intervening DNA sequence. Recombination reactions of this type occur in virtually every cell, filling specialized roles that vary greatly from one species to another but share a common mechanism. Each site-specific recombination system consists of a short, unique DNA sequence (20 to 200 bp)

and a recombinase, an enzyme that acts specifically at that sequence. In some systems, additional proteins are required to facilitate or regulate the process. The result of a site-specific recombination reaction can be similar to that of the crossovers that sometimes accompany homologous recombination, but the process does not require extensive homology at recombination sites.

The DNA rearrangements promoted by site-specific recombinases appear in numerous and sometimes surprising roles. Examples range from prescribed roles in

the replication cycles of viral, plasmid, and bacterial DNAs, to key events in the life cycle of some viruses, to regulation of the expression of certain genes.

Precise DNA Rearrangements Are Promoted by Site-Specific Recombinases

The recombination sites recognized by site-specific recombinases often consist of two inverted repeats, separated by a short asymmetric (nonpalindromic) core sequence (Figure 14-1a). During recombination, the asymmetric cores of the two DNA segments are aligned in the same orientation. The recombinase binds to the symmetric repeats on either side of the core. In many systems, the sequence in the core itself can be varied without affecting its recognition by the recombinase. However, recombination itself occurs only if the sequences of the two cores are identical.

The reaction outcome depends on the location and orientation of the recombination sites (Figure 14-1b).

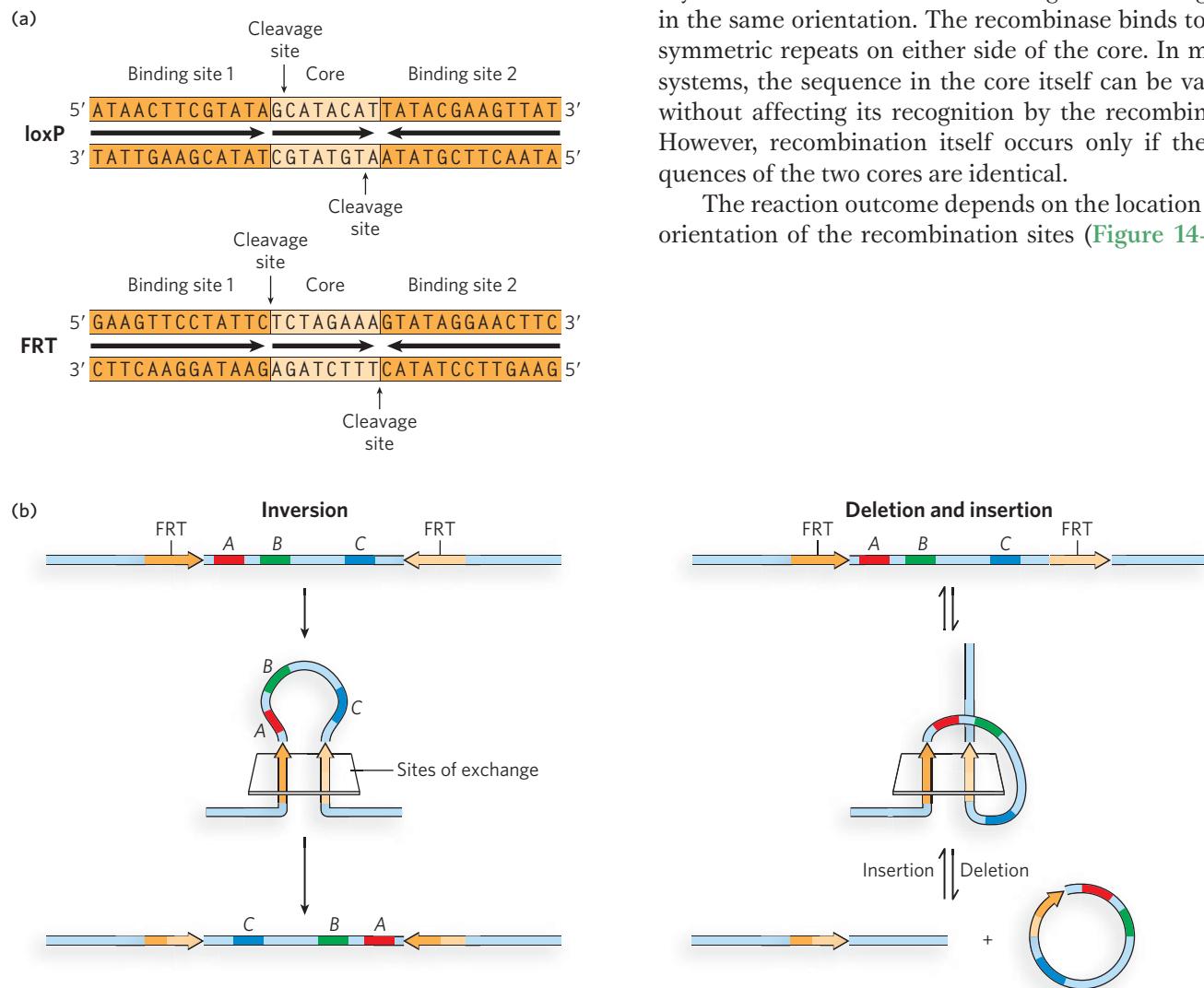


FIGURE 14-1 The structure and activity of site-specific recombination sites. (a) Shown here are two recombination sites from well-studied recombination systems, *lox* (*loxP*) and *FRT*; the inverted 13 bp repeats are binding sites for the recombinases (named Cre and Flp, respectively; these two sites and their recombinases are discussed later in the chapter). Repeats are separated by an asymmetric core sequence. The cleavage and exchange

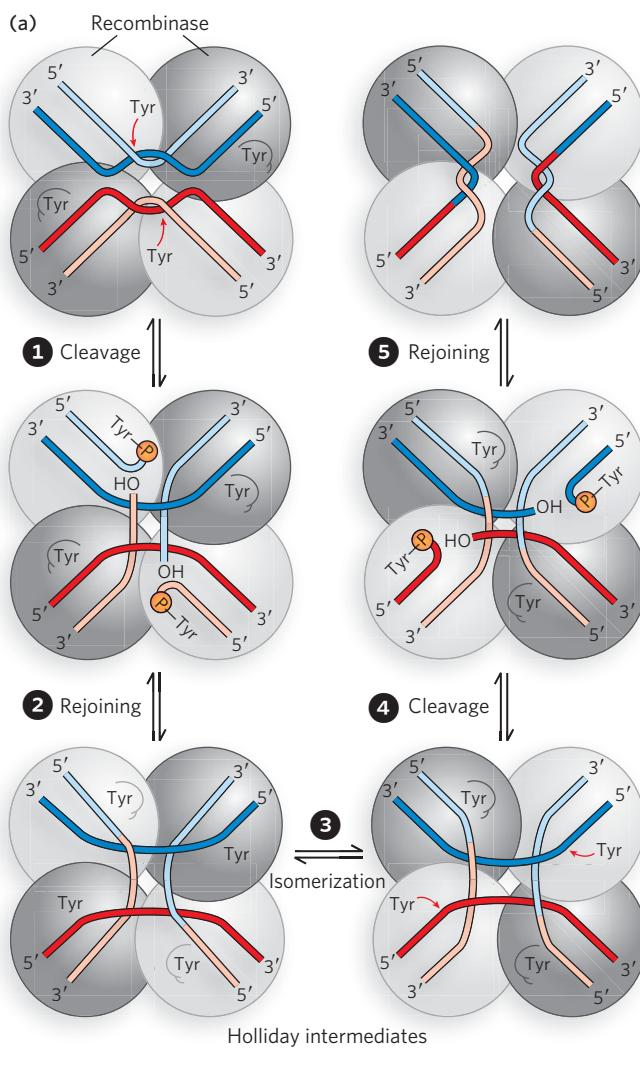
events occur at or near the ends of the core sequence. (b) Two recombination sites flank a length of DNA to be recombined. A, B, and C are imaginary genes or genetic markers. Orientation (shown by the arrowheads) refers to the asymmetric nucleotide sequence in the recombination sites, *not* the 5'→3' direction. Recombination can lead to inversion (left) or to deletion and, in the reverse process, insertion (right), as described in the text.

Recombination between two oppositely oriented sites on the same DNA molecule produces an inversion. Recombination between two sites with the same orientation on the same DNA molecule results in a deletion. If the sites are on different DNAs, the result is an insertion. Some recombinase systems are highly specific for one of these reaction types, and they act only on sites with particular orientations.

Site-specific recombination systems use either a Tyr or a Ser residue as the key nucleophile in the active site. In vitro studies of many such systems have clarified the fundamental reaction pathway. A pair of recombinases recognizes and binds to each recombination site. The two pairs come together to form a synaptic complex that incorporates four recombinase subunits and two aligned cores—the recombination target sites. If the two recombination sites are on the same DNA molecule, the intervening core DNA is bent into a loop as the sites are brought together (see Figure 14-1b, left). The recombinase subunits carry out the reaction by cleaving the DNA at the recombination sites and precisely religating the ends.

The site-specific recombination reaction is best understood for the tyrosine-class recombinases (Figure 14-2a). One DNA strand in each site is cleaved at a specific point, and one recombinase subunit becomes covalently linked to the DNA at each cleavage site through a phosphotyrosine bond (step 1). The transient protein-DNA linkage ensures that the overall reaction proceeds with a minimal free-energy change, so high-energy cofactors such as ATP are unnecessary. The cleaved DNA strands are rejoined to new partners to form a Holliday intermediate (step 2), with new phosphodiester bonds created at the expense of the protein-DNA linkage. An isomerization then occurs in the protein complex (step 3). This step includes a branch migration through the core sequence (a step that is blocked if the cores are not identical), coupled to a conformational change that brings the active sites of the two recombinase subunits that did not participate

in the first two steps into register with the phosphodiester bonds they must act on to complete the reaction. The cleavage and rejoicing reactions are then repeated, at this second point, within each of the two recombination sites (steps 4 and 5).



(b) Holliday intermediate

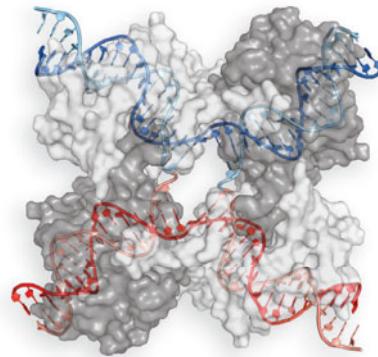


FIGURE 14-2 A site-specific recombination reaction.

(a) The reaction proceeds within a tetramer of identical recombinase subunits. The subunits bind to a specific sequence, the recombination site, and catalyze the recombination in several steps, as described in the text. The light gray subunits are the active subunits in each panel, with the active-site Tyr residue poised to react or covalently linked to the DNA. (b) A surface contour model of Flp recombinase, showing the four subunits bound to a Holliday intermediate; this is equivalent to the product of step 2 in (a). The protein is made transparent so that the bound DNA is visible.

[Source: (b) PDB ID 3CRX.]

In systems that use an active-site Ser residue (see Moment of Discovery), both strands of each recombination site are cut concurrently and rejoined to new partners, without the Holliday intermediate. All four recombinase subunits participate, each forming a phosphoserine covalent intermediate at the cleavage sites. In both types of system (serine and tyrosine), the exchange is always reciprocal and precise, regenerating a pair of recombination sites when the reaction is complete.

Many mechanistic details of these reactions have become clear with the structural elucidation of recombinases caught at different steps of the process. The four recombinase subunits and the four DNA arms in the synaptic complex take up a square planar arrangement (Figure 14-2b). As shown by the crystal structure, the tyrosine-class recombinases are not in perfect four-fold symmetry. Instead, alternating subunits are in slightly different conformations—two active (with the active-site Tyr residues positioned near the phosphodiester bonds to be cleaved) and two inactive. After formation of the covalent 3'-phosphotyrosyl linkage (see Figure 14-2a, step 1), the cleaved DNA 5' ends migrate to complete the formation of a Holliday intermediate (step 2). The isomerization then occurs within the complex (step 3), coupled to subtle conformational changes that convert the active recombinase subunits to the inactive state, and inactive subunits to active, to permit completion of the reaction (steps 4 and 5).

The overall process is closely related to the reaction mechanism promoted by topoisomerases (see Figure 9-19). For both topoisomerases and site-specific recombinases, the reaction begins with the formation of a protein-DNA phosphotyrosine or phosphoserine linkage at the expense of a phosphodiester bond in the DNA. In the case of topoisomerases, the same phosphodiester bond is re-created after the DNA topology has been changed. In the case of site-specific recombinases, each end of the cleaved phosphodiester bond is joined to a new partner. The different outcomes are brought about by the different architectures of the proteins promoting the two reactions, resulting in different and very precise movements of DNA segments between the phosphodiester cleavage and re-formation steps.

Site-Specific Recombination Complements Replication

Replication of the circular chromosomes of viruses, plasmids, and many bacteria poses a unique set of challenges. As noted in Chapter 13, recombinational DNA repair of stalled replication forks can give rise to contiguous dimeric chromosomes. A specialized site-specific

recombination system in *E. coli* converts the dimeric chromosomes to monomeric chromosomes so that cell division can proceed. The reaction is a site-specific deletion reaction catalyzed by a tyrosine-class recombinase, XerCD. As described here, site-specific recombination can also be used as an elegant mechanism to generate more than two copies of a chromosome during one replication cycle.

A common plasmid in *Saccharomyces cerevisiae*, called the 2 μ (2 micron) plasmid, has a site-specific recombination system. The recombinase, known as Flp, is encoded by the plasmid. In this system, site-specific recombination is used to amplify plasmid copy number in the cell whenever necessary. When the copy number becomes too low, Flp is activated to promote the recombination reaction. The key to copy-number amplification is the timing of the recombination. The replication origin of the plasmid is situated such that one Flp recombination target (FRT) site is replicated well before the other (Figure 14-3). If recombination occurs when only one FRT has been replicated, the result is the inversion not just of one segment of DNA but of one replication fork relative to the other. Instead of meeting at the opposite side of the circle, the two forks begin to follow each other around the circle, promoting an extended rolling-circle replication. This generates multiple tandem copies of the plasmid, instead of just two, from one replication initiation. The multimeric plasmid is then broken down into plasmid monomers by subsequent Flp-mediated recombination events carried out between FRT sites in the same orientation.

Site-Specific Recombination Can Be a Stage in a Viral Infection Cycle

Bacteriophages such as P1 and λ have played important roles in the development of molecular biology and biotechnology (see How We Know). When it enters a cell, the DNA of these phages has two potential fates (Figure 14-4). A **lysogenic pathway** involves incorporation of the phage genome as part of the host genome, either by integration into the host chromosome or as an autonomously replicating plasmid. In either case, phage genes are largely repressed, and the phage DNA is replicated passively by host enzymes. Lysogenized bacteriophage genomes are referred to as **prophages**, and the parasitic infection is benign as long as the phage remains in this state. In the alternative, **lytic pathway**, the bacteriophage DNA is replicated and packaged into new phage heads, and the host cell is destroyed by lysis to disperse the progeny. The specific mechanisms used in the P1 life cycle feature site-specific recombination in some key steps.

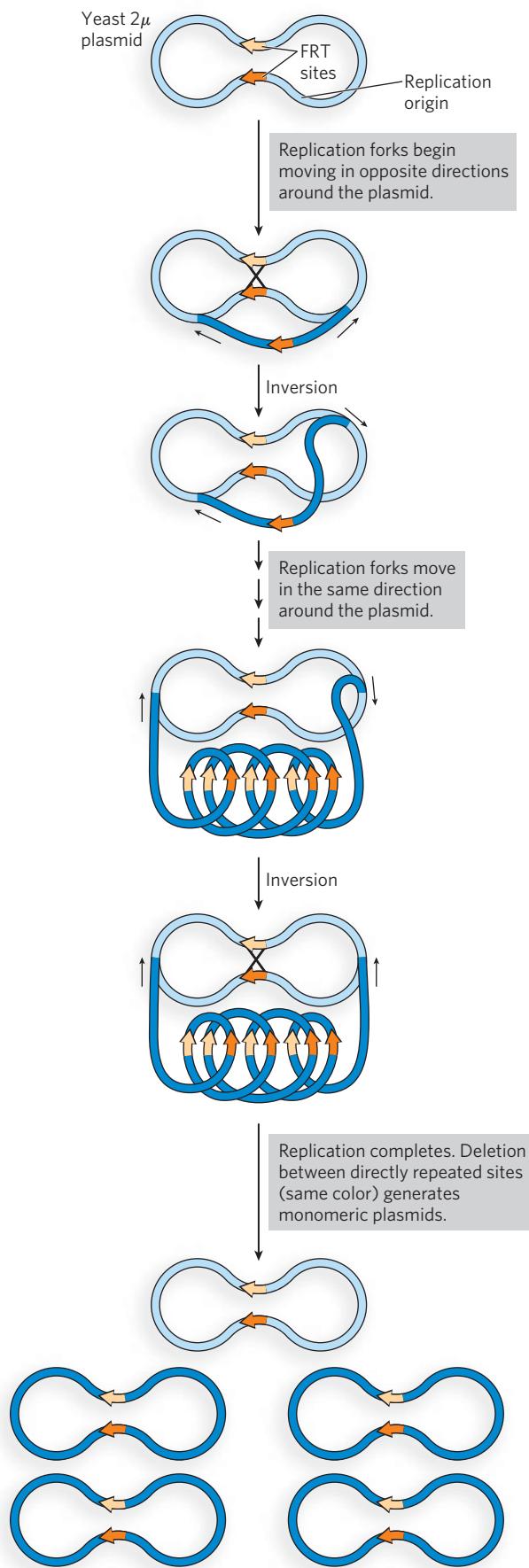


FIGURE 14-3 Coupling site-specific recombination to extensive replication in a yeast plasmid. The yeast 2μ plasmid, a circular DNA, has two FRT sites (orange) on opposite sides of the circular DNA molecule and inverted relative to each other. FRT sites are targeted by the plasmid-encoded Flp recombinase. The recombination reaction inverts the DNA in the sequences of about a half of the plasmid relative to those of the other. The inversion also changes the direction of one replication fork relative to the other. Inversion thus leads to a double rolling-circle replication that can produce multiple copies of the plasmid in one replication cycle, increasing the plasmid copy number in the cell.

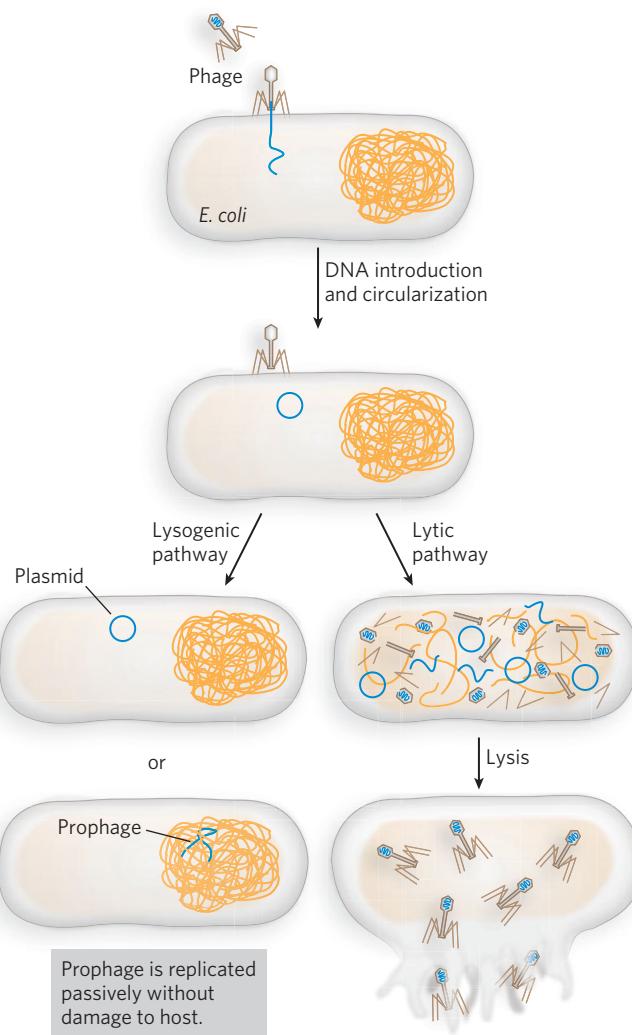


FIGURE 14-4 Two possible fates for a phage-infected host cell. Several types of bacteriophage introduce their DNA into cells in a linear form, which is circularized inside the cell. The lysogenic pathway involves either integration of the DNA (now referred to as a prophage) into the host chromosome or its passive replication as a plasmid. The alternative, lytic pathway eventually destroys the host cell and releases phage progeny.

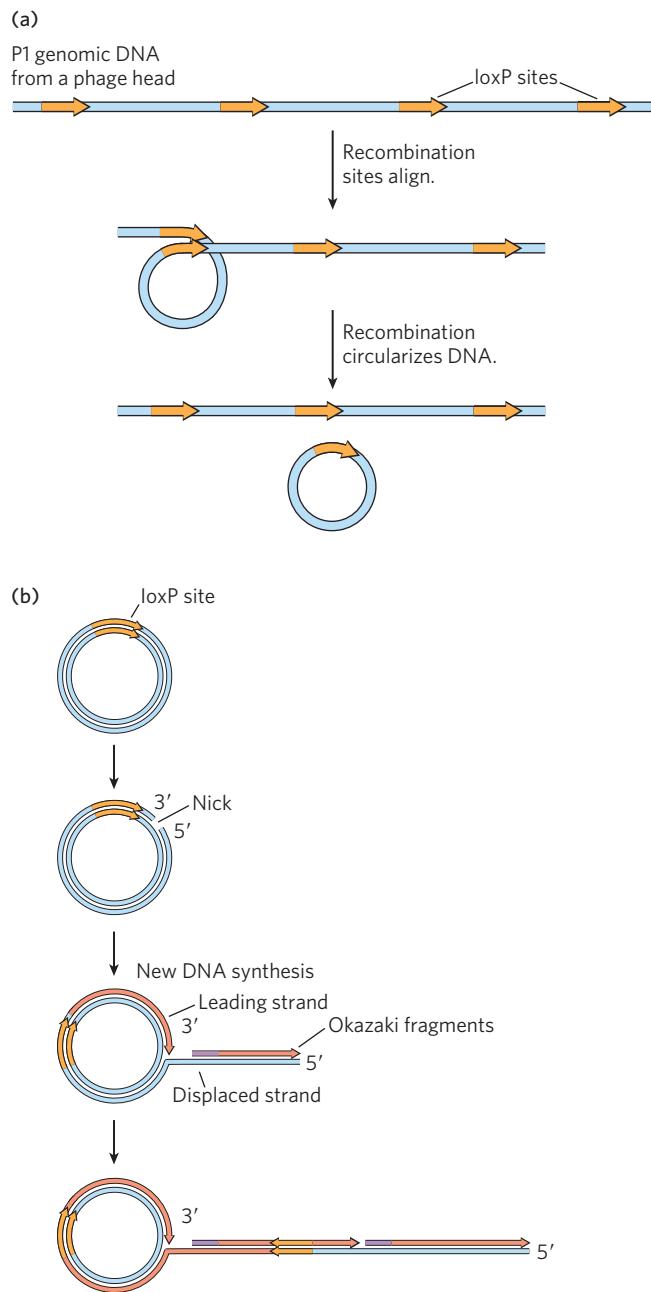


FIGURE 14-5 Circularization of P1 DNA. (a) P1 phage contains multiple copies of its genome, packaged in its head in a contiguous tandem array. In a host cell, circularization generates monomeric, circular genomes. The orange arrows are loxP sites, and serve to define one monomeric genome equivalent. Circularization may involve Cre-mediated recombination, or homologous recombination at other locations. (b) Following circularization, the P1 genome can undergo rolling-circle replication, producing many copies from one template.

P1 enters a bacterial cell as a linear DNA molecule containing multiple contiguous copies of the 90 kbp genome. In the host, the DNA is rapidly circularized

to produce multiple genome-length circular DNAs (Figure 14-5a). The circularization can occur by homologous recombination, or it can be promoted by a phage-encoded site-specific recombination system. The latter system, known as **Cre-lox**, involves recombination sites called loxP sites (*locus of crossover (x), phage*)—more often known simply as lox sites—and the recombinase Cre (*cyclization recombination*). The circularized DNA can enter a lysogenic state, maintaining the same copy number in the host cell. In addition to circularization, the Cre-lox recombination system aids in plasmid segregation at cell division by resolving any plasmid dimers to monomers. In the lytic pathway, P1 replicates in a rolling-circle mode in which the replication fork travels unidirectionally around the circularized chromosome (Figure 14-5b). This generates long, linear DNAs with many contiguous copies of the P1 genome. The DNAs are cut and the genomes incorporated into phage heads before cell lysis. Occasionally, large pieces of host DNA are also incorporated into phage heads. This low-frequency event allows P1 to be used as an experimental vehicle to move bacterial genes from one cell to another in a process known as **bacterial transduction**.

Gene Expression Can Be Regulated by Site-Specific Recombination

Salmonella typhimurium, which inhabits the mammalian intestine, moves by rotating the flagella on its cell surface. The many copies of the protein flagellin (M_r 53,000) that make up the flagella are prominent targets of mammalian immune systems. But *Salmonella* cells have a mechanism that evades the immune response: they switch between two distinct flagellin proteins (FljB and FljC) roughly once every 1,000 generations, using the process of **phase variation**.

The switch is accomplished by periodic inversion of a segment of DNA containing the promoter for a flagellin gene. The inversion is a site-specific recombination reaction mediated by the Hin recombinase at specific 14 bp sequences (*hix* sequences) at either end of the DNA segment. When the DNA segment is in one orientation, the gene for FljB flagellin and the gene for a repressor protein (FljA) are expressed; the repressor shuts down expression of the gene for FljC flagellin (Figure 14-6a). When the DNA segment is inverted, the *fljA* and *fljB* genes are no longer transcribed, and the *fljC* gene is induced as the repressor becomes depleted (Figure 14-6b). The Hin recombinase, encoded by the *hin* gene in the DNA segment that undergoes inversion, is expressed when the DNA segment is in either orientation, so the cell can always switch from one state to the other.

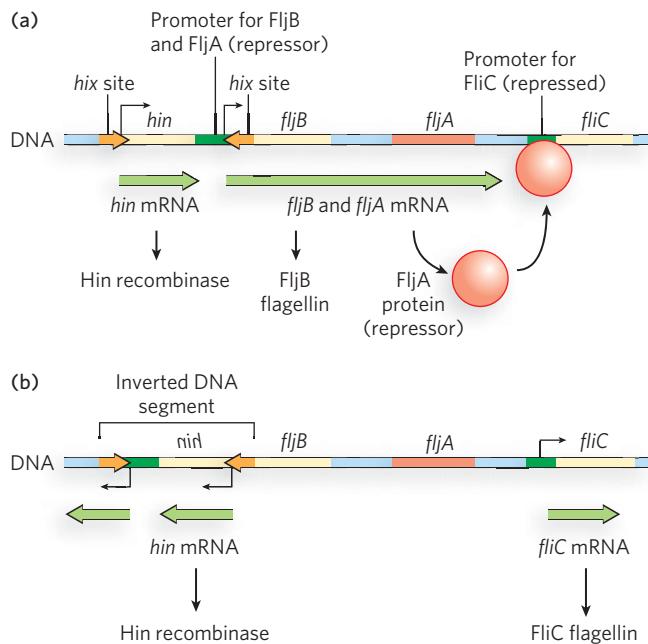


FIGURE 14-6 Phase variation in *Salmonella* flagellin genes. (a) In one orientation, *fliB* is expressed along with a repressor protein (product of the *fliA* gene) that turns off transcription of the *fliC* gene; the result is production of the flagellin FliB. (b) In the opposite orientation, the *fliA* and *fliB* genes cannot be transcribed, and only the *fliC* gene is expressed, producing flagellin FliC. *Salmonella* can flip between these two flagellin-producing systems.

The *Salmonella* system is by no means unique. Similar regulatory systems occur in other bacteria and in some bacteriophages. Gene regulation by DNA rearrangements that move genes and/or promoters is particularly common in pathogens that benefit by changing their surface proteins, thereby staying ahead of host immune systems.

Site-Specific Recombination Reactions Can Be Guided by Auxiliary Proteins

Two of the recombination systems described above, the Flp recombination system of the yeast 2μ plasmid and the Cre-lox system of bacteriophage P1, are relatively simple. In both cases, the recombination targets are well-characterized and about 34 bp long; Flp and Cre, both tyrosine-class recombinases, are the only enzymes required. The recombination can produce inversion, deletion, or insertion, depending on the placement and orientation of the recombination targets. The overall reactions promoted by Flp and Cre, as with all site-specific recombinases, are **isoenergetic**, occurring

without the input of ATP. The Flp and Cre reactions thus tend to approach an equilibrium in which substrates and products are in equal concentrations. This equilibrium can be altered in some systems with the aid of auxiliary proteins that participate in the site-specific recombination reactions.

The Hin recombinase of *Salmonella* is a serine-class recombinase. It has the notable property that it catalyzes recombination only when its *hix* recombination targets are on the same supercoiled DNA molecule and are inverted relative to each other. This specificity is accomplished through the formation of an elaborate structure involving several auxiliary proteins, which acts as a topological filter (Figure 14-7). The structure cannot form unless the DNA is supercoiled and the sites are properly oriented. The basic site-specific recombination reaction can thus be exquisitely adapted to the particular needs of the virus or cell in which it occurs.

The Cre and Flp systems also work when engineered into the cells of any organism and thus are highly useful in a wide range of biotechnological applications. A few are shown in Figure 14-8. If the requisite lox or FRT sites are engineered into plasmids or chromosomes in the proper locations, these systems

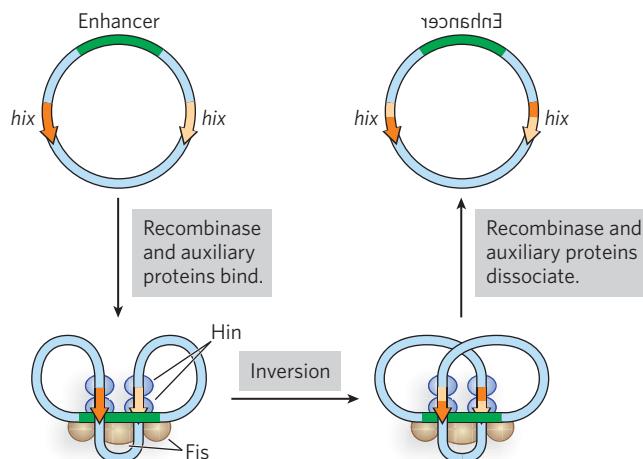


FIGURE 14-7 A topological filter in Hin-hix recombination. The Hin recombination complex consists of four Hin subunits bound in pairs to two *hix* sites, along with an enhancer sequence (green) bound by a host-encoded protein called Fis. Reaction cannot occur unless the entire complex comes together with the topology shown here. This particular alignment of *hix* sites occurs only if the sites are present in the same supercoiled DNA molecule and have the opposite orientation. [Source: Adapted from R. Harshey and M. Jayaram, *Crit. Rev. Biochem. Mol. Biol.* 41:387, 2006.]

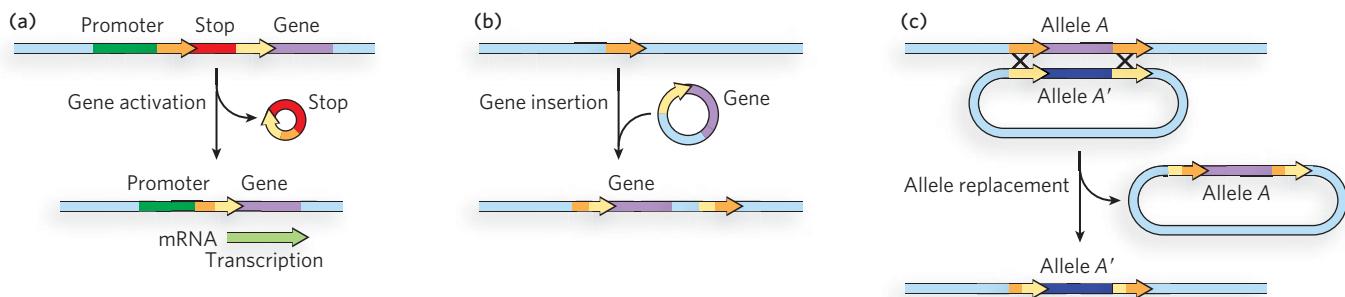


FIGURE 14-8 Some biotechnology applications of site-specific recombination. (a) Gene activation (by deletion of a transcription stop signal). (b) Gene insertion. (c) Allele replacement, using two variants of the same recombination

target site on each end of the gene (or other sequence) to be replaced. In each case, the site-specific recombination target site is engineered into the chromosome or the introduced DNA, or both, with the orientation indicated by the arrows.

can be (and have been) used to activate a particular gene, insert a new gene into a cell at a chosen location, replace one gene with another gene or an altered version of the same gene, delete a gene, or alter the linear structure of an entire chromosome. The sequence specificity of the recombinases allows all of these transactions to be promoted with extraordinary precision. Even more elaborate manipulations are possible. For example, if you tied expression of the recombinase to a promoter expressed only in a particular tissue, you could limit the recombination event to that tissue. Deleting a gene in a certain tissue at a specified time can be a powerful tool for exploring the function of that gene. A vivid example of the application of site-specific recombination in biotechnology is described in Highlight 14-1.

SECTION 14.1 SUMMARY

- Site-specific recombination entails the precise cleavage and rejoicing of DNA ends at specific and reproducible sites in the DNA.
- There are two classes of site-specific recombinases, defined by the key nucleophilic amino acid residue at their active sites: tyrosine or serine.
- Site-specific recombination can be coupled to replication, or resolving chromosomal dimers to monomers before cell division, or amplification of plasmid copy number.
- The mechanism of site-specific recombination can be used to facilitate DNA transactions critical to a viral life cycle.
- Site-specific recombination is sometimes used to regulate gene expression.

- Site-specific recombination can be rendered specific for one particular reaction outcome (integration, deletion, or inversion) by coupling the reaction to the formation of a larger, structured complex in which only one reaction outcome is possible.
- Biotechnologists have adapted site-specific recombination systems to manipulate DNA segments ranging from plasmids to genomes.

14.2 Mechanisms of Transposition

We now consider another type of recombination system: recombination that allows the movement of transposable elements, or transposons—the process of transposition. Found in virtually all cells, transposons move, or “jump,” from one place on a chromosome, the **donor site**, to another on the same or a different chromosome, the **target site**. DNA sequence homology is not usually required for this movement. In some cases the new location is determined more or less randomly, although most transposons exhibit a certain degree of target specificity, and some even make use of particular target sequences.

Insertion of a transposon in an essential gene could kill the cell (an outcome deleterious to the cell *and* the transposon), so transposition is tightly regulated and usually very infrequent. Transposons are perhaps the simplest of molecular parasites, adapted to replicate passively within the chromosomes of host cells. Sometimes they carry genes that are useful to the host cell and thus exist in a kind of symbiosis with the host. In almost all cases, they contribute in important ways to the evolution of the host.

HIGHLIGHT 14-1 TECHNOLOGY

Using Site-Specific Recombination to Trace Neurons

Site-specific recombination offers an opportunity to modify genomic DNA with exquisite precision. A vivid example, and one that illustrates its biotechnological potential, is the brainbow technology introduced by Jeff Lichtman, Jean Livet, and Joshua Sanes. Combining transgenic technology and site-specific recombination, they created a system to map neurons in the mouse brain.

The use of site-specific recombination to make genomic alterations always requires some genetic engineering. Recombination target sites must be installed where a biotechnologist wants them to be. The corresponding recombinase must also be present in the same cell and at the right time, so the systems have to be adapted to a particular purpose or experiment. The most widely used recombination systems are the Cre-lox system of bacteriophage P1 and the Flp-FRT system of the yeast 2μ plasmid. Both have relatively short recombination target sites (34 to 36 bp; see Figure 14-1a), and both rely on a single recombinase enzyme (Cre or Flp) that requires no auxiliary proteins.

Mario Capecchi, Oliver Smithies, and Martin Evans developed powerful procedures to generate transgenic mice with designed genomic alterations (Figure 1; see Chapter 7). DNA containing a gene alteration is introduced into mouse embryonic stem cells. The DNA is introduced in the form of an engineered plasmid DNA that sandwiches the desired alteration between a set of selectable markers that will allow identification of the rare cells with the desired alteration inserted at the correct location. The altered stem cells (from brown mice) are introduced into a very early embryonic stage (blastocyst) of black mice, and the embryos are implanted in a surrogate mother. The altered brown-mouse cells become part of the developing embryo and can appear in almost any tissue. Progeny with the altered brown-mouse cells in their germ line are crossed to produce all-brown progeny, signifying the presence of the desired genetic change in the germ line.

The brainbow method makes use of green fluorescent protein (GFP), the procedures for generating transgenic mice, and site-specific recombination to trace the path of the countless neurons that make up the brain (Figure 2). Inserted into the mouse genome is a gene cassette (a structured set of genes arranged for a particular biotechnological purpose) with several copies of GFP variants that encode proteins fluorescing with different colors, such as red (RFP), orange (OFP), yellow (YFP), and cyan (CFP). Variants of the loxP target site for the Cre recombinase are engineered

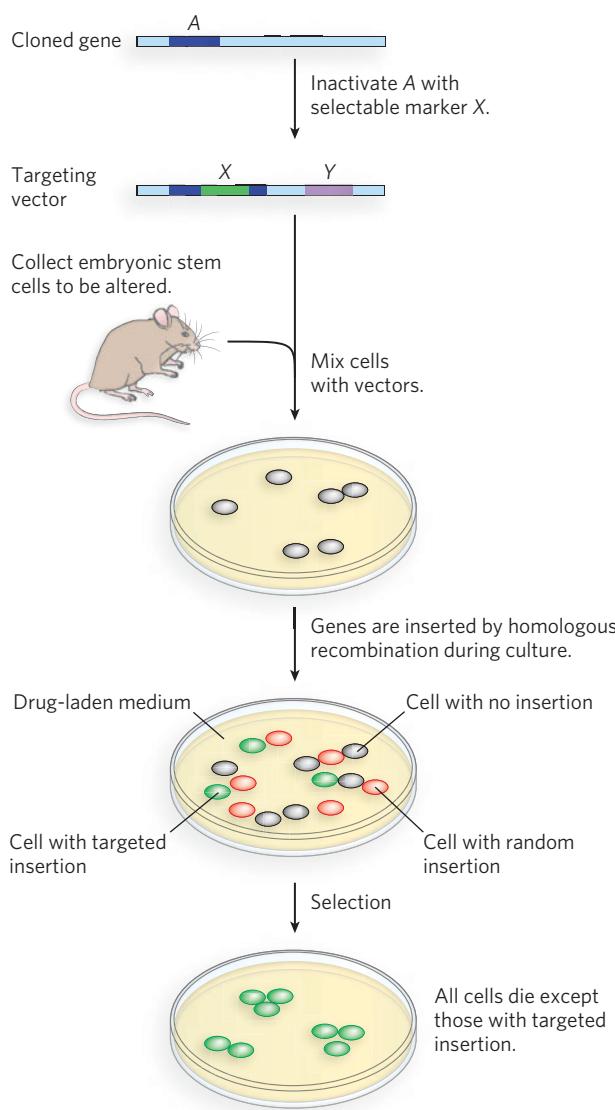


FIGURE 1 Transgenic mice are engineered by insertion of a targeting vector into embryonic stem cells. The vector contains a selectable marker (X) and the desired chromosomal alteration (such as a lox site to be introduced) sandwiched between two DNA segments complementary to the chromosomal site where the alteration is to be integrated. These chromosomal sequences can direct homologous recombination. A second selectable marker (Y) is included in the cassette, outside these homologous sequences, and is generally introduced to the chromosome only if the DNA is integrated at an incorrect (nonhomologous) chromosomal site. The targeted cells are subjected to selection *in vitro*, using drugs to select “for” cells that have selectable marker X and “against” cells that also have selectable marker Y. The surviving cells are then introduced into an early-stage embryo and become part of the tissue of the developing mouse. Genetic crosses allow the selection of mice expressing the desired alterations in their germ line.

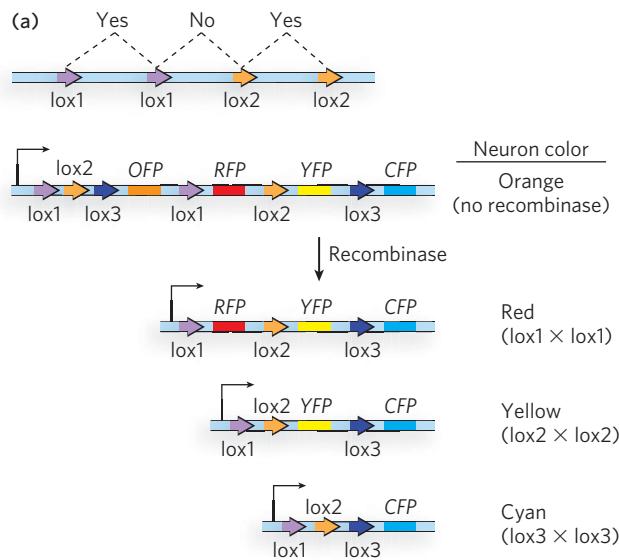


FIGURE 2 Neuronal networks can be traced by turning the network into a brainbow. (a) Transgenic mice with genes encoding GFP variants and different (modified) intervening lox sites are mated to transgenic mice with the gene encoding the Cre recombinase, leading to heterozygous progeny that carry out Cre-mediated recombination in developing neurons. The different lox sites differ in their core sequences, so lox1 reacts only with lox1, and lox2 only with lox2. Cre-mediated recombination results in different patterns of GFP variant expression, and thus a different color, in each neuron. (b) The resulting brainbow of neurons, visualized by a specialized form (epifluorescence) of light microscopy. [Source: (b) Adapted from J. W. Lichtman, J. Livet, and J. R. Sanes, *Nat. Rev. Neurosci.* 9:417–422, 2008.]

between the genes. The loxP sites are arranged so that only one of the three possible recombination events can occur in a particular cassette, and each event will result in gene expression of one of the four GFP variants. The cassette also includes a promoter that directs gene expression only in neurons. Transgenic mice have been engineered that have several of these cassettes, with the potential for expressing one GFP variant from each cassette in a given neuron.

The engineered mice with GFP cassettes are homozygous for these cassettes, and they pass them on to all their progeny. Separately, a second population of homozygous transgenic mice are engineered to express the Cre recombinase, again from a promoter directing gene expression transiently and only in developing neurons. When a mouse with the GFP cassettes is mated to a mouse expressing the Cre recombinase, all progeny are heterozygous for both

the cassette and the recombinase genes. As these mouse embryos develop, the Cre recombinase is expressed early in the development of each neuron. Recombination events occur in some or all of the cassettes in a given neuron, leading to the expression of a particular set of GFP variants. Mixing several different GFP variants in a cell increases the number of potential colors. The outcome is unpredictable for each cell. However, only one set of recombination reactions occurs in each cell, and the end result imparts a distinctive color that is expressed for the life of that neuron. Neighboring developing neurons go through the same recombination processes, but the number of possible outcomes is large and neighboring cells rarely acquire the same color. The result is a rainbowlike array of fluorescent colors in the neural network—a brainbow! Researchers use the brainbow to trace the paths of the axons through the brain.

Transposition Takes Place by Three Major Pathways

Transposition mechanisms are summarized in **Figure 14-9**. Some transposons move by a simple cut-and-paste mechanism. The element is completely excised from the donor DNA and then inserted into a target site. In an alternative, replicative pathway, the transposon is joined to the target site before it is completely excised from the donor DNA. Replication then creates two complete copies of the element, one in the donor site and one in the target site. Elements that make use of either of these pathways are sometimes simply referred to as DNA transposons. A third pathway uses an RNA intermediate to copy the element and insert the copy at a second, target site; these elements, as we'll see, are referred to as retrotransposons.

The basic transposition systems consist of the transposable DNA element and an enzyme, a **transposase**, usually encoded by a gene within the transposable

element. The reactions do not involve the formation of covalent intermediates between the transposase and the DNA. Instead, the transposase catalyzes the hydrolysis of a particular phosphodiester bond, then catalyzes the attack of the liberated 3' hydroxyl on another phosphodiester bond in a transesterification reaction (**Figure 14-10**). The reactions generally leave behind breaks or gaps in the donor DNA strands, which must be repaired by the cell. At the target site, the insertion sites on each strand are staggered to create short repeated sequences at each end of the inserted transposon (see Figure 14-9).

In cut-and-paste transposition, cuts on each side of the transposon excise it, and the transposon moves to a new location (**Figure 14-11a**). This leaves a double-strand break in the donor DNA that must be repaired. We can consider the mechanism used by the well-characterized bacterial transposon Tn5 to illustrate some additional details. Transposases bound to each end of the transposon come together to form a complex

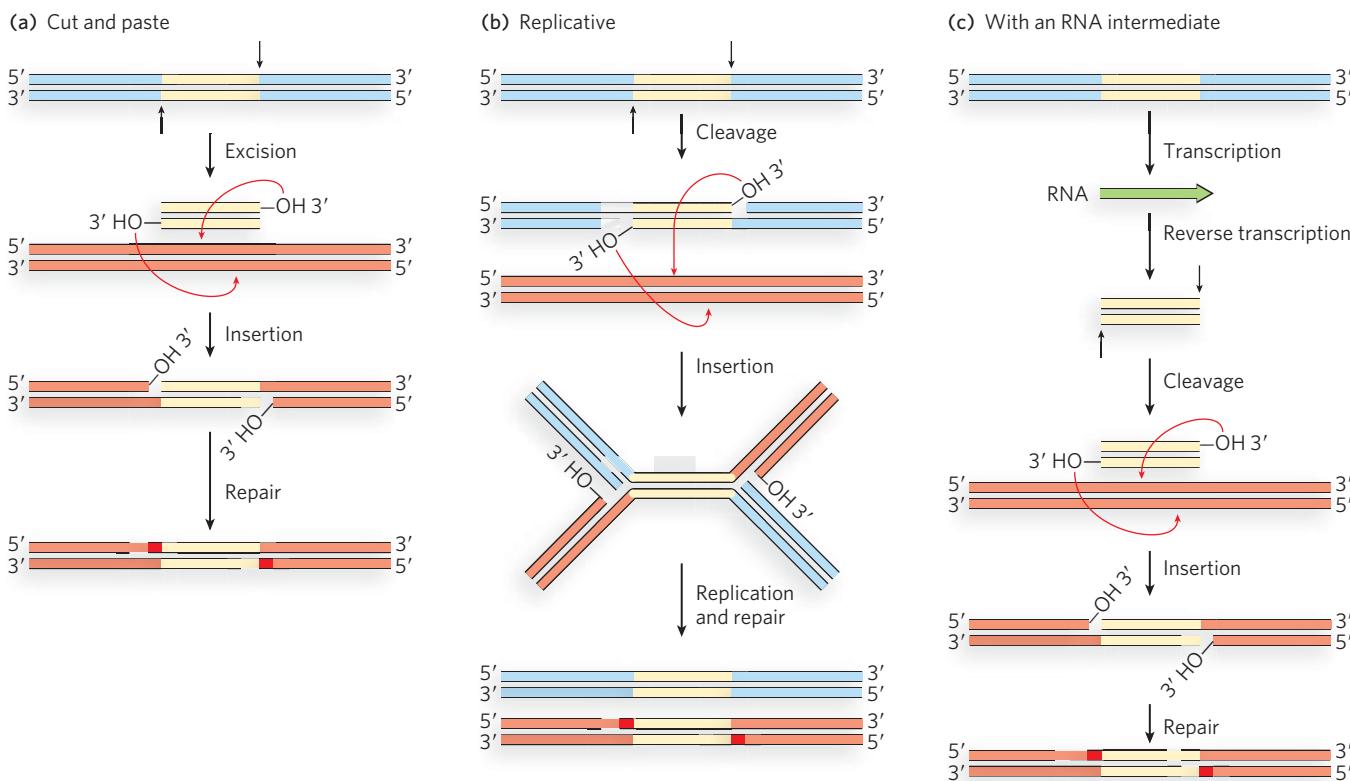


FIGURE 14-9 Three general transposition pathways. (a) Cut-and-paste transposition leaves behind a broken chromosome (double-strand break) that must be repaired. (b) Replicative transposition leaves a copy of the transposon at the original site, as does (c) transposition that uses an RNA intermediate.

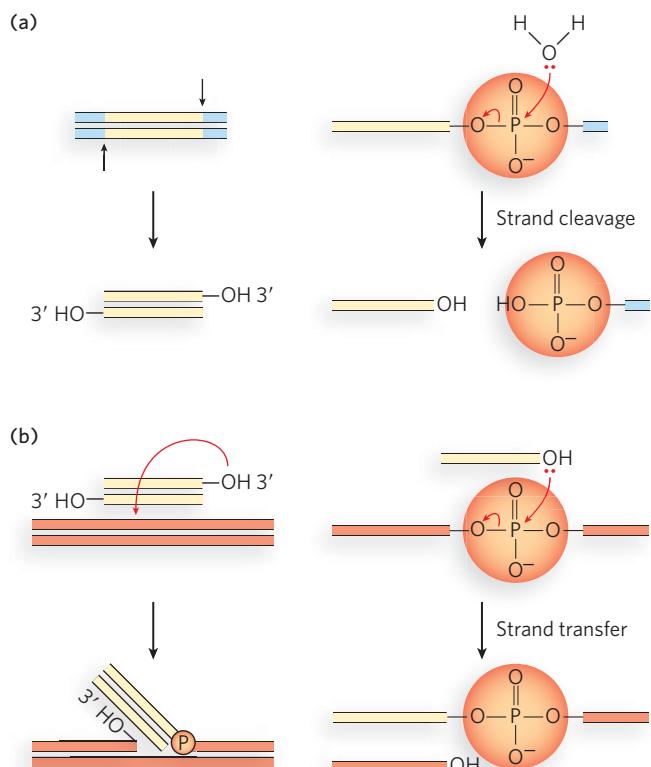


FIGURE 14-10 Hydrolytic cleavage and a transesterification reaction involved in transpositions.

These are the reactions promoted by integrases and transposases. The left side of each panel shows the fate of the DNA, and the right side shows reaction details. (a) The first step in transposition is usually the attack of a water molecule on a phosphodiester bond. (b) The 3'-OH thus liberated acts as a nucleophile, attacking another phosphodiester bond in the target DNA. The attacked bond is cleaved, and a new bond is created.

prior to a cleavage reaction. Each transposon subunit catalyzes the cleavage of the 3' end of one transposon DNA strand. The 3' end then makes a nucleophilic attack on a phosphodiester bond on the other DNA strand at each end of the element, excising the transposon with hairpin closed ends. Next, the transposase catalyzes the hydrolysis of a phosphodiester bond at each end, creating the free 3' ends required for subsequent strand transfer steps to insert the transposon at the target site. Staggered insertion points on the two DNA strands result in the duplication of a short target-derived DNA sequence at either end of the inserted element. The cleaved donor DNA is repaired by ligation or recombinational DNA repair (see Chapter 13), or it is degraded.

This pathway might seem overly elaborate, until we consider the economy involved. One transposase active site at each end of the transposon effectively cleaves two DNA strands of opposite polarity to excise the element from the donor DNA. The hairpin ends are transient intermediates, which researchers have detected in several well-studied transposition reactions. Although not detected in all transposition pathways that use cut and paste, the hairpins may well be a general feature of these mechanisms. The structure of the Tn5 transposase bound to transposon ends and poised to promote strand transfer at the target site (see Figure 14-11a, step 4) is shown in Figure 14-11b. This type of complex illustrates many of the reactions discussed in the rest of the chapter.

In replicative transposition (Figure 14-12), the transposon ends are again brought together in a complex with the transposase, but only one strand is cleaved at each end of the transposon DNA to initiate the process. The cleavage exposes the 3' end of each transposon DNA strand, and these 3' ends are then used as nucleophiles to insert the transposon at a DNA target site in the same or a different DNA molecule. The 3' ends exposed in the resulting intermediate are used to prime replication of the entire transposon. A **cointegrate** is an intermediate in this process, consisting of the donor region covalently linked to DNA at the target site. Two complete copies of the transposon are present in the cointegrate, both having the same relative orientation in the DNA. If the target site is in a different DNA molecule (plasmid or chromosome), the DNAs harboring the transposons in the donor and target sites are linked together in the cointegrate. In some well-characterized transposons, such as the bacterial Tn3, this cointegrate is resolved: the two DNAs are separated by site-specific recombination in which specialized recombinases promote the required deletion reaction. Completion of the reaction installs the transposon at the target site while leaving a copy behind at the donor location.

Transposons that produce an RNA intermediate in the process of transposition are called **retrotransposable elements**, or **retrotransposons**. These transposons can be classified according to the presence or absence of long terminal repeat (LTR) sequences at their termini. The terminal repeats range from about 100 bp to more than 5,000 bp and generally include binding sites for enzymes that function in the transposition process. Both LTR and non-LTR retrotransposon types are widely dispersed, particularly in eukaryotic organisms.

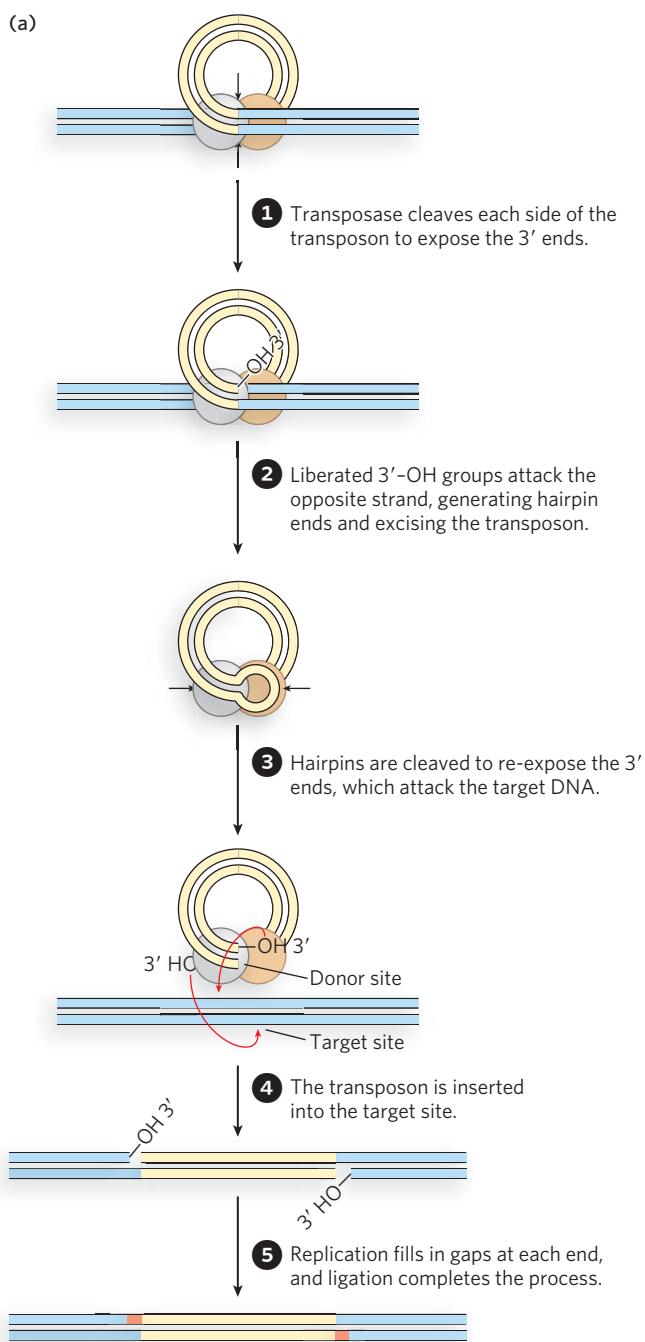
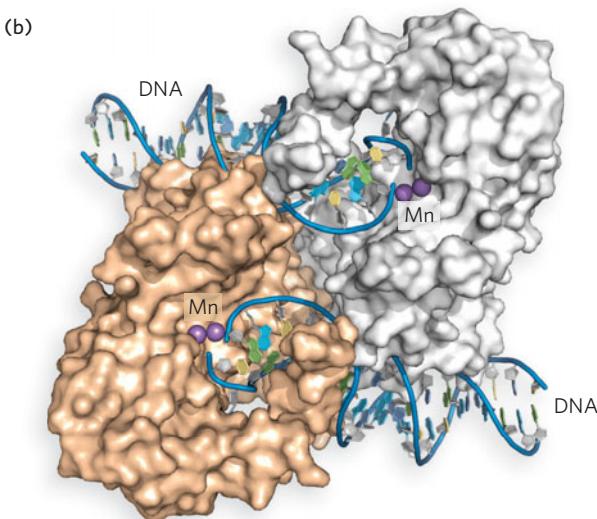


FIGURE 14-11 Cut-and-paste transposition. (a) The transposition steps. (b) Structure of the Tn5 transposase, bound to cleaved transposon ends. The manganese (Mn) ions (present during crystallization) are bound where the Mg^{2+} ions would bind in the normal reaction. [Source: (b) PDB ID 1MM8.]



Retrotransposons make use of a specialized enzyme, **reverse transcriptase**, that can use either RNA or DNA as a template to synthesize DNA. In two major steps, reverse transcriptase generates a double-stranded DNA fragment from a single strand of RNA. The RNA intermediate is first used as a template to generate a complementary DNA strand, forming an RNA-DNA hybrid duplex. The DNA strand of the duplex is then used as a template in the second step to

generate a second DNA strand and a complete double-stranded DNA. The RNA strand is degraded during this second step by a ribonuclease H (RNase H) activity that is present either as a distinct functional domain of reverse transcriptase or as a separate enzyme. RNase H is a class of nonspecific ribonuclease enzyme found in nearly all organisms.

The mechanism is similar to the one used by DNA polymerase (see Chapter 11), and DNA synthesis

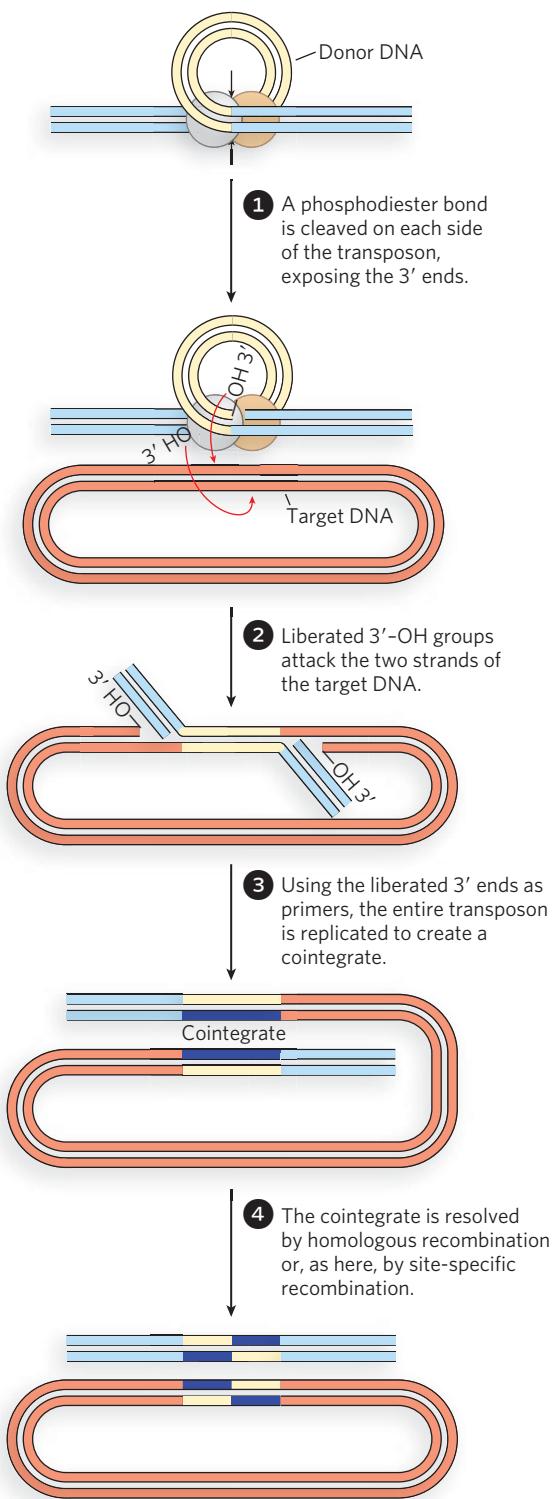


FIGURE 14-12 Replicative transposition.

proceeds in the same 5'→3' direction. Like DNA polymerase, reverse transcriptase adds nucleotides to a primer but cannot initiate DNA synthesis de novo. There are two possible sources of primer that

distinguish LTR and non-LTR retrotransposons by their mechanism of propagation, as well as by their structure, as proposed by Marlene Belfort and colleagues. For LTR retrotransposons, the priming occurs when the RNA is not linked to a chromosome. These **extrachromosomally primed (EP) retrotransposons** borrow some aspects of both cut-and-paste and replicative transposition systems (Figure 14-13a). First, a sequence at one end of the LTR retrotransposon (a promoter) directs transcription of the transposon DNA into RNA. (The process of transcription, promoted by RNA polymerases, is described in Chapter 15.) The resulting single strand of RNA is identical in sequence to one of the two strands of the transposon (with U replacing T). Reverse transcriptase, encoded by the retrotransposon, uses the new RNA strand as a template to synthesize a complementary strand of DNA. The primer for DNA synthesis varies from one class of LTR retrotransposon to another, but is often a cellular tRNA with a 3' end that is complementary to, and anneals to, the retrotransposon RNA. Once the DNA strand is completed, reverse transcriptase uses this strand to generate a DNA complement (the source of primer for this synthesis also varies), resulting in a double-stranded DNA fragment that represents a complete copy of the transposon. Another enzyme, **integrase**, catalyzes insertion of this free DNA transposon copy into a DNA target site. The integrase is related to certain transposases, and it uses the 3' ends of the transposon to attack phosphodiester bonds in the target, as we have seen for cut-and-paste and replicative transposition systems. DNA gaps left at the ends of the inserted transposon are repaired by host replication and ligation enzymes.

In the case of non-LTR retrotransposons, the RNA intermediate is brought to and sometimes linked to the DNA target site by an element-encoded enzyme, before the reverse transcriptase reaction (Figure 14-13b). The enzyme makes a cut in one strand of the target DNA

and uses the liberated 3' end as the primer for DNA synthesis. These non-LTR elements are sometimes called **target-primed (TP) retrotransposons**. A second cut liberates a primer for synthesis of the second DNA strand, a process coupled to elimination of the RNA strand. The product is a double-stranded DNA transposon joined to the target site.



Marlene Belfort [Source: Courtesy of Marlene Belfort.]

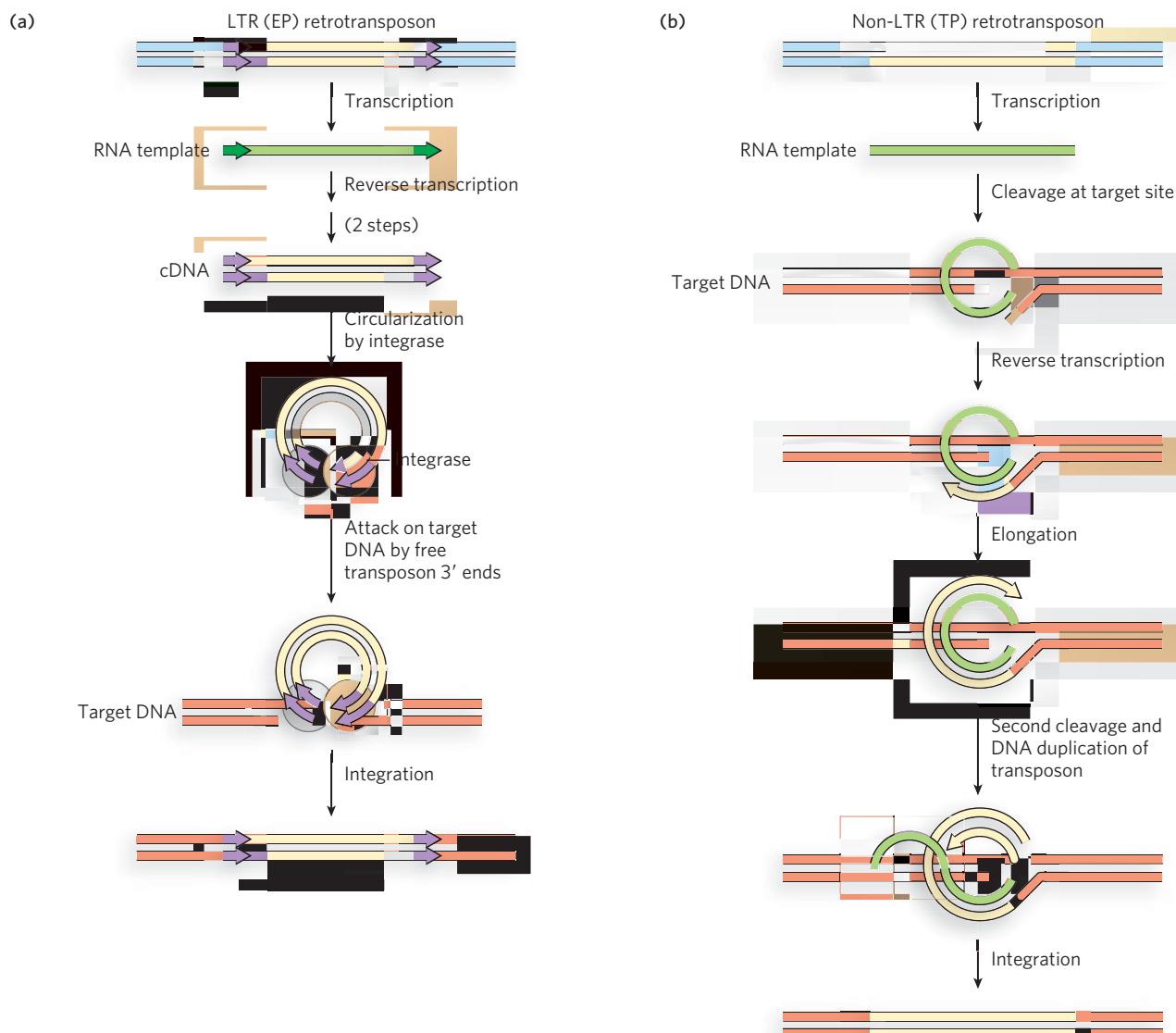


FIGURE 14-13 Retrotransposition. (a) In extrachromosomally primed (EP) retrotransposition, a tRNA or other RNA that can anneal to the transposon RNA is used as a primer for reverse transcription of the RNA to create a double-stranded DNA fragment, in two steps. This DNA fragment inserts itself into a DNA target site, as in cut-and-paste transposition. (b) In target-primed (TP) retrotransposition, the RNA migrates to

the target site, where a phosphodiester bond is cleaved hydrolytically. The exposed 3' end is used to prime reverse transcription of the RNA, which is again converted to double-stranded DNA in two steps. The insertion is completed by ligation. [Source: Adapted from A. Beauregard et al., *Annu. Rev. Genet.* 42:587–617, 2008.]

Bacteria Have Three Common Classes of Transposons

Most of the transposons found in bacteria make use of transposition pathways that do not have RNA intermediates. Bacterial transposons are broadly classified as insertion sequences, composite transposons, or complex transposons (Figure 14-14).

Insertion sequences (also called IS elements) are simple transposons that contain only the sequences required for transposition and the genes for the proteins (transposases) that promote it. **Composite transposons** contain one or more genes in addition to those needed for transposition. These extra genes might, for example, confer antibiotic resistance, thereby enhancing the survival chances of the host cell. Indeed, transposition

contributes substantially to the spread of antibiotic-resistance elements among pathogenic bacterial populations, which is rendering some antibiotics ineffective (see Highlight 9-1). **Complex transposons** have larger genomes and include genes for auxiliary proteins that activate or otherwise assist the transposase, and even some enzymes that promote processes other than transposition. Some complex transposons bridge the distinction between viruses and transposons, exhibiting a capacity not only to transpose within a cell but to migrate as viruses between different cells.

Many hundreds of distinct insertion sequences have been identified in bacteria. Some of these are found in many different species, demonstrating a capacity to cross species lines. The interspecies transfer might happen, for example, on rare occasions when a bacterium dies and pieces of its degraded DNA are taken up at random by another bacterial species. Most insertion sequences transpose by a cut-and-paste mechanism, but a few use replicative transposition. Closely related insertion sequences can be organized into subgroups or families. The IS3, IS4, and IS5 families are particularly widespread.

Composite transposons typically consist of two insertion sequences flanking several other genes. An example is the transposon Tn5, which has two IS elements called IS50 (found elsewhere in bacteria as simple insertion sequences) flanking a group of genes conferring resistance to the antibiotics kanamycin, bleomycin, and streptomycin (Figure 14-14b). Transpositions use only the outer end of each IS50 element, such that the entire composite transposon is moved by cut and paste.

Complex transposons exhibit the most variety of the bacterial elements. Several, including bacteriophage Mu (Figure 14-14c), have been studied in some detail (see How We Know). Bacteriophage Mu has a 37 kbp genome. Like bacteriophage P1, its life cycle features both lysogenic and lytic pathways. When Mu

infects a bacterial cell, a copy of its genome is generally inserted into a random site in the chromosome, probably by cut and paste, and can be replicated passively there. To promote lysis of the cell, the bacteriophage DNA not only is replicated to produce new phage particles but also undergoes rapid transposition to additional random sites in the host chromosome by replicative transposition. The insertion of Mu DNA into random sites in the chromosome can create mutations, often inactivating host genes (Mu is for *mutator*.)

Retrotransposons Are Especially Common in Eukaryotes

Eukaryotic DNA transposons are structurally similar to bacterial transposons, and they migrate by a cut-and-paste mechanism. Retrotransposons are much more richly represented in eukaryotes than are other types of transposons. In the human genome, more than 46% of the DNA in each cell consists of transposon sequences. More than 90% of that transposon DNA comes from retrotransposons. Of all the retrotransposons in the human genome, just over 20% are LTR retrotransposons (about 8% of the human genome) and the remainder are non-LTR. We'll consider first the DNA transposons that use the simple cut-and-paste mechanism, then the elements that utilize an RNA intermediate.

Eukaryotic Cut-and-Paste Transposons A family of transposons called the *Tc1/mariner* family are possibly the most phylogenetically widespread transposons in nature, found in eukaryotes ranging from fungi to plants to humans. First discovered in the early 1980s in the nematode worm *Caenorhabditis elegans* (*Tc* is derived from transposon *Caenorhabditis*), the family acquired the *mariner* moniker as the ubiquitous presence of these transposons became evident (*mariner* was

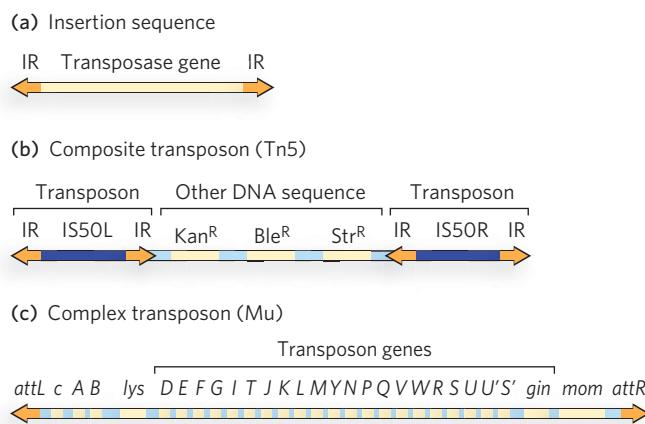


FIGURE 14-14 Three types of bacterial transposons.

(a) Insertion sequences are the simplest transposons, consisting of a transposase-encoding gene and a transposase-binding site within the inverted repeat (IR) sequence at either end. (b) Composite transposons usually consist of two IS elements flanking some additional genes. The transposon Tn5, with two IS50 elements flanking three genes specifying antibiotic resistance, is an example. (c) Complex transposons contain genes, in addition to the transposase gene, that are required for general maintenance. An example is bacteriophage Mu, which can function as either a very efficient transposon or a bacteriophage; *attL* and *attR* are the transposase-binding sites.

the name first attached to one of these transposons discovered in *Drosophila*). The Tc1/mariner elements transpose by a cut-and-paste mechanism and are closely related to the bacterial transposon family IS630.

The transposable elements in this family are 1,300 to 2,400 bp long, and each contains a gene encoding a transposase. Active Tc1/mariner transposons can move about the cellular genomes of just about any species. However, few of them are active (capable of transposition), due to mutations in the transposase genes. Transposons of this family jump into other DNA sites more or less randomly, so the potential for inactivation of essential host genes by disruption is considerable. Transposons that have successfully made the jump into a particular genome have either undergone selection to prevent further transposition or are subject to cellular silencing mechanisms that prevent transposition. Genetic engineering has been used to reactivate transposons of this family, for use in genetic research (Highlight 14-2).

Eukaryotic Retrotransposons Both the LTR (extrachromosomally primed) and non-LTR (target-primed) retrotransposon classes are abundant in eukaryotes. Among the LTR retrotransposons are several different

Ty elements of *S. cerevisiae* and the element *gypsy* of *Drosophila*. Non-LTR retrotransposons include LINEs (long interspersed nuclear elements) and SINEs (short interspersed nuclear elements), along with certain types of introns.

LTR retrotransposons are very closely related to the retroviruses discussed below. Transposition of these elements is illustrated by the Ty transposition cycle (Figure 14-15). A Ty element, integrated in the DNA chromosome of the host cell, is transcribed to produce a single-stranded mRNA molecule with the poly(A) tail characteristic of eukaryotic mRNAs (see Chapter 16). The mRNA is transported from the nucleus to the cytoplasm, where it is translated to produce several Ty proteins, including reverse transcriptase (pol), integrase (int), and a structural protein called Gag. The mRNA is encapsulated in a viruslike particle (VLP), where reverse transcriptase catalyzes formation of a complementary linear, double-stranded DNA, in two steps. The DNA is transported back into the nucleus with the aid of the VLP. Inside the nucleus, the VLP's outer shell, consisting of Gag protein, is shed, and integrase inserts the duplex DNA into the host chromosome at a new location. Almost every step is aided by one or more proteins encoded by the host cell.

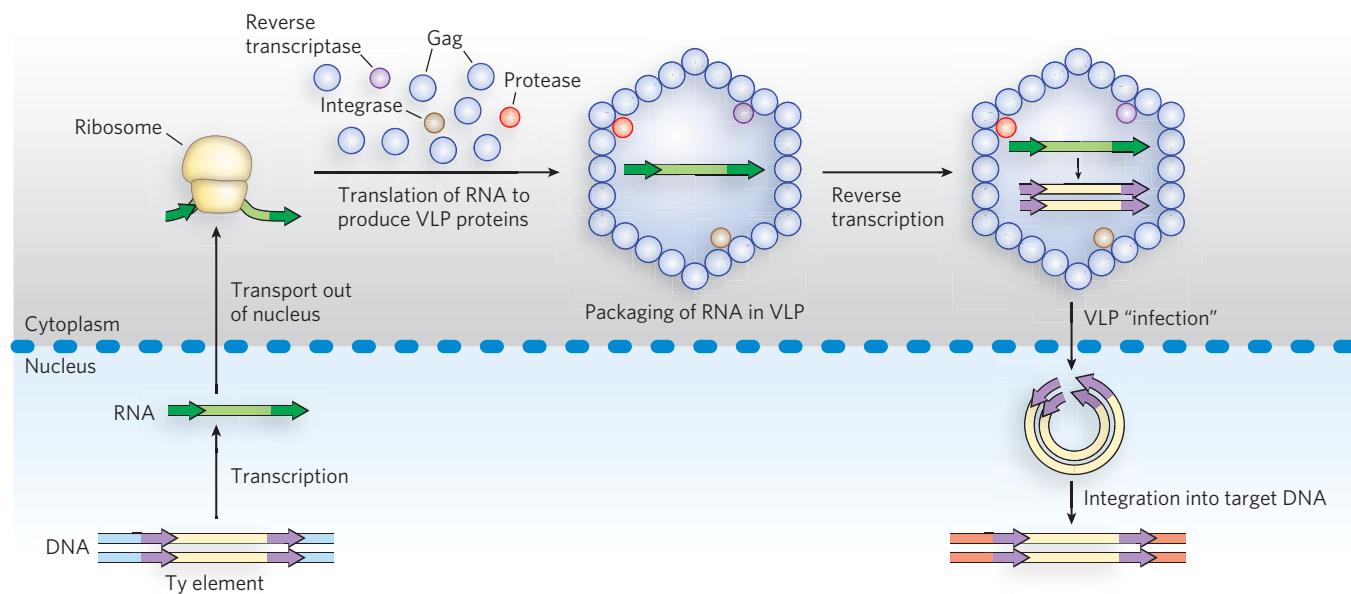


FIGURE 14-15 Retrotransposition by a Ty element in yeast.

The Ty element, within the host DNA, is transcribed to produce an mRNA, which is transported to the cell cytoplasm and translated to generate reverse transcriptase, integrase, and Gag (a structural protein). Within the viruslike particle (VLP),

reverse transcriptase converts the RNA to double-stranded DNA. The VLP is transported back into the nucleus, the Gag protein coat is shed, and the DNA transposon is integrated into a new target site in the host chromosome. [Source: Adapted from A. Beauregard et al., *Annu. Rev. Genet.* 42:587–617, 2008.]

HIGHLIGHT 14-2 EVOLUTION

Awakening Sleeping Beauty

After a transposon successfully integrates into a genome, evolution tends to favor inactivation of the transposase, preventing further transposition and its possible detrimental effects on the host. Thus, virtually all cut-and-paste transposons found in vertebrates are inactive. For a geneticist, this is not always a good thing. Many transposons, with their capacity to integrate into chromosomal sites more or less at random, have the potential to disrupt and thereby inactivate genes at random. Researchers can use this property to create libraries in which each individual organism has one gene inactivated, allowing broad explorations of gene function. To realize this potential, however, the transposons must be made to hop once again. Can these sleeping transposons be awakened?

The answer is clearly yes. For Ronald Plasterk, Zsuzanna Izsvák, and their colleagues, reconstructing an active vertebrate transposon was a classic exercise in genomics. Beginning with a set of related Tc1/mariner elements from fish, the researchers carefully compared the sequences of the embedded and inactive transposase genes (Figure 1). They used a majority-rule kind of analysis. If a particular

sequence was present in most of the transposable elements, it was likely to be functional. Sequences unique to one or a few elements were likely to represent the inactivating changes. Once the research group had established a consensus sequence for a putative active element, they began reconstructing it. Alterations were introduced into one of the inactive genes to make it identical to the consensus.

The scheme was completely successful. The engineered transposon was highly active, not just in fish but in a wide range of eukaryotic cells, including human cells. The new element was dubbed *sleeping beauty*, an apt name for an element awakened from a transposon that had probably been dormant for millennia. Researchers are continuing to study *sleeping beauty* to learn more about Tc1/mariner transposition mechanisms, and it is increasingly used as a tool to mutagenize genes in many different organisms. For example, the introduction of *sleeping beauty* into the mouse genome leads to pups that rapidly develop tumors, because of inactivation of genes controlling cell division. Particular types of tumors can be screened to identify the genes in which the engineered transposon is inserted, and a list generated of all the genes whose inactivation leads to particular tumor types.

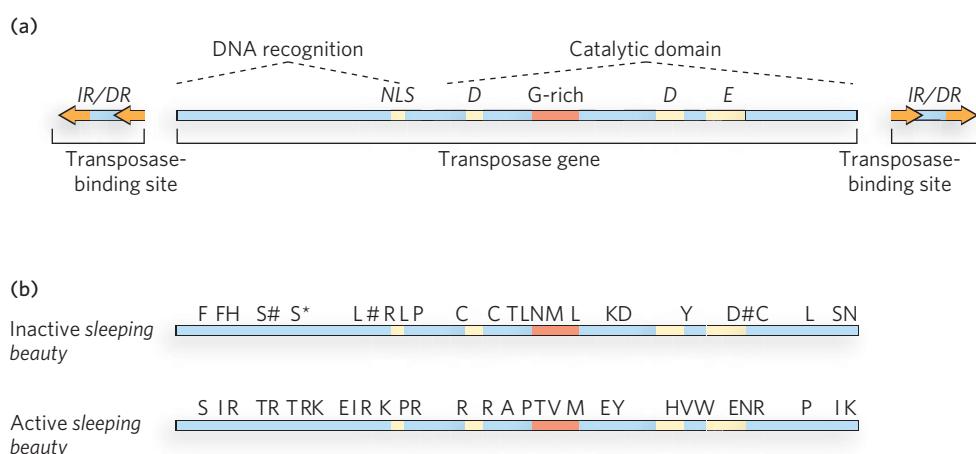


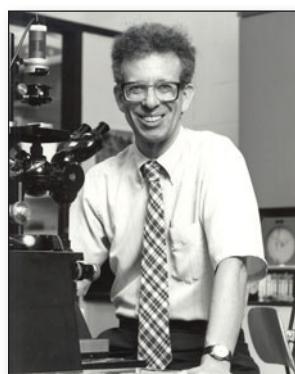
FIGURE 1 Reconstruction of an active Tc1/mariner transposase gene led to creation of the engineered transposon *sleeping beauty*. (a) The structure of an active Tc1/mariner element. (b) Amino acids that were changed to make *sleeping beauty* active again. Asterisk denotes a stop coding number signs denote frameshift mutations. [Source: Z. Ivics et al., *Cell* 91:501–510, 1997.]

The non-LTR retrotransposons also rely on reverse transcriptase, but the transposition pathways vary. The LINEs and SINEs are the most common types of transposons in the human genome. Prominent in all mammalian genomes, they also occur in other classes of eukaryotes and have played important roles in genomic evolution. The most common human LINE is L1, approximately 6,000 bp long. Present in about 520,000 copies, L1 elements account for about 17% of the entire human genome. A LINE element is first transcribed to mRNA in the nucleus. The mRNA is transported to the cytoplasm, where it is translated to produce two proteins: one has both reverse transcriptase and endonuclease functions, and the other helps form a ribonucleoprotein complex important to several steps in the cycle. The ribonucleoprotein complex is transported into the nucleus, where the endonuclease and reverse transcriptase catalyze reintegration of the duplex DNA, as described for all non-LTR (TP) retrotransposons above.

LINE transposition occurs primarily in germ-line cells, using enzymes encoded by the LINE. The transposition cycle of SINEs (<500 bp long) is similar, with one significant difference: SINEs lack the genes needed to code their own transposition and must rely on enzymes encoded by LINEs. When a transposon relies on enzymes encoded by other elements in this way, it is said to be a nonautonomous element.

Retrotransposons and Retroviruses Are Closely Related

Retroviruses are RNA viruses that contain reverse transcriptase. Most retroviruses infect animal cells. On infection, the virus's single-stranded RNA genome (~10,000 nucleotides), along with molecules of reverse transcriptase carried in the viral particle, enters the host cell. The reverse transcriptase converts the viral RNA to double-stranded DNA (Figure 14-16). The resulting viral DNA often becomes incorporated into the genome of the eukaryotic host cell. These integrated (and dormant) viral genes can be activated and transcribed, and the gene products—viral proteins and the viral RNA genome—are packaged as new viruses.



Howard Temin, 1934-1994

[Source: Courtesy of McArdle Laboratory for Cancer Research, Department of Oncology, University of Wisconsin-Madison.]

The idea that biological information could flow from RNA to DNA in the retroviral life cycle was predicted by Howard Temin in 1962. The enzyme that promotes this reaction, reverse transcriptase, was ultimately detected by Temin and, independently, by David Baltimore in 1970. This discovery aroused much attention as dogma-shaking proof that genetic information can flow “backward,” from RNA to DNA.

Retroviruses typically have three genes: *gag*, *pol*, and *env* (Figure 14-17). (The name *gag* is from the historical designation group associated antigen.) In a sense, the retrovirus is simply a retrotransposon, with one additional gene, *env*, which encodes the proteins of the viral envelope. The additional gene is critical because it gives the element the capacity to move from cell to cell instead of just within a genome. The transcript (mRNA) that contains *gag* and *pol* is translated into a long polyprotein. This single large polypeptide is cleaved into six proteins with distinct functions. The proteins derived from the *gag* gene make up the interior core of the viral particle. The *pol* gene encodes a protease that cleaves the polyprotein, an integrase that inserts the viral DNA into the host chromosome, and reverse transcriptase. Many reverse transcriptases have two subunits, *a* and *b*. The *pol* gene specifies the *b* subunit (M_r 90,000), and the *a* subunit (M_r 65,000) is simply a proteolytic fragment of the *b* subunit. At each end of the retrovirus's linear RNA genome are LTR sequences of a few hundred nucleotides, analogous to the LTR sequences found at the ends of LTR retrotransposons. Transcribed into double-stranded DNA, these sequences facilitate integration of the viral chromosome into the host DNA and contain promoters for viral gene expression.

Viral reverse transcriptases catalyze three different reactions: RNA-dependent DNA synthesis, RNA degradation (by a separate RNase H domain), and DNA-dependent DNA synthesis. For DNA synthesis to begin, the reverse transcriptase requires a primer. As seen for many LTR retrotransposons, the primer is a cellular tRNA obtained during an earlier infection and carried in the viral particle. This tRNA is base-paired at its 3' end with a complementary sequence in the viral RNA. The new DNA strand is synthesized in the 5'→3' direction, as in all RNA and DNA polymerase reactions. Reverse transcriptases, unlike DNA



David Baltimore [Source: Courtesy of Norman Seef.]

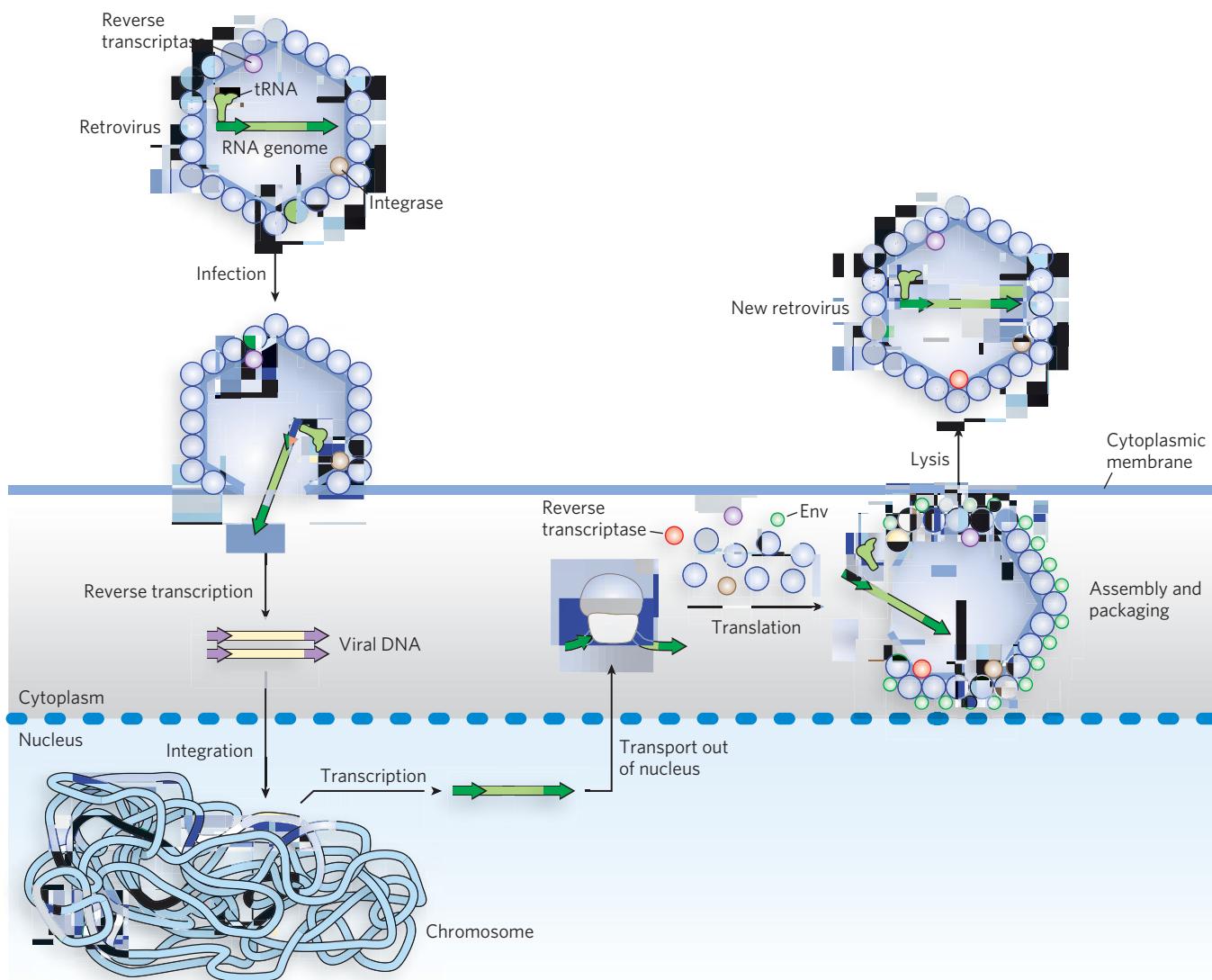


FIGURE 14-16 Retroviral infection of a mammalian cell.

Viral particles entering the host cell (left) carry reverse transcriptase and a cellular tRNA (from a previous host) already base-paired to the retroviral RNA. The tRNA facilitates immediate conversion of the RNA to double-

stranded DNA by the action of reverse transcriptase. The DNA enters the nucleus and is integrated into the host genome, a process catalyzed by a virally encoded integrase. On transcription and translation of the viral DNA, new viruses are formed and released by cell lysis (right).

polymerases, do not have 3'→5' proofreading exonucleases. They generally have error rates of about 1 per 20,000 nucleotides added. An error rate this high is extremely unusual in DNA replication and seems to be a feature of most enzymes that replicate the genomes of RNA viruses. A consequence is a higher mutation rate and a faster rate of viral evolution, which are factors in the frequent appearance of new strains of disease-causing retroviruses.

Reverse transcriptases have become important reagents in the study of DNA-RNA relationships and in DNA cloning techniques. They make possible the

synthesis of DNA complementary to an mRNA template, and synthetic DNA prepared in this manner, called **complementary DNA (cDNA)**, can be used to clone cellular genes (see Figure 7-8).

A Retrovirus Causes AIDS

The human immunodeficiency virus (HIV), which causes acquired immune deficiency syndrome (AIDS), is a retrovirus. Identified in 1983, HIV has an RNA genome with the standard retroviral genes and several other, unusual genes. The virus targets mainly the T lymphocytes (T cells) of the immune system, binding

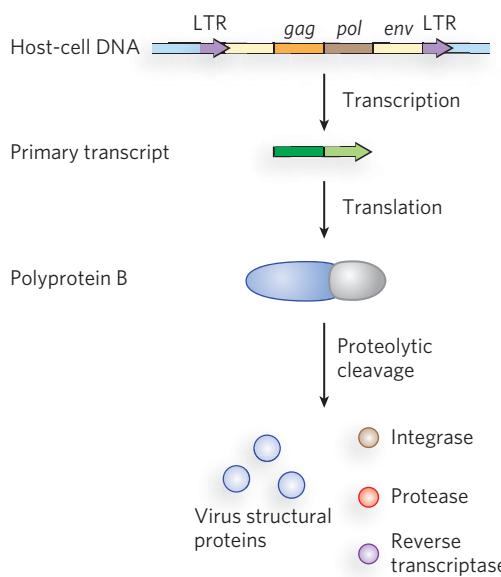


FIGURE 14-17 The structure and gene products of an integrated retroviral genome. The retroviral RNA genome is flanked by long terminal repeats (LTRs), which contain sequences needed for the regulation and initiation of transcription. The retroviral transcript encompasses the *gag*, *pol*, and *env* genes. Translation yields a polyprotein derived from the *gag* and *pol* genes, which is cleaved into six distinct proteins, and a second polyprotein derived from the *env* gene that is similarly cleaved to generate viral envelope proteins.

specifically to a receptor protein, called CD4, on their surface. Unlike many other retroviruses, HIV kills many of the cells it infects, while quietly integrating its genome into the chromosomes of other cells, where it is passively replicated. This gradually suppresses the host's immune system, eventually leading to death. The reverse transcriptase of HIV is even more—tenfold—error-prone than other known reverse transcriptases, resulting in high mutation rates. One or more errors are

HIGHLIGHT 14-3 MEDICINE

Fighting AIDS with HIV Reverse Transcriptase Inhibitors

Research into the chemistry of template-dependent nucleic acid biosynthesis, combined with modern techniques of molecular biology, elucidated the life cycle and structure of HIV, the retrovirus that causes AIDS. A few years after the isolation of HIV, this research resulted in the development of drugs that can prolong the lives of people with HIV/AIDS.

The first drug to be approved for clinical use in treating HIV infection was AZT, 3'-azido-3'-deoxythymidine (Figure 1), a structural analog of deoxythymidine. AZT was first synthesized in 1964 by Jerome P. Horwitz. It failed as an anticancer drug (the purpose for which it was made), but in 1985 it was found to be a useful treatment for AIDS. AZT is taken up by T lymphocytes (T cells), immune system cells that are particularly vulnerable to HIV infection, and converted to AZT triphosphate. (AZT triphosphate taken directly would be ineffective because it cannot cross the plasma membrane.) HIV's reverse transcriptase has a higher affinity for AZT triphosphate than for dTTP, and binding of AZT triphosphate to this enzyme competitively inhibits dTTP binding. When AZT is added to the 3' end of the growing DNA strand, lack of a 3' hydroxyl means that the DNA strand is terminated prematurely, and viral DNA synthesis grinds to a halt.

AZT triphosphate is not as toxic to T cells as it is to the virus, because cellular DNA polymerases have

a lower affinity for this compound than for dTTP. At concentrations as high as 1 to 5 mM, AZT affects HIV reverse transcription but not most cellular DNA replication. Unfortunately, AZT does seem to be toxic to the bone marrow cells that give rise to erythrocytes, and many individuals taking AZT develop anemia. AZT can increase the survival time of people with advanced AIDS by about a year, and it delays the onset of AIDS in people still in the early stages of HIV infection. Some other AIDS drugs, such as dideoxyinosine (DDI) (see Figure 1), have a similar mechanism of action.

Newer drugs target and inactivate the HIV protease. Because of the high error rate of HIV reverse transcriptase and thus the rapid evolution of HIV, the most effective treatments of HIV infection use a combination of drugs directed at both the protease and the reverse transcriptase.

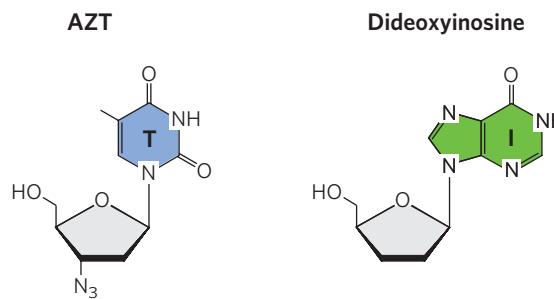


FIGURE 1 Two HIV reverse transcriptase inhibitors used in HIV/AIDS therapy: AZT and DDI.

generally made every time the viral genome is replicated, so any two viral RNA molecules are rarely identical.

Many modern antiviral vaccines contain coat proteins of the virus. These proteins are not infectious on their own but stimulate the immune system to recognize and resist subsequent viral invasions. Because of the high error rate of the HIV reverse transcriptase, however, the *env* gene (along with the rest of the HIV genome) undergoes very rapid mutation, complicating the development of an effective vaccine. In addition, integrated copies of the virus in some T cells provide a source of recurring infection. The most effective therapy currently available is directed toward the inhibition of viral enzymes, hindering the repeated cycles of cell invasion and replication needed to propagate an HIV infection. The HIV protease is targeted by a class of drugs known as protease inhibitors. Reverse transcriptase is the target of drugs widely used to treat HIV-infected individuals (Highlight 14-3).

SECTION 14.2 SUMMARY

- Transposons (transposable elements) are segments of DNA that can move, or “jump,” from one genomic location to another.
- The three general transposition mechanisms are cut and paste, replicative transposition, and transposition with an RNA intermediate. Retrotransposons use an RNA intermediate.
- Transposition makes use of transposases, specialized enzymes that cleave phosphodiester bonds and catalyze transesterification reactions in which the 3' end of a cleaved DNA strand attacks a phosphodiester bond.
- Bacterial transposons fall into three classes. Insertion sequences (IS elements) have only the gene encoding the transposase. Composite transposons contain several additional genes sandwiched between two IS elements. Complex transposons encode additional proteins that facilitate the function of the transposase, and some encode proteins with other functions.
- Eukaryotic transposons include elements that migrate by a cut-and-paste mechanism. Even more common are eukaryotic retrotransposons, some of which are closely related to retroviruses.
- Retrotransposons make use of the enzyme reverse transcriptase, a DNA polymerase that can use both RNA and DNA as a template for DNA synthesis.
- A retrovirus (HIV) is the causative agent of AIDS.

14.3 The Evolutionary Interplay of Transposons and Their Hosts

As we noted in the introduction to this chapter, transposition provides an alternative survival strategy that almost certainly has ancient roots. Evolution has given rise to many types of transposons, which have colonized the genomes of all extant organisms, and it has also given rise to other, more complex pathogens, such as viruses. Although the need to adapt to their hosts has affected transposon evolution, transposons have not always remained separate entities. Some transposable elements, and the enzymes they encode, have been appropriated by host cells and adapted to new biological tasks. The movements of transposons have driven genomic changes that have contributed in important ways to evolution.

Viruses, Transposons, and Introns Have an Interwoven Evolutionary History

Many well-characterized eukaryotic retrotransposons, from sources as diverse as yeast and fruit flies, have a structure very similar to that of retroviruses. Retrotransposons probably gave rise to retroviruses.

The evolution of virtually all retrotransposons and retroviruses can be traced through their reverse transcriptase genes. Similarly, the evolution of an even broader range of transposable elements can be linked to the evolution of retrotransposons and retroviruses through their integrase and transposase genes. Integrase and transposases catalyze very similar reactions (Figure 14-18). The most widespread class of both types of enzymes uses a set of three active-site amino acids, two aspartates and one glutamate, to promote the hydrolytic cleavage and transesterification of phosphodiester bonds. These three residues (D, D, and E) are not adjacent in the primary sequence of these enzymes, but they come together in the active site and constitute a well-recognized motif called the **DDE motif** (Figure 14-19). The close relationship between these enzymes, regardless of source, can have practical benefits. For example, the transposase of the bacterial transposon Tn5 can be used as a model to study the function of the HIV integrase, and even to rapidly test drugs that might be used to inhibit the HIV enzyme.

Transposons have a long and complex history. Their dispersal may include rare events in which DNA was transferred by some means (such as bacterial conjugation, cellular fusion, viral infection, accidental DNA uptake) among the cells of different species. When a transposon is introduced into a new genome, there is

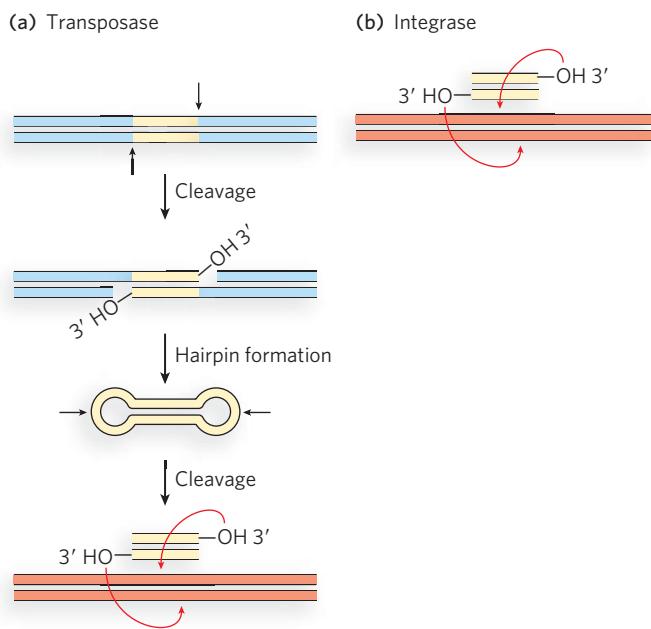


FIGURE 14-18 Transposases and integrases. (a) Transposases promote the nucleophilic attack of the 3' end of a DNA strand on a phosphodiester bond. (b) An integrase carries out the same reaction. (Transposases can also use a water molecule as the nucleophile.) The two types of enzyme have similar active sites.

often a period of many host generations during which the element transposes more or less freely. The number of inserted transposons may increase, with the resulting genomic changes being passed on whenever they do not have a deleterious effect on the host. As time passes, the transposons become subject to silencing processes, including the introduction of mutations in their transposase or integrase genes that inactivate the gene products. Alternatively, the host may find a way to shut down transposition. One common silencing mechanism

involves RNA interference (RNAi; see Chapter 22). In brief, the cell produces short RNA molecules that are complementary to the transcripts of the transposase-encoding gene. The RNA hybridizes to the gene transcripts, preventing their translation and effectively blocking the synthesis of an enzyme required for transposition of an entire class of transposons.

Linear transmission of transposons from one host generation to the next is predominant, with transfer between species occurring rarely. Thus, many transposon families are found only in certain classes of organisms. In eukaryotes, ongoing genomic sequencing efforts have revealed 12 superfamilies of DNA transposons, including *Tc1/mariner* (Table 14-1). Many of these superfamilies are found in more than one eukaryotic type. Seven are closely related to transposons found in bacteria, indicating that they appeared before the divergence of bacteria and eukaryotes.

Sometimes transposons benefit their hosts. As we've seen, the antibiotic-resistance genes encoded by the transposon *Tn5* have contributed greatly to the development of bacterial pathogens that are resistant to those antibiotics. In human cells, there are more than 1 million copies of the *Alu* transposon (a 300 bp SINE element) in the DNA, accounting for nearly 10% of the genome. These elements are so widespread that a typical human gene includes several copies in the introns of its primary transcript. Host cells use these elements as target sites for RNA editing (see Chapter 16). Other transposon genes are appropriated by the host for other purposes. Efforts to trace the evolution of mammalian genes have identified several dozen that are derived from transposons. A dramatic case of transposon domestication—occurring in immunoglobulin formation—is described shortly.

Perhaps more important is the overall impact of transposons on the evolution of the host. Genomic changes promoted by transposons come in many forms.

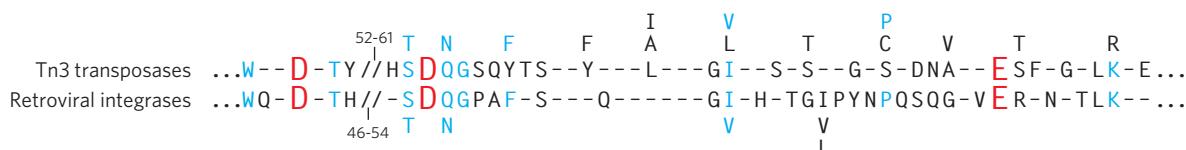


FIGURE 14-19 The DDE motif. Consensus sequences are shown for the catalytic domain of transposases of the *Tn3* family (top) and retroviral integrases (bottom). The DDE motif consists of three residues (Asp, Asp, and Glu, the DDE residues shown in red) that are generally not adjacent in the primary sequence but come together at the active site when the protein is folded. The motif is found in most transposases

and integrases. Some additional residues that are conserved in transposases and integrases are shown in blue. The residues shown are the only ones that are found in these enzymes at the positions indicated. Dashes indicate any amino acid residue; two peptide segments, with the residue numbers indicated, are omitted here. [Source: P. Polard and M. Chandler, *Mol. Microbiol.* 15:13-23, 1995.]

Table 14-1 DNA Transposons in Eukaryotes

Superfamily	Bacterial Relative	Catalytic Motif	Length (kbp)	Occurrence (groups of organisms)
Tc1/mariner hAT	IS630	DDE (or DDD)	1.2–5.0	All except diatoms and green algae
	Not determined	DDE	2.5–5.0	Vertebrates, invertebrates, plants, fungi, green algae, <i>Entamoeba</i> , <i>Phytophthora</i>
P element	Not determined	DDED	3–11	Invertebrates, green algae
MuDR/Foldback	IS256	DDE	1.3–74	Vertebrates, invertebrates, plants, fungi, diatoms, <i>Entamoeba</i>
CACTA	Not determined	Not determined	4.5–15	Invertebrates
PiggyBac	IS1380	DDE	2.3–6.3	<i>Phytophthora</i>
PIF/Harbinger	IS5	DDE	2.3–5.5	Vertebrates, invertebrates, plants, fungi, diatoms
Merlin	IS1016	DDE	1.4–3.5	Vertebrates, invertebrates, <i>Phytophthora</i>
Transib	Not determined	DDE	3–4	Invertebrates, fungi
Banshee	IS481	DDE	3–5	<i>Trichomonas</i>
Helitron	IS91	HHYY	5.5–17	All except diatoms, green algae
Maverick	None	DDE	15–25	All except plants, diatoms, green algae

Source: Adapted from C. Feschotte and E. J. Pritham, *Annu. Rev. Genet.* 41:331–68, 2007.

Transposons are set up to bring their ends together in a complex prior to any cleavage event, but this control mechanism can go awry. If transposase subunits form a complex involving two ends derived from different copies of the same transposon, on the same or different chromosomes, large genomic rearrangements can result. Genes may be captured between two transposable elements and moved to different genomic locations. If the genes are duplicated in the process, the new gene copies may evolve and acquire new functions. Transposition is not always precise; the insertion of a transposon into a gene, followed by its later excision, can add or subtract base pairs in the gene and create new alleles. Also, the insertion or excision of transposons at particular genomic sites can alter the expression of genes or sets of genes.

As we noted earlier, the transposons that seem to clutter mammalian genomes have been referred to as selfish or junk DNA, but these labels are being shed as our understanding broadens. Transposon DNA may play a key role in chromosomal structure and packaging. And far from being dormant, transposon DNA is actively transcribed in at least some cells. As new classes of functional RNAs are being discovered at a rapid pace, the RNAs produced by transposons may prove to have unexpected cellular roles.

A Hybrid Recombination Process Assembles Immunoglobulin Genes

Even though the human genome contains only about 25,000 genes, a human is capable of producing millions of different immunoglobulins (antibodies) with distinct binding specificities. During the differentiation of B lymphocytes (B cells), immunoglobulin genes are recombined so that each cell will express an antibody with a unique binding specificity. Studies of the recombination mechanism reveal a close relationship to DNA transposition and suggest that this system for generating antibody diversity evolved from an ancient cellular invasion by transposons.

Immunoglobulins consist of two heavy and two light polypeptide chains (Figure 14-20 shows the general structure of the IgG class of immunoglobulins). Each chain has two regions: a variable region, with a sequence that differs greatly from one immunoglobulin to another, and a constant region, which is virtually unchanging within a class of immunoglobulins. There are two distinct families of light chains, kappa and lambda, which differ somewhat in the sequence of their constant regions. For all three types of polypeptide chain (heavy chain, and kappa and lambda light chains), diversity in the variable regions is generated by a similar

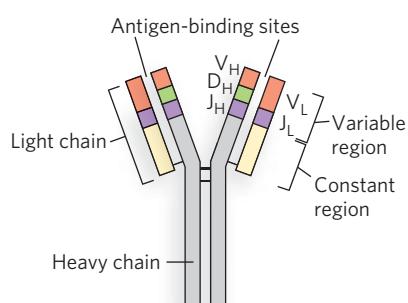


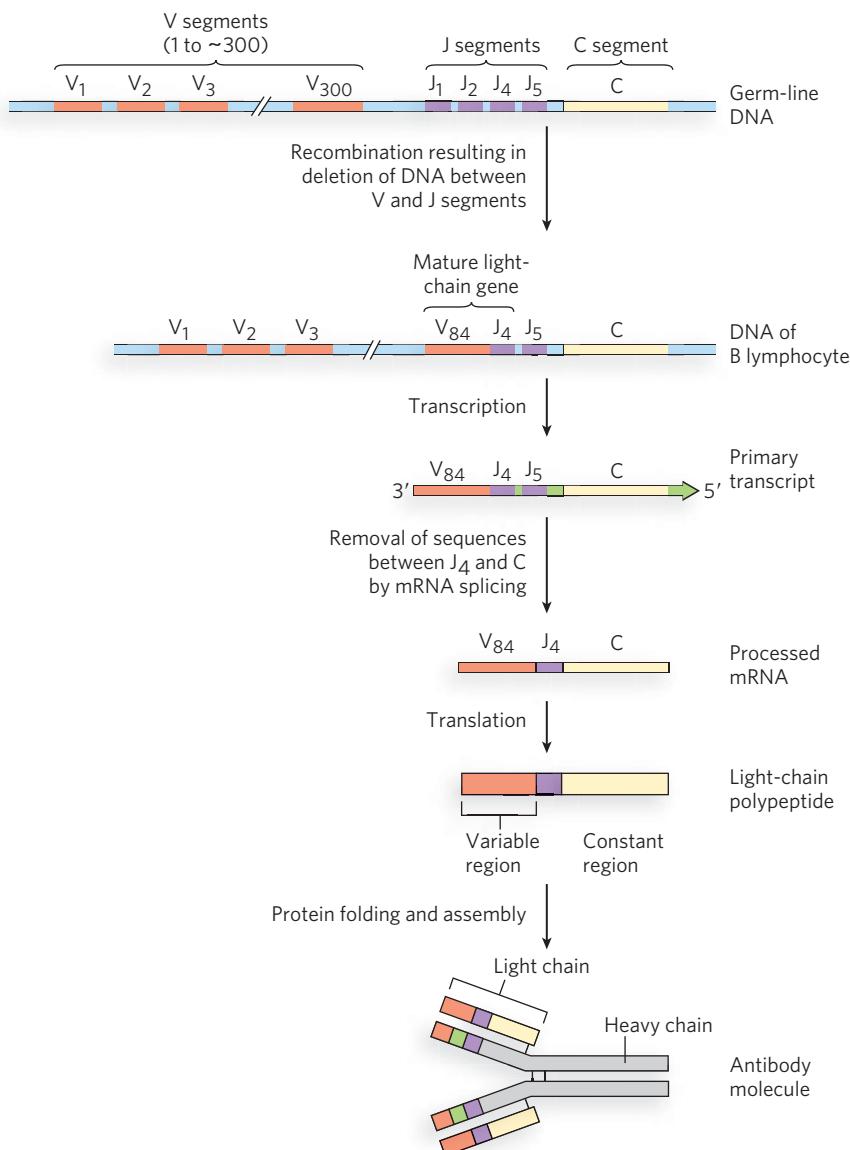
FIGURE 14-20 Immunoglobulin G (IgG). Pairs of heavy and light chains combine to form a Y-shaped molecule. Two antigen-binding sites are formed by the combination of variable domains from one light and one heavy chain.

mechanism. The genes for these polypeptides are divided into segments, and the genome contains clusters with multiple versions of each segment. The joining of one version of each gene segment creates a complete gene.

Figure 14-21 depicts the organization of the DNA encoding the kappa light chain and shows how a mature kappa light chain is generated. In undifferentiated cells, the coding information for this polypeptide is separated into three segments. The V (variable) segment encodes the first 95 amino acid residues of the variable region, the J (joining) segment encodes the remaining 12 residues of the variable region, and the C (constant) segment encodes the constant region. The genome

FIGURE 14-21 Recombination of the V and J gene segments of the human IgG kappa light chain.

This process results in antibody diversity. Shown at the top is the arrangement of IgG-coding sequences in a bone marrow stem cell. Recombination deletes the DNA between specific V and J segments. The RNA transcript is processed by RNA splicing; translation produces the light-chain polypeptide. The light chain can combine with any of 5,000 possible heavy chains to produce an antibody molecule.



contains ~ 300 different V segments, 4 different J segments, and 1 type of C segment.

As a stem cell in the bone marrow differentiates to form a mature B cell, one V segment and one J segment are brought together by a specialized recombination system (Figure 14-22). During this programmed DNA deletion, the intervening DNA is discarded. There are about $300 \times 4 = 1,200$ possible V-J combinations. Additional variation in the sequence at the V-J junction is introduced by imprecision in the recombination reaction. This increases the overall variation by a factor of at least 2.5, so the cells can generate at least $2.5 \times 1,200 = 3,000$ different V-J combinations. The final joining of the V-J combination to the C region is accomplished by an RNA-splicing reaction after transcription (see Chapter 16).

The recombination mechanism for joining the V and J segments is facilitated by recombination signal sequences (RSS) that lie just downstream of each V segment and just before each J segment. These sequences are bound by proteins called RAG1 and RAG2

(products of the recombination activating gene). The RAG proteins catalyze the formation of a double-strand break between the RSS and the V (or J) segments to be joined. The V and J segments are then joined with the aid of a second complex of proteins.

The genes for the heavy chains and the lambda light chains form by similar processes. Heavy chains have more gene segments than light chains, with more than 5,000 possible combinations. Because any heavy chain can combine with any light chain to generate an immunoglobulin, each human has at least $3,000 \times 5,000 = 1.5 \times 10^7$ possible immunoglobulins. And additional diversity is generated by high mutation rates (of unknown mechanism) in the V segments during B-cell differentiation. Each mature B cell produces only one type of antibody, but the range of antibodies produced by the B cells of an individual organism is clearly enormous.

The immune system evolved, in part, from ancient transposons. The mechanism for generation of the double-strand breaks by RAG1 and RAG2 resembles several reaction steps in transposition (see Figure 14-22). A hairpin intermediate is formed transiently at each of the ends to be joined, as in the reaction promoted by the Tn5 transposase (see Figure 14-10). In addition, the deleted DNA, with its terminal RSS, has a sequence structure found in most transposons. In the test tube, RAG1 and RAG2 can associate with this deleted DNA and insert it, transposonlike, into other DNA molecules (probably a rare reaction *in vivo*). In fact, a subtle rearrangement of the RSS, coupled with placement of the genes encoding the RAG proteins between the RSS ends, creates a DNA element that functions exactly like a transposon. The RAG1 protein is closely related in sequence to the transposases encoded by the *Transib* superfamily of eukaryotic transposons (see Table 14-1). The properties of the immunoglobulin gene rearrangement system point to an intriguing origin, in which the distinction between host and parasite has become blurred by evolution.

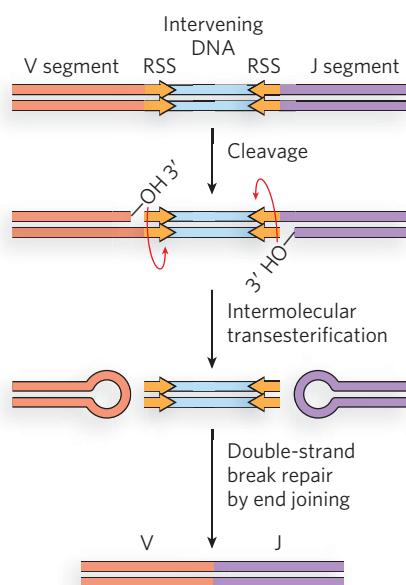


FIGURE 14-22 Immunoglobulin gene rearrangement.

Proteins RAG1 and RAG2 bind to RSS (recombination signal sequences) and cleave one DNA strand between the RSS and the V (or J) segments that are to be joined. The liberated 3' hydroxyl acts as a nucleophile, attacking a phosphodiester bond in the other strand to create a double-strand break. The resulting hairpins on the V and J segments are cleaved, and the ends are covalently linked by a complex of proteins specialized for end-joining repair of double-strand breaks, as described in Chapter 13. The steps in the generation of the double-strand break, catalyzed by RAG1 and RAG2, are chemically related to steps in transposition reactions.

SECTION 14.3 SUMMARY

- Transposons, retrotransposons, and retroviruses have a shared evolutionary history, evident in the phylogenies of the key enzymes—reverse transcriptases, transposases, and integrases—that promote these processes.
- Important elements of the vertebrate immune system, the enzymes that promote immunoglobulin gene rearrangements and thus immunoglobulin diversity, evolved from the transposase/integrase family of enzymes. RAG1 is related to the transposases of the *Transib* transposons.

Unanswered Questions

For any organism, the information required for creating a new generation is passed on through its DNA. Stable transmission is needed, yet genomes are surprisingly dynamic. Recombination processes contribute to repair and facilitate key steps of replication and cell division. A hidden world of transposons makes a home in each genome, replicating passively yet contributing to evolution in important ways. This dynamic genome still holds some secrets to unlock.

- 1. How can scientists use recombination and transposition to manipulate genomes?** Viruses and transposons invade cells and genomes through mechanisms tailored by evolution to suit the needs of these parasites. In many cases, they invade without harming the host. Site-specific recombinases promote precise genomic rearrangements on cue. Many new technologies are based on these processes and enzymes, but it seems certain that they only scratch the surface of possibilities. For curing human genetic diseases, we'll need new methods for precise replacement of dysfunctional genes, not just in a few cells but in many. Viruses have already been harnessed to carry new DNA into human cells and integrate it into the genome. We need novel techniques for targeting that integration with reliable precision. The enzymes described in this chapter (and in Chapter 13) will probably be part of the solution. A new capacity for the precise alteration of genomes will facilitate both research and medicine.
- 2. How many proteins and other factors are involved in controlling transposition?** Exploration of the elaborate interface between transposons and their hosts is only just beginning. The processes that

silence a transposon often involve genes found in both the transposon and the host. The extent of host gene involvement has not been fully explored in most cases; functional RNA molecules may do part of the work. Similarly, elaborate processes that prevent transposon integration into other transposons are only partially understood. AIDS is, as yet, an almost intractable disease, in part because of the capacity of HIV to integrate into a host genome and remain there, replicating passively. A permanent cure for HIV cannot occur as long as these silent HIV genomes provide a potential source of new infection. A better understanding of how this integration is regulated may eventually lead to genomic clean-up therapies to eliminate or permanently inactivate these pathogens. That understanding must come from work on a wide range of viruses and transposons to fully sample the variety of mechanisms they use, as well as to unearth host functions that play subtle roles.

- 3. What do retroviruses and transposons contribute to their hosts?** The evolutionary history of these pathogens is clearly not entirely shaped by their own requirements. Obvious contributions to host survival have already been described, but the sheer bulk of transposon DNA in the human genome inspires new questions about function. How do all these repeated transposon sequences affect the structure and function of chromosomes? New reports suggest that much of the genomic DNA previously labeled as junk is in fact transcribed. What are all these RNA molecules doing?

How We Know

Bacteriophage λ Provided the First Example of Site-Specific Recombination

Echols, H. 2001. *Operators and Promoters: The Story of Molecular Biology and Its Creators*. Berkeley: University of California Press.

Gottesman, M.E., and R.A. Weisberg. 2004. Little lambda, who made thee? *Microbiol. Mol. Biol. Rev.* 68:796–813.

Nash, H.A. 1975. Integrative recombination of bacteriophage lambda DNA in vitro. *Proc. Natl. Acad. Sci. USA* 72:1072–1076.

Since the 1950s, scientists have known that the DNA of bacteriophage λ is linked to its bacterial host chromosome at a specific chromosomal location. The correct explanation for how the λ DNA enters the chromosome appeared in 1962, before anyone knew that the linear λ DNA is circularized on entering a bacterial cell. Allan Campbell, then at the University of Rochester, had the novel insight that circularization, followed by recombination into the host chromosome, could explain many observations associated with λ lysogeny. Clearly, a uniquely precise recombination process was at work, one that used defined DNA sequences.

A molecular understanding of this, as yet unprecedented, reaction mechanism required an in vitro system—which came in a breakthrough reported separately by Howard Nash (integration) and by Max Gottesman and Susan Gottesman (excision) in 1975. The researchers were working in competing laboratories just a few buildings apart at the National Institutes of Health in Bethesda, Maryland. The Nash system was the more successful of the two, rapidly leading to a detailed biochemical definition of the λ integration reaction and its components.

In his in vitro system, Nash constructed an altered bacteriophage λ chromosome that included both recombination sites, by then defined and named *attB* and *attP* (*B* for bacterium and *P* for phage), separated by about 15% of the chromosome's length (Figure 1). As a source of the required enzymes, Nash used a concentrated extract derived from cells in which λ proteins were being produced. He then showed that integrative recombination between the two recombination sites would occur in cells to produce phage chromosomes 15% smaller than normal.

Phage with the shortened chromosome had the useful property that they were infectious in the presence of metal-chelating agents, whereas phage with the larger chromosome were not. After an in vitro reaction, phage introduced into bacterial cells and plated on agar containing a metal chelator started an infection cycle only after successful recombination. The infection created plaques (clear spots of killed cells in the bacterial lawn) that could be counted. After years of optimization, Nash reported the first in vitro site-specific recombination system in 1975 (the Gottesmans' report appeared three months later).



Howard Nash [Source: Courtesy of Howard Nash.]

A successful in vitro system is a powerful thing in molecular biology. Nash, soon joined at the NIH by Kiyoshi Mizuuchi, used his system to purify the λ Int protein, discover a required host protein (IHF, for *integron host factor*), and define the reaction requirements. Following further work in other labs, notably that of Art Landy at Brown University, the λ integration system gradually yielded its secrets and stimulated the search for other site-specific recombination systems, such as those described in this chapter.

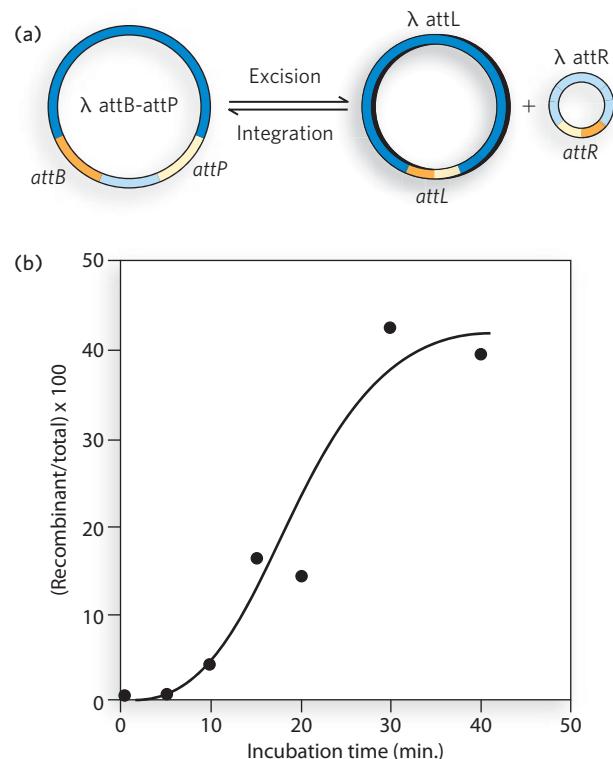


FIGURE 1 (a) The structure of the altered bacteriophage λ substrate developed by Nash, and the reaction promoted by the recombination system. (b) The number of recombinant λ phages produced (as percentage of total) as a function of incubation time with the enzyme extract. [Source: Adapted from H. A. Nash, *Proc. Natl. Acad. Sci. USA* 72:1072–1076, 1975.]

If You Leave out the Polyvinyl Alcohol, Transposition Gets Stuck

Craigie, R., and K. Mizuuchi. 1985. Mechanism of transposition of bacteriophage Mu: Structure of a transposition intermediate. *Cell* 41:867-876.



Kiyoshi Mizuuchi [Source: Courtesy of Kiyoshi Mizuuchi.]

Understanding how a process works often starts with focusing on the reactions that are most efficient and easiest to detect and study. Thus, bacteriophage Mu was chosen as a model system for studying transposition. Although Mu is a complicated transposon, its status as a preferred research subject was based on its capacity to transpose often. When it moves, it replicates itself, leaving a copy behind at the original chromosomal site and depositing a new one in the target. A few base pairs of chromosomal DNA in the target are also replicated, creating a short repeated sequence at both ends of the insertion site. The entire process seemed a little bit like magic.

In 1979, James Shapiro, at the University of Chicago, proposed a mechanism for Mu transposition (Figure 2). It involved nicking DNA strands to expose both 3' ends of the transposon, then making a staggered break in the target DNA, leaving 5' overhangs on the resulting ends. The transposon 3' ends were then joined to the target 5' ends. The remaining 3' ends would prime replication, creating two copies of the transposon and an intermediate called a cointegrate. This intermediate could be resolved by homologous or site-specific recombination to yield the final products. Other researchers proposed alternatives, but Shapiro's model eventually proved to be largely correct, except that the direct attack of transposon 3' ends on target phosphodiester bonds was not yet known, or anticipated. The key was to find the postulated reaction intermediates.

Mizuuchi, working with his associate Bob Craigie, found the intermediates. In the early 1980s, they developed an *in vitro* system that supported Mu transposition, using a plasmid that included a much-abbreviated copy of Mu with both ends of the transposon (the binding sites for the Mu transposase). The Mu proteins

required (MuA and MuB), and the *E. coli* host proteins, were made available as extracts from cells in which they were being expressed. The target DNA was another circular DNA, derived from bacteriophage φ X174 (a phage that has no transposition properties). With this system,

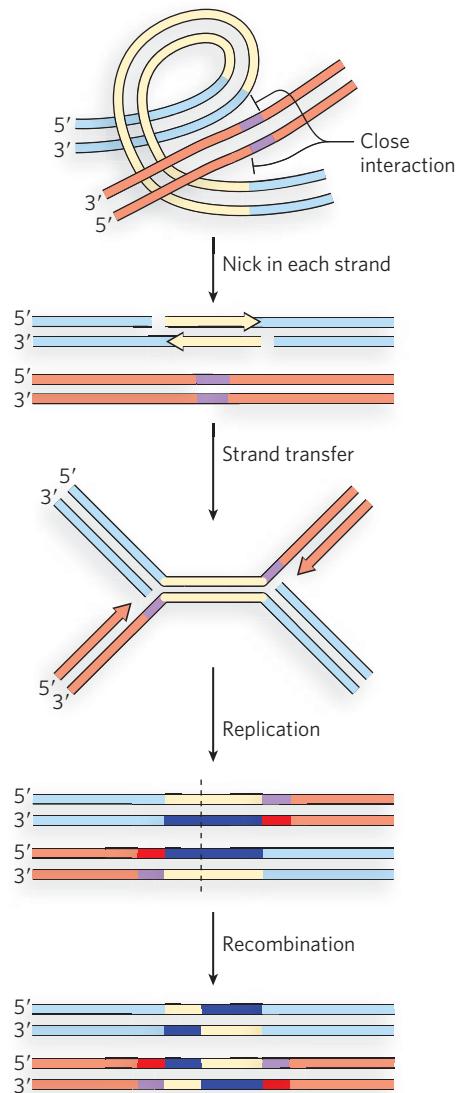


FIGURE 2 The Shapiro model for transposition. [Source: J. A. Shapiro, *Proc. Natl. Acad. Sci. USA* 76:1933-1937, 1979.]

the researchers could detect both the cointegrate and the simple insertion products where a new copy of Mu was integrated in the target DNA. Although the results supported Shapiro's model, the researchers did not detect the predicted strand-transfer intermediates before replication. Either these intermediates did not exist or they were converted to the final products of transposition too quickly for detection.

There are two major steps in the process postulated by Shapiro: DNA cleavage and strand transfer, followed by replication. We now know that the initial DNA cleavage and strand-transfer events need MuA and MuB proteins. The replication steps are more complex, requiring multiple proteins from the host cell. Completing the entire reaction required a concentrated cell extract and the addition of the polymer polyvinyl alcohol. The polymer acted as a solvent-exclusion agent, further concentrating the reaction components. In controls, Craigie and Mizuuchi left out different reaction components to demonstrate that each was needed. When they left out polyvinyl alcohol, the reaction did not generate products, but a prominent new DNA band appeared on

the agarose gels that they were using to analyze the reaction. Craigie and Mizuuchi knew how to capitalize on this bit of serendipity. Analysis of this new DNA species soon revealed that it was a strand-transfer intermediate proposed by Shapiro.

What had happened in the polyvinyl alcohol control? The absence of the polymer led to a partial reaction in which strand transfer became a dead end, with the resulting transposition intermediate building up to concentrations that made it much easier to detect and study. To confirm that this species was indeed a normal reaction intermediate (and not simply produced by unusual reaction conditions), Craigie and Mizuuchi isolated the putative intermediate DNA species, added back cell extract without MuA and MuB but with replication enzymes now aided by polyvinyl alcohol, and showed that the predicted transposition products were generated. The result was a definitive study establishing key facts about the pathway of Mu transposition. More broadly, the study played a major role in developing our current understanding of all transposition mechanisms.

Key Terms

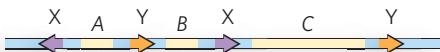
transposable element, p. 482	isoenergetic, p. 488	integrase, p. 495
transposon, p. 482	donor site, p. 489	target-primed (TP) retrotransposon, p. 495
transposition, p. 482	target site, p. 489	insertion sequence, p. 496
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lysogenic pathway, p. 485	cointegrate, p. 493	complex transposon, p. 497
prophage, p. 485	retrotransposable element, p. 493	retrovirus, p. 500
lytic pathway, p. 485	retrotransposon, p. 493	complementary DNA (cDNA), p. 501
Cre-lox, p. 487	reverse transcriptase, p. 494	DDE motif, p. 503
bacterial transduction, p. 487	extrachromosomally primed (EP)	
phase variation, p. 487	retrotransposon, p. 495	

Problems

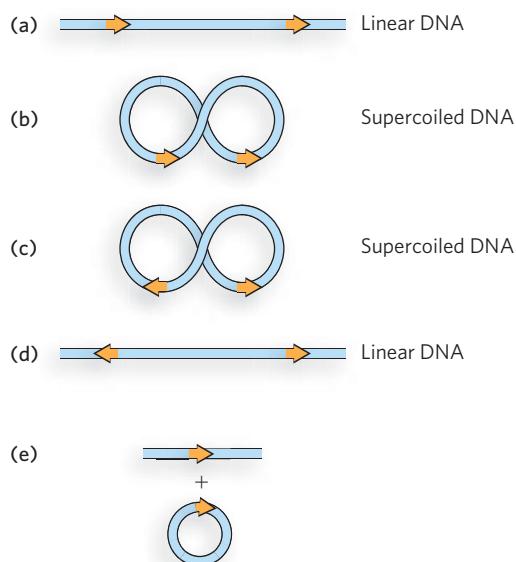
1. Holliday intermediates are associated with both homologous genetic recombination and some site-specific recombination systems. How does their formation differ in the two types of reactions?
2. Compare and contrast the DNA sequence requirements of homologous genetic recombination, site-specific recombination, and transposition.
3. Draw the products of Cre-lox mediated site-specific recombination reactions for the recombination target sites and orientations indicated by arrows in the illustrations below.



4. Draw the possible products of Flp recombinase-mediated site-specific recombination for the DNA molecule shown below. Arrowheads indicate the location and orientation of FRT sites. X and Y denote altered FRT sites that are functional but will not cross-react with each other. Draw all the products possible if recombination occurs at (a) the X sites and (b) the Y sites, and if two recombination events occur at (c) the X sites and then the Y sites, and (d) the Y sites and then the X sites.



5. Which of the following DNA molecules are appropriate substrates for the Hin recombinase? Arrowheads indicate the location and orientation of hix sites.



6. The bacterial transposon Tn3 uses a replicative transposition pathway to move from one DNA molecule to another. Tn3 encodes not only a transposase but a site-specific recombinase. Suggest a role in transposition for the site-specific recombinase in this system.
7. The Flp and Cre-lox site-specific recombination systems are widely used in biotechnology to promote genomic rearrangements in eukaryotic cells. The bacteriophage λ site-specific recombination system (see How We Know) is rarely used for this purpose, even though it was the first such system discovered. Suggest why the λ system is not used.

8. Cut-and-paste transposition results in a short repeated sequence flanking the newly inserted transposon. The DNA sequence shown below is the target site for the transposition of a cut-and-paste transposon. Indicate with arrows the phosphodiester bonds targeted for transesterification by the transposon 3' ends to generate a 5 bp repeat, 5'-AGGCT-3', at both ends of the newly inserted transposon.

5' ...ATGCAGGCTAATGGCTACCTGA...
3' ...TACGTCCGATTACCGATGGACT...

9. Many retroviruses have sequences complementary to the 3' end of one of the tRNA molecules prominent in host cells infected by the virus. What is the purpose of this complementarity?
10. Many TP (non-LTR) retrotransposons encode a reverse transcriptase that also has endonuclease activity. What is the primary function of the endonuclease activity?
11. The human genome has more than a million copies of the SINE element Alu. These transposons are found in the DNA between genes, and often in the introns of genes, but very rarely in gene exons. Explain why this is so.

Data Analysis Problem

Livet, J., T.A. Weissman, H.N. Kang, R.W. Draft, J. Lu, R.A. Bennis, J.R. Sanes, and J.W. Lichtman. 2007. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* 450:56–62.

12. Site-specific recombination and transposition are regularly used in biotechnology. An elegant use of site-specific recombination is found in a 2007 report by Livet and colleagues. One of the challenges in brain research is the sheer complexity of the neuronal network. Tracing one neuron to elucidate its connections was nearly impossible until the advent of the “brainbow” technology (see Highlight 14-1). Researchers placed genes for different colored variants of green fluorescent protein (GFP; see Figure 7-19) in cassettes, with the various GFP genes separated by lox sites recognized by the site-specific recombinase Cre (Figure 1a), and inserted the cassettes into the mouse genome. The variants in this example are red fluorescent protein (RFP), yellow fluorescent protein (YFP), and cyan fluorescent protein (CFP). Each cassette was inserted at FRT sites, using a separate site-specific recombinase, Flp. Multiple cassettes were inserted into the genomes of some mice (Figure 1b).

The Cre recombinase was inserted into the genome of a different group of mice, the cloned enzyme structured so that it was expressed uniquely in brain tissue, briefly, during neuron development. When mice containing the cassettes were mated to mice containing the cloned Cre recombinase, Cre-mediated recombination created a unique pattern of expression of GFP variants in each mature neuron of the heterozygous offspring, effectively coloring the neurons. The vivid results are shown in Highlight 14-1.

- (a) Suggest why the Flp recombination system rather than Cre-lox was used to insert the cassettes into the mouse genome.

- (b) If one GFP cassette was already present in the mouse genome, how could more cassettes be added?
- (c) In the cassette shown in Figure 1a, two different lox sites are used. What sequences in the lox sites must be different to prevent the two sites from recombining with each other?
- (d) When two or more different GFP variants are expressed in a neuron, the final color is a blend that reflects the amount of each GFP present. In mice with three cassettes, one GFP variant is expressed from each cassette (assuming that the lox sites preclude recombination between cassettes). Ten different colors are possible. Describe the combinations leading to the different colors.

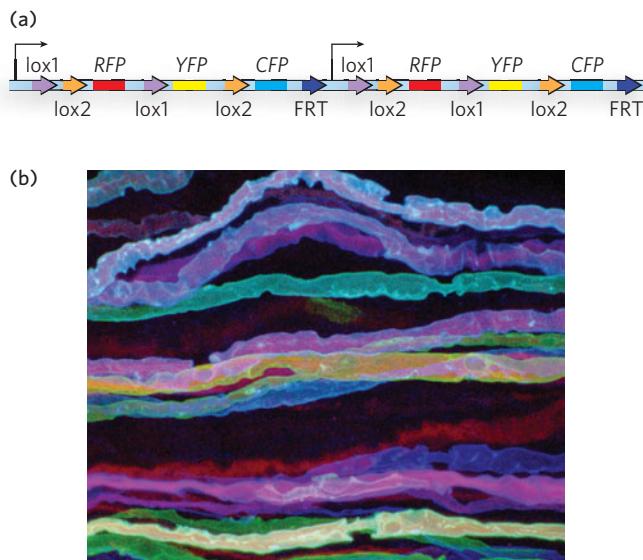


FIGURE 1 [Source: Courtesy of Jeff Lichtman.]

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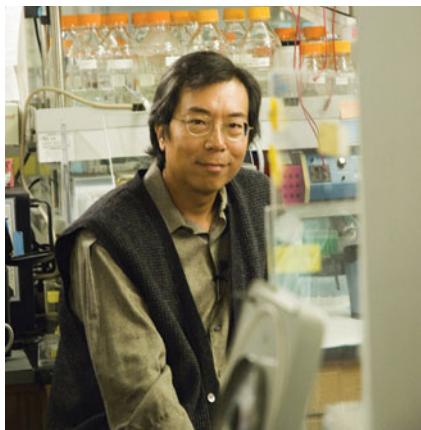
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DNA-Dependent Synthesis of RNA



Robert Tjian [Source: Bonnie Azab/UC Berkeley.]

Moment of Discovery

In the early 1980s, it was clear that specialized proteins must exist for accurate and regulated mRNA synthesis from particular genes in mammalian cells. However, nobody had been able to identify such “transcription factors” or determine how this process of transcriptional activation works. The breakthrough in my laboratory came when we found out that human cell extracts contained a factor that can discriminate between two templates and somehow pro-

gram the enzyme that reads DNA to choose the right promoter DNA and ignore all others. But how?

We decided to use a short piece of the active promoter DNA sequence as “bait” to fish out proteins that selectively bind this site. The challenge was to purify this activity away from the other ~3,000 DNA-binding proteins present in human cell extracts! After months of struggling with this problem, I vividly recall walking into the lab and my coworkers, Jim Kadonaga and Kathy Jones, saying, “We think we know which protein it is!” They had cleverly treated the human cell extract with a huge excess of sheared calf-thymus DNA to remove most nonspecific DNA-binding proteins, enriching the treated extract for the protein we wanted. Sequence-specific DNA affinity resin was then used to bind the transcription factor in the treated extracts, leading to the purification of a single protein. We called this protein specificity protein 1 (Sp1), the first of many sequence-specific transcription factors that were to prove critical for human gene regulation.

I'll never forget the feeling of profound excitement at having discovered such a fundamental protein in biology and, at the same time, having devised a new means to isolate hundreds more of these key gene-regulatory proteins.

—**Robert Tjian**, on discovering the first specific eukaryotic transcription factor

15.1 RNA Polymerases and Transcription Basics 516

15.2 Transcription in Bacteria 523

15.3 Transcription in Eukaryotes 532

Information encoded in the DNA of cells and viruses provides the instructions for making the RNA and protein molecules that carry out the activities essential for life. The first step in expression of this information is **transcription**, the enzymatic production of an exact complementary strand of RNA on a DNA template. Transcription thus involves the transfer of genetic information from DNA to RNA. For protein-coding regions of DNA, transcription begins the gene expression pathway leading to the production of protein through translation of a messenger RNA (see Chapter 18). For non-protein-coding regions of DNA, transcription produces RNA molecules that in many cases are components of RNA-protein complexes, or ribonucleoproteins. Some of these are enzymes, but the majority play non-enzymatic roles in controlling gene expression on many levels. Increasing evidence shows that a much greater proportion of an organism's transcribed DNA is non-protein-coding than protein-coding. The functions of many such transcripts are just beginning to be defined.

All RNA molecules, except for the RNA genomes of certain viruses, are derived from information stored in DNA. Transcription produces three major kinds of RNA, and many more RNAs are generated in smaller amounts. As described in Chapter 6, **messenger RNAs (mRNAs)** encode the amino acid sequence of one or more polypeptides specified by a gene or set of genes. **Transfer RNAs (tRNAs)** read the information encoded in the mRNA and provide the appropriate amino acid to a growing polypeptide chain during protein synthesis. **Ribosomal RNAs (rRNAs)** are constituents of ribosomes, the intricate cellular machines that synthesize proteins. Other specialized RNAs have regulatory or catalytic functions or are precursor forms of the three main classes of RNA (Highlight 15-1).

Unlike DNA replication, which involves copying the entire chromosome, transcription is selective. Only particular genes or groups of genes are transcribed at any one time, and some portions of the DNA genome may be transcribed rarely or not at all. The cell directs the transcription machinery to express genetic information as it is needed. Specific regulatory sequences mark the beginning and end of the DNA segments to be transcribed and designate which strand of the double-stranded DNA is to be used as the template. The regulation of transcription is an important and exciting aspect of gene expression. We'll discuss regulation in this chapter, and in further detail in Chapters 19–22.

As one of the central cellular processes studied by molecular biologists, transcription—enzymatic RNA synthesis directed by a DNA template—has been worked out in some detail, yet some fascinating puzzles remain. We begin by examining the enzymes responsible for

transcription, and then address the mechanics of transcription in bacteria and in eukaryotic cells.

15.1 RNA Polymerases and Transcription Basics

Transcription in cells and viruses is catalyzed by specialized enzymes called **RNA polymerases**. The transcription reaction resembles DNA replication in its fundamental chemical mechanism, in the direction of synthesis ($5' \rightarrow 3'$), and in the requirement for a template strand. And, like replication, transcription has initiation, elongation, and termination phases. In contrast to replication, however, transcription does not require a primer to begin RNA synthesis, and it involves defined sections of DNA rather than the entire molecule. Also, just one of the two strands of a DNA segment serves as the template for a given transcription reaction (Figure 15-1).

RNA Polymerases Differ in Details but Share Many Features

The discovery of DNA polymerase and its dependence on a DNA template triggered a search for enzymes that synthesize RNA molecules complementary to a DNA strand. Synthesis of RNA independent of a template had been discovered years earlier, but the enzyme required for this, polynucleotide phosphorylase, was shown to be involved in RNA degradation, not biosynthesis. An enzyme that added the 3'-terminal CCA to tRNAs had also been identified, but again, this was not the highly sought-after template-directed RNA polymerase. The race was on to discover an enzyme having the properties required for template-dependent RNA synthesis.

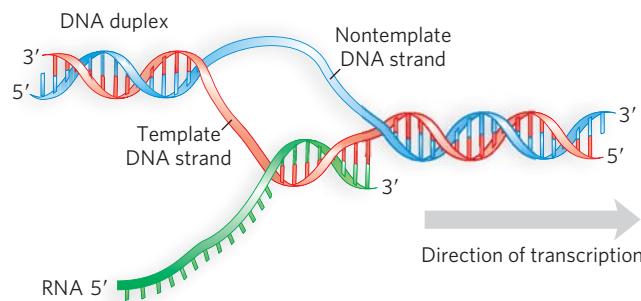


FIGURE 15-1 Transcription of DNA into RNA. The DNA duplex opens to allow a complementary RNA copy to be made from one strand (the template). Synthesis proceeds in the $5' \rightarrow 3'$ direction in the mRNA strand.

HIGHLIGHT 15-1 A CLOSER LOOK

The ABCs of RNA: Complexity of the Transcriptome

When complete eukaryotic genome sequences became available, molecular biologists were excited to discover the extent of the **transcriptome**, the entire set of transcripts produced in a cell. Initially, researchers focused on characterizing the transcription products of known genes. These included mRNAs and known stable noncoding RNAs (ncRNAs) such as rRNAs, tRNAs, small nuclear RNAs (snRNAs) involved in pre-mRNA splicing, and small nucleolar RNAs (snoRNAs), which guide chemical modifications in the ribosome.

Unexpected levels of complexity began to emerge, however, beginning with the discovery of naturally occurring interfering RNAs, such as small interfering RNAs (siRNAs) and microRNAs, which have roles in the regulation of translation (see Chapter 22). Using a combination of microarrays and RNA-Seq (see Chapter 8), researchers could detect transcripts without being biased by prior expectations. Use of these techniques showed that a lot of transcription was occurring that had previously been ignored. These new technologies revealed that the transcription landscape in higher eukaryotes is much more complex than expected. Surprisingly, a large fraction of transcripts originate from intergenic regions—regions between the coding sequences of genes—that had been thought to be silent, or from sequences that run in the opposite direction (antisense) to genes. Transcription that does not map to protein-coding genes or to known ncRNA genes also occurs in yeast.

In a parallel set of experiments, arrays of synthetic DNA oligonucleotides representing all non-repetitive sequences in human chromosomes 21 and 22 were used to map the binding sites for three human transcription factors—Sp1, cMyc, and p53—that activate the transcription of many protein-coding genes involved in cell growth and differentiation. The experiments revealed far more transcription factor-binding sites than would be predicted from the number of protein-coding genes in these chromosomes. Of these binding sites, more than one-third lie within or immediately 3' to well-characterized genes and seem to correlate with the transcription of ncRNAs. These findings have changed our thinking about transcription: much more of it goes on than

previously suspected (Figure 1). Just what is all that RNA doing?

Some possible roles of previously undetected transcripts have emerged. For example, long noncoding RNAs (lncRNAs) are produced from regions that are either intergenic or antisense to genes. The functional significance of lncRNAs is not known, although several studies suggest roles for these transcripts in gene regulation. The shorter transcripts, particularly those associated with gene promoters, fall into two somewhat arbitrary categories: molecules 20 to 200 nucleotides long are called small RNAs (sRNAs), and molecules of 200 to 1,000 nucleotides are called long RNAs (lRNAs). These categories include numerous subfamilies of transcripts defined by their abundance, longevity, and genomic origin. Although the source of these transcripts is not yet fully worked out, at least some sRNAs may result from aborted or prematurely terminated transcription. Functions for lRNAs and sRNAs are unknown, but they may be transcribed, in part, to maintain an open promoter around transcription start sites in chromatin.

Whether pervasive transcription is simply a consequence of low-level background transcription or has functional significance is a subject of active research. In animals, it is unclear whether transcripts that are initiated near a particular promoter are functional. One possibility is that promoter-associated transcripts may help maintain chromatin in an open state that is more accessible to the transcription and regulatory machinery. In addition, it could keep a pool of Pol II available for rapid deployment to make mRNAs. This will clearly be an expanding area of research, and more surprises are certainly in store.

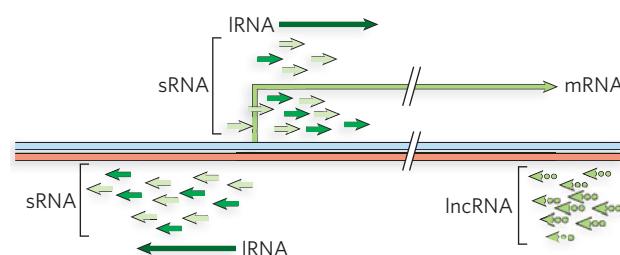


FIGURE 1 A single region of DNA can produce several types of RNA transcripts. [Source: Adapted from A. Jacquier, *Nat. Rev. Genet.* 10:833–844, 2009, Fig. 1.]

By 1960, four research groups had independently detected in cell extracts an activity that could form an RNA polymer from ribonucleoside 5'-triphosphates (rNTPs). Jerard Hurwitz and his coworkers partially purified the RNA polymerase from *E. coli* to separate it from polynucleotide phosphorylase and the CCA-adding enzyme. They performed experiments demonstrating that the RNA product of this polymerase was complementary to the sequence of DNA supplied in the reaction mix. A particularly telling experiment involved a DNA strand with an alternating (AT)_n sequence. Using different radiolabeled rNTPs, Hurwitz found that the RNA product required only ATP and UTP for complete synthesis, and not GTP or CTP (Figure 15-2). Subsequent work on the purified *E. coli* RNA polymerase, and later on bacteriophage RNA polymerases, helped define the fundamental properties of transcription.

In addition to a DNA template, DNA-dependent RNA polymerases require Mg²⁺ and all four rNTPs

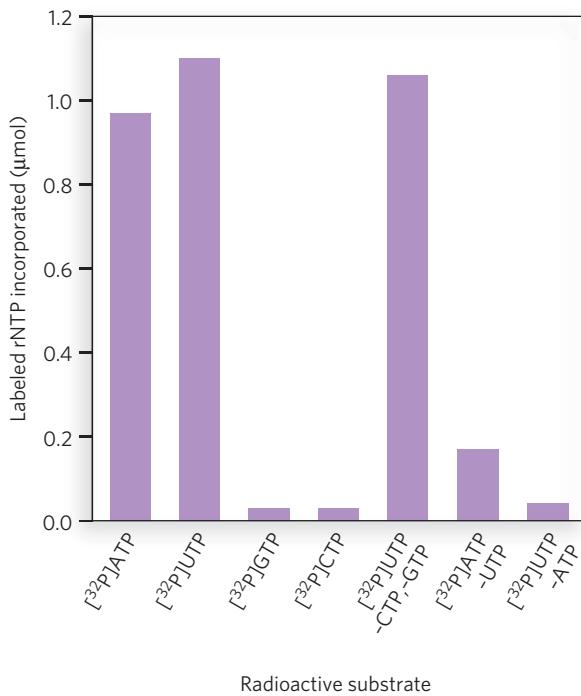
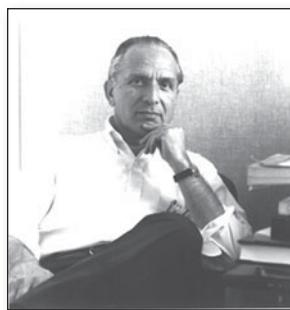


FIGURE 15-2 Initial detection of a DNA-dependent RNA polymerase. RNA polymerizing activity was partially purified from *E. coli* and analyzed for synthesis, using a template DNA of sequence (AT)_n and various rNTP substrates. Reactions took place in the presence of nonradioactive (unlabeled) rNTPs and one or more labeled rNTPs, as noted, or lacking rNTPs, as noted. Incorporation of radioactively labeled rNTP substrates was measured. [Source: Adapted from J. Hurwitz et al., *Cold Spring Harb. Symp. Quant. Biol.* 26:91-100, 1961.]



Jerard Hurwitz [Source: Courtesy of Jerard Hurwitz.]

(ATP, GTP, UTP, and CTP) as substrates for the polymerization reaction. The chemistry and mechanism of RNA synthesis closely resemble those of DNA synthesis (Figure 15-3). RNA polymerase extends an RNA strand by adding ribonucleotide units to the 3'-hydroxyl end, building RNA in the 5' → 3' direction. The 3'-hydroxyl group

makes a nucleophilic attack on the α phosphate of the incoming rNTP, with the concomitant release of pyrophosphate. As noted above, only one of the two DNA strands serves as template. The template DNA is copied in the 3' → 5' direction (antiparallel to the new RNA strand), just as in DNA replication. Each nucleotide in the newly formed RNA is selected by Watson-Crick base pairing: U residues—and not T residues, as in DNA—are inserted in the RNA to pair with A residues in the DNA template, G residues are inserted to pair with C residues, and so on (see Figure 6-11). Base-pair geometry may also play a role in nucleotide selection and the resulting fidelity of the polymerase reaction.

RNA polymerases are fascinating enzymes that continue to be actively studied in many laboratories. The simplest examples consist of one polypeptide

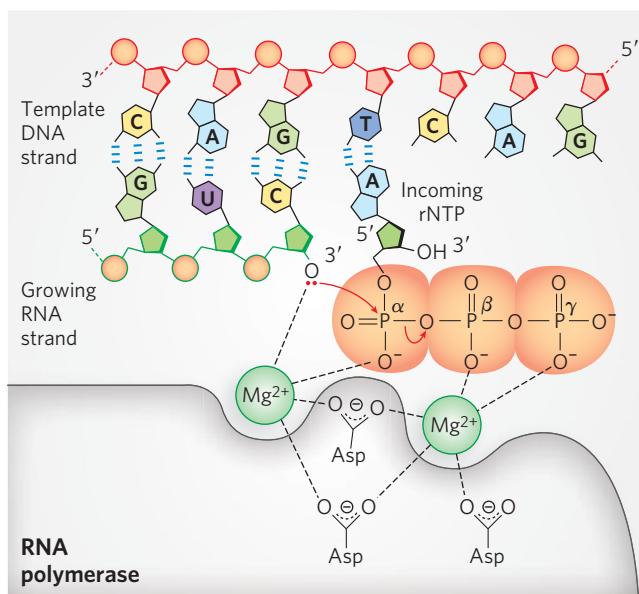


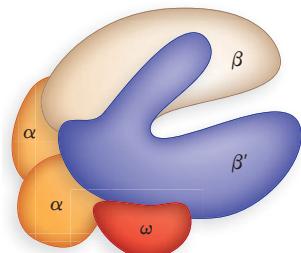
FIGURE 15-3 The chemical mechanism of RNA synthesis.

The addition of an rNTP to a growing transcript is a Mg²⁺-dependent reaction that produces a 5' → 3' phosphodiester linkage.

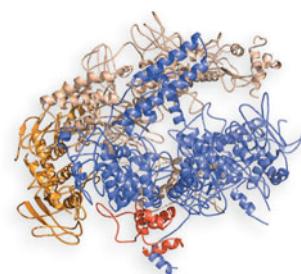
chain, such as the phage T7 and phage Sp6 polymerases. In contrast, all cellular RNA polymerases, from bacteria to humans, are composed of multiple polypeptides that fold together to create the functional enzyme. In *E. coli*, for example, the **RNA polymerase core** is a large, complex enzyme with five polypeptide subunits: two copies of the α subunit and one copy each of the β , β' , and ω subunits: $\alpha_2\beta\beta'\omega$ (M_r 390,000), as shown in **Figure 15-4a**. A sixth subunit, designated σ and known as **sigma factor**, binds transiently to the core and directs the enzyme to specific binding sites on the DNA. These six subunits constitute the **RNA polymerase holoenzyme**. Bacteria have multiple sigma factors, named according to their molecular weight; the most common is σ^{70} (M_r 70,000). Thus, the RNA polymerase holoenzyme of *E. coli* exists in several forms, depending on the type of σ subunit it contains. Sigma factors play an important role in the recognition of different types of bacterial genes (see Section 15.2).

In eukaryotic cells, three distinct RNA polymerases are responsible for transcribing RNAs with different functions. **RNA polymerase I (Pol I)** transcribes genes

(a) Bacterial RNA polymerase core



(b) Bacterial RNA polymerase



(c) Eukaryotic RNA polymerase II

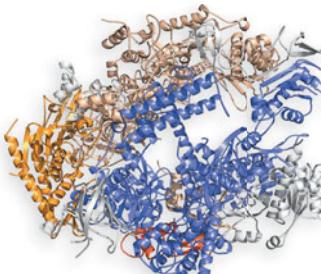


FIGURE 15-4 The architecture of RNA polymerases.

(a) The bacterial RNA polymerase core is composed of several subunits that give the enzyme the overall shape of a crab claw. The pincers are formed from the large β and β' subunits. At the start of transcription, the sigma factor (σ) associates with the core polymerase to form the holoenzyme (not shown). The crystal structures of (b) bacterial *T. aquaticus* RNA polymerase and (c) eukaryotic (*S. cerevisiae*) RNA polymerase II are also shown. [Sources: (b) Adapted from G. Zhang, *Cell* 98:811, 1999 (c) PDB ID 1I50.]

Table 15-1 The Subunits of RNA Polymerase Holoenzymes

Bacterial Core	Eukaryotic		
	Pol I	Pol II	Pol III
β	RPA1	RPB1	RPC1
β'	RPA2	RPB2	RPC2
α	RPC5/RPC9	RPB3/RPB11	RPC5/RPC9
ω	RPB6	RPB6	RPB6
	[+9 others]	[+7 others]	[+11 others]

encoding large rRNA precursors. **RNA polymerase II (Pol II)** transcribes nearly all protein-coding genes to make mRNA. **RNA polymerase III (Pol III)** transcribes genes encoding smaller functional RNAs, including tRNAs, some small nuclear RNAs (snRNAs), and 5S ribosomal RNA (the naming of rRNAs is explained in Chapter 18). These enzymes are related to bacterial RNA polymerase at the level of both sequence and structure, indicating that RNA polymerase is an ancient enzyme. However, the eukaryotic RNA polymerases are larger and contain additional proteins not found in bacteria (Table 15-1).

The molecular structures of bacterial and yeast RNA polymerases have been determined by x-ray crystallography (Figure 15-4b, c). The cleft between the two pincers of the claw contains the enzyme active site and binds two Mg^{2+} ions that facilitate RNA polymerization. The more conserved parts of the polymerase complex are in the interior, whereas regions that have varied more over the course of evolution are at the exterior of the complex.

Unlike DNA polymerase, RNA polymerase does not require a primer to initiate synthesis. RNA polymerase catalyzes RNA synthesis in three distinct phases, similar to those of the DNA polymerase reaction. **Initiation** occurs as RNA polymerase binds to specific DNA sequences called **promoters**. **Elongation** is the process of adding nucleotides to the growing RNA strand. **Termination** is the release of the product RNA when the polymerase reaches the end of a gene or other transcription unit.

The two strands of a DNA duplex have different roles in transcription. The **template strand** serves as a template for RNA synthesis, and its complement, the **nontemplate strand**, more often called the **coding strand**, is identical in base sequence to the RNA transcribed from the gene, with U in the RNA in place of T in the DNA (Figure 15-5). The coding strand for a particular gene may be located in either strand of a given chromosome.

	Start codon
Coding DNA	GACGTTAATATAAACCTGAAGATTAAACATGACTGAATCTTGTCAACTCTTGAAGAGTCCTTAAAAGAAATCGA
Template DNA	CTGCAATTATATTGGACTTCAATTGTACTTGACTTAGAAACGAGTTGAGAAACTTCTAGGAATTTCTTAGCT
RNA	GACGUUAAAUAUAAAACCUGAAGAUAAAACUGACUGAAUCUUUGCUAACUCUUUGAAGAGUCGUUAAAAGAAUCGA

FIGURE 15-5 The DNA template for RNA synthesis. The coding (nontemplate) strand of the DNA is identical in base sequence to the RNA transcribed from the gene, with U in the RNA in place of T in the DNA. The template strand is used to direct RNA synthesis by RNA polymerase. ATG in the coding strand (blue) is the initiation (start) codon.

KEY CONVENTION

The template strand of the DNA is copied during transcription, and its sequence is the complement of the RNA transcript. The coding strand of the DNA has the same sequence as the RNA transcript (except for A in the DNA and U in the RNA). Hence, for example, the start codon for transcription is 5'-ATG in the coding strand of the DNA and 5'-AUG in the mRNA. By convention, gene, promoter, and regulatory sequences in DNA are written as they appear in the coding strand.

To enable RNA polymerase to synthesize an RNA strand complementary to the template DNA strand, the DNA duplex must unwind over a short distance, forming what is known as a transcription “bubble” (Figure 15-6). During transcription, the *E. coli* RNA polymerase generally keeps about 17 bp unwound. In the elongation phase, the growing end of the new RNA strand base-pairs temporarily with the DNA template in the unwound region to form a short hybrid RNA-DNA double helix, ~8 bp in length. The RNA in this

hybrid duplex is displaced shortly after its formation as the DNA double helix re-forms. Elongation of a transcript by *E. coli* RNA polymerase proceeds at a rate of 50 to 90 nucleotides per second. Because DNA is a helix, the movement of a transcription bubble requires considerable strand rotation of the nucleic acid molecule. Consequently, a moving RNA polymerase generates waves of positive supercoils ahead of the transcription bubble and negative supercoils behind. This has been observed both in the laboratory with purified enzymes and in live bacterial cells. In the cell, the topological problems caused by transcription are relieved through the action of topoisomerases (see Chapter 9).

RNA polymerases lack a separate proofreading 3'→5' exonuclease active site, which exists in many DNA polymerases. Consequently, the error rate for transcription is higher than that for chromosomal DNA replication—approximately one error for every 10^4 to 10^5 ribonucleotides incorporated into RNA. Because many copies of a transcript are generally produced from a single gene, and all of these are eventually degraded and replaced, a mistake in an RNA molecule

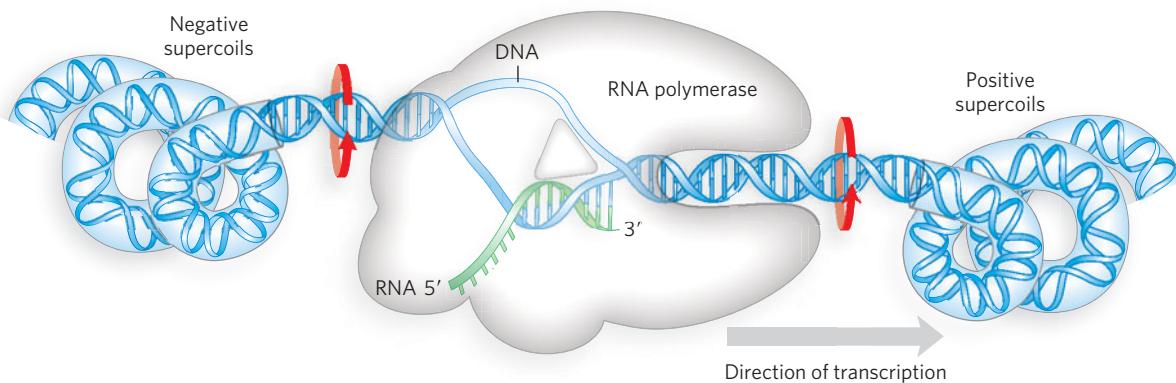


FIGURE 15-6 The transcription “bubble.” The DNA duplex is unwound for about 17 bp, forming a bubble, enabling RNA polymerase to access the template strand. DNA supercoiling occurs in front of and behind the transcription bubble.

is less consequential to the cell than a mistake in the permanent information stored in DNA. Many RNA polymerases, including bacterial RNA polymerase and the eukaryotic Pol II, pause when a mispaired base is added during transcription. In addition, they can remove mismatched nucleotides from the 3' end of a transcript by direct reversal of the polymerization reaction. However, it's unclear as yet whether this activity is a true proofreading function and to what extent it may contribute to the fidelity of transcription.

Transcription Initiation, Elongation, and Termination Occur in Discrete Steps

The steps of the transcription pathway are shown in **Figure 15-7**. The polymerase binds the promoter (step 1), forming first a **closed complex** in which the bound DNA is intact, and then an **open complex** (step 2), in which the bound DNA is partially unwound near a region ~10 bp before (upstream of) the transcription start site. Transcription is initiated within the complex (step 3), leading to a conformational change that converts the complex to the form required for elongation. **Promoter clearance**, involving movement of the transcription complex away from the promoter, leads to the formation of a tightly bound **elongation complex** (step 4). Once elongation begins, RNA polymerase becomes a highly efficient enzyme, completing synthesis of the transcript before dissociating from the DNA template (step 5), then recycling for a new round

of transcription. The steps in this pathway are conserved in bacteria and eukaryotes.

RNA synthesis is processive, which means that once RNA polymerase begins elongating a transcript, the kinetics of the polymerization reaction greatly favor the addition of the next nucleotide over premature release of the transcript. As we'll see, elongation is not a uniform process but instead occurs in fits and starts. We'll also see that specific sequences trigger termination of RNA synthesis by RNA polymerase.

DNA-Dependent RNA Polymerases Can Be Specifically Inhibited

Small molecules and polypeptides that inhibit transcription are useful both as antibiotics and as tools for research. **Actinomycin D**, one of a class of peptide antibiotics isolated from *Streptomyces* soil bacteria, inhibits transcription elongation by RNA polymerase in bacteria and eukaryotes (Figure 15-8). The planar portion of this molecule intercalates into the double-helical DNA between successive G≡C base pairs, deforming the DNA and preventing movement of the polymerase along the template. Because actinomycin D inhibits RNA elongation both in intact cells and in cell extracts, it is used in the laboratory to identify cell processes that depend on RNA synthesis. **Acridine** inhibits RNA synthesis in a similar fashion. **Rifampicin**, a small molecule isolated from *Streptomyces mediterranei*, inhibits bacterial RNA synthesis by binding to the β subunit of bacterial RNA

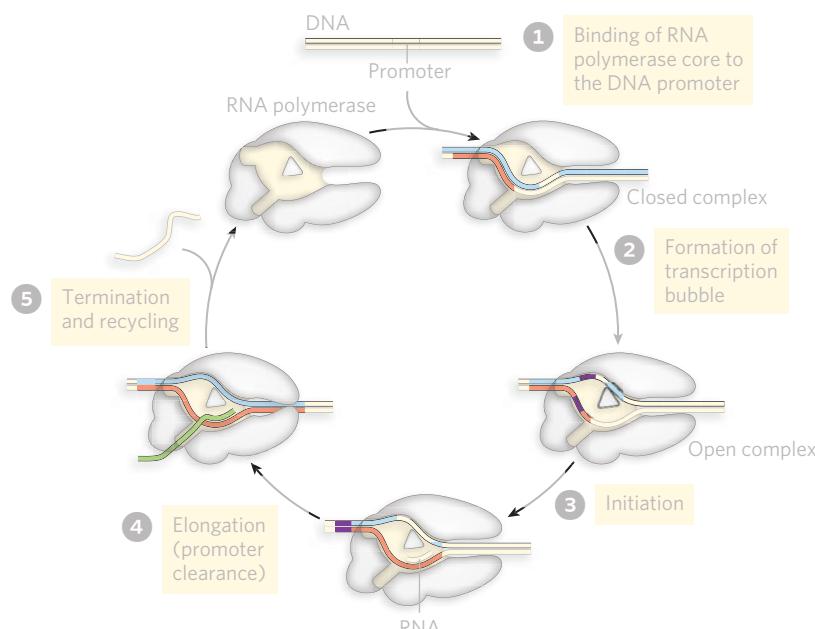


FIGURE 15-7 An overview of transcription.

DNA binding at the promoter leads to initiation of transcription by the polymerase holoenzyme, followed by elongation and termination. The steps are further described in the text.

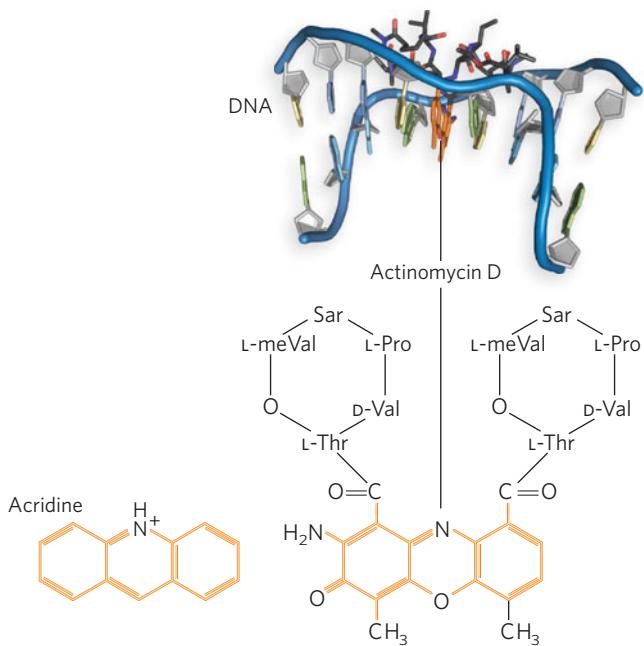


FIGURE 15-8 Inhibitors of transcription. The DNA structure can be deformed by actinomycin D, which contains a heterocyclic group (orange) that intercalates into the DNA, inhibiting transcript elongation. (Sar is sarcosine; meVal is methylvaline.) Acridine, also containing a heterocyclic group, has a similar inhibitory effect. [Source: PDB ID 1DSC.]

polymerase, preventing promoter clearance. Because it does not affect the function of eukaryotic polymerases, rifampicin is sometimes used as an antibiotic to treat such diseases as tuberculosis and leprosy.

Some species rely on transcription inhibitors for natural biodefense. For example, the mushroom *Amanita phalloides* (Figure 15-9a) produces **α-amanitin**, a cyclic polypeptide that disrupts eukaryotic mRNA synthesis by blocking Pol II and, at higher concentrations, Pol III (Figure 15-9b). The binding position of α-amanitin in Pol II (Figure 15-9c) prevents the flexibility required for translocation of the polymerase along the DNA substrate. α-Amanitin is useful in the laboratory as a specific inhibitor of eukaryotic Pol II, or to determine the polymerase responsible for transcribing a particular gene. This can be demonstrated in an experiment monitoring RNA synthesis when an RNA polymerase and rNTPs are combined with a DNA template (Figure 15-9d). Under normal conditions, Pol I produces rRNA, Pol II produces mRNA, and Pol III produces tRNA. The addition of α-amanitin inhibits the synthesis of mRNA, but not that of rRNA or tRNA. Indeed, neither Pol I nor bacterial RNA polymerase is sensitive to α-amanitin—nor is the RNA polymerase II of *A. phalloides* itself!

Transcriptional Regulation Is a Central Mechanism in the Control of Gene Expression

Transcription is the first step in the complicated and energy-intensive pathway of protein synthesis, so much of the regulation of protein levels in both bacterial and eukaryotic cells occurs during transcription, particularly its early stages. Because requirements for any gene product vary according to cellular conditions or developmental stage, cells and viruses control transcription so that gene products are made only when they are needed, and in the required proportions. Regulation can occur at any step in transcription, but much of it is directed at the promoter-binding and initiation steps.

The DNA sequence in the promoter region affects the efficiency of RNA polymerase binding and initiation of transcription. However, differences in promoter sequences are just one of several levels of control during initiation. The binding of additional proteins to sequences both near to and distant from the promoter can also affect transcription levels. Protein binding can *activate* transcription by facilitating RNA polymerase binding or later steps in the initiation process, or it can *repress* transcription by blocking polymerase activity (see Chapter 20 and Chapter 21).

SECTION 15.1 SUMMARY

- Transcription is catalyzed by DNA-dependent RNA polymerases, which use ribonucleoside 5'-triphosphates to synthesize RNA complementary to the template strand of duplex DNA. The steps of transcription consist of binding of RNA polymerase to a promoter on DNA to form a closed complex, opening of the complex by local DNA unwinding near the promoter, initiation, elongation, and termination.
- The simplest RNA polymerases, with one polypeptide chain, are found in some bacteriophages. All cellular RNA polymerases are composed of multiple polypeptides that fold together to create the functional enzyme.
- Bacterial RNA polymerase uses a sigma (σ) factor to recognize and bind the promoter during initiation.
- Eukaryotic cells have three types of RNA polymerases. Pol I and Pol III transcribe genes encoding rRNAs and small functional RNAs such as tRNA, respectively. Pol II transcribes protein-coding genes to make mRNA.
- Once an elongation complex forms on a DNA template, RNA polymerase completes the synthesis of the transcript before dissociating from the DNA.

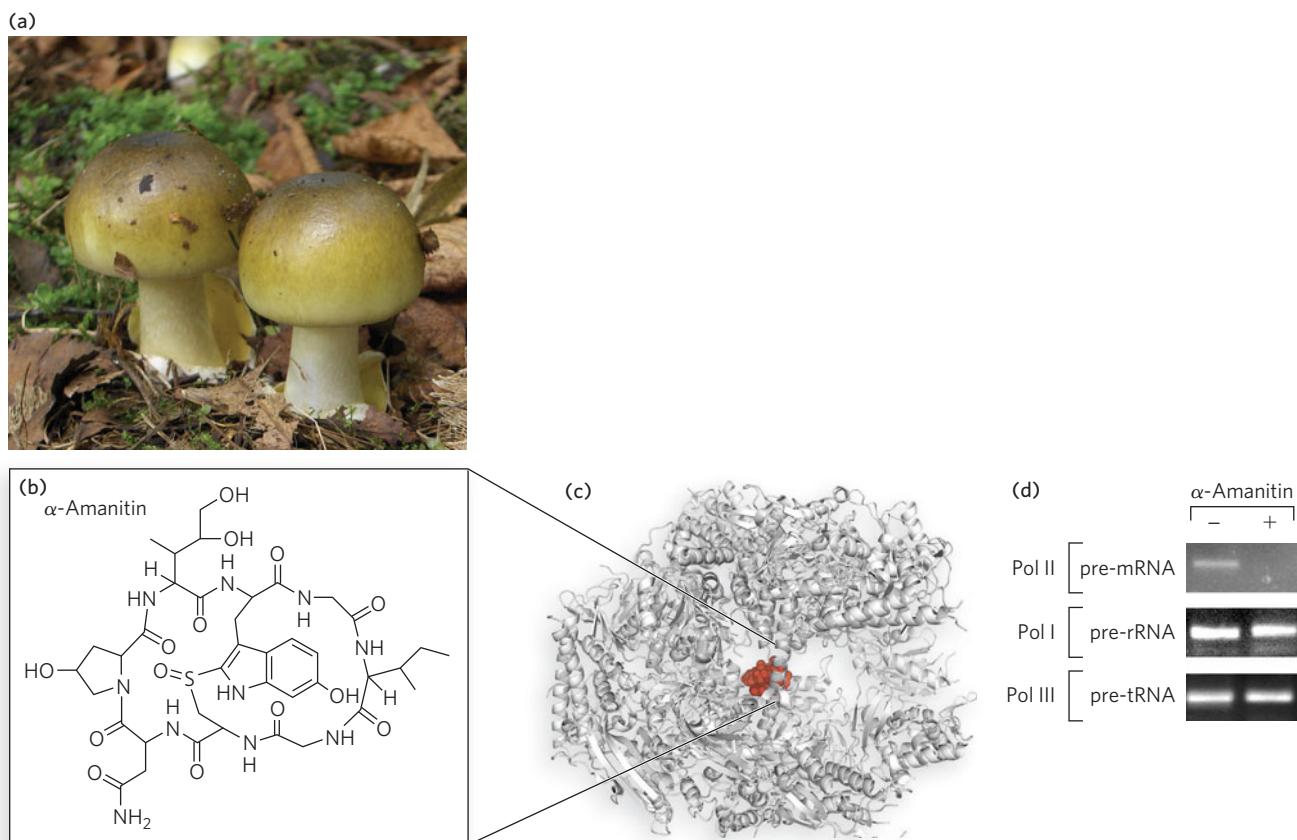


FIGURE 15-9 Inhibition of transcription by α -amanitin.

(a) A mushroom to avoid! *Amanita phalloides* is poisonous to eukaryotes because it produces α -amanitin. (b) The chemical structure of α -amanitin. (c) Binding of α -amanitin (red) to

yeast Pol II. (d) α -Amanitin inhibits synthesis of mRNA by Pol II, but not synthesis of rRNA by Pol I or tRNA by Pol III. [Sources: (a) Stanislaw Skowron. (c) PDB ID 1K83. (d) Y. Lee et al., *EMBO J.* 23:4051, 2004, Fig. 3.]

- Various naturally occurring small molecules inhibit polymerase enzymes and can be used to detect which polymerase produces specific types of RNA.

15.2 Transcription in Bacteria

Transcription shares many fundamental properties in all organisms. Due to their relative ease of study, RNA polymerases from bacteria and bacteriophages were the focus of the first experiments that revealed the principles of how these enzymes recognize DNA and synthesize an RNA transcript. Bacterial transcription continues to be an active area of research, in part because many experimental tools already exist for analyzing polymerase function both *in vitro* and *in vivo*. One obvious difference between bacteria and eukaryotes is that bacteria have a single RNA polymerase enzyme for synthesizing all the RNA molecules in the cell, instead of the three RNA polymerases found in eukaryotes.

Promoter Sequences Alter the Strength and Frequency of Transcription

In *E. coli*, RNA polymerase binds to DNA within a 100 bp region stretching from about 70 bp before the transcription start site to about 30 bp beyond it. By convention, the DNA base pairs corresponding to the beginning of an RNA molecule are given positive numbers (+1 is the transcription start site), and those preceding the RNA start site are given negative numbers. The promoter region thus extends between positions -70 and +30.

The most common sigma factor in *E. coli* is σ^{70} . Analyses and comparisons of the bacterial promoters recognized by a σ^{70} -containing RNA polymerase holoenzyme have revealed similarities in two short sequences centered about positions -10 and -35 (Figure 15-10). These sequences are important interaction sites for σ^{70} . Although the sequences are not identical for all bacterial promoters in this class, certain nucleotides that are particularly common at each

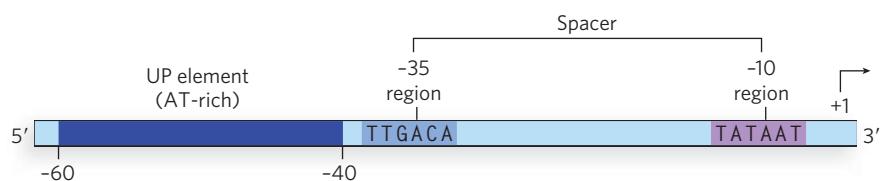


FIGURE 15-10 Features of bacterial promoters recognized by σ^{70} . Different sigma factors recognize distinct promoter elements. The σ^{70} factor recognizes the -10 sequence, -35 sequence, and UP element. Promoters recognized by other sigma factors have other consensus sequences in the promoter region.

position form a **consensus sequence**. The consensus sequence centering at the -10 region is 5'-TATAAT-3'; the consensus sequence at the -35 region is 5'-TTGACA-3'. A third AT-rich recognition element, the **upstream promoter (UP) element**, occurs between positions -40 and -60 in the promoters of certain highly expressed genes. The UP element is bound by an α subunit of RNA polymerase. The efficiency with which an RNA polymerase binds to a promoter and initiates transcription is determined in large measure by these sequences, the spacing between them, and their distance from the transcription start site.

Many independent lines of evidence attest to the functional importance of the sequences in the -35 and -10 regions. Mutations that affect the function of a given promoter often involve a single base pair in these regions. Variations in the consensus sequence also affect the efficiency of RNA polymerase binding and transcription initiation. A change in just one base pair can decrease the rate of binding by several orders of magnitude. The promoter sequence thus establishes a basal level of transcription that can vary greatly from one *E. coli* gene to the next.

Experiments with the Lac promoter in *E. coli* demonstrated the importance of promoter sequence

(a)

	Spacer		
	-35 region		-10 region
Consensus sequence	TTGACA		TATAAT
Wild-type Lac promoter	GGCTTTACACTTATGCTTCGGCTCG	TATGTT	GTTGTGGAATT
Mutant 1	GGCTTTACACTTATG- TTCCGGCTCG TATGTT	GTTGTGGAATT	
Mutant 2	GGCTTTACACTTATGCTTCGGCTCG TAT AATGTTGTGGAATT		
Mutant 3	GGCTTTACACTTATG- TTCCGGCTCG TAT AAT GTTGTGGAATT		
Mutant 4	GGCTT GAC ACTTATG- TTCCGGCTCG TAT AAT GTTGTGGAATT		

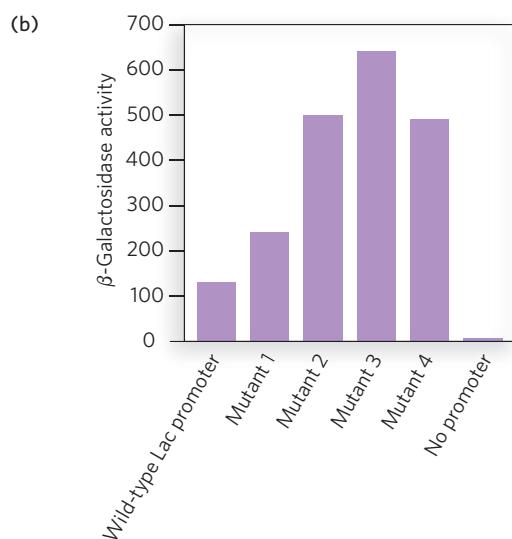


FIGURE 15-11 Mutational analysis of a bacterial promoter.

The Lac promoter, driving the *lac* operon, has a sequence close to the consensus sequence. (a) Mutations are created that make the promoter conform to the consensus.

(b) This results in increased expression and activity of β -galactosidase. [Source: Adapted from M. H. Caruthers, *J. Bacteriol.* 164:1353-1355, 1985.]

to gene expression (Figure 15-11). The Lac promoter drives the expression of genes in the *lac* operon, which encodes proteins that metabolize the sugar lactose. In fact, the classic experiments that first revealed transcription to be a regulated event were performed on the *lac* operon (see Chapter 20). The Lac promoter sequence is close to, but not exactly the same as, the bacterial consensus promoter sequence. In the experiment shown in Figure 15-11, mutations were introduced into the Lac promoter to make it conform to the consensus, and the mutated promoters were used to drive the expression of the enzyme β -galactosidase (β -gal, one of the *lac* operon proteins). Promoter activity was evaluated by a reaction in which β -gal converts a colorless substrate into a chemical with blue color: the more β -gal expressed, the deeper the blue color produced. The results showed that the mutations bringing the promoter into consensus tended to confer greater levels of protein expression.

Sigma Factors Specify Polymerase Binding to Particular Promoters

Escherichia coli has at least seven different kinds of sigma factors (Table 15-2); the number varies in other bacteria. Each RNA polymerase molecule contains only one σ subunit, which directs the polymerase enzyme to bind a specific type of promoter sequence. The σ^{70} factor binds reversibly to RNA polymerase and is essential for general transcription in exponentially growing cells. However, it can be replaced by

alternative sigma factors that trigger the transcription of genes involved in diverse functions, including stress responses, changes in cell shape, and iron uptake. A sigma factor only transiently associates with the RNA polymerase core, separating from it after transcription initiation.

Through their interactions with specific promoter classes, sigma factors direct RNA polymerase holoenzymes to the transcription start sites of genes associated with particular promoters, depending on the needs of the cell. All sigma factors recognize specific variants of the -10 and -35 regions of the promoter—except for σ^{54} , which binds to sequences in the -24 and -12 regions. For example, when cells experience a sudden temperature increase or other environmental stress, RNA polymerase containing a σ^{32} (M_r 32,000) subunit binds to so-called heat shock promoters and enhances the transcription of heat shock genes. By using different σ subunits, the cell can coordinate the expression of sets of genes, permitting major changes in cell physiology.

Because different sigma factor proteins are similarly organized, researchers can exchange bits of sequence between proteins to examine the effect on promoter recognition and transcription activation. Carol Gross and her colleagues at the University of California, San Francisco, used this approach to dissect the mechanisms by which “housekeeping” sigma factors (σ^{70} class) compare with specialized sigma factors, such as σ^{32} . Gross’s work showed that σ^{32} , and other specialized sigma factors, have an altered 17 amino acid segment that reduces binding affinity for the promoter. As

Table 15-2 Some *E. coli* Sigma Factors

Subunit Class	Function of Genes Activated	Consensus Sequence		
		-35	Spacer (bp)	-10
σ^{70} (RpoD)	“Housekeeping” genes expressed in all growing cells	TTGACA	16–18	TATAAT
σ^{38} (RpoS)	Starvation/stationary growth phase	[None]		CTATACT
σ^{28} (RpoF)	Flagellar structure and movement	TAAA	15	GCCGATAA
σ^{32} (RpoH)	Heat shock	CTTAA	12–14	GGGTAT
σ^{24} (RpoE)	Extracytoplasmic stress	GGAAC TT	16	TCAAA
σ^{54} (RpoN)	Nitrogen uptake and metabolism	-24	Spacer (bp)	-12
		TGGCAC	5	TTGC

Sources: For σ^{70} family (RpoD, RpoS, RpoF, RpoH) and σ^{54} (RpoN) consensus sequences: M. M. Wösten, *FEMS Microbiol. Rev.* 22:127–150, online, January 17, 2006. For σ^{24} (RpoE): K. M. Thompson, V. A. Rhodius, and S. Gottesman, *J. Bacteriol.* 189:4243–4256, 2007, online, April 6, 2007, doi: 10.1128/JB.00020-07.



Carol Gross [Source: Photo by Rebecca Bartlett. Courtesy of Carol Gross.]

in housekeeping sigma factors such as σ^{70} decreased the requirement for -10 and -35 promoter conservation and increased transcription initiation at nonoptimal promoters.

Some sigma factors, such as σ^{38} , direct RNA polymerase to genes that respond to cellular stresses, including osmotic shock, temperature changes, and starvation; an example is the gene *osmY*. The process can be monitored using a combination of DNA “footprinting” and an assay for mRNA levels (Figure 15-12a). DNA footprint analysis can identify a region of DNA bound by a protein, such as a sigma factor or transcription factor (see Moment of Discovery). In this kind of experiment, DNA thought to contain sequences recognized by a DNA-binding protein is isolated and radiolabeled at one end (see Figure 15-12a, step 1). A DNA-binding protein is added to a sample of the DNA (step 2; in this case, σ^{38} and σ^{70} are the test DNA-binding proteins), and chemical or enzymatic reagents are used to cleave the DNA randomly in the samples with and without the DNA-binding protein (step 3), averaging one cut per molecule. When the sets of cleavage products are compared side by side after separation by gel electrophoresis (step 4), a relatively uniform ladder of fragments appears for the sample not treated with the DNA-binding protein. A gap, or “footprint,” in the ladder of DNA fragments in the protein-containing sample identifies the region of DNA bound by the protein and thus protected from cleavage. In the case of *osmY*, the promoter DNA can be bound by RNA polymerase with σ^{38} , but not σ^{70} . Monitoring of the transcription of the *osmY* gene reveals that on increasing the osmotic strength of the culture medium, the promoter is activated to allow transcription, whereas a control promoter that uses σ^{70} (the *lacUV5* promoter) is not activated (Figure 15-12b). DNA footprinting is a valuable tool for mapping the binding sites of transcription factors and other DNA-binding proteins (see How We Know).

a result, these sigma factors require the exact consensus sequences at the -10 and -35 regions. Because many promoters deviate from the consensus, the specialized sigma factors bind only to the much smaller subset of promoters that contain the optimal consensus upstream sequences. Gross and colleagues found that converting the σ^{32} amino acid sequence to the same as that

Structural Changes Lead to Formation of the Transcription-Competent Open Complex

The major type of bacterial RNA polymerase holoenzyme, as mentioned above, contains a sigma factor of the σ^{70} class. The most common variant of the holoenzyme contains an unrelated sigma factor, σ^{54} , which is the sole representative of the σ^{54} class. The process of transcription initiation by σ^{70} -containing versus σ^{54} -containing polymerase holoenzymes is mechanistically distinct. In both cases, the holoenzyme binds to its promoter to form what is initially a closed complex, with the DNA maintaining its double-stranded structure. Formation of the closed complex is readily reversible. In complexes with the σ^{70} -class factor, the closed complex can spontaneously convert to a transcription-competent open complex, in a process of isomerization (Figure 15-13a). In contrast, σ^{54} -containing holoenzymes require specialized activator proteins of the AAA+ family (see Chapter 5 and Chapter 11) to catalyze conversion to the open complex, with concomitant ATP hydrolysis (Figure 15-13b). In both cases, energetically favorable conformational changes in the RNA polymerase accompany an opening of the DNA duplex, exposing the template and coding strands in the -11 to $+3$ region. In contrast to the σ^{70} open complex, formation of the σ^{54} open complex is irreversible and ensures that transcription will initiate.

To understand the structural basis for closed-to-open complex isomerization, it is helpful to examine the molecular structure of the RNA polymerase holoenzyme. In the open complex, several channels provide access to the core of the enzyme (Figure 15-14). One channel enables rNTPs to enter the catalytic site, and another allows the growing RNA polynucleotide to exit the enzyme during the elongation phase of transcription. A third channel provides space for DNA to enter the catalytic center in double-stranded form, between the two pincers of the claw-shaped complex. The two strands separate and are held apart by a cleft, or pin, in the polymerase structure that helps keep the transcription bubble open within the enzyme. But by the time the DNA exits the RNA polymerase, it is duplex DNA again.

Two significant structural changes result from the closed-to-open complex conversion. First, the pincers close around the DNA downstream from the transcription start site. Second, the negatively charged N-terminus of the sigma factor moves from the active-site cleft of the polymerase (where the chemical reaction occurs) to an exterior position ~ 50 Å away,

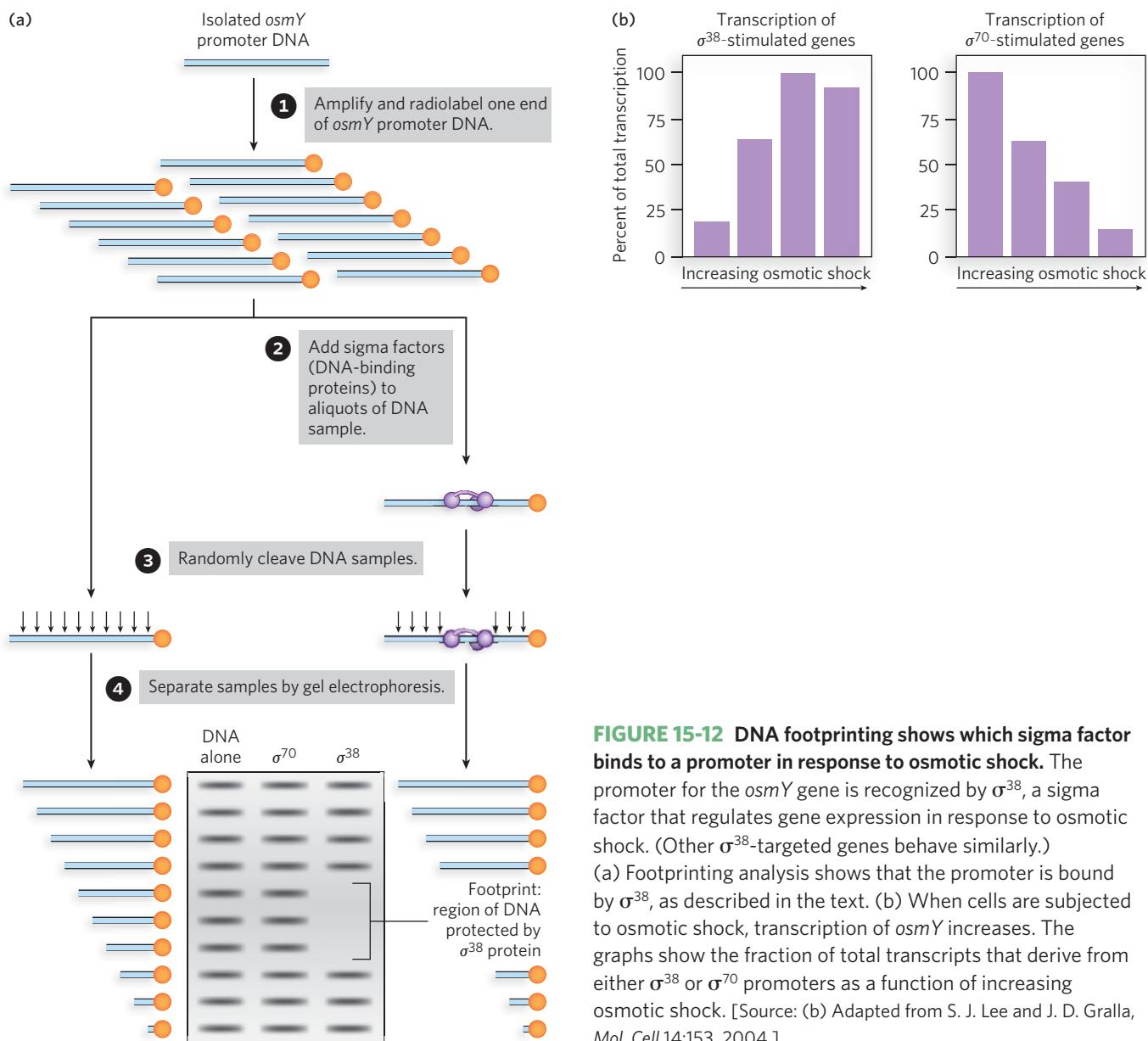


FIGURE 15-12 DNA footprinting shows which sigma factor binds to a promoter in response to osmotic shock. The promoter for the *osmY* gene is recognized by σ^{38} , a sigma factor that regulates gene expression in response to osmotic shock. (Other σ^{38} -targeted genes behave similarly.) (a) Footprinting analysis shows that the promoter is bound by σ^{38} , as described in the text. (b) When cells are subjected to osmotic shock, transcription of *osmY* increases. The graphs show the fraction of total transcripts that derive from either σ^{38} or σ^{70} promoters as a function of increasing osmotic shock. [Source: (b) Adapted from S. J. Lee and J. D. Gralla, *Mol. Cell* 14:153, 2004.]

allowing the DNA template strand to take its place. In the open position, the RNA polymerase is ready to begin RNA synthesis.

Initiation Is Primer-Independent and Produces Short, Abortive Transcripts

In contrast to DNA polymerases, which require an oligonucleotide to prime DNA synthesis by base pairing with the template strand, RNA polymerases can begin

transcription with a single nucleotide—they do not depend on a preexisting strand from which to extend new RNA. RNA polymerases must therefore bind and hold two nucleotides in place on the DNA template for long enough, and in the correct orientation, to catalyze phosphodiester bond formation between them. Once this occurs, and for the first 8 to 10 phosphodiester bonds formed, there is a high probability that the polymerase will release the transcript from the template without extending it further. If this happens, the

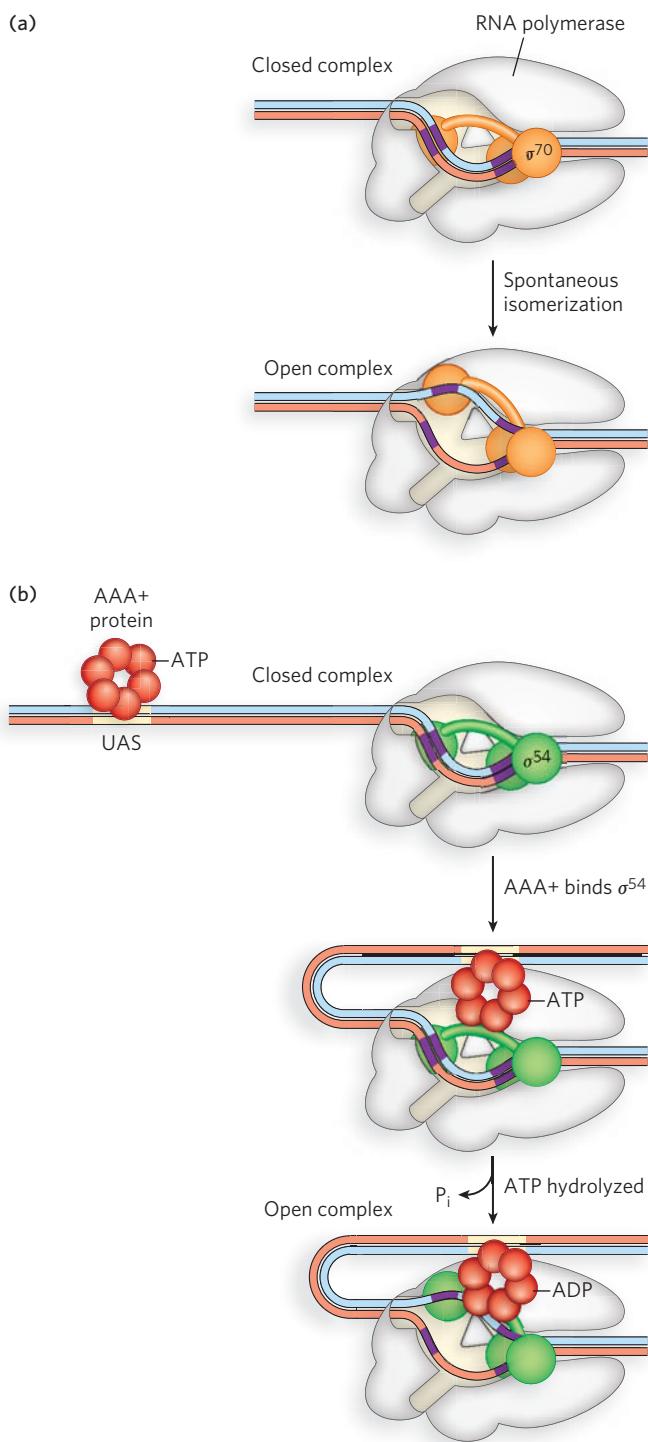


FIGURE 15-13 Conversion of the closed complex to the open complex by sigma factors. (a) σ^{70} induces strand opening and formation of the open complex, without the need for ATP or other factors. All other sigma factors except σ^{54} function in the same way. (b) σ^{54} requires an AAA+ protein to form the open complex at a promoter. The AAA+ protein is a hexamer that binds an upstream activator sequence (UAS), then also binds σ^{54} in the closed complex, creating a DNA loop. AAA+ hydrolyzes a bound ATP, providing the energy to form the open complex.

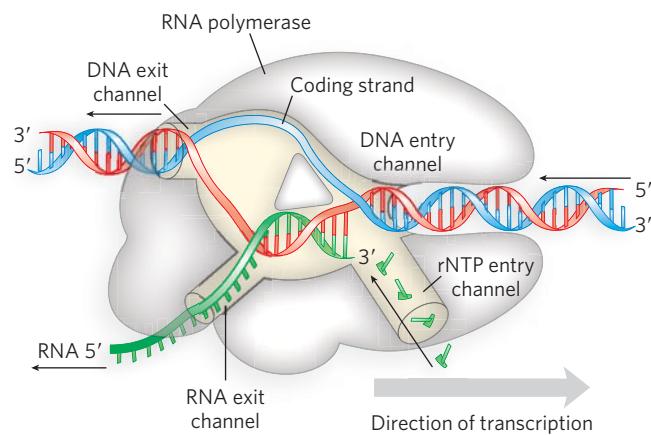


FIGURE 15-14 RNA polymerase channels. Distinct channels in RNA polymerase allow the DNA to enter as double-stranded DNA and to peel apart within the polymerase so that 8 bp form between the template strand and the growing RNA transcript. Two other channels provide entry for rNTPs and an exit for the transcript.

assembled polymerase holoenzyme begins RNA synthesis again on the same template. This process is called **abortive initiation** (Figure 15-15). Occasionally, the polymerase holds on to the transcript long enough to extend it beyond 10 nucleotides, at which point the RNA becomes stable. After a successful initiation, transcription enters the elongation phase and continues along the DNA template until it reaches a termination signal.

Interestingly, these two properties—beginning without a primer and initially producing abortive transcripts—are universal characteristics of DNA-dependent RNA polymerases. Even the single-subunit bacteriophage polymerases exhibit these properties. The molecular structures of the phage T7 RNA polymerase and the bacterial RNA polymerase suggest explanations for both of these characteristics. Abortive initiation seems to occur because, early in initiation, the RNA exit channel (see Figure 15-14) is blocked—either by a part of the polymerase itself, in the case of T7 polymerase, or by part of the sigma factor. For a transcript to extend beyond about 10 nucleotides, a structural transition must take place to unblock the RNA exit channel, a process that occurs only occasionally during the initial stage of transcription. In bacteria, this process may weaken the affinity of the sigma factor within the polymerase complex, explaining why the σ subunit often falls off the complex during elongation of the transcript. Although we don't know why abortive initiation occurs, it may help polymerase fidelity by providing opportunities for transcription termination prior to the polymerase engaging a template.

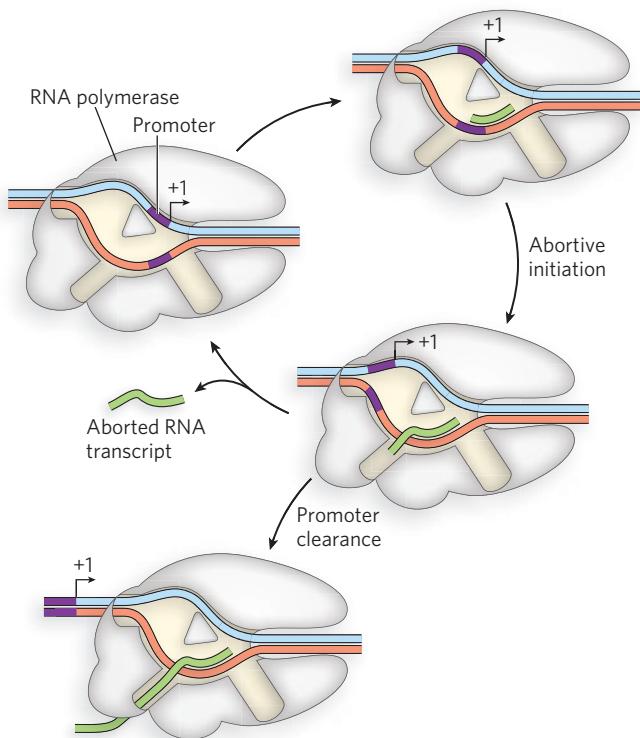


FIGURE 15-15 Abortive initiation. RNA polymerase undergoes a cycle of abortive initiation in which short RNA transcripts are synthesized and released, until the promoter site is cleared. The nascent transcript is then in position in the RNA exit channel, and initiation is successfully completed.

Transcription Elongation Is Continuous until Termination

Once RNA polymerase enters the elongation phase, the enzyme does not release the DNA template until it encounters a termination sequence. In this mode the polymerase is said to be processive, moving smoothly along the template, synthesizing the complementary RNA strand and dissociating only when the transcript is complete. During transcript elongation, the DNA moves through the polymerase active site, as observed in the polymerase open complex (see Figure 15-14). The strands of the DNA double helix separate just before the site of catalysis, held apart by a structural protrusion within the polymerase that allows the two strands to re-form a double helix upon exiting the polymerase interior. At any given time during elongation, 8 to 9 nucleotides of the RNA transcript remain base-paired with the DNA template, while the rest of the transcript is stripped off and directed out through the RNA exit channel.

During elongation, the polymerase attempts to ensure the accuracy of transcription by **pyrophosphorylation**, in which the catalytic reaction runs in reverse

whenever the polymerase stalls along the DNA. This process, known as **kinetic proofreading**, works because the polymerase tends to stall after incorporating a mismatched base into the growing RNA chain, thus enabling pyrophosphorylation to remove the incorrect base (Figure 15-16a). Pyrophosphorylation is also used in the proofreading that occurs during DNA synthesis (see Chapter 11).

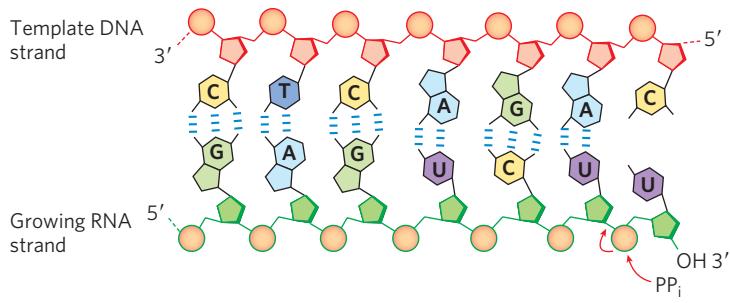
RNA polymerase contains an inherent nuclease activity, providing another means of correcting newly synthesized transcripts by hydrolysis. In this process, known as **nucleolytic proofreading**, the polymerase reverses direction by one or a few nucleotides and breaks the RNA phosphodiester bond upstream from a mismatched base, removing the error-containing strand (Figure 15-16b).

Despite these proofreading mechanisms, RNA polymerases are generally less accurate than DNA polymerases. Whereas, on average, DNA polymerase makes an error only once per 10^6 nucleotides incorporated, RNA polymerase makes an error about once per 10^4 to 10^5 nucleotides added. This can be tolerated because most transcripts are made in many copies, and one or two error-containing transcripts will be vastly outnumbered by correct transcripts in the cell. In RNA viruses, error-prone transcription can be a survival advantage, enabling the emergence of mutants that can escape detection by host cell immunity.

Bacterial RNA polymerase can synthesize RNA at a rate of 50 to 90 nucleotides per second. Rather than transcribing DNA at a constant pace, however, the enzyme pauses at times throughout the process. Pausing can be detected using single-molecule methods (see How We Know). Backtracking is a mechanism of pausing in which the RNA polymerase moves backward and peels the 3' end of the RNA off the DNA template by inserting the end into the rNTP entry channel (see Figure 15-14). This temporarily blocks further movement of the polymerase. Transcription restarts when the enzyme cleaves the peeled-back 3' end of the transcript, using its intrinsic nuclease activity. Pausing has several possible functions, including providing time for the RNA transcript to fold properly and to be translated synchronously with transcription.

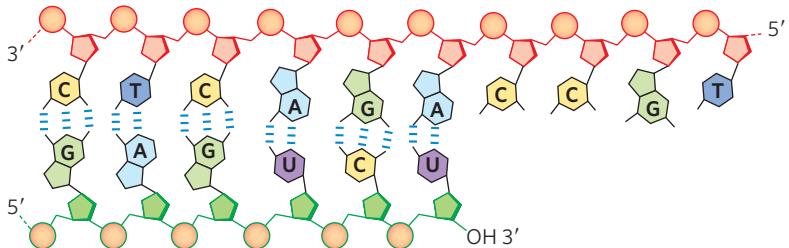
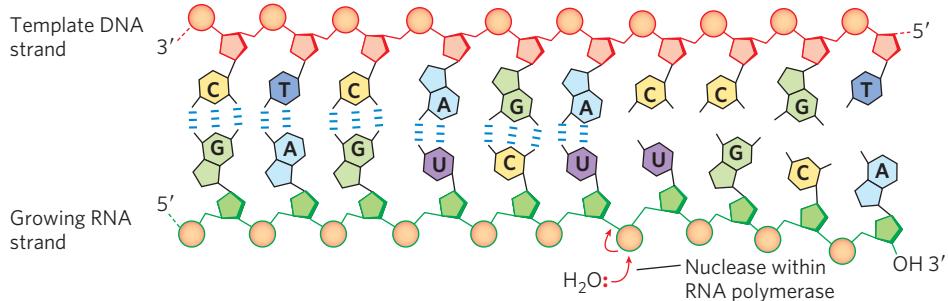
During transcription, fast and slow elongation complexes have been shown to initially coexist on the DNA. Slower, more hesitant elongation complexes are selectively removed from the template by pause sites in the DNA. As the slower complexes pause, the efficient formation of an RNA hairpin leads to dissociation of the polymerase, release of the mRNA, and thus termination of transcription. When faster complexes encounter a pause site, formation of the RNA hairpin is less efficient and the mRNA is not released.

(a) Kinetic proofreading

**FIGURE 15-16 Proofreading by RNA polymerase.**

(a) In kinetic proofreading, the polymerase stalls after incorporating a mismatched base into the growing RNA chain, enabling pyrophosphorolysis to remove the incorrect base. (b) In nucleolytic proofreading, the polymerase backtracks on the DNA, melting several nucleotides of the RNA (i.e., breaking the DNA-RNA base pairs), then an intrinsic nuclease removes the section of melted RNA.

(b) Nucleolytic proofreading



Specific Sequences in the Template Strand Cause Transcription to Stop

Transcription stops when the RNA polymerase transcribes through certain sequences in the DNA template. At this point, the polymerase releases the finished transcript and dissociates from the template. *E. coli* DNA has at least two classes of such **termination sequences**, one class that relies primarily on structures that form in the RNA transcript and another that requires an accessory protein factor called rho (ρ).

Most ρ -independent termination sequences have two distinguishing features. The first is a region that produces an RNA transcript with self-complementary

sequences, permitting the formation of a hairpin structure centered 15 to 20 nucleotides before the projected end of the RNA strand, as shown in **Figure 15-17a**. (Note that these regions occur only once at the end of the transcript, whereas the pause sites described above occur at multiple places within the transcript.) The second feature is a highly conserved segment of three A residues in the template strand that are transcribed into U residues near the 3' end of the hairpin. When a polymerase arrives at such a termination sequence, it stalls. Formation of the hairpin in the newly transcribed RNA disrupts several A=U base pairs in the RNA-DNA hybrid segment and may disturb important interactions between

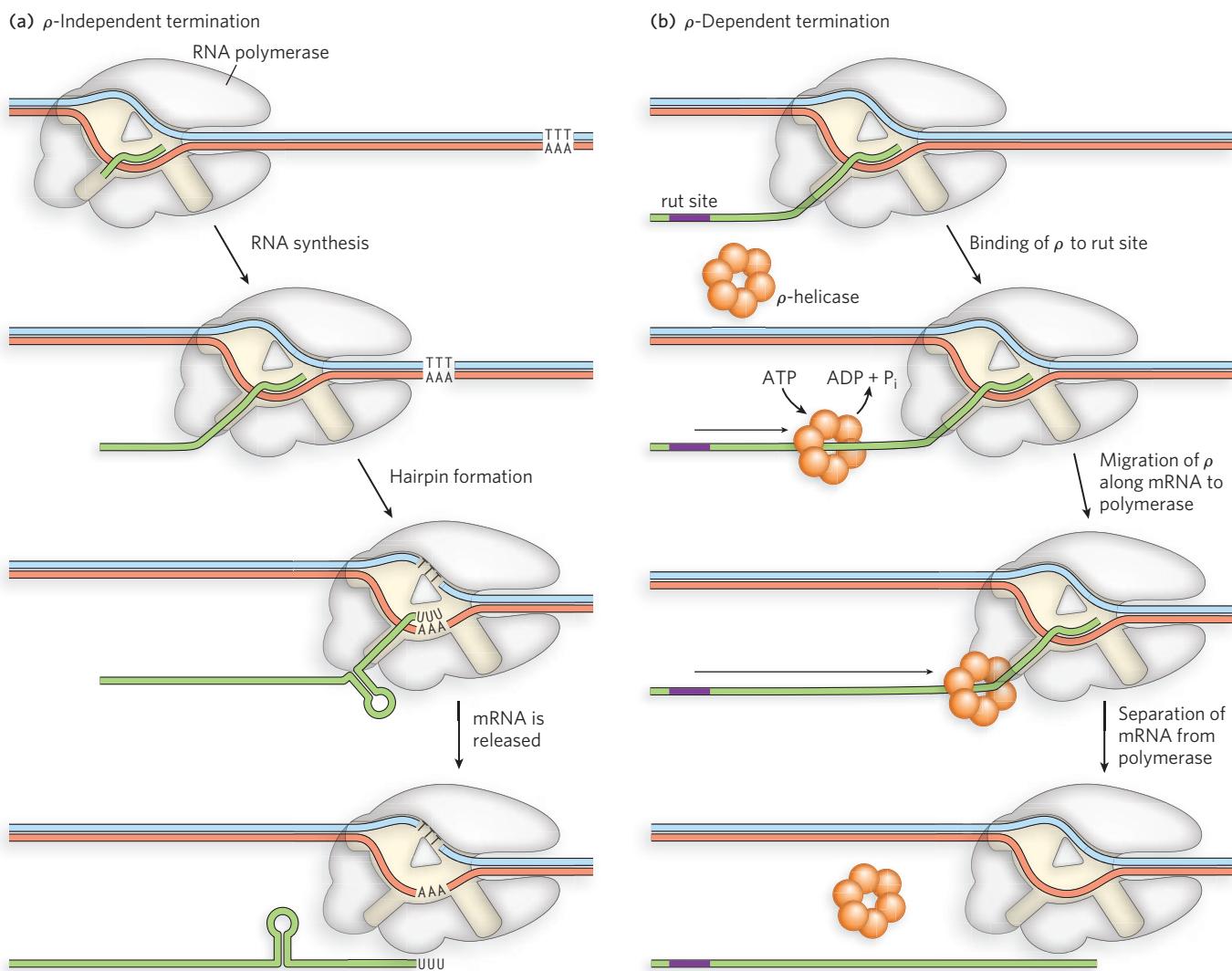


FIGURE 15-17 Termination of transcription. (a) In ρ -independent termination, an mRNA sequence forms a hairpin, followed by three U residues, stalling the polymerase and separating it from the mRNA. (b) RNAs that include a rut site (purple) recruit the ρ helicase, which migrates in the 5' → 3' direction along the mRNA and separates it from the polymerase.

RNA and the RNA polymerase, leading to dissociation of the transcript.

The ρ -dependent terminators lack the sequence of repeated A residues in the template strand but typically include a CA-rich sequence called a rut (*rho utilization*) site. The ρ factor, a hexameric helicase, associates with RNA preferentially at rut sites and migrates along the RNA in the 5' → 3' direction until it encounters a stalled transcription complex (Figure 15-17b). Here it contributes to release of the RNA transcript from both the DNA template and the polymerase. ATP-dependent RNA-DNA unwinding activity intrinsic to the ρ factor promotes its translocation along the RNA, and the ρ protein hydrolyzes ATP during the termination process.

SECTION 15.2 SUMMARY

- Transcription begins at specific promoter sequences upstream from the coding sequence in the DNA template. A sigma factor, of which there are several classes in bacteria, binds to the polymerase holoenzyme and recruits it to a particular type of promoter, enabling transcription at subsets of genes in response to environmental stimuli and the needs of the cell.
- RNA polymerase first forms a closed complex on promoter DNA, a readily reversible state that is not yet capable of transcription.
- On conversion to a transcription-competent open complex, either through spontaneous isomerization or by ATP-dependent conformational change, RNA polymerase begins RNA synthesis without requiring a primer.
- Transcription initiation requires promoter clearance, in which the RNA polymerase moves beyond the promoter region of the DNA to begin rapid elongation of the transcript.
- During elongation, the RNA polymerase is highly processive, synthesizing transcripts without dissociating from the DNA template.
- RNA polymerase corrects errors in newly synthesized transcripts through the use of nucleolytic proofreading, in which the polymerase reverses direction by one or a few nucleotides and hydrolyzes the RNA phosphodiester bond upstream of a mismatched base, removing the error-containing strand.
- Termination occurs when the polymerase transcribes through certain DNA sequences in a process that sometimes requires an accessory factor, ρ .

15.3 Transcription in Eukaryotes

In eukaryotic cells, three distinct RNA polymerases—Pol I, II, and III—carry out DNA-dependent synthesis of RNA. Although the properties of these polymerases resemble those of bacterial RNA polymerase in many ways, the eukaryotic polymerases require many additional proteins, or **transcription factors**, to begin efficient transcription at promoter sequences. Like bacterial sigma factors, each eukaryotic transcription factor binds to a specific promoter sequence and to a particular RNA polymerase, bridging the two to initiate transcription. Using a variety of general transcription factors, eukaryotic cells promote the transcription of many sets of genes under varying conditions. Specific transcription factors bind DNA at a long distance upstream from the promoter, at sequences known as enhancers, and can stimulate or repress transcription in various ways. Transcription factors, both general and specific, have important roles in gene regulation and cell development (see Chapter 21). Indeed, recent studies show that differentiated cells, once believed to be committed to a particular cell type, can be converted to another cell type simply by manipulating the expression of transcription factors (Highlight 15-2).

Because transcription is a fundamental process in all cells, it is not surprising that some eukaryotic RNA polymerase subunits are homologous to those of bacterial polymerase. Some subunits are common to all three of the eukaryotic polymerases (see Table 15-1). Relative to bacteria, eukaryotes require additional factors to help the RNA polymerases find and access promoters in the cell nucleus. This is because eukaryotic DNA is packaged into chromatin through the formation of nucleosomes (see Chapter 10). In addition, the sheer size of eukaryotic genomes, and the large number of promoters to be sorted through, probably requires additional transcription machinery.

We begin with a brief discussion of Pol I and Pol III, and then focus on Pol II transcription. As the polymerase responsible for transcribing the genes that encode proteins, Pol II is the most extensively studied of the three eukaryotic polymerases.

Eukaryotic Polymerases Recognize Characteristic Promoters

Each of the three types of RNA polymerase that make up the eukaryotic transcription machinery transcribes only certain classes of genes, and thus each type binds to specific and distinct promoter sequences. Pol I binds to a single type of promoter that controls the expression of the pre-ribosomal RNA (pre-rRNA)

HIGHLIGHT 15-2 MEDICINE

Using Transcription Factors to Reprogram Cells

Patterns of gene transcription largely control how cells develop into specific cell types. This process is of great importance in medicine, because the possibility of reprogramming cells to carry out specific functions could revolutionize the treatment of patients with degenerative diseases. Experimental attempts to reprogram cells began several decades ago with the discovery that an oocyte (egg cell) engineered to contain the nucleus of an adult cell can cause the nucleus to revert to an undifferentiated state. This process, called somatic cell nuclear transfer (SCNT), can produce an embryo and embryonic stem cells with the genetic makeup of an adult cell. Presumably, these results come about through the reprogramming of transcription in the composite cells.

This idea was tested and validated in 2006, when researchers found that fibroblasts can be induced to undergo a dramatic cell-fate reversal to an undifferentiated state, known as induced pluripotent stem cells, by transiently expressing four master-regulatory transcription factors in the fibroblasts. The next step was to see whether fibroblasts might be more generally susceptible to reprogramming into different kinds of cells—if the right set of transcription factors could be identified.

To test this possibility, Marius Wernig and colleagues at Stanford University set out to convert mouse fibroblasts into neurons. Reasoning that multiple transcription factors were probably necessary to reprogram fibroblasts to a neuronal fate, the researchers cloned 19 genes that encode transcription factors that are expressed specifically in neural tissues or function during neural development. The genes were cloned into lentiviruses, viral vectors that could be used to introduce the genes into mouse fibroblasts by infection. To detect changes in cell fate, the researchers used fibroblasts derived from mouse embryos and tail tips of newborn or adult mice that had been genetically altered to express a green fluorescent protein marker when the gene for the protein Tau was turned on. Because the Tau gene is specifically expressed in neurons, cells that had acquired at least this property of neurons could be easily identified.

When all 19 of the candidate transcription factors were introduced into the fibroblasts, some of the cells turned green. By a process of elimination, the researchers eventually found that a combination of

only three transcription factors was sufficient to convert fibroblasts into neurons (Figure 1). These factors—Ascl1, Brn2, and Myt1l (or Zic)—caused cells to express a variety of neuronal markers and become capable of firing action potentials, a basic function of neurons. Furthermore, when cultured together with bona fide mouse neuronal cells, the reprogrammed cells received both excitatory and inhibitory synaptic connections from the mouse neurons, and could form synapses with each other.

Beyond its implications for understanding transcriptional activation and regulation, this discovery offers the intriguing possibility of creating cell types at will. If such transcription-based reprogramming proves feasible in human cells, it could be used to generate neurons that mimic particular disease states for use in drug development. Researchers are now eager to find out how many different cell types can be produced by activating distinct combinations of lineage-specific transcription factors.

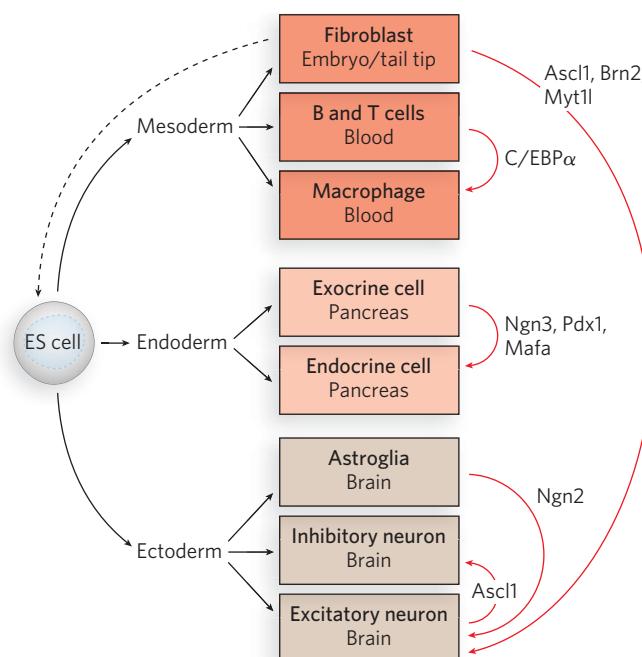


FIGURE 1 An embryonic stem (ES) cell has the potential to develop into various cell types (black arrows). Activation of specific transcription factors can convert one differentiated cell type to another (red arrows) or even to an undifferentiated state (dotted arrow). Mesoderm, endoderm, and ectoderm are the three embryonic layers from which all cells and tissues develop; astroglia are non-neuron cells of nerve tissue. [Source: Adapted from C. R. Nicholas and A. R. Kriegstein, *Nature* 463:1031–1032, 2010, Fig. 1.]

transcript, from which rRNAs are derived. Pol II, which synthesizes mRNAs, microRNAs, and some other noncoding RNAs, can recognize thousands of promoters that vary greatly in sequence. Pol III recognizes well-characterized promoter sequences for tRNAs, the 5S rRNA, and some other small regulatory RNAs that in many cases are located *within* the gene itself rather than in more conventional locations upstream from the RNA start site.

Although each polymerase works with its own unique set of transcription factors, all three types use a factor called the **TATA-binding protein (TBP)**. This protein, so-named because of its binding to a 5'-TATAAA sequence (known as the TATA box) near position -30 , plays a major role in transcription initiation. Genomic sequencing studies have shown that only about a quarter of human genes include a TATA box in the core promoter, the region responsible for recruiting the essential transcription machinery. Nonetheless, TBP is used for transcription initiation of all genes, and in most of those that lack a TATA box, TBP binds without sequence specificity. A summary of the eukaryotic polymerases and the types of RNA produced by each, along with their promoter elements, is shown in Table 15-3.

RNA Polymerase I Promoters Synthesis of pre-rRNA accounts for nearly half of all the transcription in eukaryotic cells. The precursor rRNA transcript produced by mammalian Pol I is processed into the mature 5.8S, 18S, and 28S rRNAs, which, together with the 5S rRNA transcribed by Pol III, are the major catalytic and architectural components of the ribosome (see Chapter 18). Transcription of rRNA genes, which occurs in the nucleolus, begins with the recruitment and assembly of Pol I and transcription factors into a multiprotein complex at the rRNA gene promoter. The promoter includes a core sequence, essential for accurate transcription initiation, and an upstream control element (UCE),

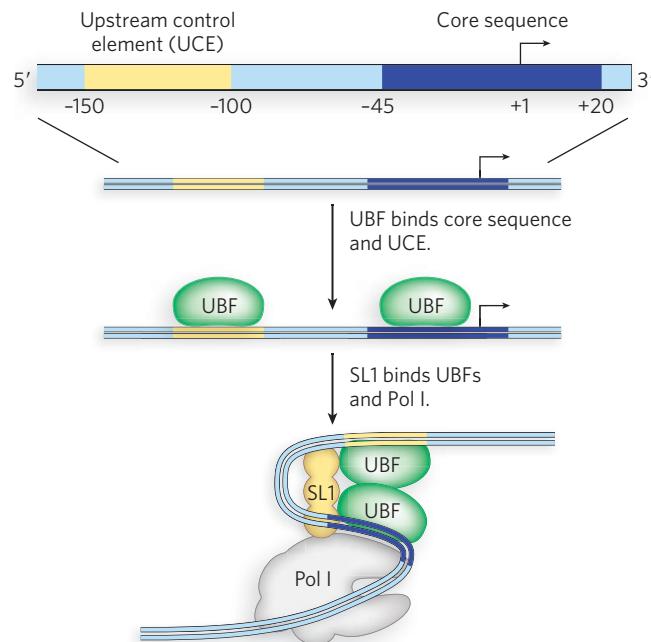


FIGURE 15-18 The Pol I promoter. The upstream binding factor (UBF) binds to the core sequence and the upstream control element. SL1, a protein complex that includes the TATA-binding protein, binds to UBF and Pol I, promoting transcription initiation.

located 100 to 150 bp upstream from the transcription start site (Figure 15-18).

The number of rRNA genes varies among organisms. Pol I promoter sequences also vary, but within a species, all Pol I promoters are the same. Low levels of transcription can be observed in the presence of a pre-initiation complex comprising Pol I and human selectivity factor 1 (SL1), which is a complex of TBP and three other TBP-associated factors (TAFs). Higher levels of transcription require, in addition to Pol I and SL1, an upstream binding factor (UBF). UBF binds to both the UCE and the core promoter and to SL1, stabilizing the complex with Pol I and helping recruit the polymerase to the promoter.

RNA Polymerase II Promoters Many Pol II promoters share certain sequence features, including a TATA box near -30 and an initiator sequence (Inr) near the RNA start site at $+1$ (Figure 15-19). Pol II promoters also sometimes include a sequence upstream from the TATA box, called a TFIIB recognition element (BRE), and a sequence downstream from the initiator, the downstream promoter element (DPE). These sequences comprise the **core promoter**. Other sequences are also needed for efficient Pol II recognition and transcription in the cell, such as upstream promoter elements and enhancers. These **regulatory sequences**, which can be

Table 15-3 Eukaryotic RNA Polymerases and Promoter Elements

Polymerase	RNA Products	Promoter Elements
Pol I	18S, 25S, and 5.8S rRNAs	UCE, core sequence
Pol II	mRNA, microRNAs, some noncoding RNAs	BRE, TATA box, Inr, DPE
Pol III	tRNA 5S rRNA 7SL RNA	Box A, Box B Box A, Box C TATA box

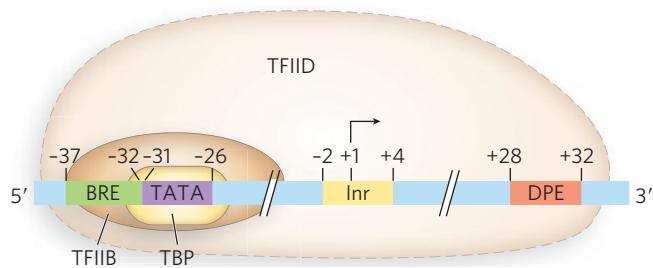


FIGURE 15-19 The Pol II core promoter. The TATA box and Inr are required for transcription by Pol II. The TFIIB recognition element (BRE) and downstream promoter element (DPE) may also be involved in initiation.

located many thousands of base pairs away from the promoter they influence, bind a variety of specific transcription factors that either activate or repress transcription, depending on various stimuli.

KEY CONVENTION

The nomenclature for transcription factors indicates which RNA polymerase is involved. TFII is a transcription factor for RNA polymerase II, and TFIII is a transcription factor for RNA polymerase III. Individual factors are distinguished by an appended A, B, C, and so on (e.g., TFIIIA, TFIIB).

RNA Polymerase III Promoters Pol III is the largest RNA polymerase with the greatest number of subunits. All of its transcription products are short, untranslated RNAs, most less than 300 nucleotides long. In addition to 5S rRNA, they include tRNAs; 7SL RNA, which is required for introducing proteins into membranes as part of the signal recognition particle; and several RNAs involved in mRNA, tRNA, and rRNA processing. Perhaps reflecting their varied gene products, Pol III promoters differ in sequence and in components. The promoters of tRNA genes include two segments, Box A and Box B, located a short distance apart *within* the tRNA-coding sequence (Figure 15-20a). The 5S rRNA gene promoter includes Box A and Box C (Figure 15-20b); other promoters contain a sequence, the TATA box, to which TBP can bind directly, just as for Pol II promoters.

Like the other eukaryotic polymerases, Pol III requires transcription factors. The tRNA genes require TFIIIB and TFIIIC, whereas the 5S rRNA gene requires TFIIIB, TFIIIC, and TFIIIA. Transcription of tRNA genes begins when TFIIIB, which includes TBP, recognizes the DNA just upstream from the transcription start site; TFIIIC binds to the promoter boxes within the gene. Together, these factors recruit Pol III to the transcription start site; TFIIIC is displaced as the polymerase transcribes through its binding site in the DNA. In 5S

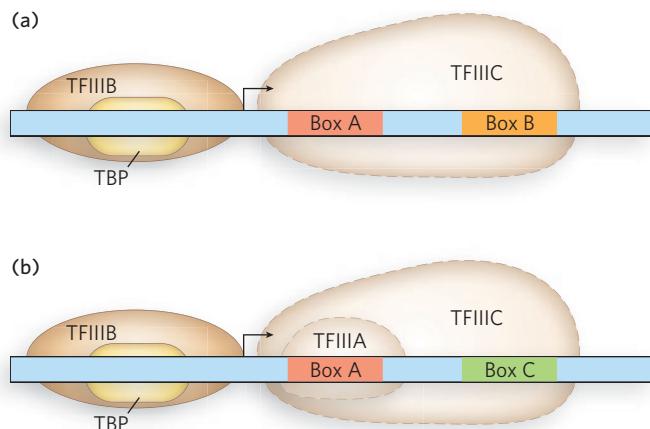


FIGURE 15-20 Pol III promoters. Pol III promoters are found within genes. (a) The Pol III tRNA promoter uses the Box A and Box B sequence elements and is bound by transcription factors TFIIIB and TFIIIC. (b) The Pol III rRNA promoter uses Box A and Box C, as well as TFIIIB, TFIIIC, and TFIIIA. Together, these factors recruit Pol III to the transcription start site.

rRNA transcription, TFIIIA binds to the DNA within the transcribed region and helps recruit TFIIIC.

Pol II Transcription Parallels Bacterial RNA Transcription

Pol II-catalyzed transcription is responsible for producing all mRNAs in the eukaryotic cell, as well as transcripts, such as microRNAs, that can base-pair with mRNAs and help regulate their expression (see Chapter 22). Consisting of 12 subunits, Pol II is strikingly more complex than its bacterial counterpart, yet it has remarkable similarities in structure, function, and mechanism (Figure 15-21). The largest subunit (RBP1) exhibits a high degree of homology to the β' subunit of bacterial RNA polymerase. Another subunit (RBP2) is structurally similar to the bacterial β subunit, and two others (RBP3 and RBP11) show some structural homology to the two bacterial α subunits (see Table 15-1). Pol II must function with genomes that have multiple chromosomes and with DNA molecules more elaborately packaged than those in bacteria. The need for protein-protein interactions with the numerous other protein factors required to navigate this labyrinth largely accounts for the added complexity of Pol II and the other eukaryotic polymerases.

Playing a role much like that of sigma factors in helping bacterial RNA polymerase recognize and bind promoter sequences, transcription factors associate with promoter DNA and recruit Pol II to form a **preinitiation complex**, as shown in Figure 15-22 (step 1). The preinitiation complex is converted to an initiation complex by unwinding the DNA (step 2). During

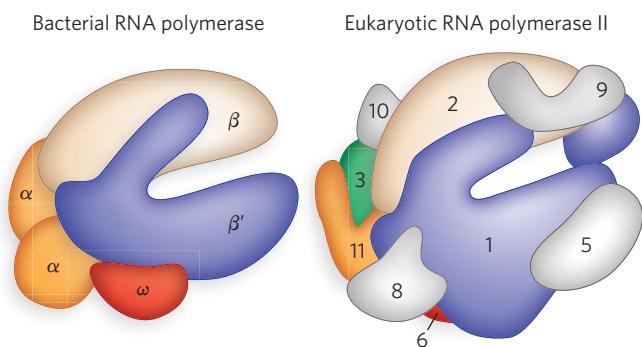


FIGURE 15-21 Bacterial RNA polymerase and eukaryotic Pol II structural elements. Although Pol II has more subunits with additional components, it has obvious structural similarities to bacterial RNA polymerase. The numbers on the Pol II subunits indicate RBP1, RBP2, and so forth.

initiation (step 3), the C-terminal domain (CTD) of Pol II is phosphorylated and some transcription factors are released. Elongation (step 4) proceeds as in bacteria. Transcription is terminated (step 5) by dephosphorylation of the Pol II CTD. Each step is associated with characteristic proteins.

Transcription Factors Play Specific Roles in the Transcription Process

The transcription initiation mechanism has been most extensively studied for Pol II. Recruitment begins with binding of the TATA box by TFIID. Like many transcription factors, TFIID is a multiprotein complex, which includes TBP and TAFs. The TAFs fine-tune TFIID by changing the affinity of TBP for DNA, helping the transcription factor bind certain promoters. Once the TBP-DNA interaction is stabilized, other transcription factors, and Pol II itself, can stably associate with the promoter to form the preinitiation complex.

The discovery of TBP and its importance as a general transcription factor required for all Pol II transcription raised questions about how and why it binds so specifically to the TATA element. This mystery of the transcription initiation process was solved when the research groups of Paul Sigler and Stephen Burley independently determined the molecular structure of TBP bound to DNA, using x-ray crystallography. The structure revealed that TBP sits on the DNA double helix much like a saddle, with an extended β sheet and loop “stirrups” in contact with the minor groove of the

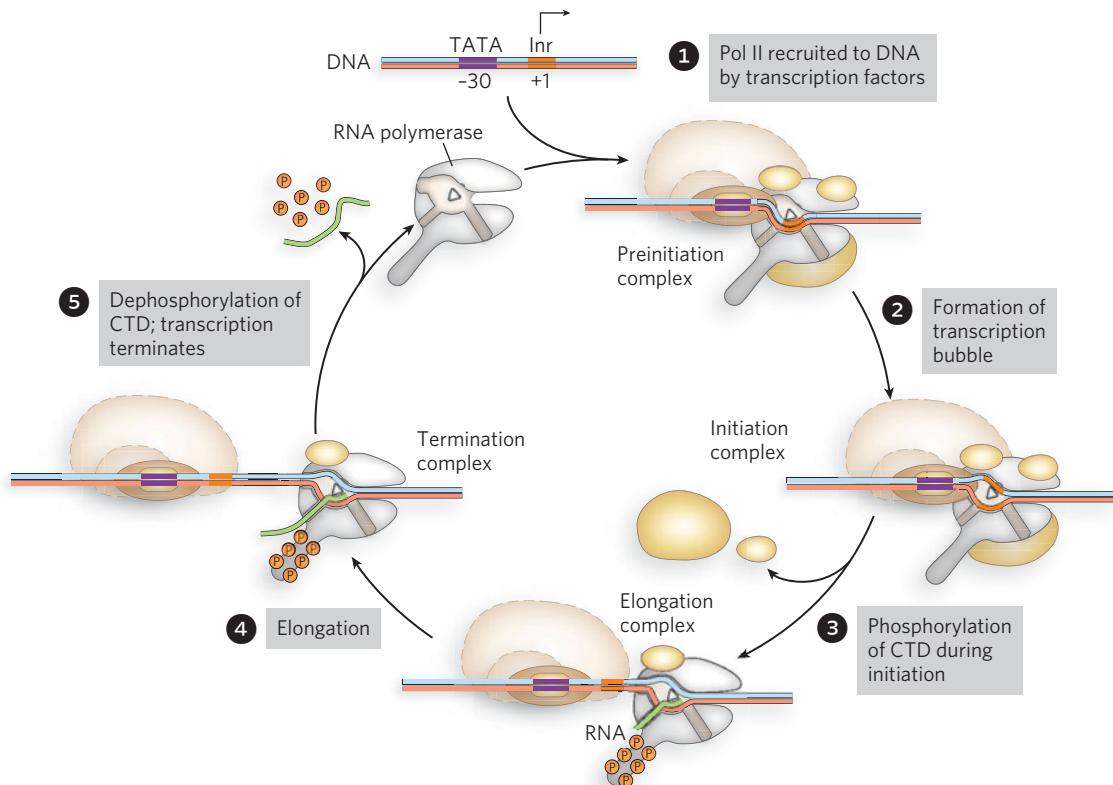


FIGURE 15-22 Transcription at Pol II promoters. The phases of transcription by Pol II—assembly, initiation, elongation, and termination—are associated with characteristic proteins, as described in the text. The ordered assembly and dissociation of these factors drives the process forward.

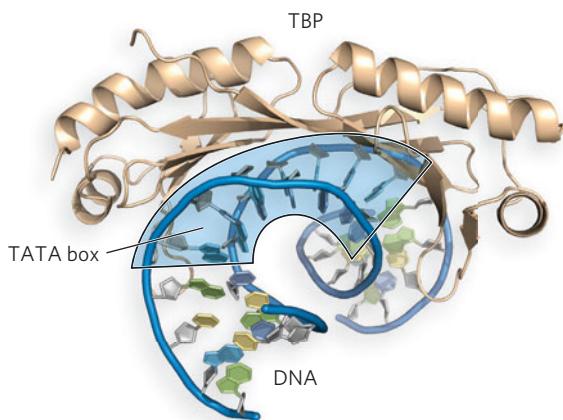


FIGURE 15-23 The crystal structure of a TBP-DNA complex. TATA-binding protein (TBP) bends the TATA box sequence, opening the minor groove to allow sequence-specific hydrogen bonding. [Source: PDB ID 1CDW.]

TATA box sequence (Figure 15-23). This unconventional mode of DNA recognition—most DNA-binding proteins recognize DNA by inserting α helices into the major groove—bends the DNA by positioning two pairs of phenylalanine side chains between base pairs at each end of the recognition sequence. The bending opens and widens the minor groove, enabling hydrogen bonding between protein side chains and the minor groove edges of the DNA bases. The observed helical bending explains why A=T base pairs are favored: they are more easily distorted to allow opening of the minor groove. Because TBP is used by all three classes of eukaryotic polymerases, a similar mechanism may account for promoter recognition in all cases.

In addition to TBP, Pol II requires an array of transcription factors to form an active transcription complex. The general transcription factors required at every Pol II promoter are highly conserved in all eukaryotes. Using cell-free systems pioneered by

Robert Roeder at Rockefeller University, in which purified proteins were added back to the reaction mix to reconstitute active transcription complexes, it was possible to determine the identity and order of proteins needed for transcription initiation. When TBP, as part of TFIID, binds to the TATA box, it is bound in turn by the transcription factor TFIIB, which binds a larger site on the DNA than TBP alone. A third transcription factor, TFIIA, is not always essential but can provide stability to the complex, and can be important at nonconsensus promoters where TBP binding is relatively weak. Pol II is bound to the complex through a mutual interaction with TFIIF. Finally, TFIIE and TFIIH bind to create the closed complex, analogous to the closed complex described for bacterial RNA polymerase (see Section 15.2). TFIIH has DNA helicase activity that promotes unwinding of the DNA near the transcription start site. This unwinding creates an open complex that is competent to begin transcription. Counting all the subunits of the various essential factors (excluding TFIIA), this minimal active assemblage has more than 30 polypeptides!

Once assembled on a promoter, Pol II typically produces a few abortive transcripts before entering the elongation phase of transcription—behavior similar to that of bacterial RNA polymerase. In contrast to bacterial polymerase, however, Pol II must be chemically modified by the addition of phosphate groups to its CTD to disengage from the promoter and begin elongating a transcript. The CTD, part of the largest polymerase subunit, consists of multiple repeats of the seven amino acid sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, which are substrates for kinases, enzymes that catalyze phosphorylation of one or more of these residues. The kinases play a key regulatory role in transcription by converting Pol II into a multiply phosphorylated form that can efficiently synthesize RNA.

TFIIE and TFIIH are released during synthesis of the initial 60 to 70 nucleotides of RNA, as Pol II enters the elongation phase of transcription. Notably, Pol II CTD phosphorylation also influences downstream processing of the RNA transcript, providing a mechanism for coupling transcription to RNA splicing and intracellular transport (see Chapter 16).

In the elongation phase, polymerase activity is greatly enhanced by elongation factors. They suppress pausing during transcription, enhance polymerase editing of



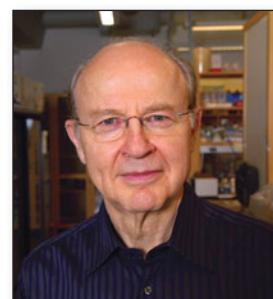
Paul Sigler, 1934–2000

[Source: Courtesy of Michael Marsland/Yale University.]



Stephen Burley [Source:

Courtesy of Rockefeller University.]



Robert Roeder [Source:

Zach Veilleux/Rockefeller University.]

misincorporated bases by hydrolysis (as for bacterial RNA polymerase), and recruit protein complexes involved in posttranscriptional processing of the mRNA. Once the RNA transcript is completed, transcription is terminated. In eukaryotes, termination is often triggered by endonucleases that recognize and cleave specific sequences in the newly synthesized RNA, leading to transcription complex disassembly and dissociation. Pol II is then dephosphorylated and recycled, readying it to initiate another transcript (see Figure 15-22).

Transcription Initiation In Vivo Requires the Mediator Complex

The initiation and control of eukaryotic mRNA synthesis requires a large set of evolutionarily conserved general transcription factors that function at most, if not all, genes. These include initiation factors TFIIB, TFIID (which includes the TATA binding protein), TFIIE, TFIIF, and TFIIH—which comprise the minimal set of helper proteins necessary and sufficient for in vitro selective binding and accurate transcription initiation by Pol II from core promoters. In vivo, in yeast cells, the multiprotein **Mediator complex** is required for the regulated transcription of nearly all Pol II-dependent genes. Its presence also in humans implies a similar central role in Pol II-catalyzed transcription.

Mediator functions as an intermediary between specific transcription factors bound at upstream promoter elements and at enhancers and the Pol II complex and general initiation factors bound at the core promoter (Figure 15-24a). First discovered and purified from yeast by Roger Kornberg and colleagues, Mediator was found to be required for transcriptional activation by specific activators in vitro, using a reconstituted enzyme system containing purified Pol II and general initiation factors. Yeast Mediator has 20 subunits in three distinct subdomains, referred to as the head, middle, and tail modules (Figure 15-24b). An additional module, which includes a kinase enzyme complex, is associated with a subset of yeast Mediator complexes. The presence of the kinase corresponds to repression of a subset of genes, suggesting a role for Mediator in transcriptional down-regulation as well as in activation.

Human Mediator contains a set of consensus subunits similar to those in yeast. As in yeast, multiple forms of Mediator seem to function differently in the transcriptional control of different sets of genes. In particular, the kinase module can exert a repressive effect when associated with the mammalian Mediator, whereas other auxiliary proteins are associated with an activating form of Mediator.

The mechanisms by which Mediator complexes control mRNA synthesis involve direct interactions with DNA-binding transcription activators bound at

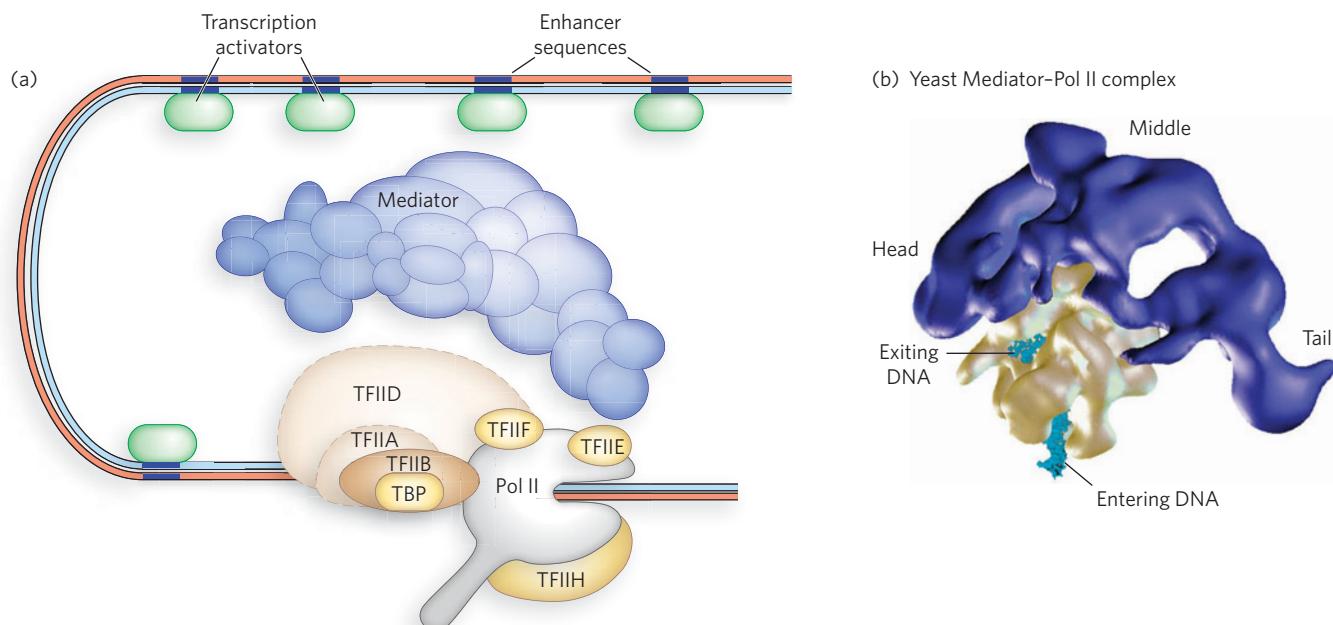


FIGURE 15-24 The Mediator complex. (a) Mediator helps to bridge distant proteins bound to enhancer sequences and Pol II and its general transcription factors, bound near the transcription start site. (b) Mediator bound to Pol II. The Mediator complex consists of 20 proteins and has three subdomains. [Source: (b) Adapted from J. A Davis et al., *Mol. Cell* 10:409–415, 2002.]

upstream promoter elements and enhancers, with Pol II, and with one or more of the general initiation factors bound at the core promoter. Mediator supports transcriptional activation, at least in part, by increasing the rate and/or efficiency of assembly of the Pol II pre-initiation complex. Mammalian Mediator complexes influence several steps during this assembly, including the recruitment of TFIID (or TBP), Pol II, and the other general initiation factors to the core promoter.

Termination Mechanisms Vary among RNA Polymerases

The three RNA polymerases use different strategies for terminating transcription, although these mechanisms have some aspects in common. The Pol III and Pol I termination pathways seem to be simpler than that of Pol II. Pol III terminates transcription at T-rich sequences located a short distance from the 3' end of the mature RNA, assisted by just a few protein factors. Pol I terminates at a terminator site located downstream from the rRNA precursor sequence and requires terminator recognition by specific protein factors.

In contrast, Pol II termination does not occur at a conserved site or at a constant distance from the 3' end of mature RNAs. In mammals, it occurs anywhere from a few base pairs to several kilobase pairs downstream from the 3' end of the mature transcript. The 3' end includes a stretch of A nucleotides called a polyadenylation signal, or poly(A) tail, which is essential for translation into protein (see Chapter 18). Pol II termination is coupled to 3'-end processing of precursor mRNA transcripts, and an intact polyadenylation signal is necessary for transcription termination of protein-coding genes in human and yeast cells.

Two different models were proposed more than two decades ago to explain how 3'-end processing contributes to Pol II transcription termination. The first, known as the allosteric or antiterminator model, proposes that transcription through the poly(A) site triggers conformational changes in the Pol II elongation complex by the dissociation of elongation factors and/or association of termination factors. This is analogous to the hairpin model of termination in bacteria (see Section 15.2). The second model, the torpedo model, suggests that the observed rapid degradation of RNA from the 3' end after cleavage at the poly(A) site creates an entry site for a 5'→3' exonuclease to degrade the RNA (Figure 15-25). The exonuclease is thought to promote Pol II release after catching up with the elongation complex. This is similar to ρ-dependent termination in bacteria. Both models are likely to be part of the true termination mechanism of Pol II.

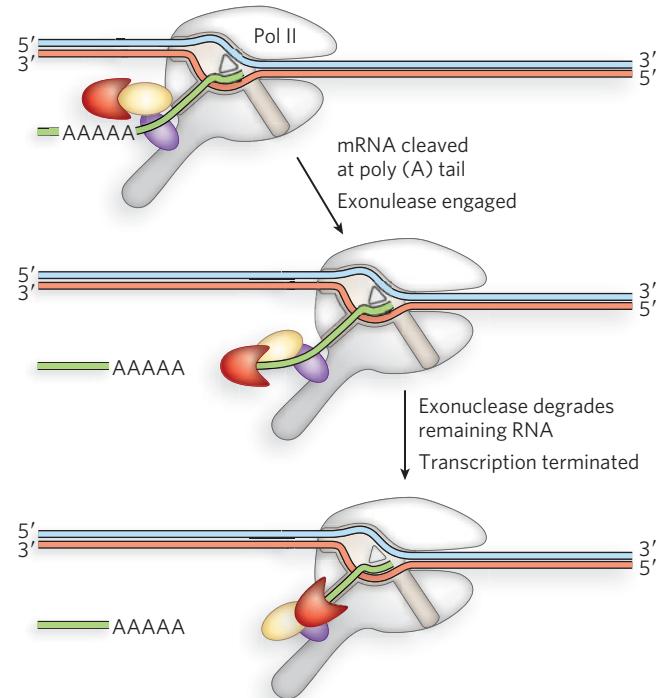


FIGURE 15-25 Torpedo model for transcription

termination by Pol II. The torpedo model hypothesizes that the mRNA transcript is cleaved downstream from the poly(A) site. An exonuclease binds the RNA remaining on the polymerase and degrades the RNA in the 5'→3' direction, moving closer to the polymerase and eventually causing it to release the mRNA.

Transcription Is Coupled to DNA Repair, RNA Processing, and mRNA Transport

In eukaryotes, transcription is coupled to other activities, including the repair of damaged DNA and various kinds of RNA processing and transport events. Researchers noticed that DNA damage repair and mRNA processing and transport are more efficient for genes that are actively being transcribed. Furthermore, DNA lesions in the template strand are repaired somewhat more efficiently than lesions in the coding (nontemplate) strand. For DNA repair, these remarkable observations are explained by the alternative functions of the TFIIH subunits. Not only does TFIIH participate in forming the closed complex during assembly of a transcription complex, but some of its subunits are also essential components of the separate nucleotide excision repair complex (see Chapter 12). When Pol II transcription stalls at the site of a DNA lesion, TFIIH can interact with the lesion and recruit the entire nucleotide excision repair complex. Genetic deletion of certain TFIIH subunits produces human diseases. Two examples are xeroderma pigmentosum, with its associated photosensitivity and

tumor susceptibility, and Cockayne syndrome, which is characterized by arrested growth, photosensitivity, and neurological disorders.

Eukaryotic mRNA is processed in a variety of ways before it is shipped across the nuclear membrane to the cytoplasm for translation. We'll discuss the mechanisms of these processing events in Chapter 16, but it is important to note here that like DNA repair, mRNA processing is naturally linked to transcription. This is possible because some of the same proteins required for elongating RNA transcripts are also required for 5'-end processing (5' capping) of RNA. Because these activities are coupled, transcripts can be processed as they are synthesized.

SECTION 15.3 SUMMARY

- The RNA polymerases of eukaryotes (Pol I, II, and III) share some structural and functional features with bacterial RNA polymerase, but they are much larger and require additional proteins—transcription factors—to begin efficient transcription at promoter sequences.
- Pol I, II, and III recognize distinct promoter sequences and require unique sets of transcription factors, with the exception of TATA-binding protein (TBP), which is used by all three polymerases.
- As in bacteria, transcription initiation in eukaryotes is highly regulated and includes multiple steps that lead to assembly of an active polymerase complex at a promoter. Pol II transcription (the most studied) proceeds through distinct phases of assembly, initiation, elongation, and termination.
- In eukaryotes, the Pol II C-terminal domain must be phosphorylated before transcription can proceed from initiation to elongation.
- Transcriptional regulation in eukaryotes is enhanced by Mediator, a large protein complex that binds simultaneously with general transcription factors associated with Pol II and specific transcription factors associated with upstream promoter elements.
- Two hypotheses for transcription termination suggest a role for mRNA sequence elements and for exonucleases, respectively.
- TFIIH, a eukaryotic transcription initiation factor, can start nucleotide excision repair of DNA when

Pol II encounters a lesion in the template strand. Transcription and processing of mRNA are coupled, because some Pol II transcription factors are also required for pre-mRNA processing events.

Unanswered Questions

Many details of transcription mechanisms are known, but future challenges include discovering how, where, and when transcripts are made and how they are used in cells for functions beyond encoding and translating proteins.

1. **How does RNA polymerase coordinate with other enzymes and regulators during gene expression in bacteria?** Pausing by RNA polymerase during transcription is thought to help the enzyme coordinate with other steps in the protein-producing pathway. How does this work, and do proteins such as RNA-modifying enzymes recognize paused transcripts as substrates? Understanding these mechanisms will not only provide basic information about transcription but also help define steps that could be disrupted to block bacterial growth, thus serving as good antibacterial drug targets.
2. **What are the structures of Pol I and Pol III?** Exquisite insights about polymerase function have come from crystallographic structural determinations. The structures of Pol I and Pol III are largely unknown, partly because of their size and complexity, but also because scientists have focused primarily on Pol II, given its central role in gene expression and gene regulation. Knowing the structural details of the other two eukaryotic polymerases will go a long way toward explaining their abilities to transcribe specific kinds of genes. These structures will also certainly lead to a better understanding of the evolutionary relationships among polymerases.
3. **What kinds of promoters are recognized by Pol II for transcription of non-protein-coding RNAs?** Pol II seems to transcribe much of the human genome at low levels. How is the polymerase recruited to the DNA for this purpose? Perhaps Pol II uses its weak, nonspecific DNA-binding affinity, or perhaps it can transcribe past termination signals at some frequency. How is such transcription controlled?

How We Know

RNA Polymerase Is Recruited to Promoter Sequences

Dynan, W.S., and R. Tjian. 1983. Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell* 32:669–680.

Dynan, W.S., and R. Tjian. 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* 35:79–87.



William Dynan [Source: Courtesy of Georgia Research Alliance.]

One of the first experiments to demonstrate how promoters are recognized by RNA polymerase II involved separating the contents of cultured human HeLa cells into the components required for accurate gene transcription *in vitro*. Bill Dynan and Bob Tjian used this system to find that Sp1 is a promoter-specific transcription factor required to recruit Pol II to only certain kinds of genes. Using genes from two different mammalian viruses, the monkey virus SV40 and the human adenovirus, Dynan and Tjian found that Sp1 recruited Pol II to the SV40 but not to the adenovirus genes (Figure 1). When SV40 and adenovirus DNA templates were present together in an *in vitro* transcription reaction, addition of Sp1 stimulated early promoter transcription of the SV40 DNA 40-fold, whereas promoter transcription of adenovirus DNA was inhibited 40%. This finding suggested that Sp1 is involved in promoter selection and is not merely a stimulatory general transcription factor.

Further experiments using deletion mutants of the SV40 promoter showed that transcriptional activation by Sp1 required sequences within tandem 21 bp repeats located 70 to 110 bp upstream from the transcription initiation site. DNA footprinting revealed that DNA

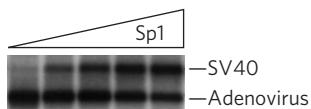


FIGURE 1 Sp1 activates transcription of SV40 DNA, but not human adenovirus DNA. Purified RNA polymerase and increasing amounts of Sp1 were added to a mixture of DNA containing either an SV40 promoter or an adenovirus promoter; transcripts initiated from each promoter were separated and analyzed by gel electrophoresis. [Source: Adapted from W. S. Dynan and R. Tjian, *Cell* 32:669, 1983, Fig. 5.]

sequences within the 21 bp repeat region were bound by Sp1 (Figure 2). In this experiment, SV40 promoter-containing DNA was incubated with increasing amounts of a cell extract enriched with the Sp1 protein. DNase I, a nuclease, was then added to digest any DNA not protected by bound protein. As the protein concentration increased, a pronounced region of the DNA around the 21 bp repeat became resistant to DNase I digestion, revealing the “footprint” left by the binding of Sp1 to the DNA.

This was an exciting result, because it indicated the presence of a specific site for Sp1 binding. Furthermore, there was a correlation between this promoter-binding activity and consequent transcription stimulation. The results suggested that Sp1 activated transcription by Pol II at the SV40 early promoter by direct binding of the Sp1 to sequences in the upstream activator sequence.

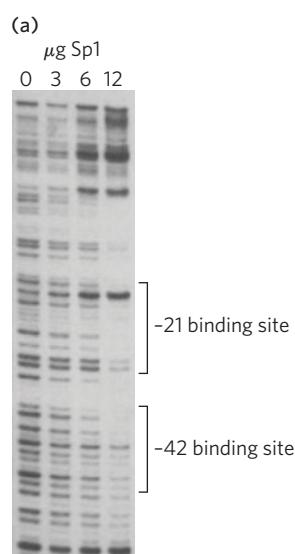
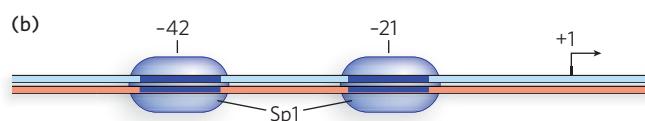


FIGURE 2 Sp1 leaves its footprint on a promoter. (a) The Sp1 footprint is visible at -21 and -42 with increasing concentrations of Sp1. (b) Sp1 binds SV40 DNA at -21 and -42. [Source: W. S. Dynan and R. Tjian, *Cell* 35:79–87, 1983.]



RNA Polymerases Are Both Fast and Slow

Neuman, K., E. Abbondanzieri, R. Landick, J. Gelles, and S.M. Block. 2003. Ubiquitous transcriptional pausing is independent of RNA polymerase backtracking. *Cell* 115:437–447.

Shaevitz, J.W., E.A. Abbondanzieri, R. Landick, and S.M. Block. 2003. Backtracking by single RNA polymerase molecules observed at near-base-pair resolution. *Nature* 426:684–687.

Molecular biologists have noticed that rather than transcribing DNA at a constant pace, an RNA polymerase hesitates at certain sites as it moves along the template. However, because the individual polymerases in a solution are not synchronized, the kinetics of pausing are difficult to study.

To circumvent this problem, Stephen Block, Bob Landick, and their coworkers chemically attached transcription elongation complexes to polystyrene beads, one polymerase to a bead. They used antibodies to attach one end of the template DNA to the surface of a microscope stage (Figure 3a), and used a laser trap to keep the bead (and RNA polymerase) in a fixed position while moving the stage (and the DNA) away, pulling the DNA taut through this constant force. They monitored the motion of the bead (and polymerase) with respect to the stage surface as the DNA was threaded through the elongation complex. This system was used to assess the force on the bead required to counteract the motion of the RNA polymerase. From these measurements, pause and arrest sites on the DNA could be mapped, and the maximal speed reached by RNA polymerase between two pause sites was measured.

Although the experiments were conducted on single molecules, thousands of recordings were made, enabling

the investigators to compare individual polymerase complexes. The experiments showed that RNA polymerase molecules alternate between constant-velocity transcription and pausing. The velocities of individual polymerase molecules typically displayed a bimodal distribution, with one peak corresponding to the rate of transcription between pauses and a second peak, near 0 bp/s, corresponding to the pauses themselves (Figure 3b). This study of individual elongation complexes provided direct evidence that RNA polymerases have different intrinsic speeds. The coexistence of slower and faster polymerases might explain how regulatory proteins modulate the behavior of elongation complexes during transcription, increasing or decreasing overall transcription rates in response to the needs of the cell.

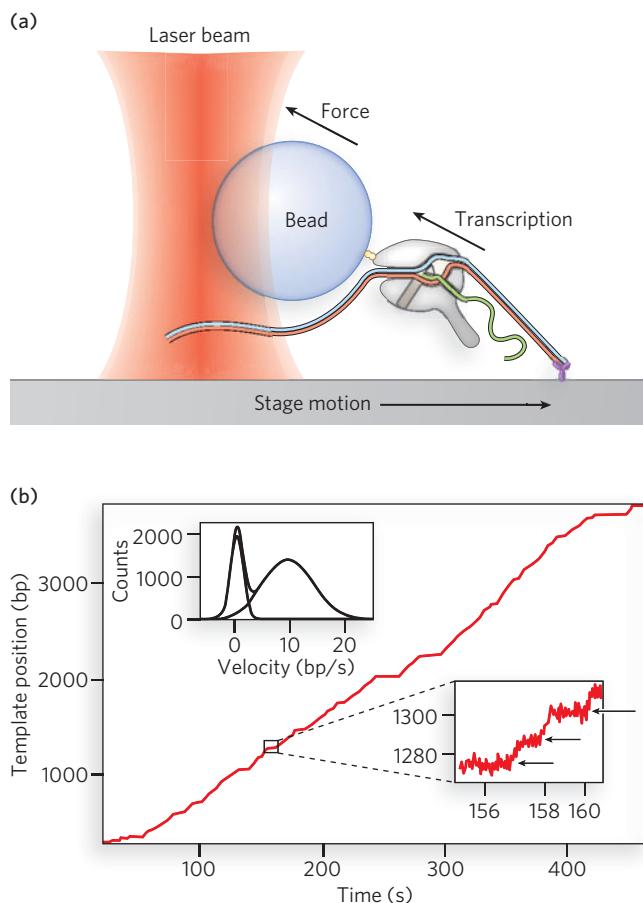


FIGURE 3 Single-molecule analysis determines the velocity of an RNA polymerase along a DNA, and monitors pausing.
(a) Representation of the experimental setup (not to scale). Transcribing RNA polymerase (white) with nascent RNA (green) is chemically attached to a polystyrene bead, and the upstream end of the duplex DNA is attached through an antibody linkage (purple) to a moveable microscope stage. The bead is held by a laser trap at a predetermined position, which results in a restoring force exerted on the bead.
(b) Representative record of position and velocity for a single polymerase molecule transcribing a 3,500 bp DNA template with <18 piconewtons of hindering force. Pausing occurs on multiple timescales; distinct pauses of seconds-long duration can be seen in the traces, and shorter pauses of about 1 second can be detected in the expanded portion of the trace (right inset, arrows). Negative values of instantaneous velocity are due to Brownian motion of the bead in the optical trap. [Source: Adapted from K. Neuman et al., *Cell* 115:437–447, 2003.]

Key Terms

transcription, p. 516	initiation, p. 519	pyrophosphorolysis, p. 529
messenger RNA (mRNA), p. 516	promoter, p. 519	kinetic proofreading, p. 529
transfer RNA (tRNA), p. 516	elongation, p. 519	nucleolytic proofreading, p. 529
ribosomal RNA (rRNA), p. 516	termination, p. 519	termination sequence, p. 531
RNA polymerase, p. 516	template strand, p. 519	transcription factor, p. 532
sigma factor, p. 519	coding strand, p. 519	TATA-binding protein (TBP), p. 534
RNA polymerase I (Pol I), p. 519	closed complex, p. 521	core promoter, p. 534
RNA polymerase II (Pol II), p. 519	open complex, p. 521	preinitiation complex, p. 535
RNA polymerase III (Pol III), p. 519	elongation complex, p. 521	Mediator complex, p. 538

Problems

1. The -10 and -35 sequences in bacterial promoters are separated by about two turns of the DNA double helix. How would transcription be affected if a deletion were introduced in the promoter region that moved the -35 sequence to the -29 position?
2. The gene encoding the *E. coli* enzyme β -galactosidase begins with the sequence ATGACCATGATTACG. What is the sequence of the mRNA transcript specified by this part of the gene?
3. The gene for β -galactosidase has 3,075 bp. How long would it take for the *E. coli* RNA polymerase to transcribe this gene, assuming initiation has occurred upstream prior to its encounter with the gene?
4. The sequence of the consensus -10 region is TATAAT. If two genes, *tesA* and *tesB*, have identical promoter sequences except in the -10 region, where the *tesA* sequence is TAATAT and the *tesB* sequence is TGTCTGA, which gene do you expect to be more efficiently transcribed, and why?
5. If a Pol II promoter were replaced with a promoter specific for Pol III in a human cell, what do you expect would happen to the number of transcripts produced?
6. Name the three major steps, and indicate their relative rates, in the transcription of a typical bacterial gene.
7. Working in a research lab, you wish to examine the kinetics of the initiation phase of bacterial RNA polymerase, as a function of promoter sequence. You want to prevent the reaction from entering the elongation phase. How many nucleotides can be added to an RNA polymer in the initiation phase? Suggest a simple reaction strategy to limit the reaction to initiation, based on the sequence of the template and the components added to the reaction mix. With your experimental design, what would the reaction products be? Assume you have an assay to measure the production of short RNA oligonucleotides (you don't need to describe the assay).
8. People who ingest *Amanita phalloides* (the source of α -amanitin) initially experience gastrointestinal distress caused by other toxins also produced by this mushroom. α -Amanitin shuts down the action of RNA polymerase II, but death does not occur until about 48 hours after ingestion and usually involves liver dysfunction. Suggest a reason for the delay in lethality.
9. A drug company has discovered a natural product, cupramycin, that efficiently intercalates into DNA. How might this compound affect transcription?
10. How might an investigator search for Pol II promoters in the DNA sequence of an entire organism? Is it possible to find all such promoters computationally?
11. Gene *A* encodes protein *A*. A genetic engineer excises a promoter sequence for gene *A* from the DNA and reinserts it at the other end of gene *A*, oriented so that an RNA polymerase binding at the promoter will transcribe across gene *A*. Will the mRNA synthesized by the RNA polymerase still possess a sequence that produces a functional protein *A*? Why or why not?
12. In bacteria, there are many examples of two (or even more) genes being transcribed from one promoter—for example, the promoter is followed by gene *A* and then gene *B*, with both genes transcribed into a single mRNA. In some cases, the first gene in the linear sequence is transcribed at much higher levels than the second gene (i.e., many but not all of the mRNAs do not include gene *B*). What kind of DNA sequences might be present between the first and second genes to account for the lower level of transcription of gene *B*?

Data Analysis Problem

Grossman, A.D., J.W. Erickson, and C.A. Gross. 1984. The *htpR* gene product of *Escherichia coli* is a sigma factor for heat shock promoters. *Cell* 38:383–390.

13. In *E. coli*, σ^{70} is the major but not the only sigma factor. Several other sigma factors direct RNA polymerase to bind to different sets of promoters. The second sigma factor to be discovered was σ^{32} , which participates in the expression of genes involved in the heat shock response. When the environmental temperature suddenly rises, cellular production of about 20 proteins increases. These proteins help protect the cell from any ill effects of the higher temperature. Some of the proteins are chaperones that facilitate protein folding (see Chapter 4). In the 1970s, a gene required for normal heat shock response was discovered and named *htpR*. Carol Gross and colleagues later

identified its protein product, HtpR, as the first alternative sigma factor in *E. coli* (several had previously been discovered in *Bacillus subtilis*); they renamed the gene *rpoH* (*rpo* genes encode RNA polymerase subunits) and named the protein σ^{32} . Was this renaming justified?

Gross and coworkers purified the 32 kDa HtpR protein. At a late stage in the purification, they ran the protein preparation over a cation-exchange column. For each fraction they collected, they ran a sample on a polyacrylamide gel (Figure 1a) and assayed the fraction for RNA synthesis directed by a heat shock promoter called P_{HS} ; the RNA product of this assay was run on a polyacrylamide gel (Figure 1b). Fraction numbers are shown above the gel lanes. (The M lane has molecular weight markers; the H lane has purified RNA polymerase.)

- Which fractions contain visible amounts of σ^{70} ?
- Which fractions contain visible amounts of the 32 kDa protein (the putative σ^{32})?
- Which fractions contain other RNA polymerase subunits?
- Which fractions produced the most transcription from the P_{HS} promoter?
- What conclusion can you draw from this experiment?

The investigators then reconstituted the RNA polymerase, using purified RNA polymerase subunits but replacing σ^{70} with the 32 kDa protein that was now fairly pure. They used σ^{70} -containing RNA polymerase as a control. To find out whether the 32 kDa protein could direct RNA synthesis from more than one heat shock promoter, they constructed a plasmid with two such promoters (P1 and P2), then cut

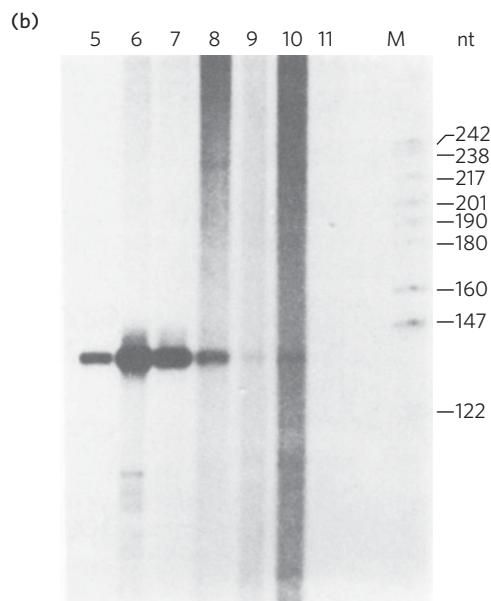
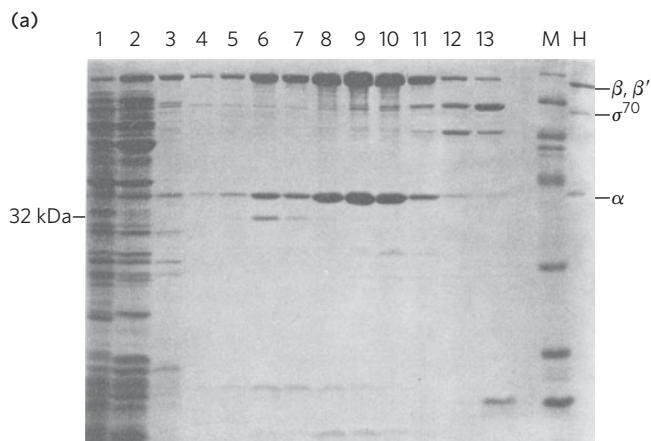


FIGURE 1 Source: Courtesy of Carol Gross.

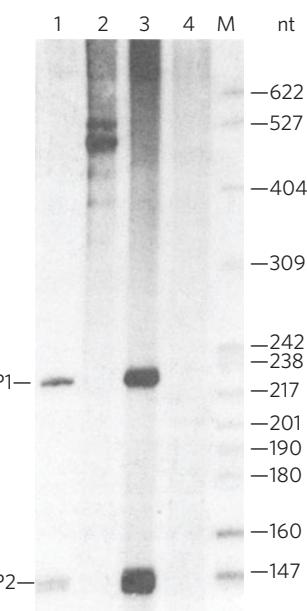


FIGURE 2 Source: Courtesy of Carol Gross.

the plasmid with restriction enzymes. Transcription from the promoters would generate RNA transcripts with lengths of 215 and 140 nucleotides, respectively. The results are shown in **Figure 2**. Lanes 1 and 3 show RNA synthesis by the polymerase with the 32 kDa protein; lanes 2 and 4 show synthesis by the polymerase with σ^{70} .

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- (f) What conclusion can you draw from these data?
- (g) What is the advantage to the cell of having specialized sigma factors that recognize unique promoter sequences?

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RNA Processing



Melissa Jurica [Source: Courtesy of Melissa Jurica.]

my purified samples tended to clump together when spotted onto the copper grids used for sample analysis by electron microscopy, making the images I saw uninterpretable.

Then late one night, while working alone, I developed some micrographs of a new spliceosome sample that showed uniform individual particles that finally looked like macromolecules—the spliceosome! I was so excited and moved that I felt ready to cry—but there was nobody around to show my result to! I posted the wet negative of the micrograph on my advisor Nico Grigorieff's door, and when he saw it the next morning we shared the joy of this breakthrough together.

The key to this result was a somewhat serendipitous discovery that the addition of EDTA, a magnesium ion-chelating agent, could suppress the tendency of the spliceosomes to aggregate. I think this is because SR proteins, which are associated with the spliceosome and tend to be sticky, require magnesium ions to bind to each other. Because we used EDTA to remove magnesium ions, the SR proteins lost the ability to associate tightly with the spliceosomes or each other. I still recall that overwhelming feeling of excitement mixed with happiness, and I use it as a well of motivation to this day, as my lab continues to push forward studies of the spliceosome.

—**Melissa Jurica**, on determining the first electron microscopic structures of spliceosomes

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Our genome is not as large as we once imagined. Humans have only about 25,000 protein-coding genes, almost twice as many as a fruit fly (14,000) and fewer than a rice plant (38,000). It turns out that much of the complexity in multicellular organisms is derived not from the number of genes, but from the number of different proteins produced and the cell's ability to regulate gene expression at the level of RNA. Some bacterial RNA molecules, and virtually all eukaryotic RNAs, are synthesized as biologically inactive precursors that must be chemically modified during or immediately following transcription. These precursors, called **primary transcripts**, are processed into mature, biologically functional molecules by specialized enzymes. RNA processing events are some of the most interesting molecular activities in RNA metabolism, because they govern how, when, and whether the RNA will be used in the cell.

Although many different kinds of RNA are processed, all processing events consist of the same sort of chemical reactions: cleavage and/or joining of RNA strands and, sometimes, modification of the nucleotides themselves. RNA processing remains an active and engaging area of research, because these seemingly

simple reactions lie at the heart of gene expression and gene regulation, and when they go awry, diseases can result. For example, errors in splicing can lead to cystic fibrosis and spinal muscular atrophy.

Studies of RNA processing mechanisms have also shed new light on evolution and the origin of life. Some of these processing reactions are catalyzed by RNAs, not by proteins, as was once believed. The discovery of catalytic RNAs and the ability of RNA to catalyze cleavage and ligation reactions in other RNAs has provided support for the theory that life began with an RNA molecule.

RNA metabolism is much more complex in eukaryotes than in bacteria (Figure 16-1). Most bacterial mRNAs are not processed before being translated into protein. Indeed, they are sometimes translated even while transcription is occurring. In contrast, eukaryotic mRNAs are chemically modified in the cell's nucleus before being transported into the cytoplasm for translation on the ribosomes (located in the cytosol or on the endoplasmic reticulum; see Chapter 18). A protective cap, made from a modified guanosine, is added to the 5' end of this primary transcript. And the 3' end is modified by cleavage and the addition of a string of adenylate residues to create a 3' poly(A) "tail."

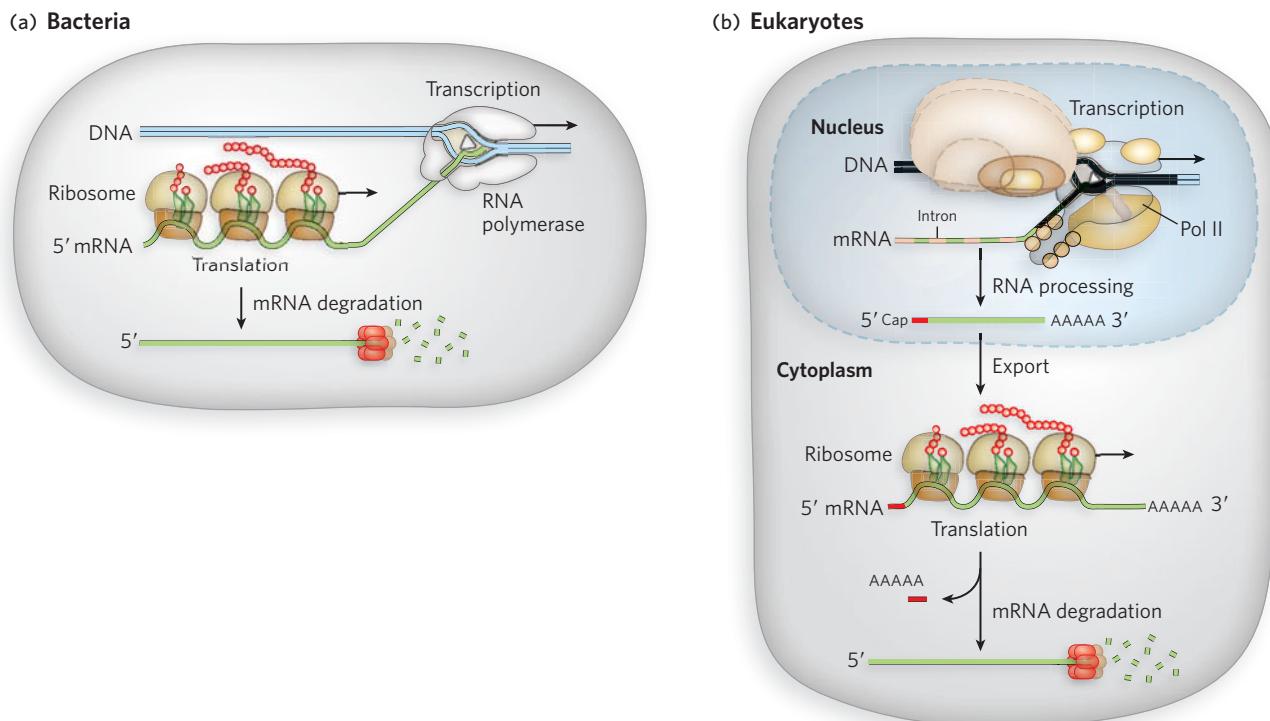


FIGURE 16-1 Messenger RNA processing in bacteria and eukaryotes. Eukaryotic mRNA undergoes several more steps than bacterial mRNA before translation. (a) Because bacterial cells lack a nucleus, transcription and translation are coupled.

(b) In eukaryotic cells, transcription and pre-mRNA (precursor mRNA) processing occur in the nucleus, followed by export to the cytoplasm and subsequent translation. In both types of cells, the mRNA is eventually degraded.

Each primary transcript for a eukaryotic mRNA typically contains sequences encompassing just one gene, although the segments encoding the polypeptide may not be contiguous. Noncoding regions that interrupt the coding region of the transcript are known as **introns**, and the coding segments are known as **exons**. In the process of splicing, the introns are removed from the primary transcript and the exons are covalently connected (ligated) to form a continuous sequence that specifies a functional polypeptide.

The enzymes that catalyze each of these three mRNA processing reactions—capping, polyadenylation, and splicing—do not operate independently. Instead, they seem to interact with one another and with the phosphorylated C-terminal domain (CTD) of RNA polymerase II (see Figure 15-22), such that each enzyme affects the function of the others. Processing of the transcript is also coupled to its transport from the nucleus to the cytoplasm. In this way, the cell keeps track of mRNAs that have been properly processed and can put them to use immediately for translation into protein. Once an mRNA transcript has reached the end of its utility in the cell, it is subject to a final type of RNA processing—degradation. Specific enzymes remove the 5' cap and the 3' poly(A) tail, and the rest of the RNA is broken down by cleavage of the phosphodiester backbone.

In this chapter, we examine how and when RNA molecules are processed and how these processes are coupled. We begin with mRNA processing, one of the earliest discovered and most ubiquitous kinds of precursor-to-mature RNA transformation that occurs in cells, describing the mechanisms of capping, polyadenylation, and splicing. Next we explore a fourth type of RNA modification known as RNA editing, then turn our attention to the transport of mRNA from the nucleus to the cytoplasm and eventual RNA degradation. We discuss the processing of non-protein-coding RNAs, including tRNA, rRNA, and tiny regulatory RNAs called microRNAs, and, finally, RNA catalysis and its implications for life's origins.

16.1 Messenger RNA Capping and Polyadenylation

In bacteria, mRNAs are often translated into protein as they are being transcribed, typically without any post-transcriptional modification. In eukaryotes, however, transcription occurs in the nucleus, and mRNAs must be transported to the cytoplasm before translation can occur. This uncoupling of transcription and translation provides opportunities for the cell to regulate when and where mRNAs are translated, and also requires that

cells guard against premature degradation of the mRNA. One important way that eukaryotic cells achieve both regulation and protection of mRNAs is by chemically capping the 5' end and adding a string of adenosines to the 3' end. In addition to preventing mRNA degradation, these modifications provide chemical handles for subsequent steps in pre-mRNA (precursor mRNA) processing events and nuclear export.

Eukaryotic mRNAs Are Capped at the 5' End

RNA polymerase II (Pol II) synthesizes naked, single-stranded ribonucleic acids that are vulnerable to degradation at either end by ribonucleases. The first modification made to a pre-mRNA transcript is the addition of a **5' cap**, which prevents degradation from the 5' end. The 5' cap is a residue of 7-methylguanosine (7-meG) linked to the 5'-terminal residue of the mRNA through an unusual 5',5'-triphosphate linkage. The 5' cap helps protect mRNA by preventing recognition of the 5' end by exoribonucleases, which digest RNA sequentially from a free 5' or 3' end.

The 5' cap is formed by condensation of a molecule of GTP with the triphosphate at the 5' end of the transcript. The guanine is subsequently methylated at N-7, and additional methyl groups are often added at the 2' hydroxyls of the first and second nucleotides adjacent to the cap (Figure 16-2a). The methyl groups are derived from S-adenosylmethionine. All of these reactions occur very early during transcription, after the first 20 to 30 nucleotides of the transcript have been added (Figure 16-2b). Four different variants of the capping enzyme, guanylyltransferase, carry out the capping reaction. Only mRNAs are capped, because all four guanylyltransferase enzymes are associated with the CTD of Pol II (not with RNA polymerase I or III).

In the 1970s, experiments by Aaron Shatkin and his colleagues showed that the 5' cap also plays a critical role in binding an mRNA to the ribosome. Using mRNAs isolated from reovirus, a human virus that can cause stomach flu, Shatkin's research group found that only mRNAs containing a 5' cap are competent to initiate protein synthesis. Viral mRNAs lacking a 5'-methylated terminus are never part of actively translating ribosomes, because they fail to bind to 40S ribosomal subunits during the translation initiation process (we discuss the details of protein synthesis in Chapter 18). Shatkin and colleagues used the reovirus RNA polymerase to synthesize reoviral mRNAs, using [^{32}P]GTP and [^3H]methyl-S-adenosylmethionine. Some of the resulting transcripts contained a 5' 7-meG cap

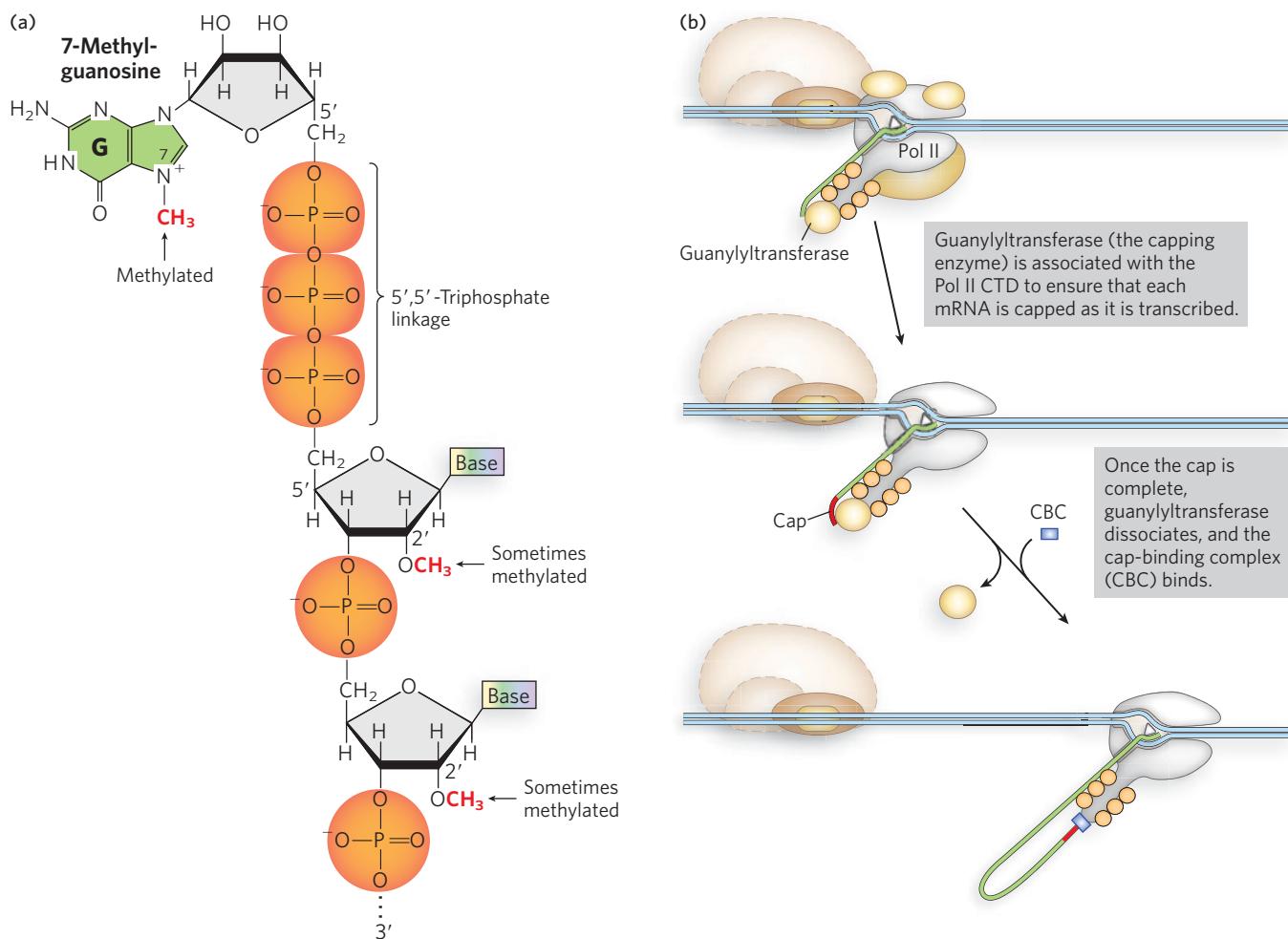


FIGURE 16-2 The mRNA 5' cap. (a) The chemical structure of the 5' cap of mRNA, showing the 7-methylguanosine and adjacent residues. (b) The cap is synthesized by the capping enzyme, guanylyltransferase, which is associated with the C-terminal domain of RNA polymerase II.

(and thus were ^3H - and ^{32}P -labeled) and the rest lacked a cap (^{32}P -labeled only). These mRNAs were incubated with wheat germ cell extract to allow the mRNAs to bind to ribosomes. The samples were then deposited at the top of a sucrose density gradient and spun in a centrifuge, so that the samples migrated into the gradient

according to their density (Figure 16-3). When the resulting gradients were fractionated and each fraction was measured for its content of ^3H - and ^{32}P -containing material, the capped mRNAs were found in the dense part of the gradient where ribosomes were located, indicating that the capped mRNAs



Aaron Shatkin [Source: Courtesy of Aaron Shatkin.]

were bound to ribosomes. In contrast, the uncapped mRNAs (containing only ^{32}P) migrated only to the top of the gradient. As we discuss in Chapter 18, the 5' cap binds the **cap-binding complex (CBC)** (see Figure 16-2b), a specific complex of proteins that recruits capped mRNAs to the ribosome to initiate translation.

Eukaryotic mRNAs Have a Distinctive 3'-End Structure

Like the 5' end, the 3' end of mRNAs must be protected from nucleolytic degradation. As we discussed in Chapter 15, mRNAs contain a polyadenylation signal embedded in the sequence transcribed by RNA polymerase. An enzyme associated with the C-terminal domain of Pol II cleaves the transcript after the polyadenylation signal, and the mRNA is polyadenylated by other

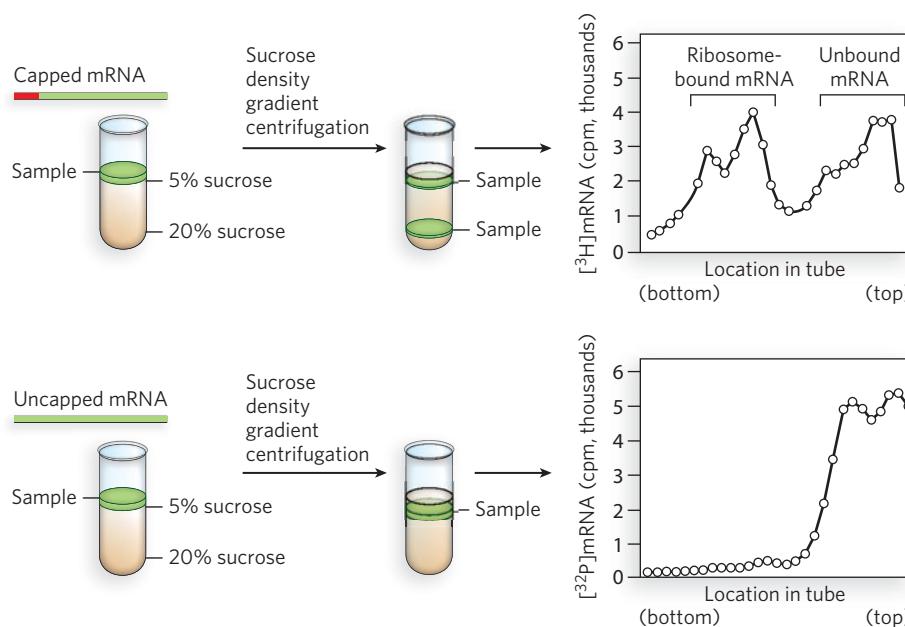


FIGURE 16-3 The 5' cap requirement for ribosome binding and translation. Radiolabeled mRNAs with or without a 5'-methyl cap were incubated with ribosomes, and the complexes were separated from free mRNA by sucrose density gradient centrifugation. Denser particles migrate farther in the gradient, as shown on the left in the graphs. Radioactivity levels (in counts per minute) of capped (top) and uncapped (bottom) mRNAs show that only the mRNA with a 5' cap migrates with ribosomes. [Source: Adapted from G. W. Both et al., *Cell* 6:185–195, 1975. Copyright 1975, MIT.]

CTD-associated factors. The rest of the transcript dissociates from the polymerase and is degraded.

The **3' poly(A) tail**, typically 80 to 250 A residues, serves as a binding site for one or more specific proteins that help protect mRNA from enzymatic destruction, by physically blocking the access of ribonucleases to the 3' end. One way this was first demonstrated was by injecting mRNAs with or without a 3' poly(A) tail into frog's eggs and testing how long the mRNAs remained intact. The 3'-polyadenylated mRNAs survived for much longer, with a remarkable 40% remaining intact 48 hours after injection. Other experiments with mRNAs injected into egg cells or incubated in cell extracts showed that, like the 5' cap, the poly(A) tail and its protein-binding partners interact with the cap-binding complex during mRNA recruitment to the ribosome.

The poly(A) tail is added in multiple catalytic steps involving polyadenylation factors, polyadenylate polymerase (PAP) and poly(A) binding protein (PABP) (Figure 16-4). First, Pol II extends the transcript beyond the site where the poly(A) tail is to be added. The transcript is cleaved at the **poly(A) addition site** by an endonuclease component of a large enzyme complex, again associated with the CTD of Pol II. The mRNA site where cleavage occurs is marked by two sequence elements: the highly conserved sequence 5'-AAUAAA located 10 to 30 nucleotides on the 5' side (upstream) of the cleavage site, and a less well-defined sequence rich in G and U residues located 20 to 40 nucleotides downstream from the cleavage site. Cleavage generates the free 3'-hydroxyl group that defines the end of the

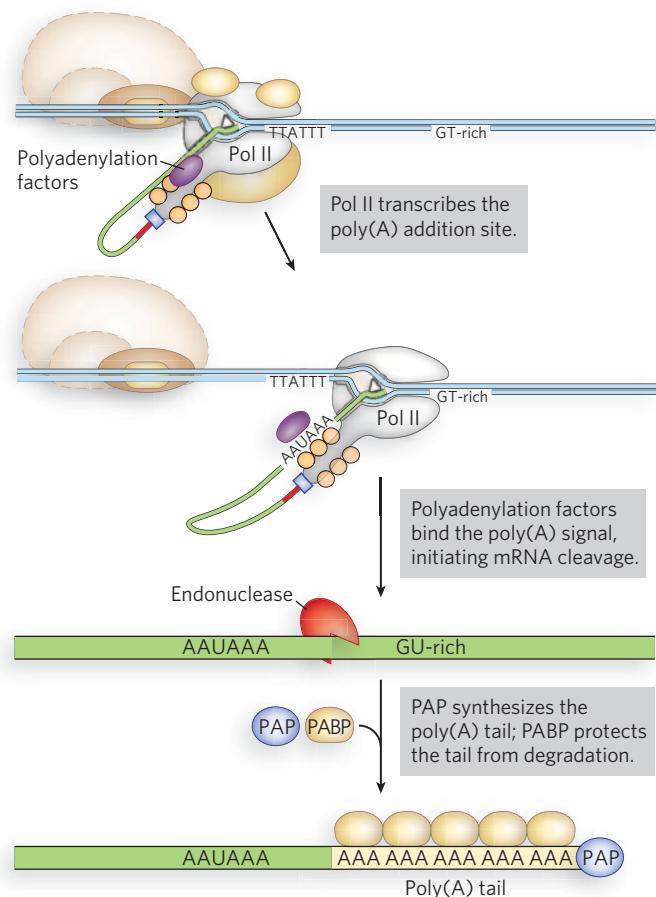


FIGURE 16-4 Addition of the 3' poly(A) tail to the transcript. Polyadenylation factors are associated with Pol II during transcription. After transcription of the 5'-AAUAAA sequence, these proteins transfer to the transcript and help recruit cleavage factors and poly(A) binding protein (PABP) before dissociating. The poly(A) tail is synthesized by polyadenylate polymerase (PAP).

HIGHLIGHT 16-1 EVOLUTION

Eukaryotic mRNA with Unusual 3' Tails

Unlike most eukaryotic mRNAs, those that encode histones—the small basic proteins that help package eukaryotic chromosomal DNA (see Chapter 10)—lack 3' poly(A) tails. Instead, the 3' ends contain a self-complementary sequence that forms a stem-loop structure, creating the binding site for a protein

complex that includes the stem-loop binding protein (SLBP) (Figure 1). SLBP both stabilizes the 3' end of histone mRNAs, preventing degradation, and provides an alternative way of recruiting the mRNA to ribosomes during translation initiation. Nascent histone-encoding transcripts have 3' extensions that are cleaved at a site five nucleotides after the conserved stem-loop,

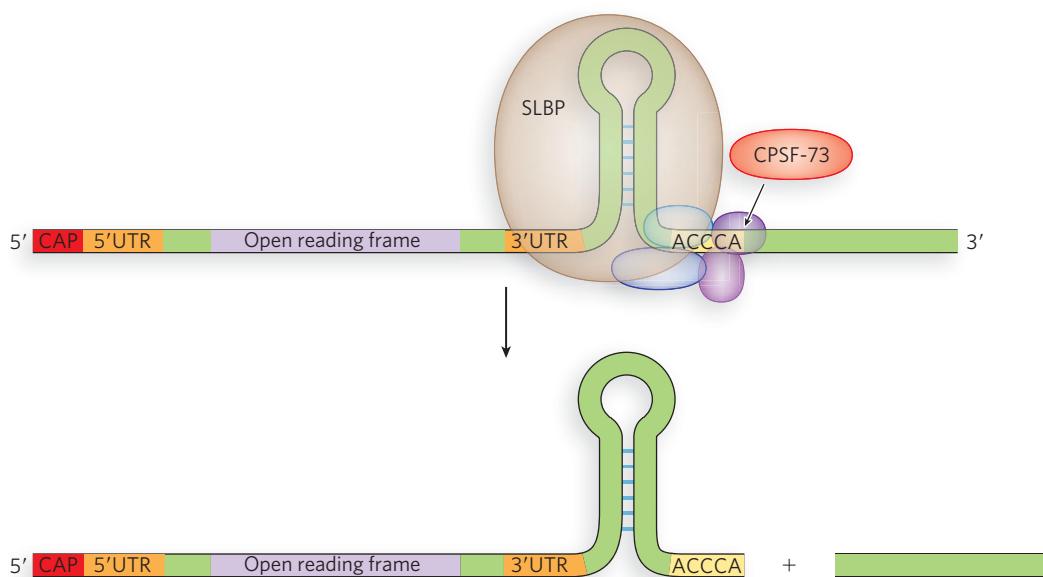


FIGURE 1 The 3' stem-loop structure of a histone pre-mRNA binds the protein SLBP, which then assembles with CPSF-73 and other splicing factors to process the 3' end. The open reading frame is the coding part of the gene. [Source: Adapted from W. Marzluff et al., *Nat. Rev. Genet.* 9:843–854, 2008, Fig. 1.]

mRNA, to which A residues are immediately added in the PAP-catalyzed reaction:



where $n = 80$ to 250. The number of A residues in the poly(A) tail varies from one species to another; it is often written simply as $(\text{A})_n$. The polyadenylation enzyme complex does not require a template, but does require cleavage of the mRNA. A few types of eukaryotic mRNA, such as those that encode histones, are cleaved but have a different kind of 3' tail that allows them to be regulated separately from the bulk of the cellular mRNA (Highlight 16-1).

mRNA Capping, Polyadenylation, and Splicing Are Coordinately Regulated during Transcription

The regulation of mRNA capping, polyadenylation, and splicing is coordinated through the association of these processes with RNA polymerase II. Capping, splicing, and polyadenylation factors are associated with the Pol II C-terminal domain, and these factors transfer to the nascent RNA at the appropriate sequences. This enables RNA processing to occur simultaneously with transcription, ensuring that mature mRNAs are processed rapidly as they emerge from the Pol II active site (Figure 16-5). Once the 5' end of the transcript has

a process catalyzed by a specific endonuclease that cleaves internal bonds in the phosphodiester backbone. The upstream cleavage product corresponds to the mature histone mRNA, and the downstream product is degraded by an exonuclease, which cleaves RNA from the 5' or 3' end.

To identify the ribonucleases responsible for these processing events, William Marzluff and his colleagues

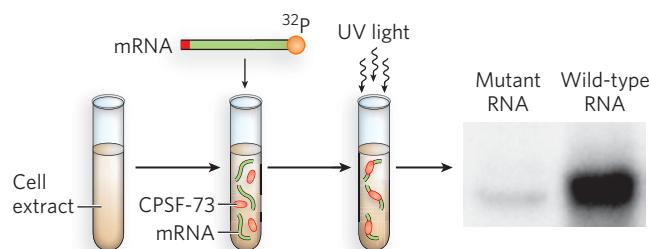


FIGURE 2 Cross-linking experiment to determine the protein bound to the radiolabeled stem-loop of histone mRNA. The cross-linked species were separated by denaturing gel electrophoresis, based on their slower migration relative to non-cross-linked molecules, revealing a prominent species that was found to contain CPSF-73. The cross-linked species corresponding to CPSF-73-pre-mRNA was observed only with the wild-type RNA, not with a mutant defective for 3'-end processing. [Source: Z. Dominski et al., *Cell* 123:37–48, 2005. © Elsevier. By permission of Z. Dominski.]

been capped, it is released from the capping enzymes and bound by the cap-binding complex, which itself associates with the Pol II CTD.

In addition, some components of the splicing apparatus appear to be tethered to the Pol II CTD, suggesting an interesting model for the splicing reaction. As the first splice junction is synthesized, it is bound by a protein splicing factor. The second splice junction is then captured by this complex as it passes by, facilitating juxtaposition of the intron ends and the subsequent splicing process. After splicing, the intron remains in the nucleus and is eventually degraded (see Figure 16-5).

at the University of North Carolina conducted a cross-linking experiment (Figure 2). Histone pre-mRNA was synthesized in the lab and incubated in the presence of a total protein extract from human cells. The preparation was then exposed to ultraviolet light to induce formation of chemical bonds between the mRNA and any proteins that might have bound to it. Remarkably, the researchers found the so-called cleavage and polyadenylation specificity factor (CPSF-73), a known protein component of the machinery responsible for cleavage and polyadenylation of the vast majority of eukaryotic mRNAs. These studies suggested that CPSF-73 is both the endonuclease and the 5' → 3' exonuclease involved in histone pre-mRNA processing. The crystal structure of human CPSF-73, along with biochemical experiments on the purified protein, showed that CPSF-73 is also the enzyme responsible for cleaving the 3' ends of all pre-mRNAs prior to polyadenylation. These findings revealed an unexpected biochemical connection between 3'-end formation in histone mRNAs and in other, polyadenylated mRNAs, implying a common ancestral mechanism for processing the 3' ends of all animal mRNA transcripts.



William Marzluff [Source: Courtesy of William Marzluff.]

SECTION 16.1 SUMMARY

- Eukaryotic mRNAs are modified by the addition of a 7-methylguanosine residue at the 5' end and by cleavage and polyadenylation at the 3' end to form a poly(A) tail.
- Modification of the ends protects the transcript from ribonucleases and is required for subsequent steps in pre-mRNA processing, and for export from the nucleus.
- Messenger RNA capping, polyadenylation, and splicing are coupled to transcription through the physical association of the enzymes with RNA polymerase II.

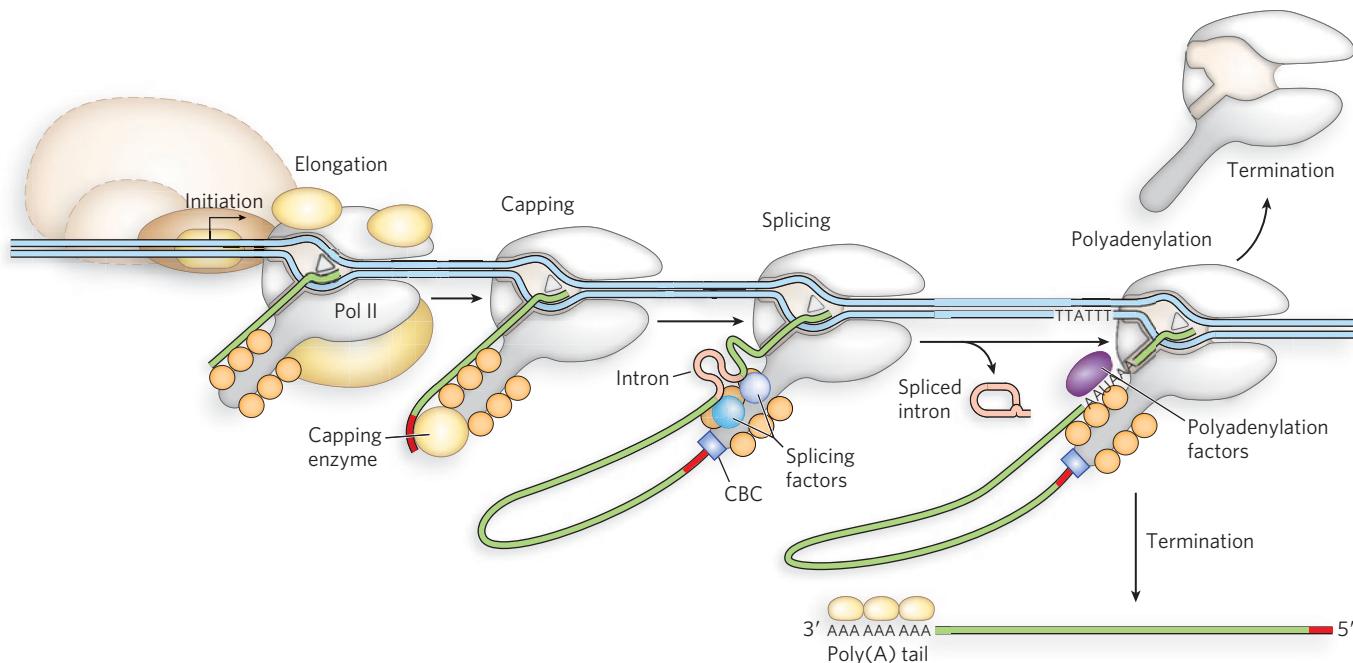


FIGURE 16-5 Coordination of transcription and pre-mRNA processing. mRNA-processing proteins associate with the C-terminal domain of Pol II. As the mRNA is synthesized, the capping enzyme (guanylyltransferase), splicing factors, and polyadenylation factors transfer from the Pol II CTD to the mRNA and process the mRNA as it is transcribed. The mature transcript is used for multiple rounds of protein synthesis, then degraded.

16.2 Pre-mRNA Splicing

Beyond capping and polyadenylation, eukaryotic transcripts are further processed in a series of reactions referred to as **RNA splicing**, producing the mature, translation-competent form of the mRNA. Pre-mRNA splicing is one of the most important distinctions between gene expression in bacteria and in eukaryotes, and hence has attracted a lot of research interest. Splicing may be a relic of the way that protein-coding sequences originally evolved, providing a glimpse of how modern genes arose. Furthermore, some human diseases, including Duchenne muscular dystrophy and cystic fibrosis, can be caused by aberrant pre-mRNA splicing, suggesting possibilities for therapies if the underlying splicing mechanisms can be understood.

All pre-mRNA splicing mechanisms consist of the ordered breaking and joining of specific phosphodiester bonds to achieve the precise excision of introns. Accurate and efficient splicing relies on base pairing between the pre-mRNA and the splicing machinery to specify the bonds to be broken or formed. In all cases, splicing must be carried out quickly and correctly to produce the mRNAs required for protein production.

A complex of RNA and proteins called the spliceosome is responsible for most pre-mRNA splicing. A

small number of introns are self-splicing, catalyzing their own excision from a primary transcript without help from proteins. The discovery of self-splicing introns led to a profound change in our understanding of modern biology, because it revealed that proteins are not the only biological catalysts in cells. The realization that RNA can function both as a carrier of genetic information and as a catalyst led to the idea of an “RNA world” that might have given rise to modern cells and organisms (see Section 16.6).

Eukaryotic mRNAs Are Synthesized as Precursors Containing Introns

In bacteria, a polypeptide chain is generally encoded by a DNA sequence that is colinear with the amino acid sequence, continuing along the DNA template without interruption until the information needed to specify the polypeptide is complete. This might seem the most efficient way to encode genetic information, but primary transcripts isolated from the nuclei of mammalian cells are much larger (2,000 to 20,000 nucleotides long) than the proteins they encode. Rapid-radiolabeling experiments showed that most of the RNA in these primary transcripts is degraded before it leaves the nucleus. Independent investigations by Phillip Sharp and Richard Roberts in the late 1970s showed that many



Phillip Sharp [Source: Courtesy of Phillip Sharp.]

eukaryotic protein-coding genes are interrupted by noncoding sequences, the introns. Electron microscopy studies of annealed chromosomal DNA and corresponding mRNA revealed regions of complementarity interspersed with looped-out regions in the DNA that did not base-pair with sequences in the mature mRNA

(Figure 16-6). This was the first indication that genes can include both coding regions and noncoding segments that are removed after transcription. As later became apparent, to form mature mRNA, introns must be removed from precursor transcripts and the remaining protein-coding segments, the exons, covalently connected (Figure 16-7).

Subsequent studies revealed that the vast majority of genes in vertebrates contain introns; those that encode histones are among the few exceptions. The occurrence of introns in other eukaryotes varies. Most genes in baker's yeast, *Saccharomyces cerevisiae*, lack introns, although in other yeast species introns are more common. Introns are also found in a few bacterial and archaeal genes, and even occur within the genes of certain bacteriophages. Although the evolutionary sig-



Richard Roberts [Source: Courtesy of Richard Roberts.]

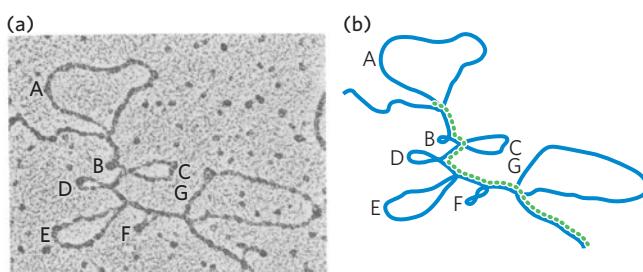


FIGURE 16-6 A DNA-mRNA hybrid revealing the presence of introns. (a) An electron micrograph and (b) drawing show the ovalbumin gene (blue in the drawing) hybridized to its mRNA (green). The DNA contains several loops and regions (A through G) that do not base-pair with the cognate mRNA, indicating regions of RNA that are processed out of the mRNA transcript. [Source: (a) A. Dugaiczyk et al., Proc. Natl. Acad. Sci. USA 76:2253–2257, 1979, Fig. 5. By permission of Bert O'Malley.]

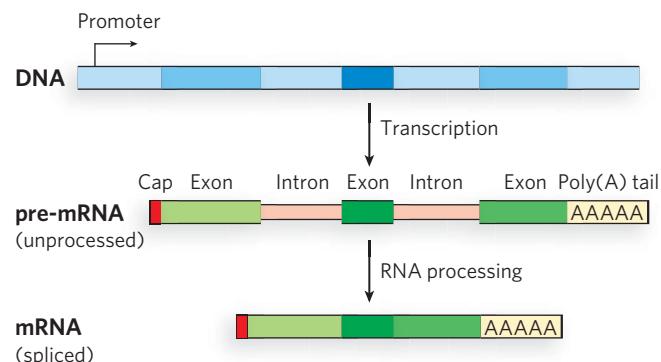


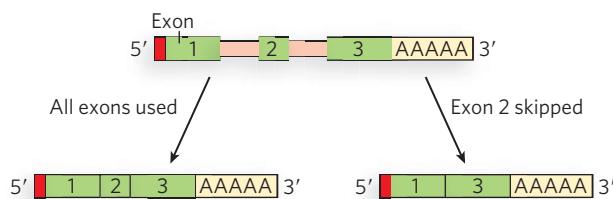
FIGURE 16-7 Interrupted genes. Introns are non-protein-coding sequences in the DNA and transcribed mRNA that are removed from the RNA during processing to form a contiguous, exon-only, protein-coding mRNA.

nificance of introns is not clear, they can play roles in regulating the amount of mature mRNA produced. For example, some introns include open reading frames that encode regulatory proteins, and others are further processed after splicing into small RNAs that base-pair with complementary mRNAs to regulate their stability or translation (see Chapter 22). Splicing also prepares mRNAs to be recognized by proteins that export them from the nucleus and promote their translation by the ribosome.

Introns in DNA are transcribed along with the rest of the gene by RNA polymerases. Then, as noted above, introns in the primary RNA transcript are excised and the exons are joined to form the mature RNA. A typical mammalian pre-mRNA includes eight introns with an aggregate length 5 to 10 times that of the flanking exons. In general, introns of animal pre-mRNAs vary in size from 50 to 20,000 nucleotides, and most exons are less than 1,000 nucleotides long, with many having just 100 to 200 nucleotides and encoding polypeptide segments of 30 to 60 amino acids. Genes of higher eukaryotes, including humans, have much more DNA devoted to introns than to exons. Some genes have hundreds of introns.

A Gene Can Give Rise to Multiple Products by Alternative RNA Splicing

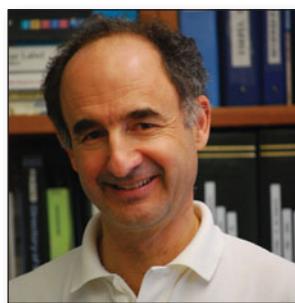
The transcription of introns might seem to consume cellular resources and energy without returning any benefit to the organism, but introns may confer an advantage. They may be vestiges of an ancient molecular parasite not unlike transposons. They may offer unique advantages to complex organisms, because they provide a means of greatly increasing the number of

**FIGURE 16-8** Different ways of assembling exons.

Alternative splicing can generate multiple products from one gene.

different protein-coding sequences that can be produced, in principle, from a single gene. Comparison of an organism's genome with the complement of proteins in a given cell type—its **proteome**—shows that in many cases, the number of different proteins greatly exceeds the number of identified genes. In some cases, at least, this discrepancy is due to **alternative splicing**, a process in which exons in the primary transcript from a single gene are spliced together in various combinations to produce *different* mRNAs and thus different polypeptides (Figure 16-8). High-throughput sequencing technology such as RNA-Seq (see Figure 8-12) has revealed that more than 90% of human genes undergo alternative splicing!

Certain exons are selected for inclusion and others are not, but the order of the exons does not change relative to the primary transcript. In fruit flies, for example, the gene *Dscam* encodes an immunoglobulin (Ig) superfamily protein, a transmembrane protein required for the formation of neuronal connections. Through alternative splicing, *Dscam* potentially gives rise to 19,008 different extracellular domains linked to one of two alternative transmembrane segments, resulting in 38,016 different possible forms (isoforms) of the *Dscam* protein! *Dscam* variants share the same domain structure but contain different amino acid sequences within three Ig domains in the extracellular region. Using *Dscam* proteins that could be recognized and distinguished by specific antibodies, Larry Zipursky and colleagues



Larry Zipursky [Source: Courtesy of Larry Zipursky.]

found that each *Dscam* binds to molecules of the same isoform, but does not bind or binds poorly to other isoforms, contributing to the formation of complex patterns of neuronal connections. Although the *Dscam* gene is an extreme example, many mammalian genes have two or more alternatively

spliced mRNAs derived from the same gene, greatly increasing the complexity of the genome and providing opportunities for regulation at the level of pre-mRNA processing.

Mechanisms of alternative splicing are not yet well understood, but we know that **splice sites**, nucleotide sequences within the intron and at the borders between introns and exons, play an important role in determining whether an exon is included in the mature mRNA. In some cases, splice sites can be masked in particular cell types, leading to intron skipping and consequent loss of protein production. For example, research on fruit flies in Don Rio's laboratory showed that in all cells except egg or sperm precursors, the protein Psi binds to a "decoy" splice site adjacent to the true splice site in a gene encoding the enzyme transposase. This prevents proper splicing of the transposase mRNA, thereby preventing transposase production in adult cells where it is not needed and could harm the integrity of the genome by disrupting gene or regulatory sequences.



Don Rio [Source: Courtesy of Don Rio.]

Regulation by alternative splicing is implicated in some human disease pathways. For example, the inclusion of specific exons in the spliced mRNAs encoding the cell surface molecule CD44 is associated with the progression of certain tumors to an invasive phenotype. Thus, understanding what determines exon choice during alternative splicing might lead to new therapeutic strategies to treat or prevent some cancers.

Complex transcripts can have more than one site for cleavage and polyadenylation, or for alternative splicing patterns, or both. If there are two or more sites for cleavage and polyadenylation, using the one closest to the 5' end will remove more of the primary transcript sequence. This mechanism, called **poly(A) site choice**, generates diversity in the variable domains of immunoglobulin heavy chains, which is required for an efficient immune response (see Chapter 14). In fruit flies, alternative splicing patterns produce, from a common primary transcript, three different forms of the myosin heavy chain at different stages of fly development. In rats, *both* mechanisms (alternative splicing and poly(A) site choice) come into play when a single RNA transcript is processed differently to produce two different hormones: the calcium-regulating hormone calcitonin in thyroid and the calcitonin-gene-related peptide (CGRP) in the brain (Figure 16-9).

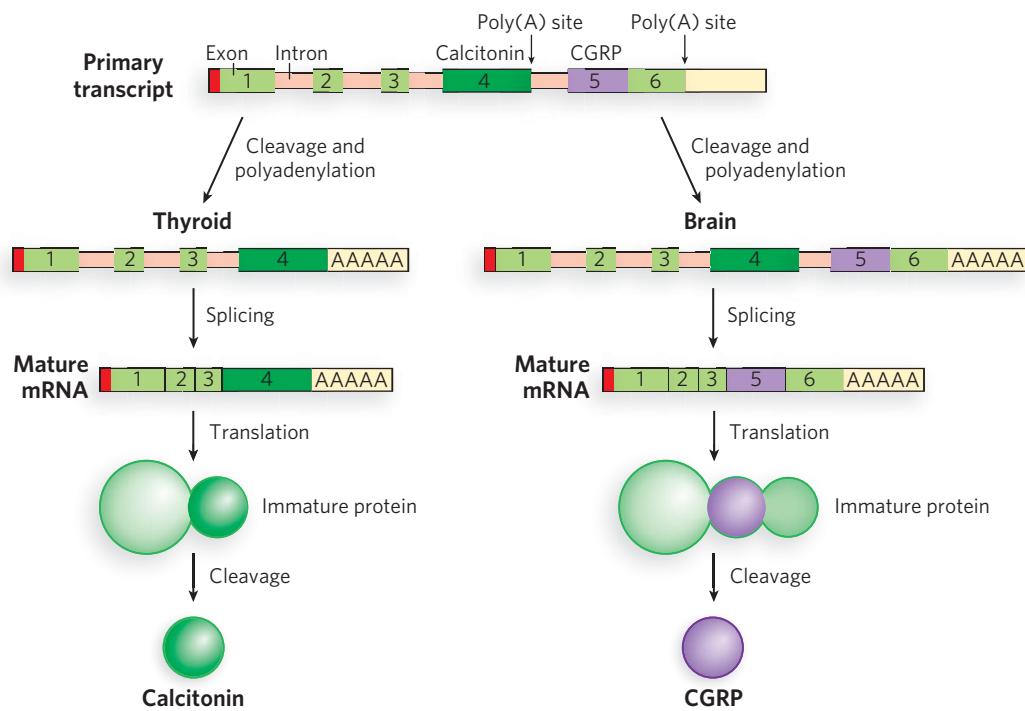


FIGURE 16-9 Alternative processing of the rat calcitonin gene transcript. Calcitonin and calcitonin-gene-related peptide (CGRP) have completely different protein sequences but are encoded by the same gene. The primary transcript contains two poly(A) sites: one used in the thyroid and the

other used in the brain. In the thyroid, exon 4 is retained; in the brain, exon 4 is eliminated and exons 5 and 6 are retained. The resulting mRNAs encode different polypeptides that are further processed to yield the final hormone products: calcitonin in the thyroid, and CGRP in the brain.

The Spliceosome Catalyzes Most Pre-mRNA Splicing

Each intron consists of a 5' splice site, a 3' splice site, and an internal A residue just upstream of the 3' splice site, at the **branch point** (Figure 16-10). Splicing involves a cascade of phosphodiester exchange reactions. In the first step, the 2'-OH of the branch-point A residue attacks the phosphate at the 5' splice site. In the second step, the 3'-OH of the released exon attacks the phosphate at the 3' splice site, thus joining the exons and separating them from the intron. These reactions are catalyzed by ribonucleoproteins (RNPs), complexes of non-protein-coding RNAs and proteins. The RNA components of the RNPs base-pair with the mRNA at the 5' splice site, 3' splice site, and branch point, positioning the associated proteins for catalysis.

Most introns in eukaryotic pre-mRNAs are removed by the **spliceosome**, a complex of five **small nuclear ribonucleoproteins**, or **snRNPs** (pronounced “snurps”), and hundreds of additional protein components. At the heart of each snRNP is a single **small nuclear RNA (snRNA)** belonging to a class of non-protein-coding eukaryotic RNAs 100 to 200 nucleo-

tides long (Figure 16-11a). Five snRNAs (U1, U2, U4, U5, and U6) involved in splicing reactions are abundant in eukaryotic nuclei. The RNAs and proteins found in snRNPs are highly conserved in eukaryotes from yeast to humans, suggesting that the splicing machinery, and hence introns themselves, were present in the earliest eukaryotes.

Each snRNA includes a binding site for a set of proteins, Sm proteins, that are common to all snRNPs (see How We Know). Seven Sm proteins (SmB, D1, D2, D3, E, F, and G) form a ringlike structure that binds adjacent to a hairpin fold near the 3' end of the snRNA (see Figure 16-11a). In addition, the snRNA contains sequences that are uniquely recognized by proteins specific to that snRNP. The snRNA-Sm protein complex forms a structure called the Sm core domain. Electron microscopy and x-ray crystallography have been used to visualize individual snRNP proteins and the Sm core (Figure 16-11b), as well as intact snRNPs and assembled spliceosomes (see Moment of Discovery).

Spliceosomes assemble from snRNPs and include other proteins that are not specifically associated with any snRNA. Chief among these are the SR proteins, so named for their serine/arginine-rich region, which play

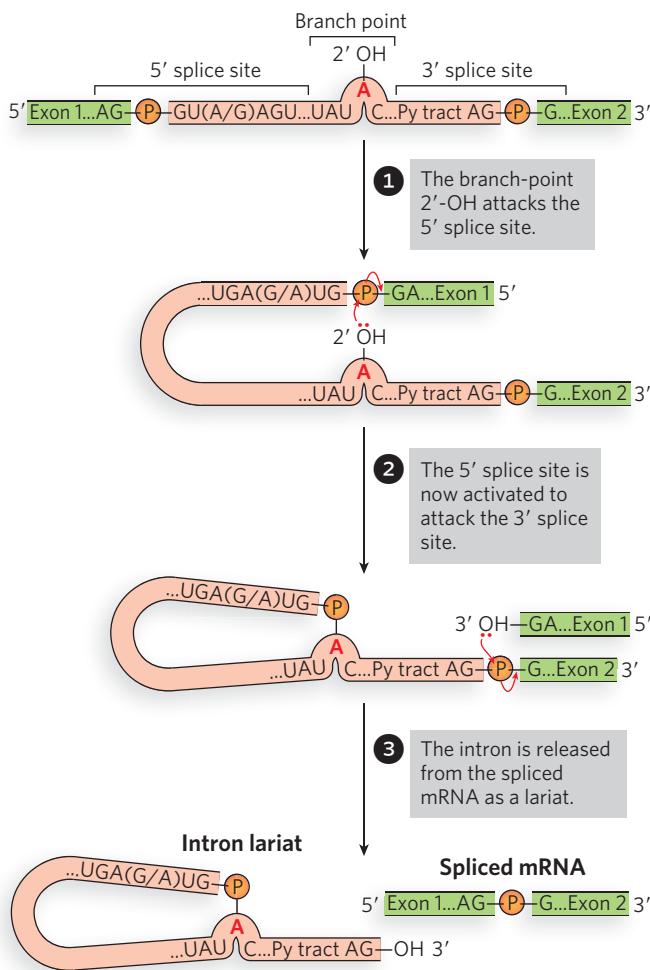


FIGURE 16-10 An overview of the splicing reaction.

Pre-mRNA splicing occurs through two site-specific transesterification reactions that result in phosphodiester bond cleavage and ligation. The 5' and 3' splice sites (indicated by brackets) consist of the conserved sequence elements shown; the Py tract in the intron is a string of pyrimidine residues.

central roles in selecting and regulating splice sites in pre-mRNAs. SR proteins have a common architecture consisting of an RS domain containing repeats of Arg and Ser, and an RNA-recognition motif (RRM). The RS domain is a site of protein-protein interactions that can be enhanced by the phosphorylation of Ser residues. The RRM domain binds to sequences in the pre-mRNA, often within the exons.

One of the key questions to be addressed by biochemical and structural studies is how introns are recognized and cleaved by the spliceosome. Introns have the dinucleotide sequences GU and AG at their 5' and 3' ends, respectively, and these sequences mark the sites where splicing occurs. However, these dinucleotide sequences at splice junctions are not sufficiently

information-rich, by themselves, to specify splice sites accurately. The surrounding sequences and perhaps the structure of the pre-mRNA itself must play a role in the selection of splice sites, and the ability of snRNPs to bind to these sequences affects the likelihood of splicing. However, the details of how this occurs in the cell are as yet undetermined. Researchers must verify splice sites by comparing the genomic sequence with the corresponding sequence of the mRNA or protein it encodes.

Cells use base pairing between the snRNAs of the spliceosome and the pre-mRNA to select correct splice sites (Figure 16-12). First, the U1-containing snRNP (referred to simply as U1 snRNP) binds to the GU sequence at the 5' splice site, along with accessory proteins, including U2AF (U2 auxiliary factor), which binds to the AG and flanking sequences at the 3' splice site. In an ATP-dependent next step, U2AF recruits U2 snRNP to the branch point. The 2'-OH of the adenosine in the branch point becomes the nucleophile that attacks the phosphodiester bond at the 5' splice site. Then the U4-U6-U5 trimeric snRNP complex binds, with U6 binding to U2. Next, U1 snRNP is released, U5 snRNP base pairs with the 5' exon, and U6 snRNP moves to the 5' splice site. Once U4 snRNP is released, the U6 and U2 snRNPs catalyze nucleophilic attack of the 2'-OH of the branch-point adenosine on the phosphodiester bond at the 5' splice site, cleaving the 5' exon-intron junction and shifting the U5 snRNP to the 3' splice site.

Formation of the simultaneous 2' and 3' phosphodiester linkages at the branch-point adenosine results in a lariat-shaped RNA containing the intron and the 3' exon. The U2-U6-U5 complex remains bound to the lariat and catalyzes nucleophilic attack of the 3'-OH of the 5' exon on the phosphodiester bond linking the intron to the 3' exon, resulting in intron excision with concomitant joining of the 5' and 3' exons. ATP hydrolysis is required, presumably for unwinding the spliceosomal RNAs and proteins, as well as helping the snRNAs base-pair with each other and with the pre-mRNA.

Some pre-mRNA introns are spliced by a less common type of spliceosome, in which the U1 and U2 snRNPs are replaced by the U11 and U12 snRNPs. Whereas U1- and U2-containing spliceosomes remove introns with 5'-GU and AG-3' terminal sequences, the U11- and U12-containing spliceosomes remove a rare class of introns that have 5'-AU and AC-3' terminal sequences at the intron splice sites. There is no obvious pattern for the use of these so-called AT-AC introns in genes, leading to speculation that they arose in a process parallel to the evolution of the majority of introns.

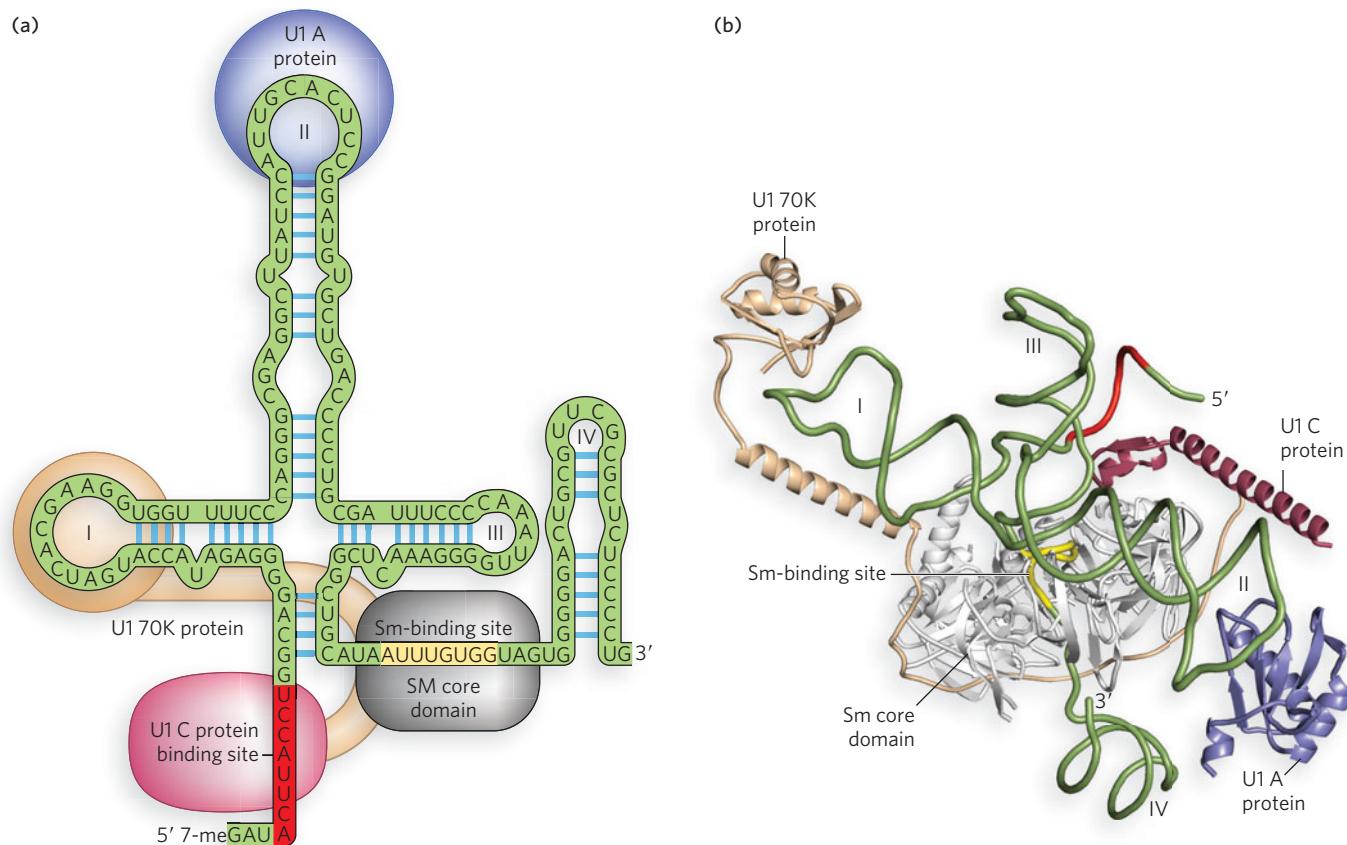


FIGURE 16-11 The structure of U1 snRNP. Each snRNP consists of an snRNA (U1 snRNA is shown here), with an Sm-binding site, as well as unique sequences and secondary structures. (a) Two-dimensional and (b) three-dimensional representations of the U1 snRNP. Sm proteins bind to the Sm-binding site to form the Sm core domain; unique proteins bind elsewhere in the snRNA to form the complete snRNP. [Source: (b) PDB ID 3CW1.]

Some Introns Can Self-Splice without Protein or Spliceosome Assistance

Although researchers initially assumed that all introns would be removed by protein-catalyzed reactions, during the early 1980s, Thomas Cech and his coworkers at the University of Colorado discovered that some introns have self-splicing capability. In an *in vitro* system, Cech and colleagues transcribed an intron-containing piece of DNA isolated from the ciliated protozoan *Tetrahymena thermophila*, using purified bacterial RNA polymerase. Remarkably, the resulting transcript spliced itself accurately without requiring any *Tetrahymena* protein enzymes (Figure 16-13). Other RNAs were also found to be capable of functioning as catalysts of RNA processing reactions (see Section 16.6). This exciting discovery was a milestone in our understanding of biological systems. The existence of catalytic RNA molecules implies that RNA competent to both carry and copy genetic information might have

formed the chemical basis for early life on Earth, before the evolution of DNA or proteins.

Subsequent research in many laboratories revealed the existence of two distinct classes of self-splicing introns, known as group I and group II. Although the details of their splicing mechanisms differ, group I and group II introns share the ability to self-splice without the involvement of any protein enzymes. Furthermore, neither class requires a high-energy cofactor such as ATP for splicing. It is important to note that group I and group II introns do require proteins for splicing *in vivo*—not for catalysis, but for forcing the pre-RNA into the correct conformation for splicing to occur.



Thomas Cech [Source: Courtesy of Bruce Weller.]

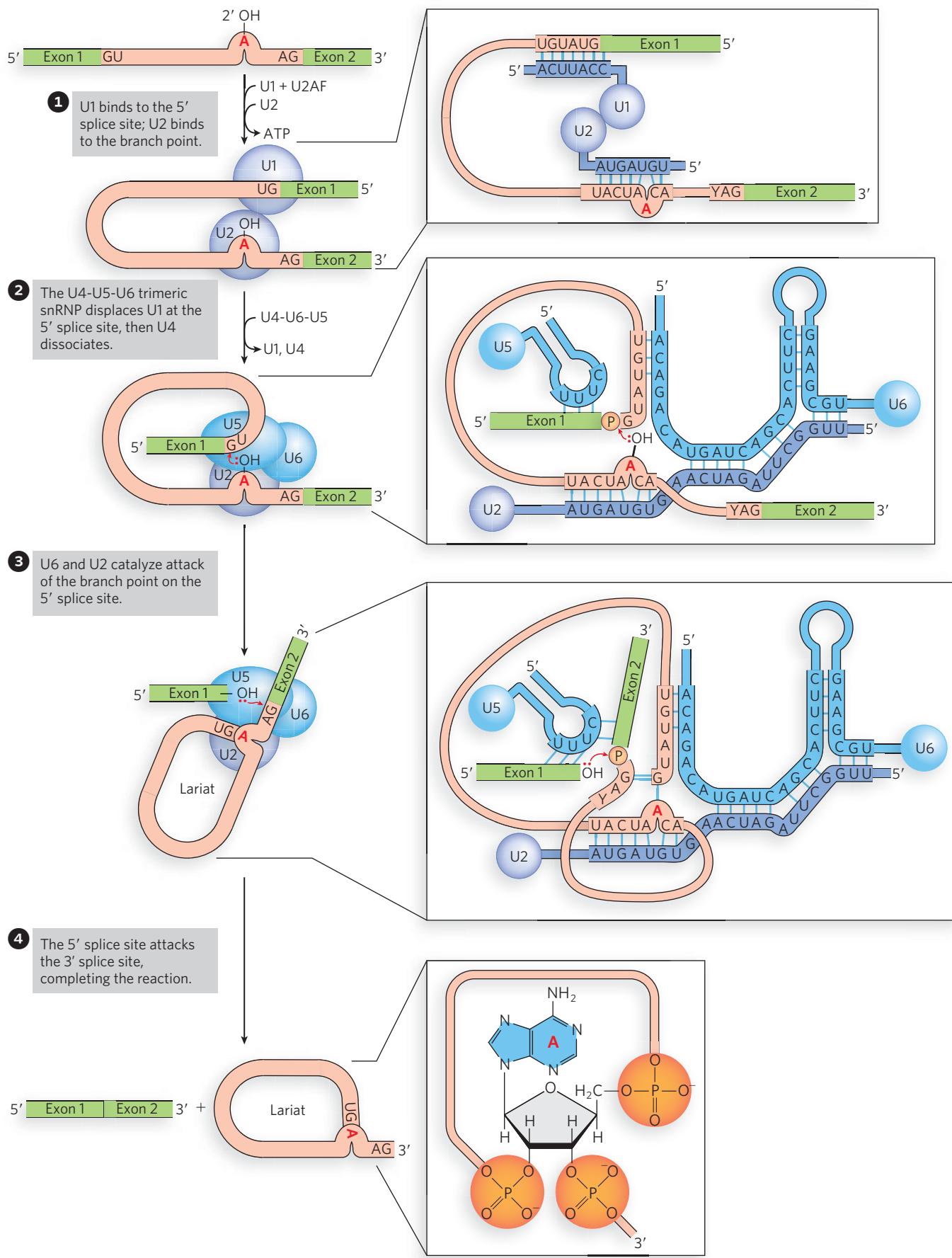


FIGURE 16-12 Spliceosome assembly on pre-mRNAs involving base pairing to snRNAs. The splicing process is described in the text. Notice that, in step 2, U6 snRNA base-pairs near the 5' exon binding site where U1 snRNA was formerly bound. As U4 dissociates, the U2-U6-U5 complex remains assembled on the pre-mRNA. U5 base-pairs to both sides of the splice junction to align the RNA for the splicing reaction, and U2 and U6 base-pair to each other. [Source: Adapted from K. Nagai et al., *Biochem. Soc. Trans.* 29:15–26, 2001.]

Group I Introns The catalytic activities and molecular structures of several **group I introns**, such as the original example discovered in *Tetrahymena*, have been studied in exquisite detail (Figure 16-14). Although the group I splicing reaction requires a guanine nucleoside or nucleotide cofactor, the cofactor is not used as a source of energy. Instead, the 3'-hydroxyl group of the guanosine is the nucleophile in the first step of the

splicing pathway (Figure 16-15). The guanosine 3'-OH forms a normal 3',5'-phosphodiester bond with the 5' end of the intron. This transesterification reaction releases the 3' end of the first exon, which then attacks the phosphate of the 3' splice site, using its 3'-OH in a second transesterification reaction. The intron is released in linear form, with the extra G residue added to its 5' end. The result is precise excision of the intron and concurrent joining of the exons.

Self-splicing group I introns share several other properties with enzymes, besides accelerating the reaction rate, including their kinetic behavior and specificity. The intron is precise in its excision reaction, largely due to a short sequence—the internal guide sequence—that can base-pair with exon sequences near the 5' splice site. This pairing promotes the alignment of specific bonds to be cleaved and rejoined.

Because the intron itself is chemically altered during the splicing reaction—its ends are cleaved—it initially appeared to lack one key enzymatic property: the ability to catalyze the same reaction in multiple substrate molecules. *In vivo*, the intron (414 nucleotides) from *Tetrahymena* rRNA is quickly degraded after its excision. Experiments have shown, however, that *in vitro*, the intron can act as a true enzyme. A series of intramolecular cyclization and cleavage reactions in the excised intron remove 15 to 19 nucleotides from its 5' end. The remaining linear RNA promotes nucleotidyl transfer reactions in which some oligonucleotides are lengthened at the expense of others. Although not known to be important in cells, this capability indicates that the RNA can catalyze RNA polymerization. Such activity hints at the possibility that RNA could catalyze its own replication—a key to the “RNA world” hypothesis (see Section 16.6).

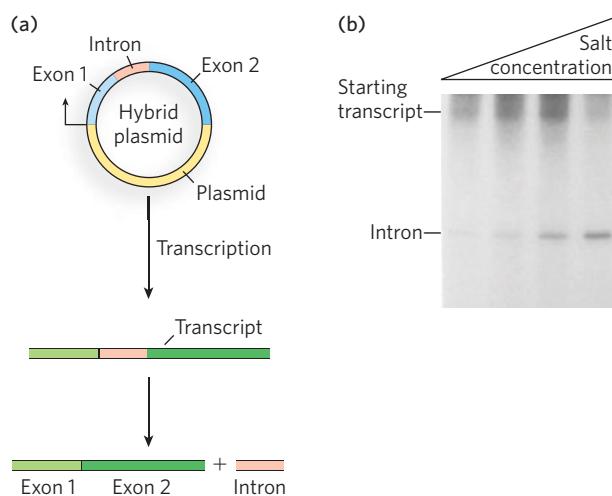


FIGURE 16-13 Self-splicing of the transcripts of the *Tetrahymena* large rRNA gene in vitro. (a) A DNA fragment including the intron (red) and flanking sequences (blue) was cloned into a plasmid, which was then transcribed in a test tube by purified RNA polymerase. (b) Samples of pre-rRNA purified from this transcription reaction were incubated in buffer solutions containing sodium and magnesium salts. The pre-rRNA was capable of self-splicing to release the intron, as shown in this agarose gel. The (shorter) intron RNA moves through the gel matrix faster than the (longer) starting transcript. Increasing salt concentration (shown at the top) facilitates the splicing reaction because salts stabilize the folded, catalytically active form of the RNA. [Sources: (a) Adapted from K. Kruger et al., *Cell* 31:147–157, 1982. Copyright 1982, MIT. (b) T. Cech et al., *Cell* 27:487–496, 1981, Fig. 1a.]

Group II Introns Though they have very few conserved sequences, **group II introns** share a common secondary structure consisting of six base-paired regions referred to as domains I through VI (Figure 16-16a). Domain I contains the binding sites for the 5' and 3' splice sites, and domain VI contains an adenosine that functions as the nucleophile to initiate the splicing reaction. Domain V contains sequences critical for the splicing reaction to occur efficiently.

The chemistry of splicing by group II introns is the same as that used by the spliceosome during splicing of eukaryotic pre-mRNAs (Figure 16-17). Here, however, the structure of the RNA itself, rather than the assembly of multiple snRNPs, creates an active site for catalysis, in which the 2'-OH of the branch-point A residue in the intron is directed to attack the phosphodiester bond at the 5' splice site. As in the spliceosomal reactions, a branched

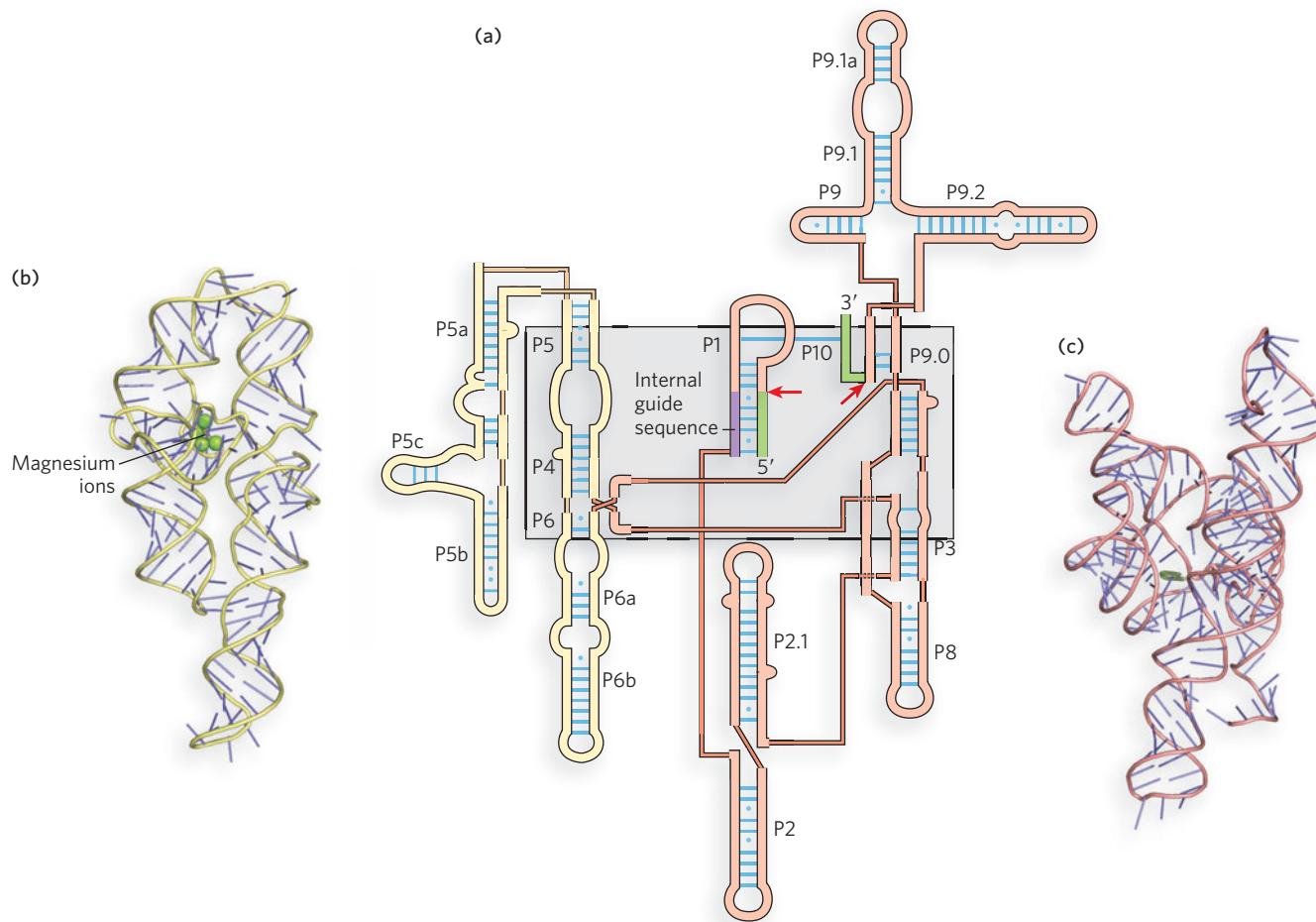


FIGURE 16-14 Secondary and tertiary structures of group I introns.

introns. (a) Group I introns share a common secondary structure in which short segments of the RNA strand fold back on themselves to form base-paired segments (exons shown in green; red arrows indicate splice sites). P segments are base-paired regions, numbered sequentially in the secondary structure; connections between P segments are shown by thinner lines indicating connectivity only, not actual sequence.

The gray shaded box indicates parts of the structure that form the catalytic core, or active site, of the intron. (b) The crystal structure of the *Tetrahymena* group I intron P4-P6 domain (shown in yellow in (a)), the first large RNA structure to be solved, showing how the helices pack together to form a three-dimensional structure. (c) The complete group I intron forms an active site for substrate binding and transesterification. [Sources: (b) PDB ID 1GID. (c) PDB ID 1U6B.]

lariat structure forms as an intermediate. After the second step produces the joined exons, the lariat intron can be linearized and degraded or can catalyze further reactions. Thus, like group I introns, group II introns have the ability to catalyze multiple reactions.

The catalytic properties of group II introns have sparked great interest among molecular biologists. The similarity between the mechanisms of self-splicing by group II introns and spliceosome-catalyzed splicing led to the hypothesis that the two processes are evolutionarily related. Perhaps group II introns are renegade spliceosomal RNAs that found a way to survive within genomes as “selfish” genetic elements. Or perhaps group II introns are ancient precursors of the spliceosome, containing just the core RNA components re-

quired for catalysis. Either scenario is consistent with the discovery that many group II introns can readily spread to new genes and new organisms by forming a catalytic complex with a protein encoded within the intron itself (Highlight 16-2). Such a mechanism for self-propagation may have enabled a functional subset of spliceosomal RNA to escape from the spliceosome, or it may have maintained the existence of a primitive self-splicing intron long after it could have been supplanted by the spliceosome.

Although spliceosomal introns seem to be limited to eukaryotes, the self-splicing intron classes are not. Genes with group I and II introns have also been found in bacteria and bacterial viruses. Bacteriophage T4, for example, has several protein-coding genes containing group

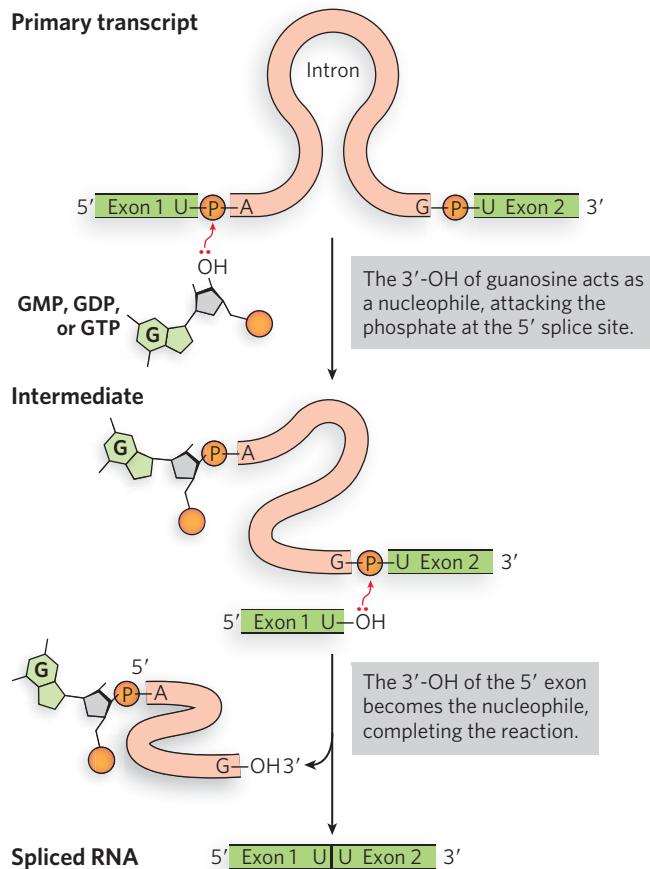


FIGURE 16-15 The self-splicing mechanism of group I introns. Group I introns use an exogenous guanosine (of a guanine nucleotide) to initiate two transesterification steps in self-splicing, as described in the text. The group I intron is released as a linear (not circular, or lariat) structure.

I introns. Group I introns are found in some nuclear, mitochondrial, and chloroplast genes coding for rRNAs, mRNAs, and tRNAs. Group II introns are generally found in the primary transcripts of mitochondrial or chloroplast mRNAs in fungi, algae, and plants. This prevalence is perhaps explained by the observation that both groups of introns are mobile genetic elements, capable of moving between bacterial strains and species (see Chapter 14).

Exons from Different RNA Molecules Can Be Fused by Trans-Splicing

Primary transcripts are sometimes covalently linked to a separate piece of RNA as introns are removed, a process referred to as **trans-splicing**. Although not known to occur in humans and most other eukaryotes, trans-splicing is the predominant mechanism of mRNA maturation in nematode worms. Genomic sequence data from several different organisms showed that trans-splicing is evolutionarily conserved in the nematode

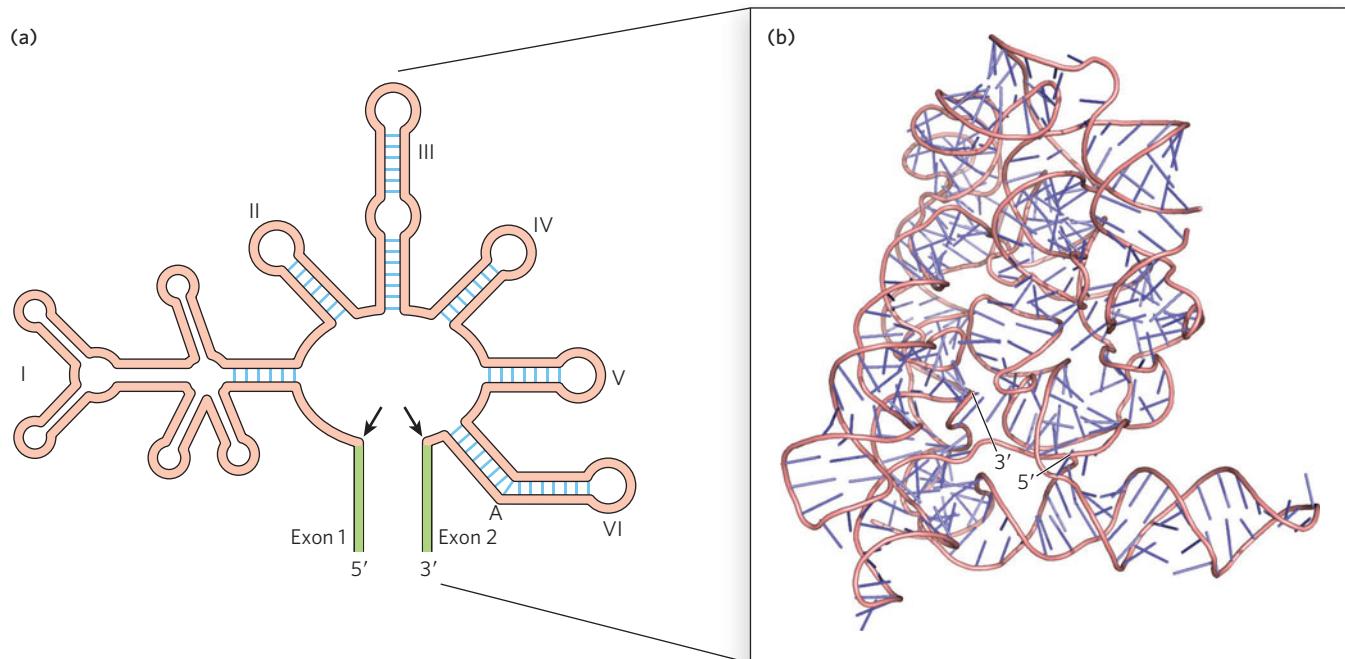


FIGURE 16-16 Secondary and tertiary structures of group II introns. (a) The overall secondary structure of group II introns is conserved, although many introns contain variable-length insertions in the loops of each base-paired segment. Black arrows indicate the splice sites marking the boundaries of the intron; green segments are the exons. The branch-point A is in domain VI. (b) The crystal structure of a spliced group II intron. [Source: (b) PDB ID 3EOH.]

HIGHLIGHT 16-2 EVOLUTION

The Origin of Introns

The origin of introns remains one of the mysteries of modern biology. Because many exons correspond to protein structural domains, it has been argued that introns are relics of early genes, in which sequences were stitched together randomly from shorter segments and only those with useful functions were maintained. According to this line of thinking, bacteria lack introns because competition for rapid growth, and consequent genome streamlining, led to intron loss from all but a few, rare bacterial and bacteriophage genes. Alternatively, introns could have arisen more recently on the evolutionary timeline. Analyses of intron positions in related genes from many different species, made possible with the arrival of online whole-genome sequence databases in the 1990s, show that in many cases, the introns and their positions within genes are not conserved. This might mean that introns were introduced relatively late in the evolution of modern genomes, or that introns are highly mobile.

The group II introns found in bacteria and in mitochondrial and chloroplast DNA are examples of mobile introns. Like retrotransposons, the introns encode proteins with both endonuclease and reverse transcriptase activities, allowing them to splice themselves back into DNA. In a transposition process termed **retrohoming**, the encoded protein forms a complex with the intron RNA after the intron is spliced from the primary transcript (Figure 1). Normally, the intron moves from one copy (allele) of a gene to an identical site in another copy of the same gene that lacks the intron. The initial insertion steps reprise the splicing mechanism, but in reverse. Once the RNA strand has been integrated into the DNA, the endonuclease cleaves the opposite DNA strand, and the inserted RNA is copied by the reverse transcriptase function associated with the endonuclease. The RNA is removed and replaced by DNA, converting the RNA intron to an inserted segment of DNA.

Over time, every copy of a particular gene in a population may acquire the intron. Much more rarely,

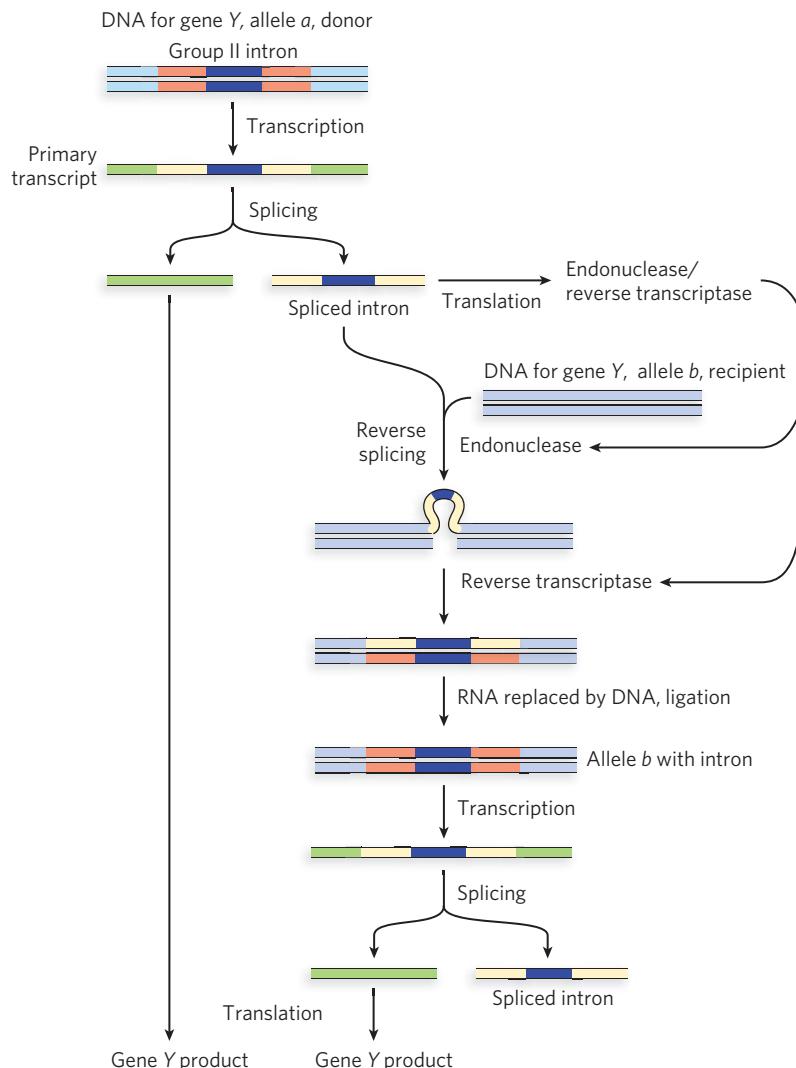


FIGURE 1 Mobile group II introns encode the enzyme activities needed to propagate the intron to new areas of the genome and to new hosts. Two alleles of a gene (*Y*) differ by the presence (allele *a*) or absence (allele *b*) of an intron. In retrohoming, the excised RNA intron can be translated to produce an enzyme with endonuclease activity, used to cleave the gene *Y*, allele *b* DNA at the site of intron insertion, and reverse transcriptase activity, used to make a cDNA copy of the intron; the copy is then ligated to create a new, intron-containing allele of gene *Y*.

the intron may insert itself into a new location in an unrelated gene. If this event does not kill the host cell, it can lead to the evolution and distribution of an intron in a new location. These mobile group II introns are thought to be the evolutionary precursors of the more widespread (and nonmobile) group II introns found in many eukaryotic genes.

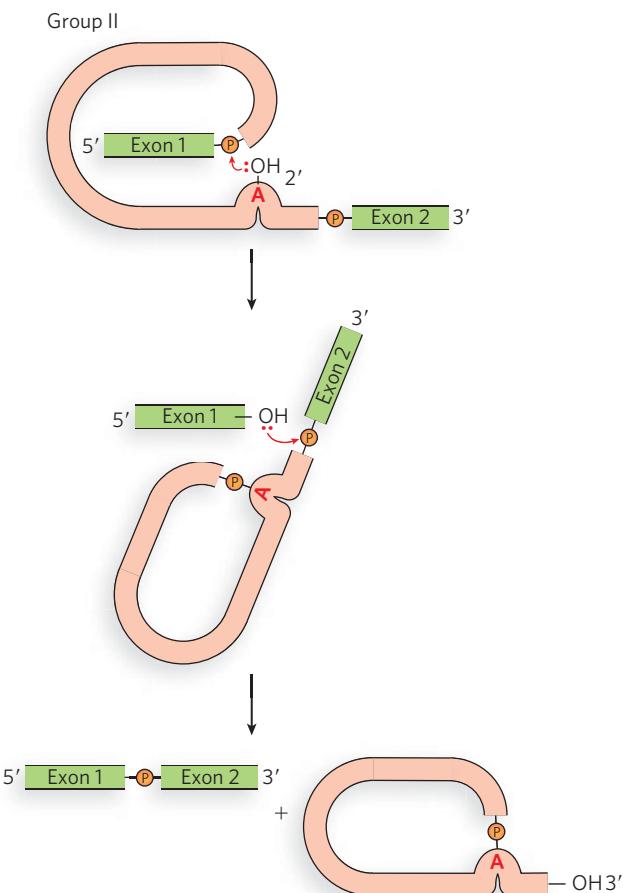


FIGURE 16-17 Self-splicing mechanism of group II introns. Group II introns self-splice by a pathway similar to that of spliceosome-catalyzed splicing, as described in the text. The group II intron is released as a lariat structure.

family. Maturation of primary transcripts involves *trans-splicing* of a short leader sequence called SL1 or SL2 onto the 5' end of the coding sequence for each individual gene (Figure 16-18).

The mechanism resembles those previously described (*cis-splicing*), using two transesterification steps. The SL (spliced leader) is donated by SL RNA (100 nucleotides). The 5' splice site is on the SL RNA, and the site of SL addition, the *trans-splice* site, is the 3' splice site on the pre-mRNA. The reaction proceeds by way of a branched intermediate similar to the lariat of *cis-splicing*. *Trans-splicing* is catalyzed by spliceosomes, including U2, U4, U5, and U6 snRNPs, but not U1. The spliced leader sequence is not translated into protein, but instead plays a regulatory role in coordinating gene expression. In *Caenorhabditis elegans*, the SL tends to be spliced adjacent to the initiation codon, AUG (often immediately next to it), so the SL is thought to play a role in initiating protein synthesis.

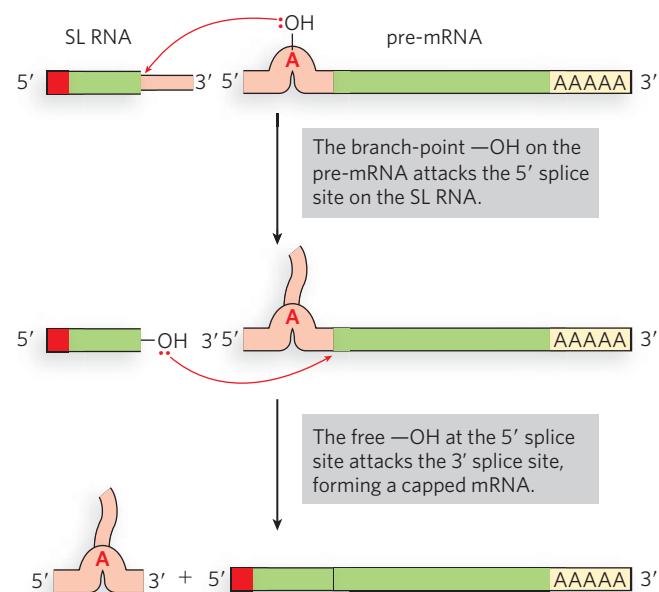


FIGURE 16-18 Trans-splicing. In nematodes, a short, 5'-capped leader sequence (SL RNA) is spliced onto a primary transcript to produce the mature mRNA.

SECTION 16.2 SUMMARY

- A few bacterial and most eukaryotic pre-mRNAs contain introns, intervening sequences that are removed as the flanking exon sequences are joined together in the process of splicing.
- Eukaryotic pre-mRNAs can contain dozens or hundreds of introns. In alternative splicing, the splicing of different sets of introns can produce a series of different mRNAs from the same initial transcript.
- Most introns are spliced by the spliceosome, a complex of five small nuclear ribonucleoproteins (snRNPs); sequences within pre-mRNAs mark the exon-intron boundaries.
- Some introns are capable of self-splicing without assistance from any proteins; both kinds of self-splicing introns, group I and group II, also occur in bacterial and mitochondrial RNAs.
- In some organisms, *trans-splicing* produces mRNAs in which exon sequences derive from different primary transcripts.

16.3 RNA Editing

In addition to being capped, polyadenylated, and spliced, the nucleotide sequence of some eukaryotic mRNAs is chemically altered by a process known as

RNA editing. Like RNA splicing, editing can expand the coding capacity of the genome by creating mRNAs that are not directly encoded by the DNA. Unlike the RNA processing reactions discussed above, however, RNA editing mechanisms seem to have been acquired in recent evolutionary history and to have arisen independently in different groups of organisms. There are two types of RNA editing: insertion or deletion, which uses a guide RNA as a template to add or remove bases from the mRNA, and substitution, in which one base is exchanged for another.

RNA Editing Can Involve the Insertion or Deletion of Bases

A particularly dramatic example of RNA editing occurs in the mitochondrial pre-mRNAs of trypanosomes, parasitic protozoa that cause human diseases such as sleeping sickness. The pre-mRNAs are edited after synthesis by an enzyme that inserts some U residues and deletes others. Like other kinds of RNA processing, editing of trypanosomal pre-mRNAs was initially discovered by comparing mtDNA sequences with corresponding mRNA sequences. RNA editing activity can be detected by isolating RNA from cells harvested at different times, converting the RNA to DNA with reverse transcriptase (see Chapter 14), then producing many copies of the DNA by the polymerase chain reaction. Comparing the sizes of DNA fragments by gel electrophoresis provides a measure of the relative amounts of edited and pre-edited targets.

RNA editing by insertion and deletion is catalyzed by the **editosome**, a complex of at least 16 proteins. A key editosome enzyme, RNA-editing terminal uridylyl-transferase (TUTase), catalyzes the uridylate addition reaction. The structure of the TUTase ensures that only U residues are added to the mRNA. Because TUTase is essential for the survival of trypanosomes in the bloodstream, it is a potential target for drug therapy.

The editosome uses small guide RNAs (gRNAs) that are partially complementary to the pre-mRNA regions to be changed (Figure 16-19a). Guide RNAs (35 to 75 nucleotides) are encoded by the trypanosomal mtDNA. Each gRNA contains three functionally important regions: an “anchor” sequence complementary to a target sequence in the pre-edited RNA, a middle segment containing the editing information, and a 3'-terminal oligo(U) (5 to 25 nucleotides) extension. The gRNAs base-pair with the target pre-mRNA, and the sequence differences are copied (by templating) from the gRNA to the pre-mRNA.

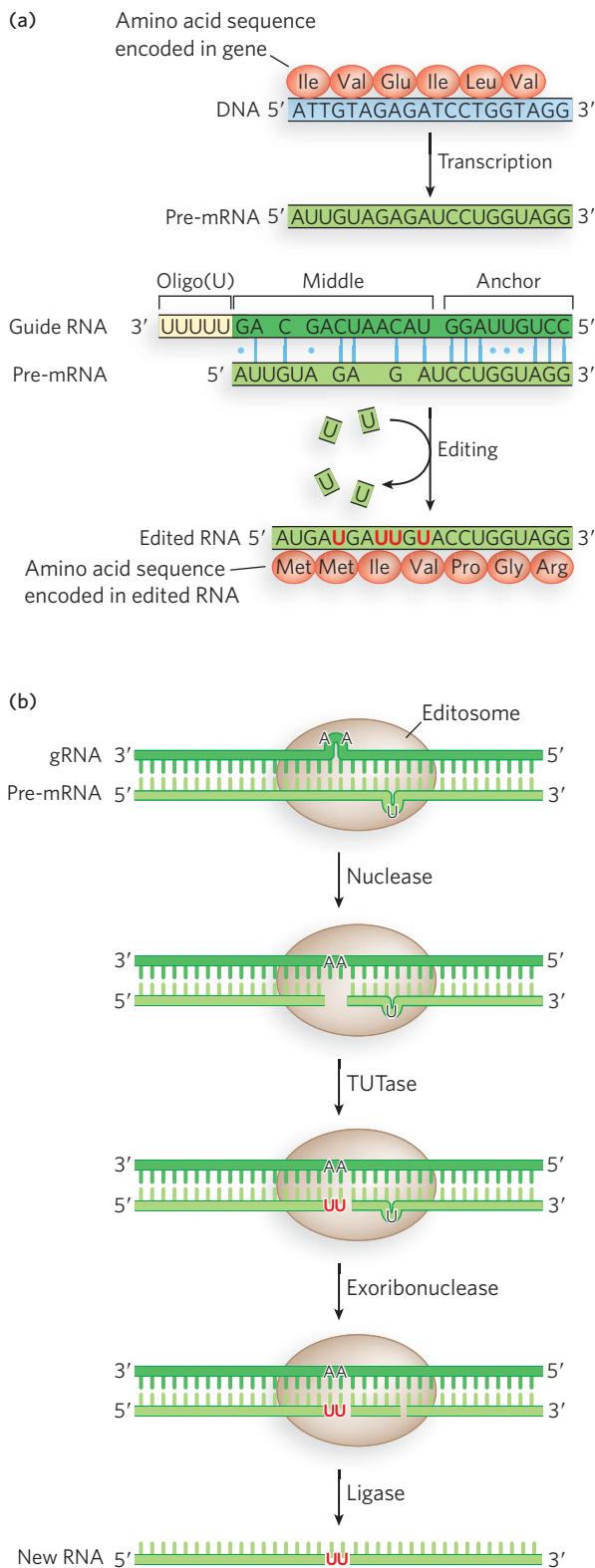
The mechanism of the editosome involves an endonucleolytic cut at the mismatch point between the

gRNA and the unedited transcript (Figure 16-19b). The insertion step is catalyzed by one of the enzymes in the complex, a TUTase that adds U residues from UTP to the 3' end of the mRNA. The opened ends are held in place by other proteins in the complex. Another enzyme, a U-specific exoribonuclease, removes any unpaired U residues. After editing has made the mRNA complementary to the gRNA, an RNA ligase rejoins the ends of the edited mRNA transcript. The resulting U insertions or deletions often create a frameshift in the edited mRNA, altering the sequence translated by the ribosome.

RNA Editing by Substitution Involves Deamination of A or C Residues

RNA editing also occurs in human cells and in the viruses that infect them. Many human pre-mRNAs are edited by **adenosine deaminase acting on RNA (ADAR)**, a fascinating enzyme that catalyzes the conversion of adenosine to inosine by removal of the amino group at C-6 on the adenine ring. Typically, ADAR converts just a few of the A residues to I residues within a very large transcript, but those changes are essential for creating the correct sequence to encode a functional protein. Many of the known substrates for ADAR are mRNAs coding for proteins that function in the central nervous system.

An interesting example of ADAR-mediated pre-mRNA editing occurs in the mRNAs encoding glutamate receptor channels (Figure 16-20). These Ca^{2+} channels, which allow the fast transmission of neural signals, are controlled by L-glutamate, the principal excitatory neurotransmitter in the brain. Two related classes of glutamate receptor channel proteins were found to differ by only a single amino acid, either an Arg or a Gln residue in a defined position of the putative channel-forming segment. However, the genomic DNA sequence encoding the channel segment of both proteins has a glutamine codon (CAG). Researchers discovered that in one set of mRNAs, ADAR converts the A of the CAG codon into an I. Because I can base-pair with C, the A-to-I conversion makes the CIG codon appear to be CGG when detected by standard sequencing reactions involving reverse transcription into DNA. Furthermore, the CIG codon is translated as a CGG codon during protein synthesis. Given that CGG encodes arginine, this editing event results in a glutamine-to-arginine substitution in the glutamate receptor channel protein. Such a change might seem subtle, but in fact the replacement of Gln by Arg in this segment profoundly alters the properties of ion flow across the channel.



A-to-I editing of pre-mRNAs, including the glutamate receptor channel pre-mRNA, often occurs at sites near exon-intron boundaries. This is because ADAR recognizes double-stranded RNA, such as the short du-

FIGURE 16-19 RNA editing by nucleotide insertion and deletion. (a) Before editing, a pre-mRNA is missing some U residues required in the mature RNA, and contains some extra U residues. A guide RNA (gRNA) in the editosome base-pairs with the pre-mRNA, guiding insertion and deletion of U residues. The insertions and deletions change the protein sequence encoded by the mRNA. (b) The editosome has several enzymatic activities: a nuclease to cleave the pre-mRNA at an insertion site, TUTase to fill in missing U residues, exoribonuclease to delete extra U residues, and ligase to seal the nick.

plexes formed in a pre-mRNA where an intron sequence folds back to base-pair with a complementary exon sequence. For this reason, editing necessarily occurs before the pre-mRNA is spliced. The C-terminal domain of Pol II enhances editing by preventing premature splicing, which would otherwise remove the intron recognition sites for ADAR. In some cases, editing occurs within longer regions of duplex RNA, including the large hairpin precursors of regulatory RNAs known as microRNAs (see Section 16.5) and duplexes arising from transcription of double-stranded viral RNAs or base-paired

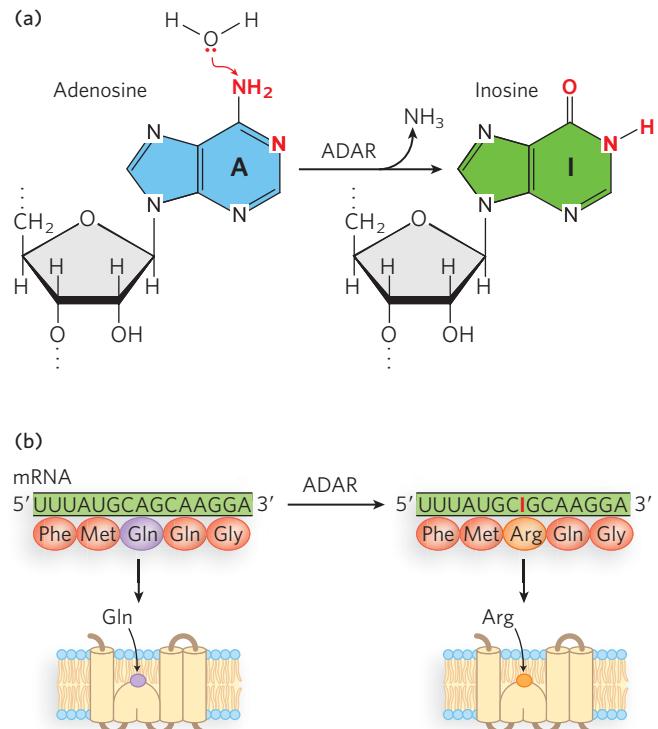
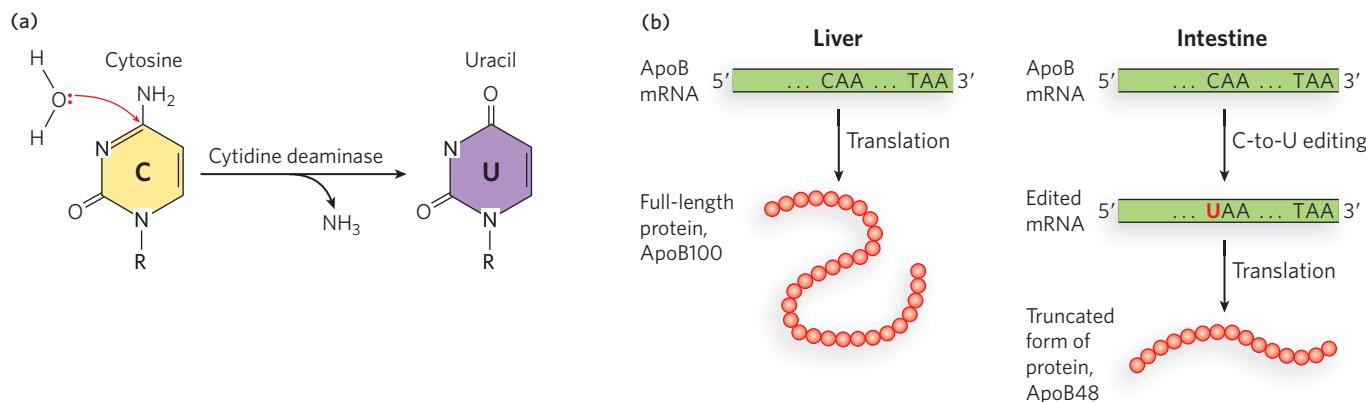


FIGURE 16-20 A-to-I editing of the mRNA for the glutamate receptor channel protein. (a) ADAR catalyzes the deamination of adenosine to inosine. (b) A-to-I editing results in replacement of a glutamine with an arginine in the protein product. The Arg residue at this position results in a protein that is much more efficient for Ca^{2+} transport.

**FIGURE 16-21** C-to-U editing of the mRNA for ApoB protein.

protein. (a) Cytidine deaminase catalyzes the deamination of cytidine to uridine. (b) The ApoB gene is expressed in the liver and intestine. In the intestine, the mRNA goes through

an additional processing step (after splicing) in which a C residue is changed to a U residue by cytidine deaminase, resulting in a stop codon and producing a smaller protein (ApoB48) that aids in the intestinal absorption of lipids.

repetitive sequences in the genome. These longer duplex RNAs can be extensively edited, with up to 50% of the adenosines converted to inosine. Sequences or structural properties of double-stranded RNA are recognized by ADAR and help specify the correct editing sites. How such editing affects RNA function is not yet known.

Another class of RNA editing enzymes, the cytidine deaminases, catalyze C-to-U conversions in mRNA substrates. Found in organisms ranging from bacteria to humans, cytidine deaminases can be critical for producing functional mRNAs. Editing involves deamination of a cytosine, converting it to uracil. Such C-to-U editing can be critical for gene expression. For example, cytidine deamination regulates expression of the apolipoprotein B (ApoB) gene in humans. ApoB is the primary apolipoprotein in low-density lipoprotein (LDL, or “bad” cholesterol), which carries cholesterol in the bloodstream. The full-length form of the protein, ApoB100, is produced in the liver. In the intestine, however, a CAA codon in the mRNA is edited to UAA, creating a stop codon that truncates the protein during translation to a shortened form, ApoB48, which is active in the gut (Figure 16-21). The truncated version is lacking a CTD, affecting the way cholesterol is metabolized in these tissues.

Some members of the cytidine deaminase enzyme family are found only in primates. These enzymes, called APOBECs, catalyze C-to-U conversions not only in cellular pre-mRNAs but in viral RNA. Some APOBEC enzymes protect human cells from infection by the human immunodeficiency virus (HIV), by editing and thereby inactivating viral RNA replication intermediates.

SECTION 16.3 SUMMARY

- The nucleotide sequence of eukaryotic mRNAs is sometimes altered by RNA editing enzymes. This can have far-reaching effects, such as altering the protein encoded by the mRNA or influencing the regulation of mRNA translation.
- One type of mRNA editing involves the insertion or deletion of U residues. Another type involves enzymatic deamination of A or C residues, converting them to I or U residues, respectively.

16.4 RNA Transport and Degradation

Once eukaryotic RNAs have been processed in the nucleus, they are ready for export to the cytoplasm. Mature mRNAs are translated into protein through the action of ribosomes, whereas other, non-protein-coding RNAs participate in various regulatory activities in the cytoplasm, or undergo further modification before reentering the nucleus. In each case, nuclear RNAs are recognized by their processed modifications as being ready for export, and they are then transported to the cytoplasm.

Like other kinds of RNA processing, degradation is a highly regulated process carried out by complex enzymatic machinery. RNA degradation enzymes are essential to cell survival because they help maintain appropriate amounts of mRNAs in response to metabolic and environmental signals. These enzymes also rid the cell of defective mRNAs containing premature stop codons.

Different Kinds of RNA Use Different Nuclear Export Pathways

Nuclear export of most non-protein-coding RNAs involves members of a conserved family of transport receptors called **importins** and **exportins**, collectively known as **karyopherins**. Exportins bind to their RNA “cargo” in the nucleus and escort it through nuclear pores to the cytoplasm, where the cargo is released. Both tRNAs and some kinds of noncoding RNAs bind directly to their respective exportin. In contrast, rRNAs are exported in pre-ribosomal particles containing ribosomal proteins, several rRNA species, and nonribosomal proteins, using their own exportin. The snRNAs are transported to the cytoplasm and remain there only transiently, for assembly into snRNP particles that re-enter the nucleus.

In each case, the exportin requires a small GTP-hydrolyzing protein called Ran that regulates cargo-receptor interactions (Figure 16-22). For nuclear export, the RNA cargo and exportin associate cooperatively with a Ran molecule bound to GTP. Once this ternary complex translocates to the cytoplasm, the Ran-bound GTP is hydrolyzed to GDP, causing release of the cargo RNA from the exportin. The Ran-GDP re-enters the nucleus, where its GDP is exchanged for GTP by a guanine nucleotide exchange factor (GEF), and the cycle can begin again.

For noncoding RNAs that are processed in the cytoplasm and returned to the nucleus, the cargo RNA and its import receptor—the importin—cross through a nuclear pore into the nucleus, where the cargo is released as the importin binds to Ran-GTP. The importin-Ran-GTP then translocates back to the cytoplasm, the importin dissociates, and GTP is hydrolyzed to GDP, restarting the cycle. Thus, export and import are reverse processes for which directionality is maintained by the presence of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm.

Spliced mRNA crosses through a nuclear pore via a Ran-independent pathway involving a different set of export factors that associate with other proteins to form a much larger complex called TREX (transcription-export). TREX couples the machineries responsible for the transcription, splicing, and export of mRNA. The TREX complex is specifically recruited to actively transcribed genes through interactions with Pol II. In human cells, the TREX component protein Aly recruits the complex to the 5' cap of nascent transcripts by interacting with the cap-binding protein CBP80. Because TREX functions as a nuclear export factor, mRNAs are transported out of the nucleus in the 5'-3' direction through a nuclear pore.

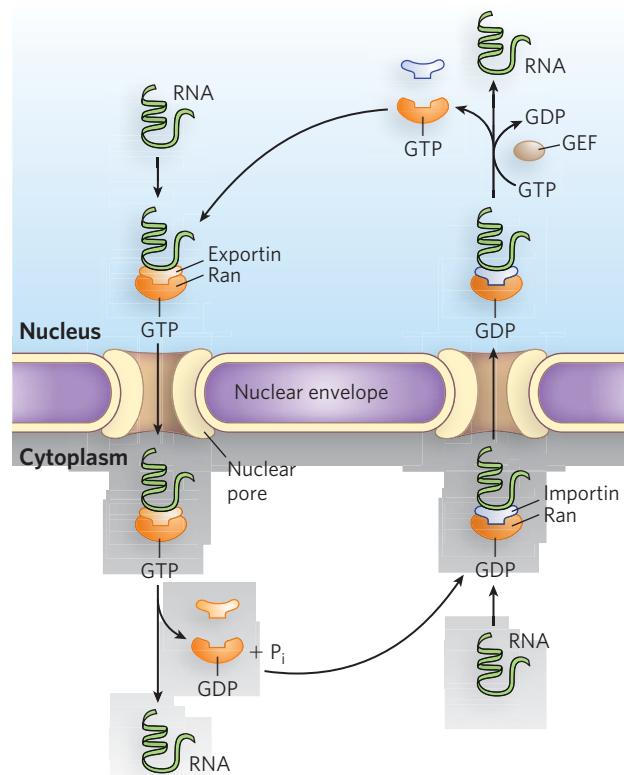


FIGURE 16-22 Nuclear export and import of RNA. The GTP-hydrolyzing protein Ran regulates RNA-receptor interactions. For nuclear export, the RNA cargo and its exportin receptor associate with Ran-GTP and the complex moves to the cytoplasm, where GTP is hydrolyzed to GDP and the cargo is released. For import, the RNA and its importin receptor cross through a nuclear pore into the nucleus, where GDP is exchanged for GTP and the RNA cargo is released.

mRNA Transport from the Nucleus to the Cytoplasm Is Coupled to Pre-mRNA Splicing

In eukaryotes, pre-mRNA splicing necessarily precedes export of the mature transcript from the nucleus, and researchers wondered whether pre-mRNA processing and nuclear export are closely coupled—which would provide a mechanism of mRNA quality control. To test this possibility, either pre-mRNA containing a single intron or the same mRNA lacking the intron were injected into the nuclei of frog's eggs. The rate of export of each type of mRNA was then measured by detecting the presence of the RNA in the cytoplasm over time. Results of this experiment showed that intron-containing pre-mRNAs, which were spliced in the nucleus, were exported much more rapidly and efficiently than the identical mRNAs

lacking the intron. Furthermore, the spliced mRNA was found to assemble with a different set of proteins than the mRNA that never contained the intron. This led to the conclusion that splicing generates a specific mRNA-protein complex that targets the mRNA for nuclear export, explaining the broader observation that an intron is required for the efficient expression of many eukaryotic protein-coding genes. In this way, only those mRNAs that have the correct end structures and spliced-exon sequence are used for protein synthesis.

Mature mRNAs produced by splicing end up in different intracellular locations and are differently translated and degraded than are otherwise identical mRNAs produced from non-intron-containing genes. The explanation is that splicing influences the set of proteins that associate with the mRNA in the nucleus to form an mRNP (mRNA ribonucleoprotein particle). These proteins in turn ensure that the mRNA interacts with exportins for shipment out of the nucleus. Chemical cross-linking experiments with cell extracts translating mRNAs with or without introns showed that several proteins bind to exon-exon junctions only as a consequence of splicing. Spliceosomes deposit a complex of proteins called the **exon junction complex (EJC)** on mRNAs at a position 20 to 24 nucleotides upstream of exon-exon junctions (Figure 16-23). At its core, EJC contains four proteins: eIF4AIII, MAGOH, Y14, and MLN51. The bound complexes accompany spliced mRNA into the cytoplasm, where they are removed during the first (“pioneer”) round of translation.

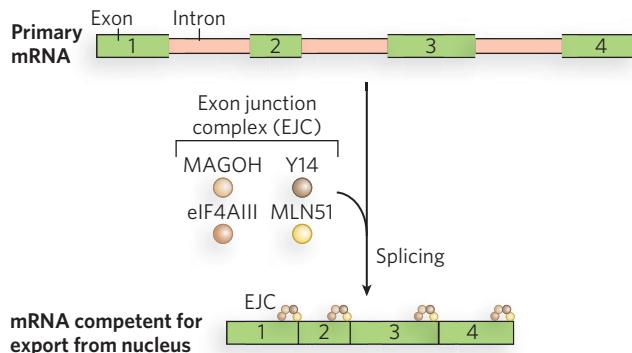


FIGURE 16-23 The exon junction complex. The EJC is a complex of four proteins responsible for mRNA quality control. An EJC is deposited on the mRNA after splicing, just upstream of each exon-exon junction. The complexes accompany the mature mRNA out of the nucleus and into the cytoplasm.

Some mRNAs Are Localized to Specific Regions of the Cytoplasm

In specialized cells, including oocytes (egg cells) and neurons, mRNAs are localized to particular sites prior to translation. The mechanism of such mRNA localization is best characterized in the fruit fly *Drosophila melanogaster*, in which maternal mRNAs are trafficked to various parts of the egg to help establish polarity during the early stages of embryo development (Figure 16-24). Shortly after the egg begins to mature, but before fertilization, mRNAs encoding proteins called Oskar and Bicoid bind to proteins that can move along microtubules, filamentous protein polymers that contribute to cell shape and structure. The *oskar* and *bicoid* mRNAs are shuttled to the parts of the egg where their protein products are required to form structures in the developing embryo. Analogous mechanisms of mRNA localization are thought to occur in neurons, in which mRNAs must be moved to parts of the cell very far from the nucleus for the localized protein synthesis required for proper neural function.

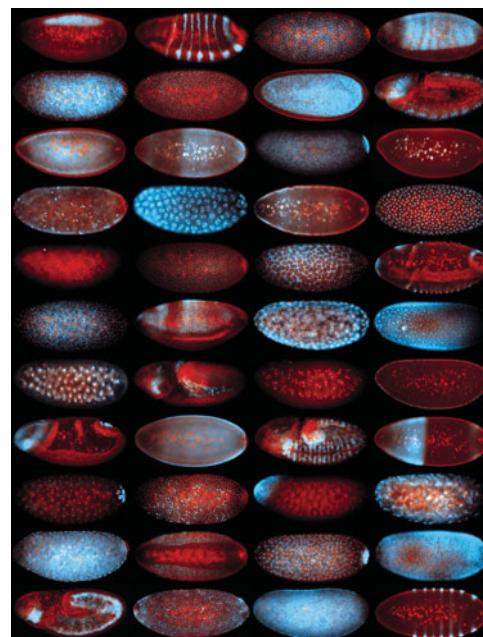


FIGURE 16-24 Transport of mRNA in the *Drosophila* egg.

The building blocks of anterior-posterior axis patterning in *Drosophila* are laid out during egg formation (oogenesis), well before the egg is fertilized and deposited. The developing egg (oocyte) is polarized by differentially localized mRNA molecules. In each photograph, a different mRNA is labeled with a blue fluorescent marker. Nuclear DNA is labeled in red. Each mRNA contributes to pattern formation in the developing embryo. [Source: E. Lecuyer et al., *Cell* 131 : 174-187, 2007. Courtesy of Eric Lecuyer.]