THE REPLICON

4.13.1 Introduction

Key Terms

The **replicon** is a unit of the genome in which DNA is replicated. Each replicon contains an origin for initiation of replication.

The **origin** is a sequence of DNA at which replication is initiated.

A **terminus** is a segment of DNA at which replication ends.

Single copy replication describes a control system in which there is only one copy of a replicon per unit bacterium. The bacterial chromosome and some plasmids have this type of regulation.

A plasmid is said to be under **multicopy control** when the control system allows the plasmid to exist in more than one copy per individual bacterial cell.

Whether a cell has only one chromosome (as in prokaryotes) or has many chromosomes (as in eukaryotes), the entire genome must be replicated precisely once for every cell division. How is the act of replication linked to the cell cycle?

Two general principles are used to compare the state of replication with the condition of the cell cycle:

- *Initiation of DNA replication commits the cell (prokaryotic or eukaryotic) to a further division.* From this standpoint, the number of descendants that a cell generates is determined by a series of decisions on whether or not to initiate DNA replication. Replication is controlled at the stage of initiation. *Once replication has started, it continues until the entire genome has been duplicated.*

- If replication proceeds, the consequent division cannot be permitted to occur until the replication event has been completed. Indeed, the completion of replication may provide a trigger for cell division. Then the duplicate genomes are segregated one to each daughter cell. The unit of segregation is the chromosome.

In prokaryotes, the initiation of replication is a single event involving a unique site on the bacterial chromosome, and the process of division is accomplished by the development of a septum that grows from the cell wall and divides the cell into two. In eukaryotic cells, initiation of replication is identified by the start of S phase, a protracted period during which DNA synthesis occurs, and which involves many individual initiation events. The act of division is accomplished by the reorganization of the cell at mitosis. In this chapter, we are concerned with the regulation of DNA replication. How is a cycle of replication initiated? What controls its progress and how is its termination signaled? In *Molecular Biology 6.29 Cell cycle and growth regulation*, we discuss the regulatory processes in eukaryotic cells that control entry into S phase and into mitosis, and also the "checkpoints" that postpone these actions until the appropriate conditions have been fulfilled.

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The unit of DNA in which an individual act of replication occurs is called the **replicon**. Each replicon "fires" once and only once in each cell cycle. The replicon is defined by its possession of the control elements needed for replication. It has an **origin** at which replication is initiated. It may also have a **terminus** at which replication stops (535).

Any sequence attached to an origin – or, more precisely, not separated from an origin by a terminus – is replicated as part of that replicon. The origin is a *cis*-acting site, able to affect only that molecule of DNA on which it resides.

(The original formulation of the replicon [in prokaryotes] viewed it as a unit possessing both the origin *and* the gene coding for the regulator protein. Now, however, "replicon" is usually applied to eukaryotic chromosomes to describe a unit of replication that contains an origin; *trans*-acting regulator protein(s) may be coded elsewhere.)

A genome in a prokaryotic cell constitutes a single replicon; so the units of replication and segregation coincide. Initiation at a single origin sponsors replication of the entire genome, once for every cell division. Each haploid bacterium has a single chromosome, so this type of replication control is called **single copy**.

Bacteria may contain additional genetic information in the form of plasmids. *A plasmid is an autonomous circular DNA genome that constitutes a separate replicon* (see **Figure 12.2**). A plasmid replicon may show single copy control, which means that it replicates once every time the bacterial chromosome replicates. Or it may be under **multicopy control**, when it is present in a greater number of copies than the bacterial chromosome. Each phage or virus DNA also constitutes a replicon, able to initiate many times during an infectious cycle. Perhaps a better way to view the prokaryotic replicon, therefore, is to reverse the definition: *any DNA molecule that contains an origin can be replicated autonomously in the cell.*

A major difference in the organization of bacterial and eukaryotic genomes is seen in their replication. Each eukaryotic chromosome contains a large number of replicons. So the unit of segregation includes many units of replication. This adds another dimension to the problem of control. All the replicons on a chromosome must be fired during one cell cycle, although they are not active simultaneously, but are activated over a fairly protracted period. *Yet each of these replicons must be activated no more than once in each cell cycle.*

Some signal must distinguish replicated from nonreplicated replicons, so that replicons do not fire a second time. And because many replicons are activated independently, another signal must exist to indicate when the entire process of replicating all replicons has been completed.

We have begun to collect information about the construction of individual replicons, but we still have little information about the relationship between replicons. We do not know whether the pattern of replication is the same in every cell cycle. Are all origins always used or are some origins sometimes silent? Do origins always fire in the same order? If there are different classes of origins, what distinguishes them?

In contrast with nuclear chromosomes, which have a single-copy type of control, the

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DNA of mitochondria and chloroplasts may be regulated more like plasmids that exist in multiple copies per bacterium. There are multiple copies of each organelle DNA per cell, and the control of organelle DNA replication must be related to the cell cycle.

In all these systems, the key question is to define the sequences that function as origins and to determine how they are recognized by the appropriate proteins of the apparatus for replication. We start by considering the basic construction of replicons and the various forms that they take; following the consideration of the origin, we turn to the question of how replication of the genome is coordinated with bacterial division, and what is responsible for segregating the genomes to daughter bacteria.

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THE REPLICON

4.13.2 Replicons can be linear or circular

Key Terms

A **replication eye** is a region in which DNA has been replicated within a longer, unreplicated region.

A **replication fork (Growing point)** is the point at which strands of parental duplex DNA are separated so that replication can proceed. A complex of proteins including DNA polymerase is found at the fork.

Unidirectional replication refers to the movement of a single replication fork from a given origin.

Bidirectional replication describes a system in which an origin generates two replication forks that proceed away from the origin in opposite directions.

Key Concepts

- A replicated region appears as an eye within nonreplicated DNA.
- A replication fork is initiated at the origin and then moves sequentially along DNA.
- Replication is unidirectional when a single replication fork is created at an origin.
- Replication is bidirectional when an origin creates two replication forks that move in opposite directions.

A molecule of DNA engaged in replication has two types of regions. **Figure 13.1** shows that when replicating DNA is viewed by electron microscopy, the replicated region appears as a **replication eye** within the nonreplicated DNA. The nonreplicated region consists of the parental duplex; this opens into the replicated region where the two daughter duplexes have formed.

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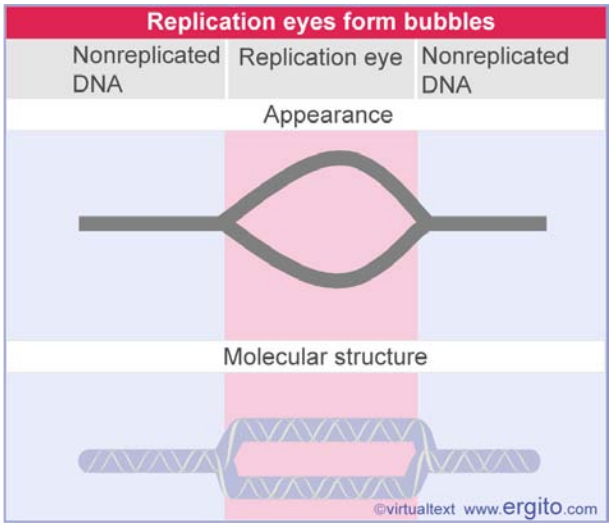

Figure 13.1 Replicated DNA is seen as a replication eye flanked by nonreplicated DNA.

The point at which replication is occurring is called the **replication fork** (sometimes also known as the **growing point**). *A replication fork moves sequentially along the DNA, from its starting point at the origin.* The origin may be used to start either **unidirectional replication** or **bidirectional replication**. The type of event is determined by whether one or two replication forks set out from the origin. In unidirectional replication, one replication fork leaves the origin and proceeds along the DNA. In bidirectional replication, two replication forks are formed; they proceed away from the origin in opposite directions.

The appearance of a replication eye does not distinguish between unidirectional and bidirectional replication. As depicted in **Figure 13.2**, the eye can represent either of two structures. If generated by unidirectional replication, the eye represents one fixed origin and one moving replication fork. If generated by bidirectional replication, the eye represents a pair of replication forks. In either case, the progress of replication expands the eye until ultimately it encompasses the whole replicon.

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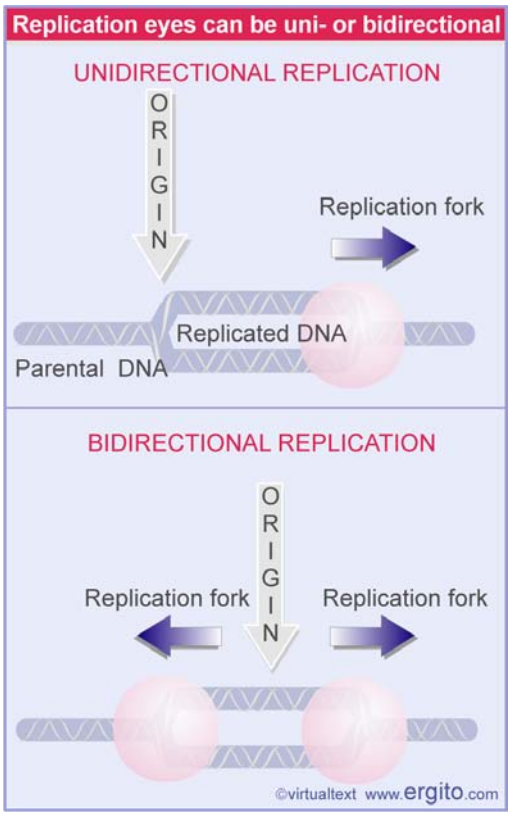

Figure 13.2 Replicons may be unidirectional or bidirectional, depending on whether one or two replication forks are formed at the origin.

When a replicon is circular, the presence of an eye forms the θ-structure drawn in **Figure 13.3**. The successive stages of replication of the circular DNA of polyoma virus are visualized by electron microscopy in **Figure 13.4**.

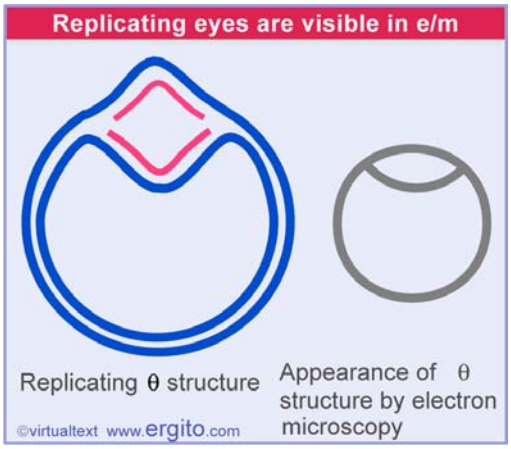

Figure 13.3 A replication eye forms a theta structure in circular DNA.

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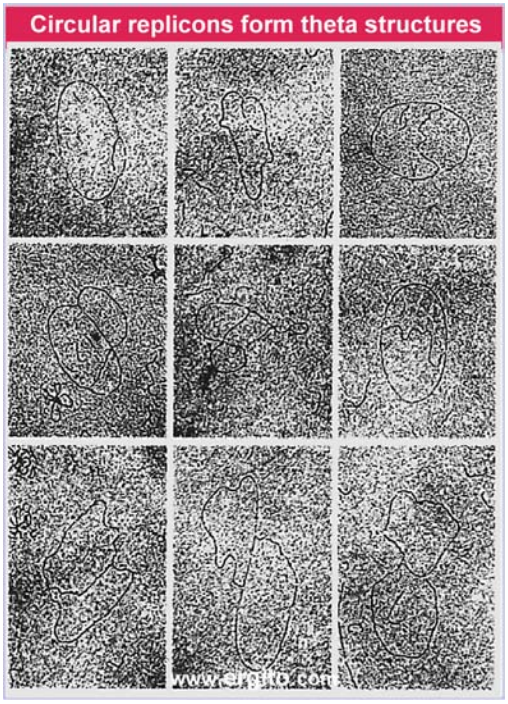

Figure 13.4 The replication eye becomes larger as the replication forks proceed along the replicon. Note that the "eye" becomes larger than the nonreplicated segment. The two sides of the eye can be defined because they are both the same length. Photograph kindly provided by Bernard Hirt.

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THE REPLICON

4.13.3 Origins can be mapped by autoradiography and electrophoresis

Key Concepts

- Replication fork movement can be detected by autoradiography using radioactive pulses.
- Replication forks create Y-shaped structures that change the electrophoretic migration of DNA fragments.

Whether a replicating eye has one or two replication forks can be determined in two ways. The choice of method depends on whether the DNA is a defined molecule or an unidentified region of a cellular genome.

With a defined linear molecule, we can use electron microscopy to measure the distance of each end of the eye from the end of the DNA. Then the positions of the ends of the eyes can be compared in molecules that have eyes of different sizes. If replication is unidirectional, only one of the ends will move; the other is the fixed origin. If replication is bidirectional, both will move; the origin is the point midway between them.

With undefined regions of large genomes, two successive pulses of radioactivity can be used to label the movement of the replication forks. If one pulse has a more intense label than the other, they can be distinguished by the relative intensities of labeling. These can be visualized by autoradiography. **Figure 13.5** shows that unidirectional replication causes one type of label to be followed by the other at *one* end of the eye. Bidirectional replication produces a (symmetrical) pattern at *both* ends of the eye. This is the pattern usually observed in replicons of eukaryotic chromosomes (537).

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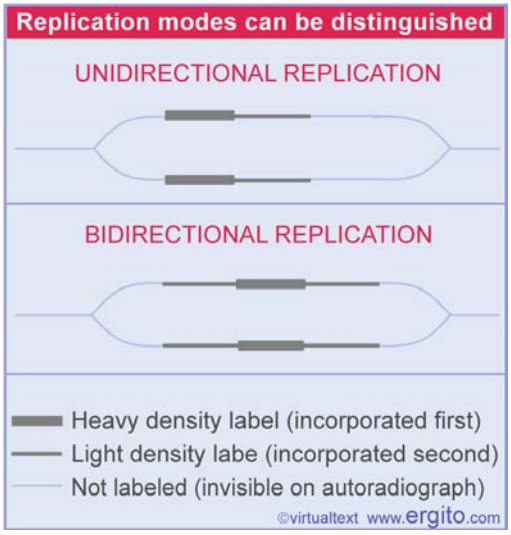

Figure 13.5 Different densities of radioactive labeling can be used to distinguish unidirectional and bidirectional replication.

A more recent method for mapping origins with greater resolution takes advantage of the effects that changes in shape have upon electrophoretic migration of DNA. **Figure 13.6** illustrates the two dimensional mapping technique, in which restriction fragments of replicating DNA are electrophoresed in a first dimension that separates by mass, and a second dimension where movement is determined more by shape. Different types of replicating molecules follow characteristic paths, measured by their deviation from the line that would be followed by a linear molecule of DNA that doubled in size.

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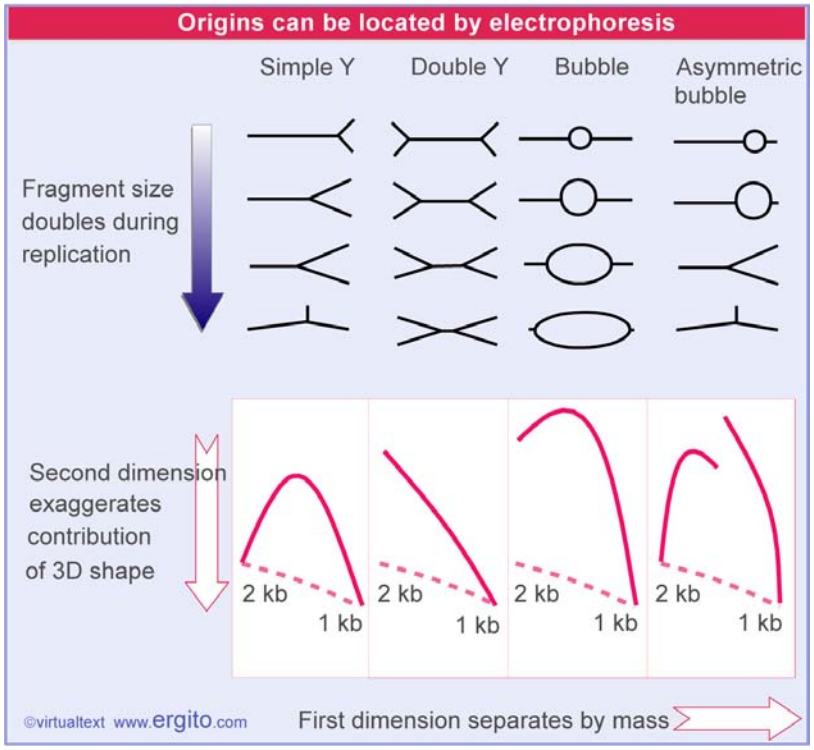

Figure 13.6 The position of the origin and the number of replicating forks determine the shape of a replicating restriction fragment, which can be followed by its electrophoretic path (solid line). The dashed line shows the path for a linear DNA.

A simple Y-structure, in which one fork moves along a linear fragment, follows a continuous path. An inflection point occurs when all three branches are the same length, and the structure therefore deviates most extensively from linear DNA. Analogous considerations determine the paths of double Y-structures or bubbles. An asymmetric bubble follows a discontinuous path, with a break at the point at which the bubble is converted to a Y-structure as one fork runs off the end.

Taken together, the various techniques for characterizing replicating DNA show that origins are most often used to initiate bidirectional replication. From this level of resolution, we must now proceed to the molecular level, to identify the *cis*-acting sequences that comprise the origin, and the *trans*-acting factors that recognize it.

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THE REPLICON

4.13.4 The bacterial genome is a single circular replicon

Key Concepts

- Bacterial replicons are usually circles that are replicated bidirectionally from a single origin.
- The origin of *E. coli*,*oriC*, is 245 bp in length.
- The two replication forks usually meet halfway round the circle, but there are *ter* sites that cause termination if they go too far.

To be properly inherited, a bacterial replicon should support several functions:

- Initiating a replication cycle.
- Controlling the frequency of initiation events.
- Segregating replicated chromosomes to daughter cells.

The first two functions both are properties of the origin. Segregation could be an independent function, but in prokaryotic systems it is usually determined by sequences in the vicinity of the origin. Origins in eukaryotes do not function in segregation, but are concerned only with replication.

As a general principle, the DNA constituting an origin can be isolated by its ability to support replication of any DNA sequence to which it is joined. When DNA from the origin is cloned into a molecule that lacks an origin, this will create a plasmid capable of autonomous replication *only if the DNA from the origin contains all the sequences needed to identify itself as an authentic origin for replication.*

Origins now have been identified in bacteria, yeast, chloroplasts, and mitochondria, although not in higher eukaryotes. A general feature is that the overall sequence composition is A·T-rich. We assume this is related to the need to melt the DNA duplex to initiate replication (539).

The genome of *E. coli* is replicated bidirectionally from a single origin, identified as the genetic locus *oriC*. The addition of *oriC* to any piece of DNA creates an artificial plasmid that can replicate in *E. coli*. By reducing the size of the cloned fragment of *oriC*, the region required to initiate replication has been equated with a fragment of 245 bp. (We discuss the properties of *oriC* and its interaction with the replication apparatus in more detail in *Molecular Biology 4.14.15 Creating the replication forks at an origin* .)

Prokaryotic replicons are usually circular, so that the DNA forms a closed circle

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with no free ends. Circular structures include the bacterial chromosome itself, all plasmids, and many bacteriophages. They are also common in chloroplast and mitochondrial DNAs. Replication of a circular molecule avoids the problem of how to replicate the ends of a linear molecule, but poses the problem of how to terminate replication (534).

The bacterial chromosome is replicated bidirectionally as a single unit from *oriC*. Two replication forks initiate at *oriC* and move around the genome (at approximately the same speed) to a meeting point. Termination occurs in a discrete region. One interesting question is what ensures that the DNA is replicated right across the region where the forks meet. Following the termination of DNA replication itself, enzymes that manipulate higher-order structure of DNA are required for the two daughter chromosomes to be physically separated (540).

Sequences that cause termination are called *ter* sites. A *ter* site contains a short (~23 bp) sequence that causes termination *in vitro*. The termination sequences function in only one orientation. The *ter* site is recognized by a protein (called Tus in *E. coli* and *RTP* in *B. subtilis*) that recognizes the consensus sequence and prevents the replication fork from proceeding (see *Molecular Biology 4.14.17 The primosome is needed to restart replication*). However, deletion of the *ter* sites does not prevent normal replication cycles from occurring (2223), although it does affect segregation of the daughter chromosomes (see *Molecular Biology 4.13.19 Chromosomal segregation may require site-specific recombination*).

Termination in *E. coli* and *B. subtilis* has the interesting features reported in **Figure 13.7**. We know that the replication forks usually meet and halt replication at a point midway round the chromosome from the origin. But two termination regions (*terE,D,A* and *terC,B* in *E. coli*, and *terI*, *terII* and also some other sites in *B. subtilis*) have been identified, located ~100 kb on either side of this meeting point. Each contains multiple terminators. Each terminus is specific for one direction of fork movement, and they are arranged in such a way that each fork would have to pass the other in order to reach the terminus to which it is susceptible. This arrangement creates a "replication fork trap". If for some reason one fork is delayed, so that the forks fail to meet at the usual central position, the more rapid fork will be trapped at the *ter* region to wait for the arrival of the slow fork.

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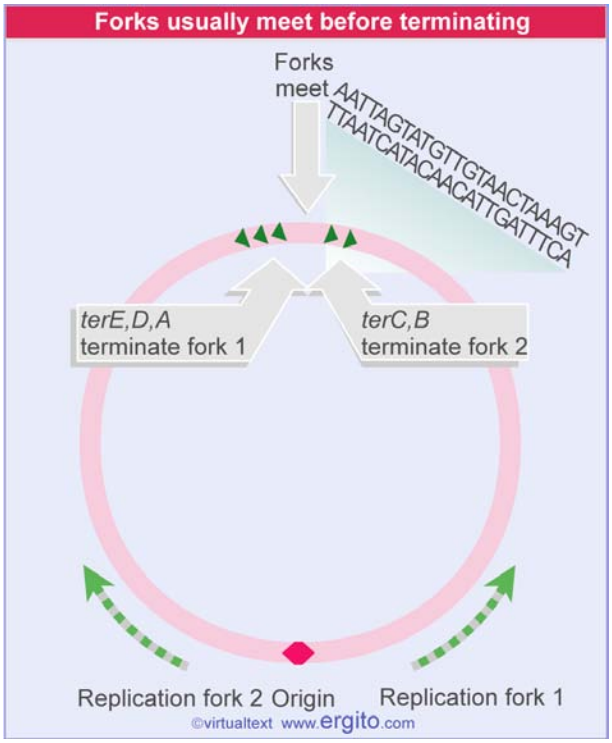

Figure 13.7 Replication termini in *E. coli* are located beyond the point at which the replication forks actually meet.

What happens when a replication fork encounters a protein bound to DNA? We assume that repressors (for example) are displaced and then reattach. A particularly interesting question is what happens when a replication fork encounters an RNA polymerase engaged in transcription. A replication fork moves >10× faster than RNA polymerase. If they are proceeding in the same direction, either the replication fork must displace the polymerase or it must slow down as it waits for the RNA polymerase to reach its terminator. It appears that a DNA polymerase moving in the same direction as an RNA polymerase can "bypass" it without disrupting transcription, but we do not understand how this happens (533; for review see 108).

A conflict arises when the replication fork meets an RNA polymerase traveling in the opposite direction, that is, toward it. Can it displace the RNA polymerase? Or do both replication and transcription come to a halt? An indication that these encounters cannot easily be resolved is provided by the organization of the *E. coli* chromosome. Almost all active transcription units are oriented so that they are expressed in the same direction as the replication fork that passes them. The exceptions all comprise small transcription units that are infrequently expressed. The difficulty of generating inversions containing highly expressed genes argues that head-on encounters between a replication fork and a series of transcribing RNA polymerases may be lethal.

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THE REPLICON

4.13.5 Each eukaryotic chromosome contains many replicons

Key Terms

S phase is the restricted part of the eukaryotic cell cycle during which synthesis of DNA occurs.

Key Concepts

- Eukaryotic replicons are 40-100 kb in length.
- A chromosome is divided into many replicons.
- Individual replicons are activated at characteristic times during S phase.
- Regional activation patterns suggest that replicons near one another are activated at the same time.

In eukaryotic cells, the replication of DNA is confined to part of the cell cycle. **S phase** usually lasts a few hours in a higher eukaryotic cell. Replication of the large amount of DNA contained in a eukaryotic chromosome is accomplished by dividing it into many individual replicons. Only some of these replicons are engaged in replication at any point in S phase. Presumably each replicon is activated at a specific time during S phase, although the evidence on this issue is not decisive (for review see 114).

The start of S phase is signaled by the activation of the first replicons. Over the next few hours, initiation events occur at other replicons in an ordered manner. Much of our knowledge about the properties of the individual replicons is derived from autoradiographic studies, generally using the types of protocols illustrated in **Figure 13.5** and **Figure 13.6**. Chromosomal replicons usually display bidirectional replication.

How large is the average replicon, and how many are there in the genome? A difficulty in characterizing the individual unit is that adjacent replicons may fuse to give large replicated eyes, as illustrated in **Figure 13.8**. The approach usually used to distinguish individual replicons from fused eyes is to rely on stretches of DNA in which several replicons can be seen to be active, presumably captured at a stage when all have initiated around the same time, but before the forks of adjacent units have met.

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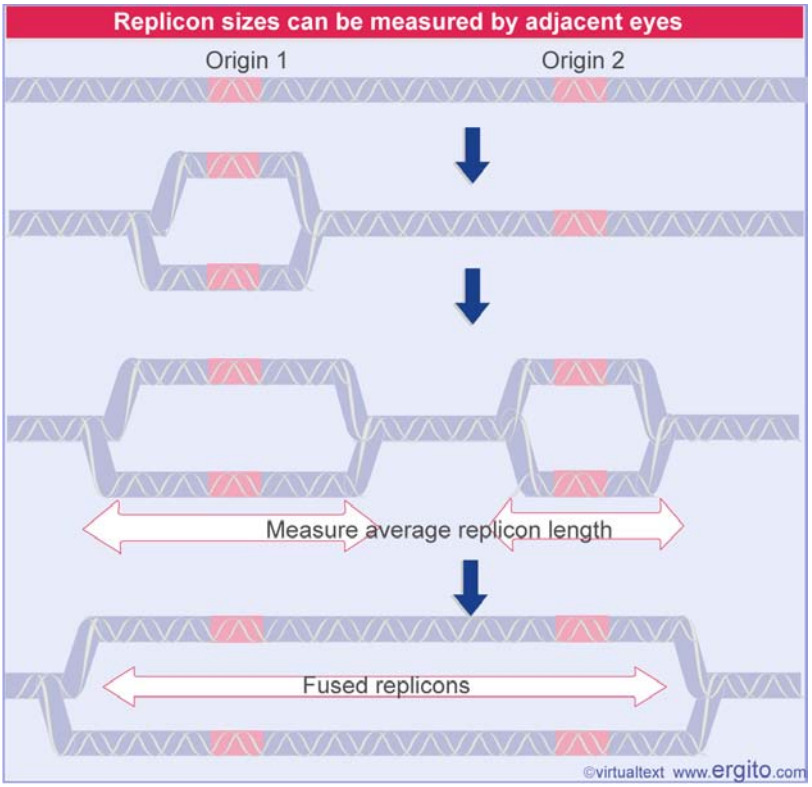

Figure 13.8 Measuring the size of the replicon requires a stretch of DNA in which adjacent replicons are active.

In groups of active replicons, the average size of the unit is measured by the distance between the origins (that is, between the midpoints of adjacent replicons). The rate at which the replication fork moves can be estimated from the maximum distance that the autoradiographic tracks travel during a given time.

Individual replicons in eukaryotic genomes are relatively small, typically ~40 kb in yeast or fly, ~100 kb in animals cells. However, they can vary >10-fold in length within a genome. The rate of replication is ~2000 bp/min, which is much slower than the 50,000 bp/min of bacterial replication fork movement.

From the speed of replication, it is evident that a mammalian genome could be replicated in ~1 hour if all replicons functioned simultaneously. But S phase actually lasts for >6 hours in a typical somatic cell, which implies that no more than 15% of the replicons are likely to be active at any given moment. There are some exceptional cases, such as the early embryonic divisions of *Drosophila* embryos, where the duration of S phase is compressed by the simultaneous functioning of a large number of replicons (558).

How are origins selected for initiation at different times during S phase? In *S. cerevisiae*, the default appears to be for origins to replicate early, but *cis*-acting sequences can cause origins linked to them to replicate at late times.

Available evidence suggests that chromosomal replicons do not have termini at which the replication forks cease movement and (presumably) dissociate from the

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DNA. It seems more likely that a replication fork continues from its origin until it meets a fork proceeding toward it from the adjacent replicon. We have already mentioned the potential topological problem of joining the newly synthesized DNA at the junction of the replication forks.

The propensity of replicons located in the same vicinity to be active at the same time could be explained by "regional" controls, in which groups of replicons are initiated more or less coordinately, as opposed to a mechanism in which individual replicons are activated one by one in dispersed areas of the genome. Two structural features suggest the possibility of large-scale organization. Quite large regions of the chromosome can be characterized as "early replicating" or "late replicating," implying that there is little interspersion of replicons that fire at early or late times. And visualization of replicating forks by labeling with DNA precursors identifies 100-300 "foci" instead of uniform staining; each focus shown in **Figure 13.9** probably contains >300 replication forks. The foci could represent fixed structures through which replicating DNA must move.

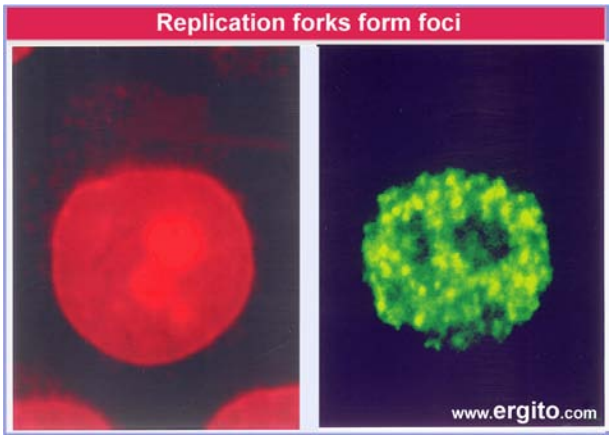

Figure 13.9 Replication forks are organized into foci in the nucleus. Cells were labeled with BrdU. The leftmost panel was stained with propidium iodide to identify bulk DNA. The right panel was stained using an antibody to BrdU to identify replicating DNA. Photographs kindly provided by A. D. Mills and Ron Laskey.

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THE REPLICON

4.13.6 Replication origins can be isolated in yeast

Key Terms

ARS (autonomous replication sequence) is an origin for replication in yeast. The common feature among different *ARS* sequences is a conserved 11 bp sequence called the A-domain.

The **A domain** is the conserved 11 bp sequence of A-T base pairs in the yeast ARS element that comprises the replication origin.

Key Concepts

- Origins in *S. cerevisiae* are short A·T-rich sequences that have an essential 11 bp sequence.
- The ORC is a complex of 6 proteins that binds to an *ARS*.

Any segment of DNA that has an origin should be able to replicate. So although plasmids are rare in eukaryotes, it may be possible to construct them by suitable manipulation *in vitro*. This has been accomplished in yeast, although not in higher eukaryotes.

S. cerevisiae mutants can be "transformed" to the wild phenotype by addition of DNA that carries a wild-type copy of the gene. The discovery of yeast origins resulted from the observation that some yeast DNA fragments (when circularized) are able to transform defective cells very efficiently. These fragments can survive in the cell in the unintegrated (autonomous) state, that is, as self-replicating plasmids.

A high-frequency transforming fragment possesses a sequence that confers the ability to replicate efficiently in yeast. This segment is called an **ARS** (for autonomously replicating sequence). *ARS* elements are derived from origins of replication.

Where *ARS* elements have been systematically mapped over extended chromosomal regions, it seems that only some of them are actually used to initiate replication. The others are silent, or possibly used only occasionally. If it is true that some origins have varying probabilities of being used, it follows that there can be no fixed termini between replicons. In this case, a given region of a chromosome could be replicated from different origins in different cell cycles.

An *ARS* element consists of an A·T-rich region that contains discrete sites in which mutations affect origin function. Base composition rather than sequence may be important in the rest of the region. **Figure 13.10** shows a systematic mutational analysis along the length of an origin. Origin function is abolished completely by mutations in a 14 bp "core" region, called the **A domain**, that contains an 11 bp consensus sequence consisting of A·T base pairs. This consensus sequence (sometimes called the *ACS* for ARS Consensus Sequence) is the only homology

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between known *ARS* elements (541).

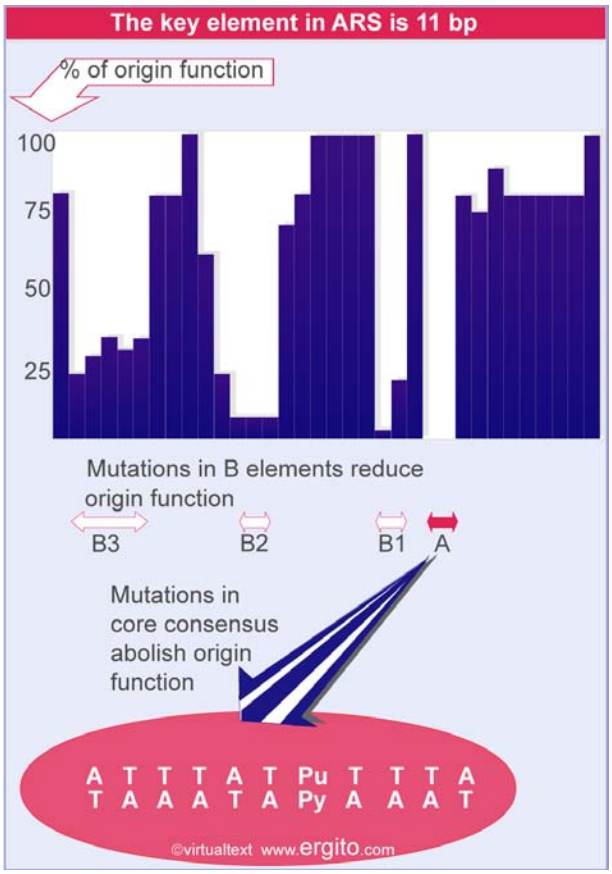

Figure 13.10 An *ARS* extends for ~50 bp and includes a consensus sequence (A) and additional elements (B1-B3).

Mutations in three adjacent elements, numbered B1-B3, reduce origin function. An origin can function effectively with any 2 of the B elements, so long as a functional A element is present. (Imperfect copies of the core consensus, typically conforming at 9/11 positions, are found close to, or overlapping with, each B element, but they do not appear to be necessary for origin function.)

The ORC (origin recognition complex) is a complex of 6 proteins with a mass of ~400 kD (for review see 2222). ORC binds to the A and B1 elements on the A·T-rich strand, and is associated with *ARS* elements throughout the cell cycle. This means that initiation depends on changes in its condition rather than *de novo* association with an origin (see *Molecular Biology 4.14.21 Licensing factor consists of MCM proteins*). By counting the number of sites to which ORC binds, we can estimate that there are about 400 origins of replication in the yeast genome (2247). This means that the average length of a replicon is ~35,000 bp. Counterparts to ORC are found in higher eukaryotic cells (2199).

ORC was first found in *S. cerevisiae* (where it is called scORC), but similar complexes have now been characterized in *S. pombe* (spORC), *Drosophila* (DmORC) and *Xenopus* (XlORC). All of the ORC complexes bind to DNA.

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Although none of the binding sites have been characterized in the same detail as in *S. cerevisiae*, in several cases they are at locations associated with the initiation of replication. It seems clear that ORC is an initiation complex whose binding identifies an origin of replication (for review see 3089). However, details of the interaction are clear only in *S. cerevisiae*; it is possible that additional components are required to recognize the origin in the other cases.

ARS elements satisfy the classic definition of an origin as a *cis*-acting sequence that causes DNA replication to initiate. Are similar elements to be found in higher eukaryotes? The conservation of the ORC suggests that origins are likely to take the same sort of form in other eukaryotes, but in spite of this, there is little conservation of sequence among putative origins in different organisms (for review see 4186).

Difficulties in finding consensus origin sequences cells suggest the possibility that origins may be more complex (or determined by features other than discrete *cis*-acting sequences). There are suggestions that some animal cell replicons may have complex patterns of initiation: in some cases, many small replication bubbles are found in one region, posing the question of whether there are alternative or multiple starts to replication, and whether there is a small discrete origin (for review see 116).

A reconciliation between this phenomenon and the use of ORCs is suggested by the discovery that environmental effects can influence the use of origins 4185). At one location where multiple bubbles are found, there is a primary origin that is used predominantly when the nucleotide supply is high. But when the nucleotide supply is limiting, many secondary origins are also used, giving rise to a pattern of multiple bubbles. One possible molecular explanation is that ORCs dissociate from the primary origin and initiate elsewhere in the vicinity if the supply of nucleotides is insufficient for the initiation reaction to occur quickly. At all events, it now seems likely that we will be able in due course to characterize discrete sequences that function as origins of replication in higher eukaryotes.

Last updated on 9-26-2003

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THE REPLICON

4.13.7 D loops maintain mitochondrial origins

Key Terms

A **D loop** is a region within mitochondrial DNA in which a short stretch of RNA is paired with one strand of DNA, displacing the original partner DNA strand in this region. The same term is used also to describe the displacement of a region of one strand of duplex DNA by a complementary single-stranded invader.

Key Concepts

- Mitochondria use different origin sequences to initiate replication of each DNA strand.
- Replication of the H-strand is initiated in a D-loop.
- Replication of the L-strand is initiated when its origin is exposed by the movement of the first replication fork.

The origins of replicons in both prokaryotic and eukaryotic chromosomes are static structures: they comprise sequences of DNA that are recognized in duplex form and used to initiate replication at the appropriate time. Initiation requires separating the DNA strands and commencing bidirectional DNA synthesis. A different type of arrangement is found in mitochondria.

Replication starts at a specific origin in the circular duplex DNA. But initially only one of the two parental strands (the H strand in mammalian mitochondrial DNA) is used as a template for synthesis of a new strand. Synthesis proceeds for only a short distance, displacing the original partner (L) strand, which remains single-stranded, as illustrated in **Figure 13.11**. The condition of this region gives rise to its name as the *displacement* or **D loop** (for review see 105; 113).

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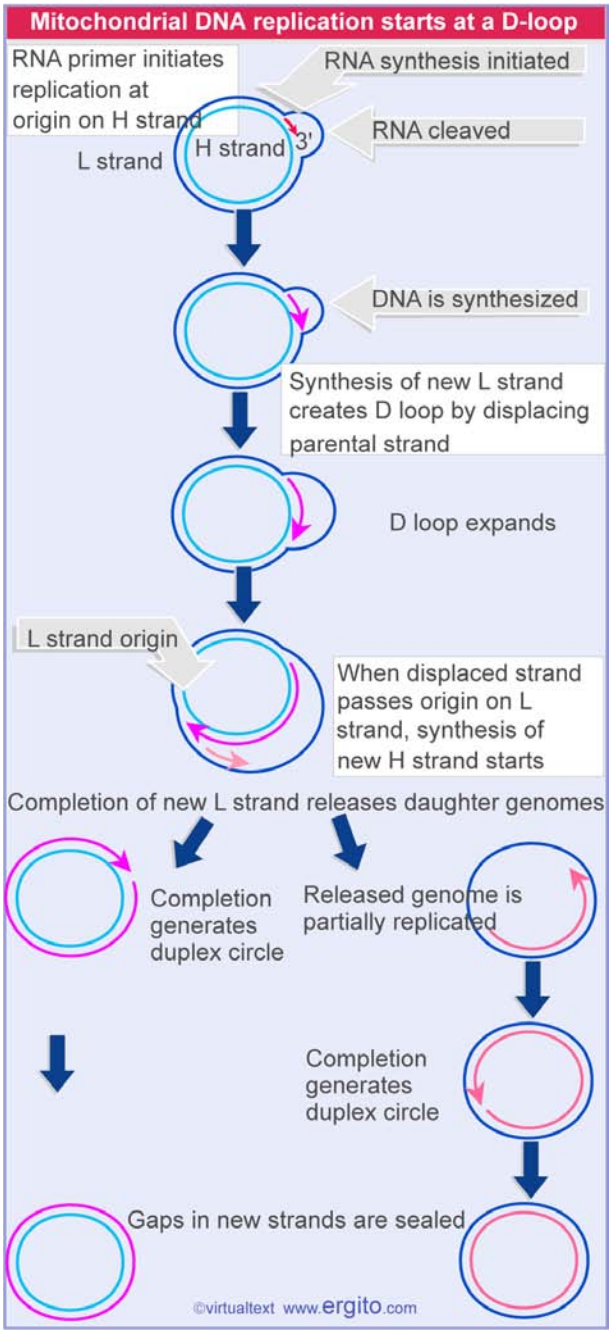

Figure 13.11 The D loop maintains an opening in mammalian mitochondrial DNA, which has separate origins for the replication of each strand.

DNA polymerases cannot initiate synthesis, but require a priming 3′ end (see *Molecular Biology 4.14.8 Priming is required to start DNA synthesis*). Replication at the H strand origin is initiated when RNA polymerase transcribes a primer. 3′ ends are generated in the primer by an endonuclease that cleaves the DNA-RNA hybrid at several discrete sites. The endonuclease is specific for the triple structure of DNA-RNA hybrid plus the displaced DNA single strand. The 3′ end is then extended into DNA by the DNA polymerase.

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A single D loop is found as an opening of 500-600 bases in mammalian mitochondria. The short strand that maintains the D loop is unstable and turns over; it is frequently degraded and resynthesized to maintain the opening of the duplex at this site. Some mitochondrial DNAs possess several D loops, reflecting the use of multiple origins. The same mechanism is employed in chloroplast DNA, where (in higher plants) there are two D loops.

To replicate mammalian mitochondrial DNA, the short strand in the D loop is extended. The displaced region of the original L strand becomes longer, expanding the D loop. This expansion continues until it reaches a point about two-thirds of the way around the circle. Replication of this region exposes an origin in the displaced L strand. Synthesis of an H strand initiates at this site, which is used by a special primase that synthesizes a short RNA. The RNA is then extended by DNA polymerase, proceeding around the displaced single-stranded L template in the opposite direction from L-strand synthesis.

Because of the lag in its start, H-strand synthesis has proceeded only a third of the way around the circle when L-strand synthesis finishes. This releases one completed duplex circle and one gapped circle, which remains partially single-stranded until synthesis of the H strand is completed. Finally, the new strands are sealed to become covalently intact (for review see 122).

The existence of D loops exposes a general principle. *An origin can be a sequence of DNA that serves to initiate DNA synthesis using one strand as template.* The opening of the duplex does not necessarily lead to the initiation of replication on the other strand. In the case of mitochondrial DNA replication, the origins for replicating the complementary strands lie at different locations. Origins that sponsor replication of only one strand are also found in the rolling circle mode of replication (see *Molecular Biology 4.13.10 Rolling circles produce multimers of a replicon*).

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THE REPLICON

4.13.8 The ends of linear DNA are a problem for replication

Key Concepts

- Special arrangements must be made to replicate the DNA strand with a 5′ end.

None of the replicons that we have considered so far have a linear end: either they are circular (as in the *E. coli* or mitochondrial genomes) or they are part of longer segregation units (as in eukaryotic chromosomes). But linear replicons occur, in some cases as single extrachromosomal units, and of course at the ends of eukaryotic chromosomes.

The ability of all known nucleic acid polymerases, DNA or RNA, to proceed only in the 5′ – 3′ direction poses a problem for synthesizing DNA at the end of a linear replicon. Consider the two parental strands depicted in **Figure 13.12**. The lower strand presents no problem: it can act as template to synthesize a daughter strand that runs right up to the end, where presumably the polymerase falls off. But to synthesize a complement at the end of the upper strand, synthesis must start right at the very last base (or else this strand would become shorter in successive cycles of replication).

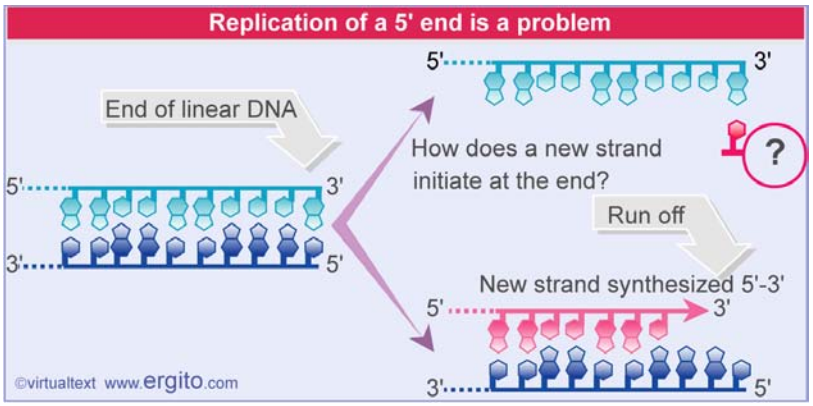

Figure 13.12 Replication could run off the 3′ end of a newly synthesized linear strand, but could it initiate at a 5′ end?

We do not know whether initiation right at the end of a linear DNA is feasible. We usually think of a polymerase as binding at a site *surrounding* the position at which a base is to be incorporated. So a special mechanism must be employed for replication at the ends of linear replicons. Several types of solution may be imagined to accommodate the need to copy a terminus:

- The problem may be circumvented by converting a linear replicon into a circular or multimeric molecule. Phages such as T4 or lambda use such mechanisms (see

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Molecular Biology 4.13.10 Rolling circles produce multimers of a replicon).

- The DNA may form an unusual structure – for example, by creating a hairpin at the terminus, so that there is no free end. Formation of a crosslink is involved in replication of the linear mitochondrial DNA of *Paramecium.*

- Instead of being precisely determined, the end may be variable. Eukaryotic chromosomes may adopt this solution, in which the number of copies of a short repeating unit at the end of the DNA changes (see *Molecular Biology 5.19.18 Telomeres are synthesized by a ribonucleoprotein enzyme*). A mechanism to add or remove units makes it unnecessary to replicate right up to the very end.

- A protein may intervene to make initiation possible at the actual terminus. Several linear viral nucleic acids have proteins that are *covalently linked to the 5′ terminal base.* The best characterized examples are adenovirus DNA, phage ϕ29 DNA, and poliovirus RNA.

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THE REPLICON

4.13.9 Terminal proteins enable initiation at the ends of viral DNAs

Key Terms

Strand displacement is a mode of replication of some viruses in which a new DNA strand grows by displacing the previous (homologous) strand of the duplex.

A **terminal protein** allows replication of a linear phage genome to start at the very end. The protein attaches to the 5′ end of the genome through a covalent bond, is associated with a DNA polymerase, and contains a cytosine residue that serves as a primer.

Key Concepts

- A terminal protein binds to the 5′ end of DNA and provides a cytidine nucleotide with a 3′–OH end that primes replication.

An example of initiation at a linear end is provided by adenovirus and ϕ29 DNAs, which actually replicate from both ends, using the mechanism of **strand displacement** illustrated in **Figure 13.13**. The same events can occur independently at either end. Synthesis of a new strand starts at one end, displacing the homologous strand that was previously paired in the duplex. When the replication fork reaches the other end of the molecule, the displaced strand is released as a free single strand. It is then replicated independently; this requires the formation of a duplex origin by base pairing between some short complementary sequences at the ends of the molecule.

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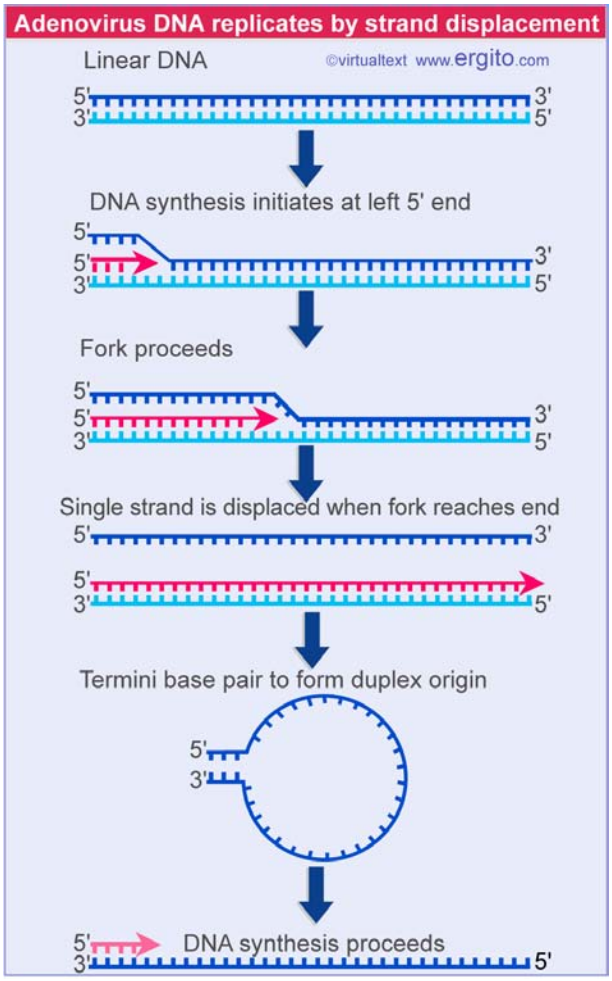

Figure 13.13 Adenovirus DNA replication is initiated separately at the two ends of the molecule and proceeds by strand displacement.

In several viruses that use such mechanisms, a protein is found covalently attached to each 5′ end. In the case of adenovirus, a **terminal protein** is linked to the mature viral DNA via a phosphodiester bond to serine, as indicated in **Figure 13.14**.

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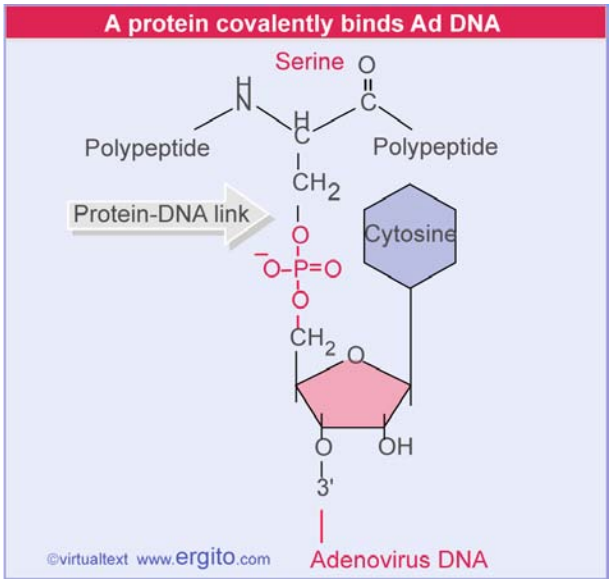

Figure 13.14 The 5′ terminal phosphate at each end of adenovirus DNA is covalently linked to serine in the 55 kD Ad-binding protein.

How does the attachment of the protein overcome the initiation problem? The terminal protein has a dual role: it carries a cytidine nucleotide that provides the primer; and it is associated with DNA polymerase. In fact, linkage of terminal protein to a nucleotide is undertaken by DNA polymerase in the presence of adenovirus DNA. This suggests the model illustrated in **Figure 13.15**. The complex of polymerase and terminal protein, bearing the priming C nucleotide, binds to the end of the adenovirus DNA. The free 3′–OH end of the C nucleotide is used to prime the elongation reaction by the DNA polymerase. This generates a new strand whose 5′ end is covalently linked to the initiating C nucleotide. (The reaction actually involves displacement of protein from DNA rather than binding *de novo*. The 5′ end of adenovirus DNA is bound to the terminal protein that was used in the previous replication cycle. The old terminal protein is displaced by the new terminal protein for each new replication cycle.)

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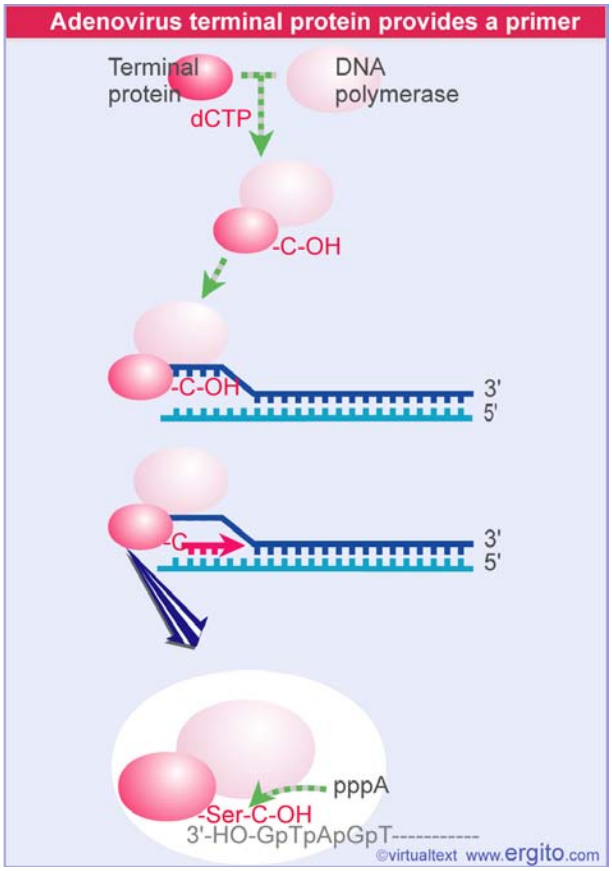

Figure 13.15 Adenovirus terminal protein binds to the 5′ end of DNA and provides a C-OH end to prime synthesis of a new DNA strand.

Terminal protein binds to the region located between 9 and 18 bp from the end of the DNA. The adjacent region, between positions 17 and 48, is essential for the binding of a host protein, nuclear factor I, which is also required for the initiation reaction. The initiation complex may therefore form between positions 9 and 48, a fixed distance from the actual end of the DNA.

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THE REPLICON

4.13.10 Rolling circles produce multimers of a replicon

Key Terms

The **rolling circle** is a mode of replication in which a replication fork proceeds around a circular template for an indefinite number of revolutions; the DNA strand newly synthesized in each revolution displaces the strand synthesized in the previous revolution, giving a tail containing a linear series of sequences complementary to the circular template strand.

Key Concepts

- A rolling circle generates single-stranded multimers of the original sequence.

The structures generated by replication depend on the relationship between the template and the replication fork. The critical features are whether the template is circular or linear, and whether the replication fork is engaged in synthesizing both strands of DNA or only one.

Replication of only one strand is used to generate copies of some circular molecules. A nick opens one strand, and then the free 3′–OH end generated by the nick is extended by the DNA polymerase. The newly synthesized strand displaces the original parental strand. The ensuing events are depicted in **Figure 13.16**.

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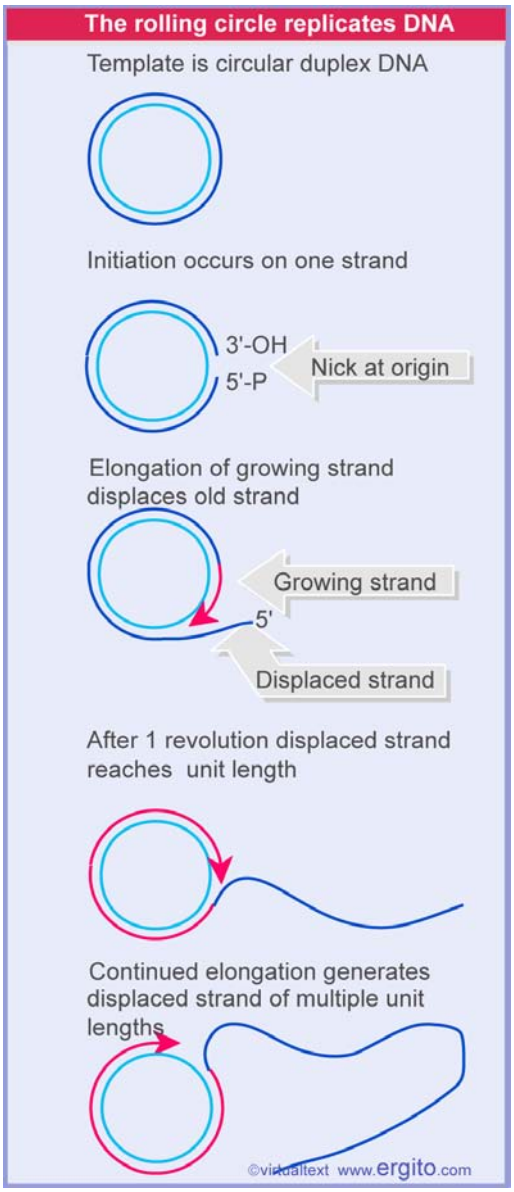

Figure 13.16 The rolling circle generates a multimeric single-stranded tail.

This type of structure is called a **rolling circle**, because the growing point can be envisaged as rolling around the circular template strand. It could in principle continue to do so indefinitely. As it moves, the replication fork extends the outer strand and displaces the previous partner (549). An example is shown in the electron micrograph of **Figure 13.17**.

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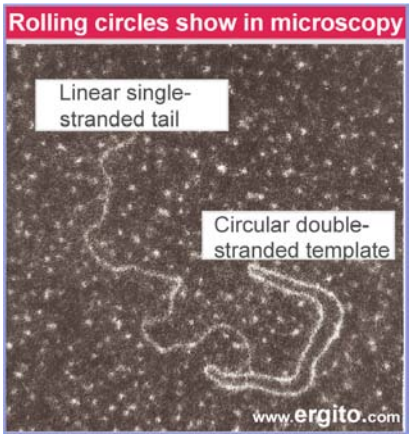

Figure 13.17 A rolling circle appears as a circular molecule with a linear tail by electron microscopy. Photograph kindly provided by David Dressler.

Because the newly synthesized material is covalently linked to the original material, the displaced strand has the original unit genome at its 5′ end. The original unit is followed by any number of unit genomes, synthesized by continuing revolutions of the template. Each revolution displaces the material synthesized in the previous cycle.

The rolling circle is put to several uses *in vivo*. Some pathways that are used to replicate DNA are depicted in **Figure 13.18**.

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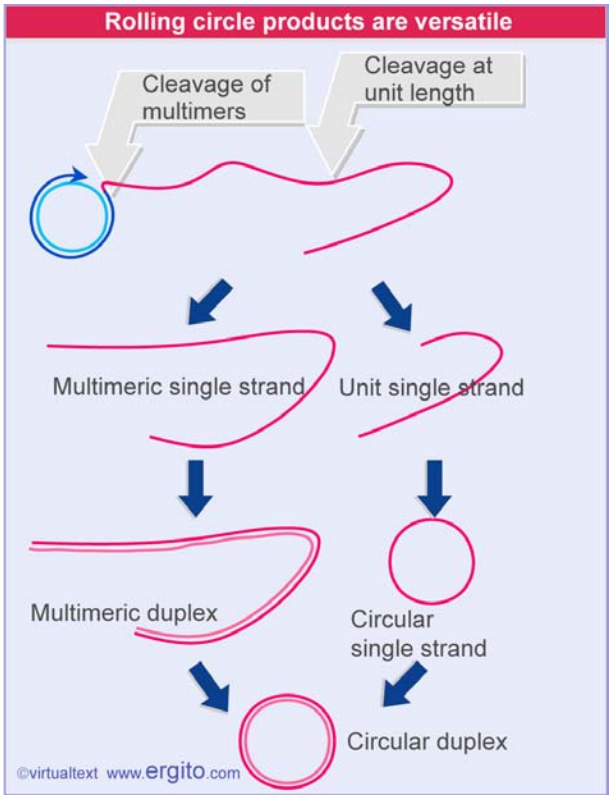

Figure 13.18 The fate of the displaced tail determines the types of products generated by rolling circles. Cleavage at unit length generates monomers, which can be converted to duplex and circular forms. Cleavage of multimers generates a series of tandemly repeated copies of the original unit. Note that the conversion to double-stranded form could occur earlier, before the tail is cleaved from the rolling circle.

Cleavage of a unit length tail generates a copy of the original circular replicon in linear form. The linear form may be maintained as a single strand or may be converted into a duplex by synthesis of the complementary strand (which is identical in sequence to the template strand of the original rolling circle).

The rolling circle provides a means for amplifying the original (unit) replicon. This mechanism is used to generate amplified rDNA in the *Xenopus* oocyte. The genes for rRNA are organized as a large number of contiguous repeats in the genome. A single repeating unit from the genome is converted into a rolling circle. The displaced tail, containing many units, is converted into duplex DNA; later it is cleaved from the circle so that the two ends can be joined together to generate a large circle of amplified rDNA. The amplified material therefore consists of a large number of identical repeating units.

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THE REPLICON

4.13.11 Rolling circles are used to replicate phage genomes

Key Terms

A **relaxase** is an enzyme that cuts one strand of DNA, and binds to the free 5′ end.

Key Concepts

- The ϕX A protein is a *cis*-acting relaxase that generates single-stranded circles from the tail produced by rolling circle replication.

Replication by rolling circles is common among bacteriophages. Unit genomes can be cleaved from the displaced tail, generating monomers that can be packaged into phage particles or used for further replication cycles. A more detailed view of a phage replication cycle that is centered on the rolling circle is given in **Figure 13.19**.

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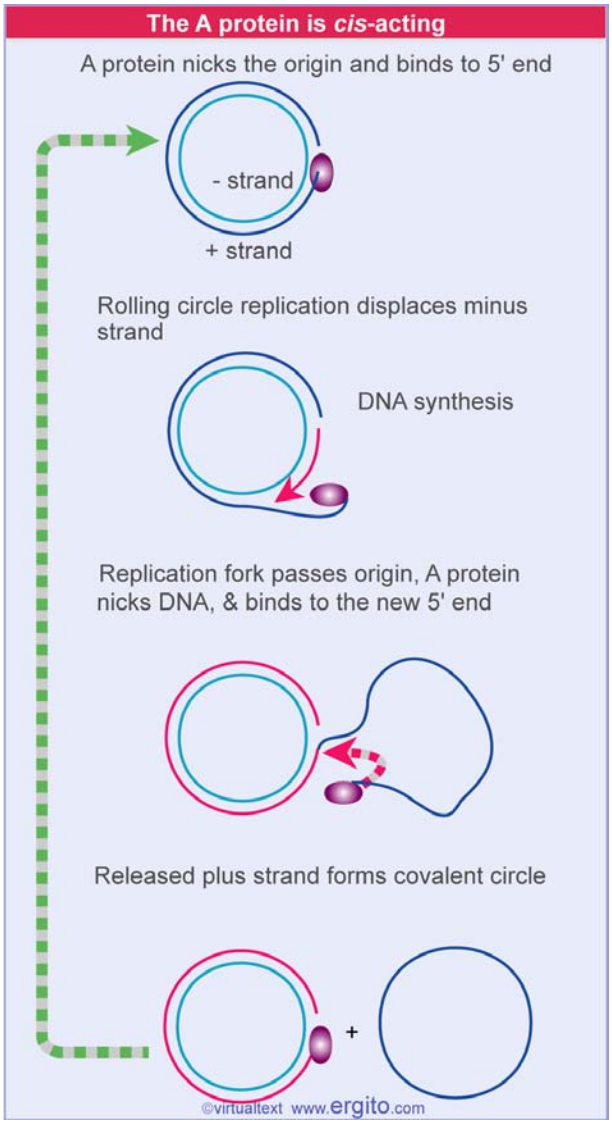

Figure 13.19 ϕ X174 RF DNA is a template for synthesizing single-stranded viral circles. The A protein remains attached to the same genome through indefinite revolutions, each time nicking the origin on the viral (+) strand and transferring to the new 5′ end. At the same time, the released viral strand is circularized.
This is a static version of an interactive figure; see http://www.ergito.com/main.jsp?bcs=MBIO.4.13.11 to view properly.

Phage ϕX174 consists of a single-stranded circular DNA, known as the plus (+) strand. A complementary strand, called the minus (–) strand, is synthesized. This action generates the duplex circle shown at the top of the figure, which is then replicated by a rolling circle mechanism.

The duplex circle is converted to a covalently closed form, which becomes supercoiled. A protein coded by the phage genome, the A protein, nicks the (+) strand of the duplex DNA at a specific site that defines the origin for replication.

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After nicking the origin, the A protein remains connected to the 5′ end that it generates, while the 3′ end is extended by DNA polymerase.

The structure of the DNA plays an important role in this reaction, for the DNA can be nicked *only when it is negatively supercoiled* (wound about its axis in space in the opposite sense from the handedness of the double helix; see *Molecular Biology 4.15.12 Supercoiling affects the structure of DNA*). The A protein is able to bind to a single-stranded decamer fragment of DNA that surrounds the site of the nick. This suggests that the supercoiling is needed to assist the formation of a single-stranded region that provides the A protein with its binding site. (An enzymatic activity in which a protein cleaves duplex DNA and binds to a released 5′ end is sometimes called a **relaxase**.) The nick generates a 3′–OH end and a 5′–phosphate end (covalently attached to the A protein), both of which have roles to play in ϕX174 replication.

Using the rolling circle, the 3′–OH end of the nick is extended into a new chain. The chain is elongated around the circular (–) strand template, until it reaches the starting point and displaces the origin. Now the A protein functions again. It remains connected with the rolling circle as well as to the 5′ end of the displaced tail, and it is therefore in the vicinity as the growing point returns past the origin. So the same A protein is available again to recognize the origin and nick it, now attaching to the end generated by the new nick. The cycle can be repeated indefinitely.

Following this nicking event, the displaced single (+) strand is freed as a circle. The A protein is involved in the circularization. In fact, the joining of the 3′ and 5′ ends of the (+) strand product is accomplished by the A protein as part of the reaction by which it is released at the end of one cycle of replication, and starts another cycle.

The A protein has an unusual property that may be connected with these activities. It is *cis*-acting *in vivo.* (This behavior is not reproduced *in vitro,* as can be seen from its activity on any DNA template in a cell-free system.) *The implication is that in vivo the A protein synthesized by a particular genome can attach only to the DNA of that genome.* We do not know how this is accomplished. However, its activity *in vitro* shows how it remains associated with the same parental (–) strand template. The A protein has two active sites; this may allow it to cleave the "new" origin while still retaining the "old" origin; then it ligates the displaced strand into a circle.

The displaced (+) strand may follow either of two fates after circularization. During the replication phase of viral infection, it may be used as a template to synthesize the complementary (–) strand. The duplex circle may then be used as a rolling circle to generate more progeny. During phage morphogenesis, the displaced (+) strand is packaged into the phage virion.

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THE REPLICON

4.13.12 The F plasmid is transferred by conjugation between bacteria

Key Terms

Conjugation is a process in which two cells come in contact and exchange genetic material. In bacteria, DNA is transferred from a donor to a recipient cell. In protozoa, DNA passes from each cell to the other.

The **F plasmid** is an episome that can be free or integrated in *E. coli*, and which in either form can sponsor conjugation.

The **transfer region** is a segment on the F plasmid that is required for bacterial conjugation.

A **pilus (pili)** is a surface appendage on a bacterium that allows the bacterium to attach to other bacterial cells. It appears like a short, thin, flexible rod. During conjugation, pili are used to transfer DNA from one bacterium to another.

Pilin is the subunit that is polymerized into the pilus in bacteria.

Key Concepts

- A free F factor is a replicon that is maintained at the level of one plasmid per bacterial chromosome.
- An F factor can integrate into the bacterial chromosome, in which case its own replication system is suppressed.
- The F factor codes for specific pili that form on the surface of the bacterium.
- An F-pilus enables an F-positive bacterium to contact an F-negative bacterium and to initiate conjugation.

Another example of a connection between replication and the propagation of a genetic unit is provided by bacterial **conjugation**, in which a plasmid genome or host chromosome is transferred from one bacterium to another.

Conjugation is mediated by the **F plasmid**, which is the classic example of an episome, an element that may exist as a free circular plasmid, or that may become integrated into the bacterial chromosome as a linear sequence (like a lysogenic bacteriophage). The F plasmid is a large circular DNA, ~100 kb in length.

The F factor can integrate at several sites in the *E. coli* chromosome, often by a recombination event involving certain sequences (called IS sequences; see *Molecular Biology 4.16.5 Transposons cause rearrangement of DNA*) that are present on both the host chromosome and F plasmid. In its free (plasmid) form, the F plasmid utilizes its own replication origin (*oriV*) and control system, and is maintained at a level of one copy per bacterial chromosome. When it is integrated into the bacterial chromosome, this system is suppressed, and F DNA is replicated as a part of the

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chromosome.

The presence of the F plasmid, whether free or integrated, has important consequences for the host bacterium. Bacteria that are F-positive are able to conjugate (or mate) with bacteria that are F-negative. Conjugation involves a contact between donor (F-positive) and recipient (F-negative) bacteria; contact is followed by transfer of the F factor. If the F factor exists as a free plasmid in the donor bacterium, it is transferred as a plasmid, and the infective process converts the F-negative recipient into an F-positive state. If the F factor is present in an integrated form in the donor, the transfer process may also cause some or all of the bacterial chromosome to be transferred. Many plasmids have conjugation systems that operate in a generally similar manner, but the F factor was the first to be discovered, and remains the paradigm for this type of genetic transfer (550).

A large (~33 kb) region of the F plasmid, called the **transfer region**, is required for conjugation. It contains ~40 genes that are required for the transmission of DNA; their organization is summarized in **Figure 13.20**. The genes are named as *tra* and *trb* loci. Most of them are expressed coordinately as part of a single 32 kb transcription unit (the *traY-I* unit). *traM* and *traJ* are expressed separately. *traJ* is a regulator that turns on both *traM* and *traY-I*. On the opposite strand, *finP* is a regulator that codes for a small antisense RNA that turns off *traJ*. Its activity requires expression of another gene, *finO*. Only four of the *tra* genes in the major transcription unit are concerned directly with the transfer of DNA; most are concerned with the properties of the bacterial cell surface and with maintaining contacts between mating bacteria.

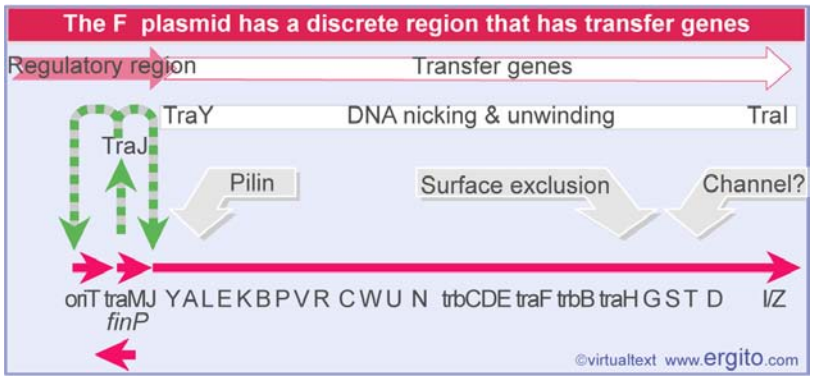

Figure 13.20 The *tra* region of the F plasmid contains the genes needed for bacterial conjugation.

F-positive bacteria possess surface appendages called pili (singular **pilus**) that are coded by the F factor. The gene *traA* codes for the single subunit protein, **pilin**, that is polymerized into the pilus. At least 12 *tra* genes are required for the modification and assembly of pilin into the pilus. The F-pili are hair-like structures, 2-3 µm long, that protrude from the bacterial surface. A typical F-positive cell has 2-3 pili. The pilin subunits are polymerized into a hollow cylinder, ~8 nm in diameter, with a 2 nm axial hole.

Mating is initiated when the tip of the F-pilus contacts the surface of the recipient cell. **Figure 13.21** shows an example of *E. coli* cells beginning to mate. A donor cell does not contact other cells carrying the F factor, because the genes *traS* and *traT*

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code for "surface exclusion" proteins that make the cell a poor recipient in such contacts. This effectively restricts donor cells to mating with F-negative cells. (And the presence of F-pili has secondary consequences; they provide the sites to which RNA phages and some single-stranded DNA phages attach, so F-positive bacteria are susceptible to infection by these phages, whereas F-negative bacteria are resistant.)

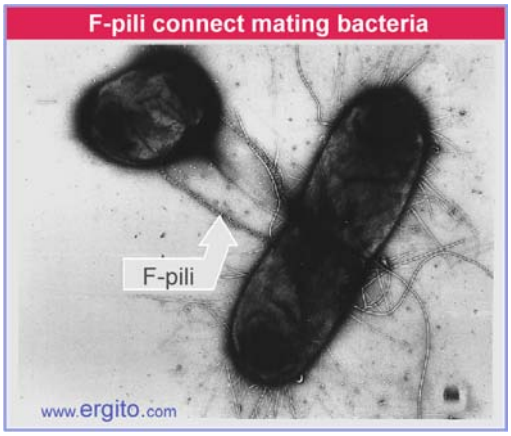

Figure 13.21 Mating bacteria are initially connected when donor F pili contact the recipient bacterium. Photograph kindly provided by Ron Skurray.

The initial contact between donor and recipient cells is easily broken, but other *tra* genes act to stabilize the association, bringing the mating cells closer together. The F pili are essential for initiating pairing, but retract or disassemble as part of the process by which the mating cells are brought into close contact. There must be a channel through which DNA is transferred, but the pilus itself does not appear to provide it. TraD is an inner membrane protein in F^+ bacteria that is necessary for transport of DNA and it may provide or be part of the channel.

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THE REPLICON

4.13.13 Conjugation transfers single-stranded DNA

Key Terms

An **Hfr** cell is a bacterium that has an integrated F plasmid within its chromosome. Hfr stands for high frequency recombination, referring to the fact that chromosomal genes are transferred from an Hfr cell to an $F^{\#}$ cell much more frequently than from an F^{+} cell.

Key Concepts

- Transfer of an F factor is initiated when rolling circle replication begins at *oriT*.
- The free 5′ end initiates transfer into the recipient bacterium.
- The transferred DNA is converted into double-stranded form in the recipient bacterium.
- When an F factor is free, conjugation "infects" the recipient bacterium with a copy of the F factor.
- When an F factor is integrated, conjugation causes transfer of the bacterial chromosome until the process is interrupted by (random) breakage of the contact between donor and recipient bacteria.

Transfer of the F factor is initiated at a site called *oriT*, the origin of transfer, which is located at one end of the transfer region. The transfer process may be initiated when TraM recognizes that a mating pair has formed. Then TraY binds near *oriT* and causes TraI to bind. TraI is a relaxase, like ϕX174 A protein. TraI nicks *oriT* at a unique site (called *nic*), and then forms a covalent link to the 5′ end that has been generated. TraI also catalyzes the unwinding of ~200 bp of DNA (this is a helicase activity; see *Molecular Biology 4.14.7 The ϕX model system shows how single-stranded DNA is generated for replication*). **Figure 13.22** shows that the freed 5′ end leads the way into the recipient bacterium. A complement for the transferred single strand is synthesized in the recipient bacterium, which as a result is converted to the F-positive state (for review see 138).

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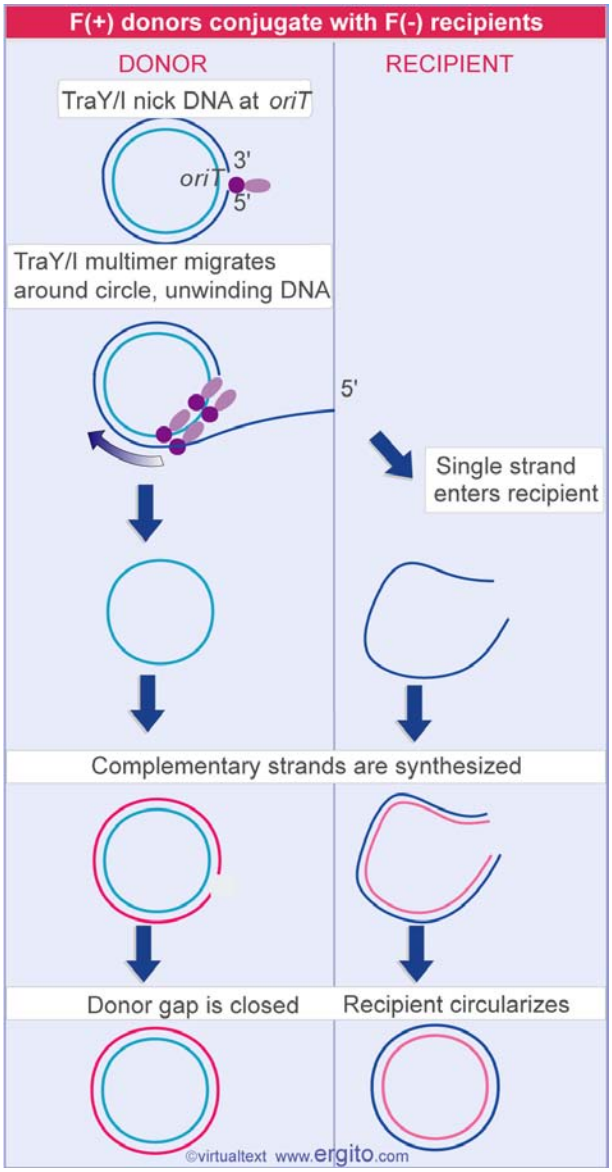

Figure 13.22 Transfer of DNA occurs when the F factor is nicked at *oriT* and a single strand is led by the 5′ end into the recipient. Only one unit length is transferred. Complementary strands are synthesized to the single strand remaining in the donor and to the strand transferred into the recipient.

A complementary strand must be synthesized in the donor bacterium to replace the strand that has been transferred. If this happens concomitantly with the transfer process, the state of the F plasmid will resemble the rolling circle of **Figure 13.16** (and will not generate the extensive single-stranded regions shown in **Figure 13.22**). Conjugating DNA usually appears like a rolling circle, but replication as such is not necessary to provide the driving energy, and single-strand transfer is independent of DNA synthesis. Only a single unit length of the F factor is transferred to the recipient bacterium. This implies that some (unidentified) feature terminates the process after one revolution, after which the covalent integrity of the F plasmid is restored (for review see 106; 111).

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When an integrated F plasmid initiates conjugation, the orientation of transfer is directed away from the transfer region, into the bacterial chromosome. **Figure 13.23** shows that, following a short leading sequence of F DNA, bacterial DNA is transferred. The process continues until it is interrupted by the breaking of contacts between the mating bacteria. It takes ~100 minutes to transfer the entire bacterial chromosome, and under standard conditions, contact is often broken before the completion of transfer (for review see 120).

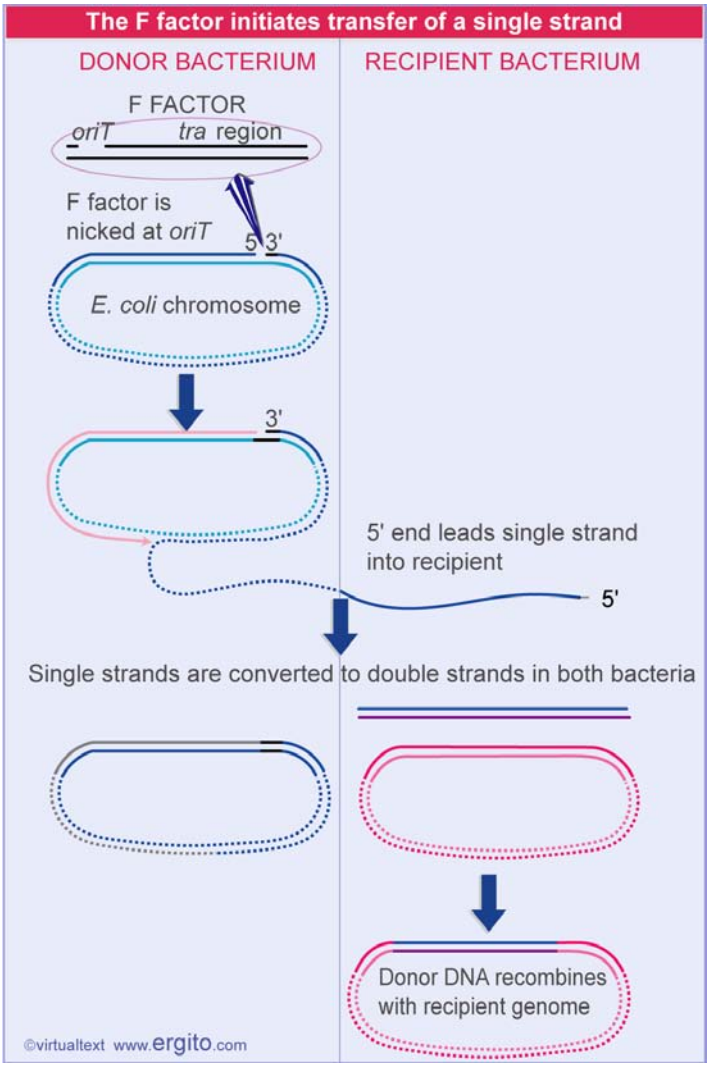

Figure 13.23 Transfer of chromosomal DNA occurs when an integrated F factor is nicked at *oriT*. Transfer of DNA starts with a short sequence of F DNA and continues until prevented by loss of contact between the bacteria.

Donor DNA that enters a recipient bacterium is converted to double-stranded form, and may recombine with the recipient chromosome. (Note that two recombination events are required to insert the donor DNA.) So conjugation affords a means to exchange genetic material between bacteria (a contrast with their usual asexual growth). A strain of *E. coli* with an integrated F factor supports such recombination at relatively high frequencies (compared to strains that lack integrated F factors);

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such strains are described as **Hfr** (for high frequency recombination). Each position of integration for the F factor gives rise to a different Hfr strain, with a characteristic pattern of transferring bacterial markers to a recipient chromosome.

Contact between conjugating bacteria is usually broken before transfer of DNA is complete. As a result, the probability that a region of the bacterial chromosome will be transferred depends upon its distance from *oriT*. Bacterial genes located close to the site of F integration (in the direction of transfer) enter recipient bacteria first, and are therefore found at greater frequencies than those located farther away that enter later. This gives rise to a gradient of transfer frequencies around the chromosome, declining from the position of F integration. Marker positions on the donor chromosome can be assayed in terms of the time at which transfer occurs, and this gave rise to the standard description of the *E. coli* chromosome as a map divided into 100 minutes. The map refers to transfer times from a particular Hfr strain; the starting point for the gradient of transfer is different for each Hfr strain, being determined by the site where the F factor has integrated into the bacterial genome.

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THE REPLICON

4.13.14 Replication is connected to the cell cycle

Key Terms

The **doubling time** is the period (usually measured in minutes) that it takes for a bacterial cell to reproduce.

A **multiforked chromosome** (in bacterium) has more than one replication fork, because a second initiation has occurred before the first cycle of replication has been completed.

The **unit cell** describes the state of an *E. coli* bacterium generated by a new division. It is 1.7 μm long and has a single replication origin.

Key Concepts

- The doubling time of *E. coli* can vary over a 10× range, depending on growth conditions.
- It requires 40 minutes to replicate the bacterial chromosome (at normal temperature).
- Completion of a replication cycle triggers a bacterial division 20 minutes later.
- If the doubling time is <60 minutes, a replication cycle is initiated before the division resulting from the previous replication cycle.
- Fast rates of growth therefore produce multiforked chromosomes.
- A replication cycle is initiated at a constant ratio of mass/number of chromosome origins.
- There is one origin per unit cell of 1.7m in length.

Bacteria have two links between replication and cell growth:

- The frequency of initiation of cycles of replication is adjusted to fit the rate at which the cell is growing.
- The completion of a replication cycle is connected with division of the cell.

The rate of bacterial growth is assessed by the **doubling time**, the period required for the number of cells to double. The shorter the doubling time, the faster the growth rate. *E. coli* cells can grow at rates ranging from doubling times as fast as 18 minutes to slower than 180 minutes. Because the bacterial chromosome is a single replicon, the frequency of replication cycles is controlled by the number of initiation events at the single origin. The replication cycle can be defined in terms of two constants:

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- *C* is the fixed time of ~40 minutes required to replicate the entire bacterial chromosome. Its duration corresponds to a rate of replication fork movement of ~50,000 bp/minute. (The rate of DNA synthesis is more or less invariant at a constant temperature; it proceeds at the same speed unless and until the supply of precursors becomes limiting.)

- *D* is the fixed time of ~20 minutes that elapses between the completion of a round of replication and the cell division with which it is connected. This period may represent the time required to assemble the components needed for division.

(The constants *C* and *D* can be viewed as representing the maximum speed with which the bacterium is capable of completing these processes. They apply for all growth rates between doubling times of 18 and 60 minutes, but both constant phases become longer when the cell cycle occupies >60 minutes.)

A cycle of chromosome replication must be initiated a fixed time before a cell division, $C + D = 60$ minutes. For bacteria dividing more frequently than every 60 minutes, a cycle of replication must be initiated before the end of the preceding division cycle.

Consider the example of cells dividing every 35 minutes. The cycle of replication connected with a division must have been initiated 25 minutes before the preceding division. This situation is illustrated in **Figure 13.24**, which shows the chromosomal complement of a bacterial cell at 5-minute intervals throughout the cycle.

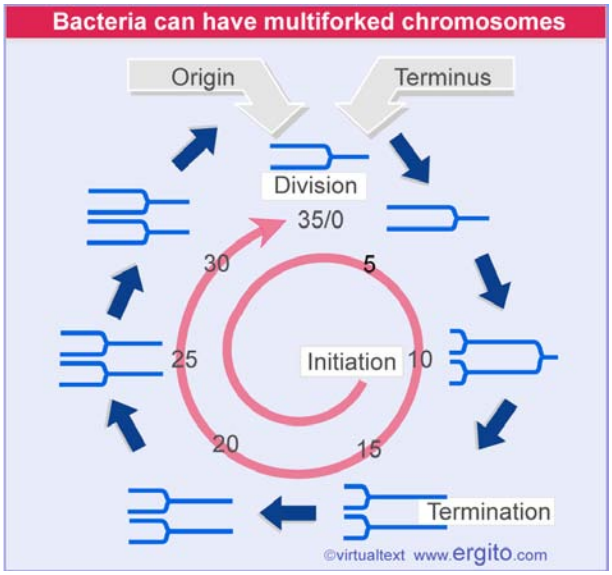

Figure 13.24 The fixed interval of 60 minutes between initiation of replication and cell division produces multiforked chromosomes in rapidly growing cells. Note that only the replication forks moving in one direction are shown; actually the chromosome is replicated symmetrically by two sets of forks moving in opposite directions on circular chromosomes.

At division (35/0 minutes), the cell receives a partially replicated chromosome. The

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replication fork continues to advance. At 10 minutes, when this "old" replication fork has not yet reached the terminus, initiation occurs at both origins on the partially replicated chromosome. The start of these "new" replication forks creates a **multiforked chromosome**.

At 15 minutes – that is, at 20 minutes before the next division – the old replication fork reaches the terminus. Its arrival allows the two daughter chromosomes to separate; each of them has already been partially replicated by the new replication forks (which now are the only replication forks). These forks continue to advance.

At the point of division, the two partially replicated chromosomes segregate. This recreates the point at which we started. The single replication fork becomes "old," it terminates at 15 minutes, and 20 minutes later there is a division. We see that the initiation event occurs $1\ ^{25}/_{35}$ cell cycles before the division event with which it is associated.

The general principle of the link between initiation and the cell cycle is that, as cells grow more rapidly (the cycle is shorter), the initiation event occurs an increasing number of cycles before the related division. There are correspondingly more chromosomes in the individual bacterium. This relationship can be viewed as the cell's response to its inability to reduce the periods of C and D to keep pace with the shorter cycle.

How does the cell know when to initiate the replication cycle? The initiation event occurs at a constant ratio of cell mass to the number of chromosome origins. Cells growing more rapidly are larger and possess a greater number of origins. The growth of the bacterium can be described in terms of the **unit cell**, an entity 1.7 µm long. A bacterium contains one origin per unit cell; a rapidly growing cell with two origins will be 1.7-3.4 µm long. In terms of **Figure 13.24**, it is at the point 10 minutes after division that the cell mass has increased sufficiently to support an initiation event at both available origins (543; 544).

How is cell mass titrated? An initiator protein could be synthesized continuously throughout the cell cycle; accumulation of a critical amount would trigger initiation. This explains why protein synthesis is needed for the initiation event. An alternative possibility is that an inhibitor protein might be synthesized at a fixed point, and diluted below an effective level by the increase in cell volume (for review see 117).

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THE REPLICON

4.13.15 The septum divides a bacterium into progeny each containing a chromosome

Key Terms

A **septum** is the structure that forms in the center of a dividing bacterium, providing the site at which the daughter bacteria will separate. The same term is used to describe the cell wall that forms between plant cells at the end of mitosis.

A **periseptal annulus** is an ring-like area where inner and outer membrane appear fused. Formed around the circumference of the bacterium, the periseptal annulus determines the location of the septum.

Key Concepts

- Septum formation is initiated at the annulus, which is a ring around the cell where the structure of the envelope is altered.
- New annuli are initiated at 50% of the distance from the septum to each end of the bacterium.
- When the bacterium divides, each daughter has an annulus at the mid center position.
- Septation starts when the cell reaches a fixed length.
- The septum consists of the same peptidoglycans that comprise the bacterial envelope.

Chromosome segregation in bacteria is especially interesting because the DNA itself is involved in the mechanism for partition. (This contrasts with eukaryotic cells, in which segregation is achieved by the complex apparatus of mitosis.) The bacterial apparatus is quite accurate, however; anucleate cells form <0.03% of a bacterial population.

The division of a bacterium into two daughter cells is accomplished by the formation of a **septum**, a structure that forms in the center of the cell as an invagination from the surrounding envelope. The septum forms an impenetrable barrier between the two parts of the cell and provides the site at which the two daughter cells eventually separate entirely. Two related questions address the role of the septum in division: what determines the location at which it forms; and what ensures that the daughter chromosomes lie on opposite sides of it?

The formation of the septum is preceded by the organization of the **periseptal annulus**. This is observed as a zone in *E. coli* or *S. typhimurium* in which the structure of the envelope is altered so that the inner membrane is connected more closely to the cell wall and outer membrane layer. As its name suggests, the annulus extends around the cell. **Figure 13.25** summarizes its development (for review see

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112).

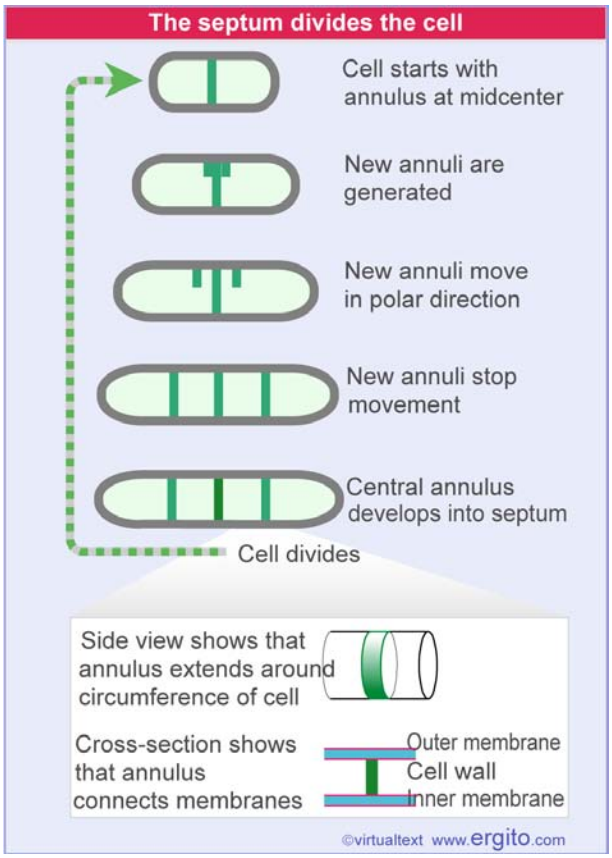

Figure 13.25 Duplication and displacement of the periseptal annulus give rise to the formation of a septum that divides the cell.
This is a static version of an interactive figure; see http://www.ergito.com/main.jsp?bcs=MBIO.4.13.15 to view properly.

The annulus starts at a central position in a new cell. As the cell grows, two events occur. A septum forms at the midcell position defined by the annulus. And new annuli form on either side of the initial annulus. These new annuli are displaced from the center and move along the cell to positions at 1/4 and 3/4 of the cell length. These will become the midcell positions after the next division. The displacement of the periseptal annulus to the correct position may be the crucial event that ensures the division of the cell into daughters of equal size. (The mechanism of movement is unknown.) Septation begins when the cell reaches a fixed length (2L), and the distance between the new annuli is always L. We do not know how the cell measures length, but the relevant parameter appears to be linear distance as such (not area or volume).

The septum consists of the same components as the cell envelope: there is a rigid layer of peptidoglycan in the periplasm, between the inner and outer membranes. The peptidoglycan is made by polymerization of tri- or pentapeptide-disaccharide units in a reaction involving connections between both types of subunit (transpeptidation and transglycosylation). The rod-like shape of the bacterium is maintained by a pair of

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activities, PBP2 and RodA. They are interacting proteins, coded by the same operon. RodA is a member of the SEDS family (SEDS stands for shape, elongation, division, and sporulation) that is present in all bacteria that have a peptidoglycan cell wall. Each SEDS protein functions together with a specific transpeptidase, which catalyzes the formation of the cross-links in the peptidoglycan. PBP2 (penicillin-binding protein 2) is the transpeptidase that interacts with RodA. Mutations in the gene for either protein cause the bacterium to lose its extended shape, becoming round (3082). This demonstrates the important principle that shape and rigidity can be determined by the simple extension of a polymeric structure. Another enzyme is responsible for generating the peptidoglycan in the septum (see *Molecular Biology 4.13.17 FtsZ is necessary for septum formation*). The septum initially forms as a double layer of peptidoglycan, and the protein EnvA is required to split the covalent links between the layers, so that the daughter cells may separate.

The behavior of the periseptal annulus suggests that the mechanism for measuring position is associated with the cell envelope. It is plausible to suppose that the envelope could also be used to ensure segregation of the chromosomes. A direct link between DNA and the membrane could account for segregation. If daughter chromosomes are attached to the membrane, they could be physically separated when the septum forms. **Figure 13.26** shows that the formation of a septum could segregate the chromosomes into the different daughter cells if the origins are connected to sites that lie on either side of the periseptal annulus.

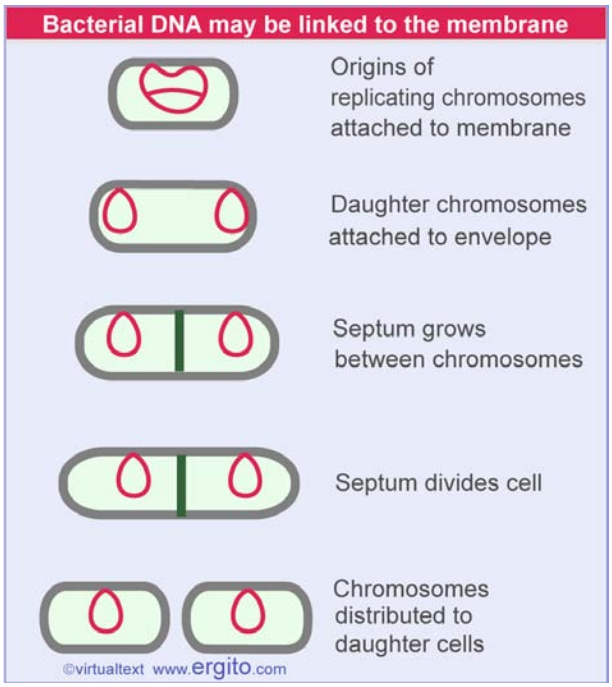

Figure 13.26 Attachment of bacterial DNA to the membrane could provide a mechanism for segregation.

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THE REPLICON

4.13.16 Mutations in division or segregation affect cell shape

Key Terms

A **minicell** is an anucleate bacterial (*E. coli*) cell produced by a division that generates a cytoplasm without a nucleus.

Anucleate bacteria lack a nucleoid, but are of similar shape to wild-type bacteria.

Key Concepts

- *fts* mutants form long filaments because the septum fails to form to divide the daughter bacteria.
- Minicells form in mutants that produce too many septa; they are small and lack DNA.
- Anucleate cells of normal size are generated by partition mutants in which the duplicate chromosomes fail to separate.

A difficulty in isolating mutants that affect cell division is that mutations in the critical functions may be lethal and/or pleiotropic. For example, if formation of the annulus occurs at a site that is essential for overall growth of the envelope, it would be difficult to distinguish mutations that specifically interfere with annulus formation from those that inhibit envelope growth generally. Most mutations in the division apparatus have been identified as conditional mutants (whose division is affected under nonpermissive conditions; typically they are temperature sensitive). Mutations that affect cell division or chromosome segregation cause striking phenotypic changes. **Figure 13.27** and **Figure 13.28** illustrate the opposite consequences of failure in the division process and failure in segregation:

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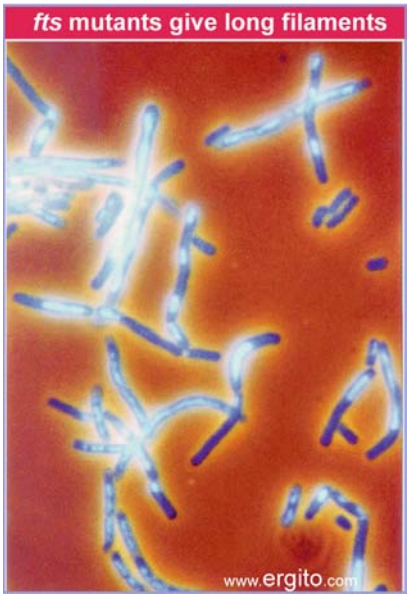

Figure 13.27 Failure of cell division generates multinucleated filaments. Photograph kindly provided by Sota Hiraga.

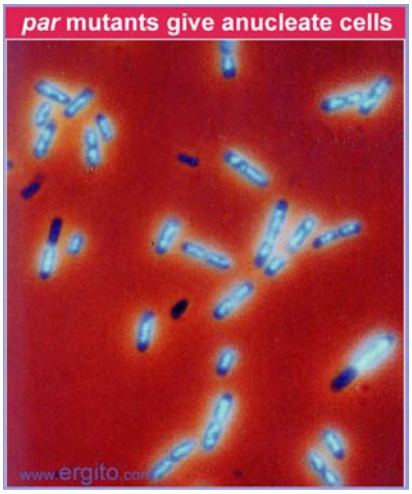

Figure 13.28 *E. coli* generate anucleate cells when chromosome segregation fails. Cells with chromosomes stain blue; daughter cells lacking chromosomes have no blue stain. This field shows cells of the *mukB* mutant; both normal and abnormal divisions can be seen. Photograph kindly provided by Sota Hiraga.

- Long *filament*s form when septum formation is inhibited, but chromosome replication is unaffected. The bacteria continue to grow, and even continue to segregate their daughter chromosomes, but septa do not form, so the cell consists of a very long filamentous structure, with the nucleoids (bacterial chromosomes)

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regularly distributed along the length of the cell. This phenotype is displayed by *fts* mutants (named for temperature-sensitive filamentation), which identify defect(s) that lie in the division process itself.

- **Minicells** form when septum formation occurs too frequently or in the wrong place, with the result that one of the new daughter cells lacks a chromosome. The minicell has a rather small size, and lacks DNA, but otherwise appears morphologically normal. **Anucleate** cells form when segregation is aberrant; like minicells, they lack a chromosome, but because septum formation is normal, their size is unaltered. This phenotype is caused by *par* (partition) mutants (named because they are defective in chromosome segregation) (542).

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THE REPLICON

4.13.17 FtsZ is necessary for septum formation

Key Terms

The **septal ring (Z-ring)** is a complex of several proteins coded by *fts* genes of *E. coli* that forms at the mid-point of the cell. It gives rise to the septum at cell division. The first of the proteins to be incorporated is FtsZ, which gave rise to the original name of the Z-ring.

Key Concepts

- The product of *ftsZ* is required for septum formation at pre-existing sites.
- FtsZ is a GTPase that forms a ring on the inside of the bacterial envelope. It is connected to other cytoskeletal components.

The gene *ftsZ* plays a central role in division. Mutations in *ftsZ* block septum formation and generate filaments. Overexpression induces minicells, by causing an increased number of septation events per unit cell mass. *ftsZ* mutants act at stages varying from the displacement of the periseptal annuli to septal morphogenesis. FtsZ is therefore required for usage of pre-existing sites for septum formation, but does not itself affect the formation of the periseptal annuli or their localization.

FtsZ functions at an early stage of septum formation. Early in the division cycle, FtsZ is localized throughout the cytoplasm. As the cell elongates and begins to constrict in the middle, FtsZ becomes localized in a ring around the circumference (3077). The structure is sometimes called the **Z-ring**. **Figure 13.29** shows that it lies in the position of the mid-center annulus of **Figure 13.25**. The formation of the Z-ring is the rate-limiting step in septum formation. In a typical division cycle, it forms in the center of cell 1-5 min after division, remains for 15 min, and then quickly constricts to pinch the cell into two.

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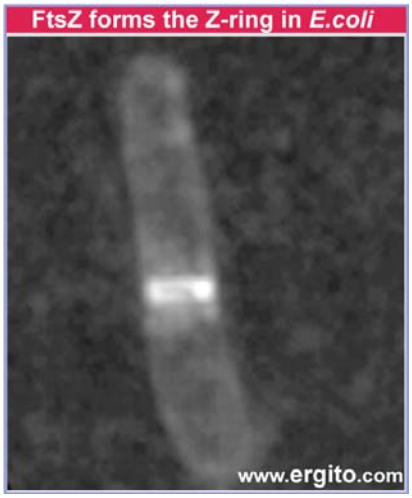

Figure 13.29 Immunofluorescence with an antibody against FtsZ shows that it is localized at the mid-cell. Photograph kindly provided by William Margolin (see 3076).

The structure of FtsZ resembles tubulin, suggesting that assembly of the ring could resemble the formation of microtubules in eukaryotic cells. FtsZ has GTPase activity, and GTP cleavage is used to support the oligomerization of FtsZ monomers into the ring structure. The Z-ring is a dynamic structure, in which there is continuous exchange of subunits with a cytoplasmic pool (3078; for reveiw see 4526).

Two other proteins needed for division, ZipA and FtsA, interact directly and independently with FtsZ (3079). ZipA is an integral membrane protein, located in the inner bacterial membrane. It provides the means for linking FtsZ to the membrane. FtsA is a cytosolic protein, but is often found associated with the membrane. The Z-ring can form in the absence of either ZipA or FtsA, but cannot form if both are absent (3080). This suggests that they have overlapping roles in stabilizing the Z-ring, and perhaps in linking it to the membrane.

The products of several other *fts* genes join the Z-ring in a defined order after FtsA has been incorporated (for review see 123; 982). They are all transmembrane proteins. The final structure is sometimes called the **septal ring**. It consists of a multiprotein complex that is presumed to have the ability to constrict the membrane. One of the last components to be incorporated into the septal ring is FtsW, which is a protein belonging to the SEDS family *ftsW* is expressed as part of an operon with *ftsI*, which codes for a transpeptidase (also called PBP3 for penicillin-binding protein 3), a membrane-bound protein that has its catalytic site in the periplasm. FtsW is responsible for incorporating FtsI into the septal ring (3081). This suggests a model for septum formation in which the transpeptidase activity then causes the peptidoglycan to grow inward, thus pushing the inner membrane and pulling the outer membrane.

FtsZ is the major cytoskeletal component of septation. It is common in bacteria, and is found also in chloroplasts. **Figure 13.30** shows the localization of the plant homologues to a ring at the mid-point of the chloroplast. Chloroplasts also have other genes related to the bacterial division genes. Consistent with the common

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evolutionary origins of bacteria and chloroplasts, the apparatus for division seems generally to have been conserved.

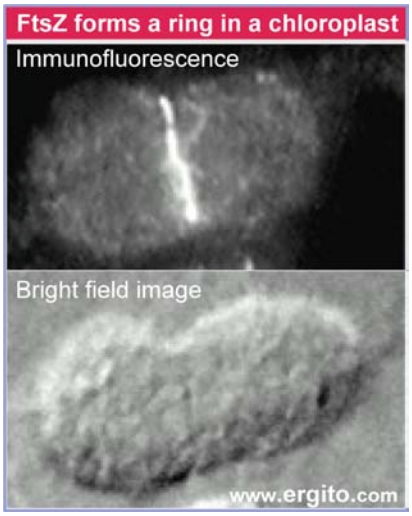

Figure 13.30 Immunofluorescence with antibodies against the *Arabidopsis* proteins FtsZ1 and FtsZ2 show that they are localized at the mid point of the chloroplast (top panel). The bright field image (lower panel) shows the outline of the chloroplast more clearly. Photograph kindly provided by Katherine Osteryoung (see 3075).

Mitochondria, which also share an evolutionary origin with bacteria, usually do not have FtsZ. Instead, they use a variant of the protein dynamin, which is involved in pinching off vesicles from membranes of eukaryotic cytoplasm (see *Molecular Biology 6.27.5 Different types of coated vesicles exist in each pathway*). This functions from the outside of the organelle, squeezing the membrane to generate a constriction.

The common feature, then, in the division of bacteria, chloroplasts, and mitochondria is the use of a cytoskeletal protein that forms a ring round the organelle, and either pulls or pushes the membrane to form a constriction.

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THE REPLICON

4.13.18 *min* genes regulate the location of the septum

Key Concepts

- The location of the septum is controlled by *minC,D,E*.
- The number and location of septa is determined by the ratio of MinE/MinC,D.
- The septum forms where MinE is able to form a ring.
- At normal concentrations, MinC/D allows a mid-center ring, but prevents additional rings of MinE from forming at the poles.

Information about the localization of the septum is provided by minicell mutants. The original minicell mutation lies in the locus *minB*; deletion of *minB* generates minicells by allowing septation to occur at the poles as well as (or instead of) at midcell. This suggests that the cell possesses the ability to initiate septum formation either at midcell or at the poles; and the role of the wild-type *minB* locus is to suppress septation at the poles. In terms of the events depicted in **Figure 13.25**, this implies that a new born cell has potential septation sites associated both with the annulus at mid-center and with the poles. One pole was formed from the septum of the previous division; the other pole represents the septum from the division before that. Perhaps the poles retain remnants of the annuli from which they were derived, and these remnants can nucleate septation.

The *minB* locus consists of three genes, *minC,D,E*. Their roles are summarized in **Figure 13.31**. The products of *minC* and *minD* form a division inhibitor. MinD is required to activate MinC, which prevents FtsZ from polymerizing into the Z-ring (3084; 3083).

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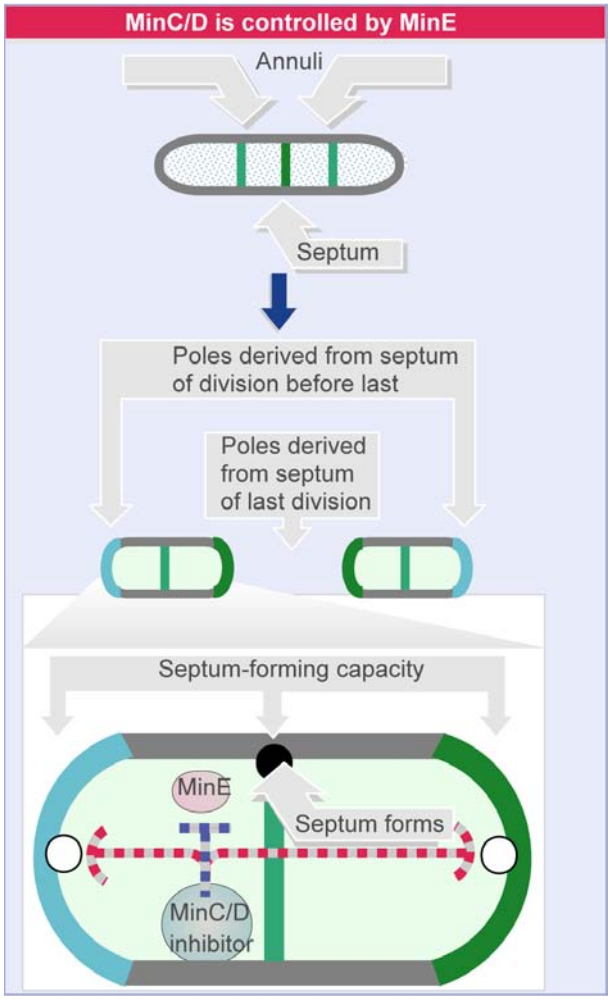

Figure 13.31 MinC/D is a division inhibitor, whose action is confined to the polar sites by MinE.

Expression of MinCD in the absence of MinE, or overexpression even in the presence of MinE, causes a generalized inhibition of division. The resulting cells grow as long filaments without septa. Expression of MinE at levels comparable to MinCD confines the inhibition to the polar regions, so restoring normal growth. MinE protects the midcell sites from inhibition. Overexpression of MinE induces minicells, because the presence of excess MinE counteracts the inhibition at the poles as well as at midcell, allowing septa to form at both locations (546).

The determinant of septation at the proper (midcell) site is therefore the ratio of MinCD to MinE. The wild-type level prevents polar septation, while permitting midcell septation. The effects of MinC/D and MinE are inversely related; absence of MinCD or too much MinE causes indiscriminate septation, forming minicells; too much MinCD or absence of MinE inhibits midcell as well as polar sites, resulting in filamentation.

MinE forms a ring at the septal position. Its accumulation suppresses the action of MinCD in the vicinity, thus allowing formation of the septal ring (which includes FtsZ and ZipA). Curiously, MinD is required for formation of the MinE ring (548).

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THE REPLICON

4.13.19 Chromosomal segregation may require site-specific recombination

Key Terms

Site-specific recombination (Specialized recombination) occurs between two specific sequences, as in phage integration/excision or resolution of cointegrate structures during transposition.

Key Concepts

- The Xer site-specific recombination system acts on a target sequence near the chromosome terminus to recreate monomers if a generalized recombination event has converted the bacterial chromosome to a dimer.

Because the multiple copies of a plasmid in a bacterium consist of the same DNA sequences, they are able to recombine. **Figure 13.32** demonstrates the consequences. A single intermolecular recombination event between two circles generates a dimeric circle; further recombination can generate higher multimeric forms. Such an event reduces the number of physically segregating units. In the extreme case of a single-copy plasmid that has just replicated, formation of a dimer by recombination means that the cell only has one unit to segregate, and the plasmid therefore must inevitably be lost from one daughter cell. To counteract this effect, plasmids often have **site-specific recombination** systems that act upon particular sequences to sponsor an intramolecular recombination that restores the monomeric condition.

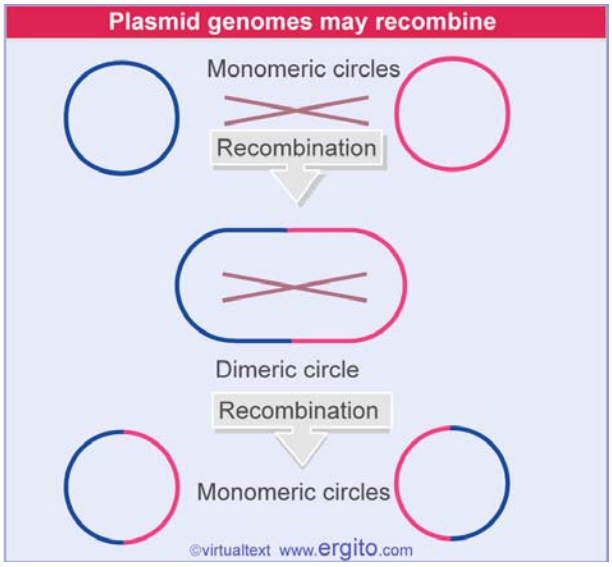

Figure 13.32 Intermolecular recombination merges monomers into dimers, and intramolecular recombination releases individual units from oligomers.

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The same types of event can occur with the bacterial chromosome, and **Figure 13.33** shows how they affect its segregation If no recombination occurs, there is no problem, and the separate daughter chromosomes can segregate to the daughter cells. But a dimer will be produced if homologous recombination occurs between the daughter chromosomes produced by a replication cycle. If there has been such a recombination event, the daughter chromosomes cannot separate. In this case, a second recombination is required to achieve resolution in the same way as a plasmid dimer.

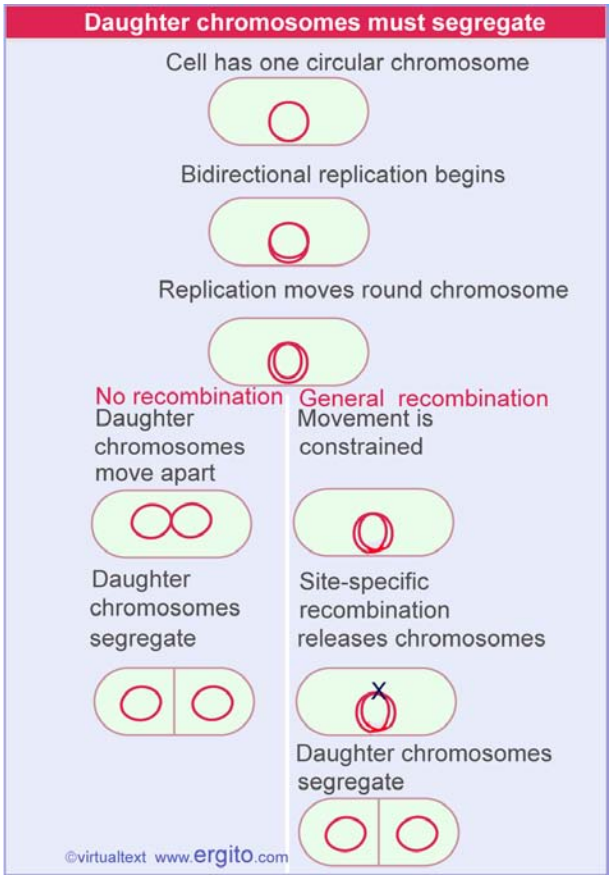

Figure 13.33 A circular chromosome replicates to produce two monomeric daughters that segregate to daughter cells. However, a generalized recombination event generates a single dimeric molecule. This can be resolved into two monomers by a site-specific recombination.

Most bacteria with circular chromosomes posses the Xer site-specific recombination system. In *E. coli,* this consists of two recombinases, XerC and XerD, which act on a 28 bp target site, called *dif*, that is located in the terminus region of the chromosome (3127). The use of the Xer system is related in an interesting way to cell division. The relevant events are summarized in **Figure 13.34**. XerC can bind to a pair of *dif* sequences and form a Holliday junction between them. The complex may form soon after the replication fork passes over the *dif* sequence, which explains how the two copies of the target sequence can find one another consistently. However, resolution of the junction to give recombinants occurs only in the presence of FtsK, a protein located in the septum that is required for chromosome segregation and cell division

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(1671). Also, the *dif* target sequence must be located in a region of ~30 kb; if it is moved outside of this region, it cannot support the reaction.

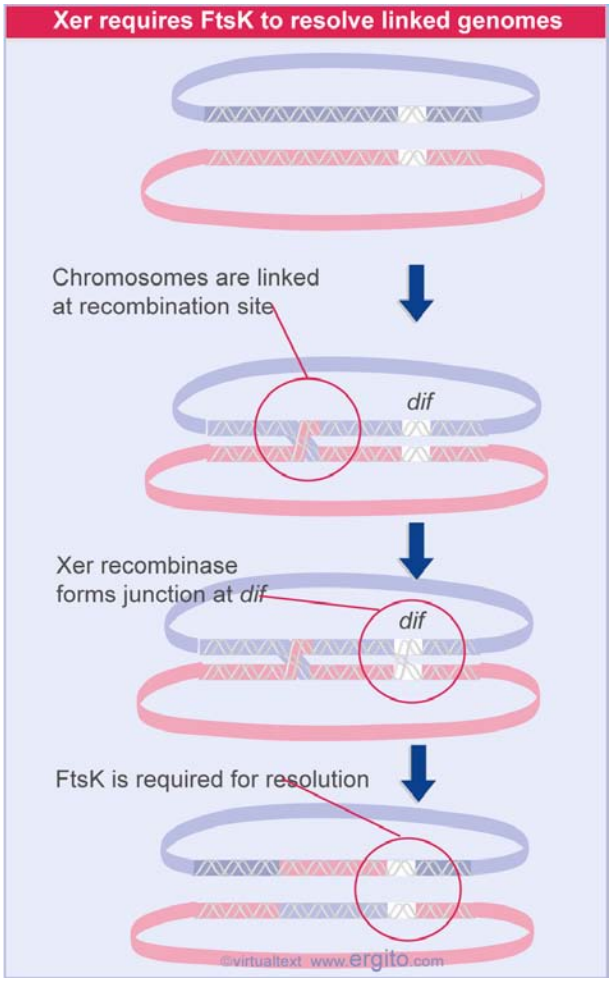

Figure 13.34 A recombination event creates two linked chromosomes. Xer creates a Holliday junction at the *dif* site, but can resolve it only in the presence of FtsK.

So there is a site-specific recombination available when the terminus sequence of the chromosome is close to the septum. But the bacterium wants to have a recombination only when there has already been a general recombination event to generate a dimer. (Otherwise the site-specific recombination would create the dimer!) How does the system know whether the daughter chromosomes exist as independent monomers or have been recombined into a dimer?

The answer may be that segregation of chromosomes starts soon after replication. If there has been no recombination, the two chromosomes move apart from one another. But the ability of the relevant sequences to move apart from one another may be constrained if a dimer has been formed. This forces them to remain in the vicinity of the septum, where they are exposed to the Xer system.

Bacteria that have the Xer system always have an FtsK homolog, and vice-versa,

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which suggests that the system has evolved so that resolution is connected to the septum. FtsK is a large transmembrane protein. Its N-terminal domain is associated with the membrane, and causes it to be localized to the septum. Its C-terminal domain has two functions (2400). One is to causes Xer to resolve a dimer into two monomers. It also has an ATPase activity, which it can use to translocate along DNA *in vitro*. This could be used to pump DNA through the septum (in the same way that SpoIIIE transports DNA from the mother compartment into the prespore during sporulation (see *Molecular Biology 4.13.20 Partitioning involves separation of the chromosomes*).

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THE REPLICON

4.13.20 Partitioning involves separation of the chromosomes

Key Concepts

- Replicon origins may be attached to the inner bacterial membrane
- Chromosomes make abrupt movements from the mid-center to the ¼ and ¾ positions.

Partitioning is the process by which the two daughter chromosomes find themselves on either side of the position at which the septum forms. Two types of event are required for proper partitioning:

- The two daughter chromosomes must be released from one another so that they can segregate following termination. This requires disentangling of DNA regions that are coiled around each other in the vicinity of the terminus. Most mutations affecting partitioning map in genes coding for topoisomerases-enzymes with the ability to pass DNA strands through one another. The mutations prevent the daughter chromosomes from segregating, with the result that the DNA is located in a single large mass at midcell. Septum formation then releases an anucleate cell and a cell containing both daughter chromosomes. This tells us that the bacterium must be able to disentangle its chromosomes topologically in order to be able to segregate them into different daughter cells.
- Mutations that affect the partition process itself are rare. We expect to find two classes. *cis*-acting mutations should occur in DNA sequences that are the targets for the partition process. *trans*-acting mutations should occur in genes that code for the protein(s) that cause segregation, which could include proteins that bind to DNA or activities that control the locations on the envelope to which DNA might be attached. Both types of mutation have been found in the systems responsible for partitioning plasmids but only *trans*-acting functions have been found in the bacterial chromosome (for review see 115; 121). In addition, mutations in plasmid site-specific recombination systems increase plasmid loss (because the dividing cell has only one dimer to partition instead of two monomers), and therefore have a phenotype that is similar to partition mutants.

The original form of the model for chromosome segregation shown in **Figure 13.26** suggested that the envelope grows by insertion of material between the attachment sites of the two chromosomes, thus pushing them apart. But in fact the cell wall and membrane grow heterogeneously over the whole cell surface. Furthermore, the replicated chromosomes are capable of abrupt movements to their final positions at ¼ and ¾ cell length. If protein synthesis is inhibited before the termination of replication, the chromosomes fail to segregate and remain close to the midcell position. But when protein synthesis is allowed to resume, the chromosomes move to the quarter positions in the absence of any further envelope elongation. This suggests

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that an active process, requiring protein synthesis, may move the chromosomes to specific locations (for review see 3126; 3125).

Segregation is interrupted by mutations of the *muk* class, which give rise to anucleate progeny at a much increased frequency: both daughter chromosomes remain on the same side of the septum instead of segregating. Mutations in the *muk* genes are not lethal, and may identify components of the apparatus that segregates the chromosomes. The gene *mukA* is identical with the gene for a known outer membrane protein (*tolC*), whose product could be involved with attaching the chromosome to the envelope. The gene *mukB* codes for a large (180 kD) globular protein, which has the same general type of organization as the two groups of SMC proteins that are involved in condensing and in holding together eukaryotic chromosomes (see *Molecular Biology 5.23.18 Chromosome condensation is caused by condensins*). SMC-like proteins have also been found in other bacteria. (MukB also has some sequence relationship to the mechanochemical enzyme dynamin, which provides a "motor" for microtubule-associated objects, but it is now thought that this is not significant.)

The insight into the role of MukB was the discovery that some mutations in *mukB* can be suppressed by mutations in *topA*, the gene coding for topoisomerase I (2461). This led to the model that the function of MukBEF proteins is to condense the nucleoid. A defect in this function is the cause of failure to segregate properly. The defect can be compensated by preventing topoisomerases from relaxing negative supercoils; the resulting increase in supercoil density helps to restore the proper state of condensation and thus to allow segregation.

We still do not understand how genomes are positioned in the cell, but the process may be connected with condensation. **Figure 13.35** shows a current model. The parental genome is centrally positioned. It must be decondensed in order to pass through the replication apparatus. The daughter chromosomes emerge from replication, are disentangled by topoisomerases, and then passed in an uncondensed state to MukBEF, which causes them to form condensed masses at the positions that will become the centers of the daughter cells.

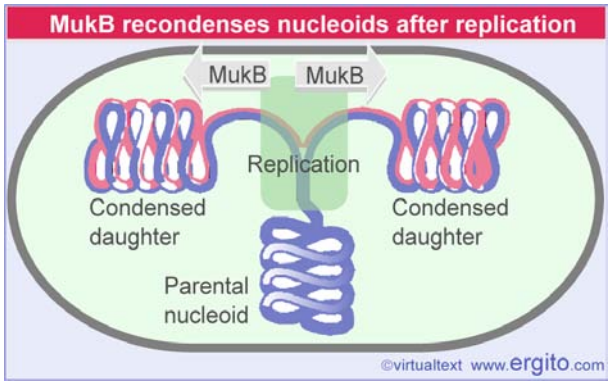

Figure 13.35 The DNA of a single parental nucleoid becomes decondensed during replication. MukB is an essential component of the apparatus that recondenses the daughter nucleoids. (After 2462.)

There have been suspicions for years that a physical link exists between bacterial

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DNA and the membrane, but the evidence remains indirect. Bacterial DNA can be found in membrane fractions, which tend to be enriched in genetic markers near the origin, the replication fork, and the terminus. The proteins present in these membrane fractions may be affected by mutations that interfere with the initiation of replication. The growth site could be a structure on the membrane to which the origin must be attached for initiation (536).

During sporulation in *B. subtilis*, one daughter chromosome must be segregated into the small forespore compartment (see **Figure 9.41**). This is an unusual process that involves transfer of the chromosome across the nascent septum. One of the sporulation genes, *spoIIIE*, is required for this process. The SpoIIIE protein is located at the septum and is probably has a translocation function that pumps DNA through (2224; for review see 3448).

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THE REPLICON

4.13.21 Single-copy plasmids have a partitioning system

Key Terms

The **copy number** is the number of copies of a plasmid that is maintained in a bacterium (relative to the number of copies of the origin of the bacterial chromosome).

An **addiction system** is a survival mechanism used by plasmids. The mechanism kills the bacterium upon loss of the plasmid.

Key Concepts

- Single copy plasmids exist at one plasmid copy per bacterial chromosome origin.
- Multicopy plasmids exist at >1 plasmid copy per bacterial chromosome origin.
- Homologous recombination between circular plasmids generates dimers and higher multimers.
- Plasmids have site-specific recombination systems that undertake intramolecular recombination to regenerate monomers.
- Partition systems ensure that duplicate plasmids are segregated to different daughter cells produced by a division.

The type of system that a plasmid uses to ensure that it is distributed to both daughter cells at division depends upon its type of replication system. Each type of plasmid is maintained in its bacterial host at a characteristic **copy number**:

- Single-copy control systems resemble that of the bacterial chromosome and result in one replication per cell division. A single-copy plasmid effectively maintains parity with the bacterial chromosome.
- Multicopy control systems allow multiple initiation events per cell cycle, with the result that there are several copies of the plasmid per bacterium. Multicopy plasmids exist in a characteristic number (typically 10-20) per bacterial chromosome.

Copy number is primarily a consequence of the type of replication control mechanism. The system responsible for initiating replication determines how many origins can be present in the bacterium. Since each plasmid consists of a single replicon, the number of origins is the same as the number of plasmid molecules.

Single-copy plasmids have a system for replication control whose consequences are similar to that governing the bacterial chromosome. A single origin can be replicated once; then the daughter origins are segregated to the different daughter cells.

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Multicopy plasmids have a replication system that allows a pool of origins to exist. If the number is great enough (in practice >10 per bacterium), an active segregation system becomes unnecessary, because even a statistical distribution of plasmids to daughter cells will result in the loss of plasmids at frequencies $<10^{-6}$.

Plasmids are maintained in bacterial populations with very low rates of loss ($<10^{-7}$ per cell division is typical, even for a single-copy plasmid). The systems that control plasmid segregation can be identified by mutations that increase the frequency of loss, but that do not act upon replication itself. Several types of mechanism are used to ensure the survival of a plasmid in a bacterial population. It is common for a plasmid to carry several systems, often of different types, all acting independently to ensure its survival. Some of these systems act indirectly, while others are concerned directly with regulating the partition event. However, in terms of evolution, all serve the same purpose: to help ensure perpetuation of the plasmid to the maximum number of progeny bacteria.

Single-copy plasmids require partitioning systems to ensure that the duplicate copies find themselves on opposite sides of the septum at cell division, and are therefore segregated to a different daughter cell. In fact, functions involved in partitioning were first identified in plasmids. The components of a common system are summarized in **Figure 13.36**. Typically there are two *trans*-acting loci (*parA* and *parB*)and a *cis*-acting element (*parS*) located just downstream of the two genes. ParA is an ATPase. It binds to ParB, which binds to the *parS* site on DNA (3086). Deletions of any of the three loci prevent proper partition of the plasmid. Systems of this type have been characterized for the plasmids F, P1, and R1. In spite of their overall similarities, there are no significant sequence homologies between the corresponding genes or *cis*-acting sites (for review see 115; 3126; 3125).

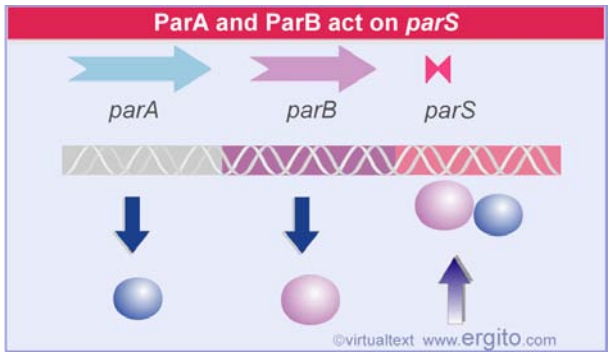

Figure 13.36 A common segregation system consists of genes *parA* and *parB* and the target site *parS*.

parS plays a role for the plasmid that is equivalent to the centromere in a eukaryotic cell. Binding of the ParB protein to it creates a structure that segregates the plasmid copies to opposite daughter cells. A bacterial protein, IHF, also binds at this site to form part of the structure (3087). The complex of ParB and IHF with *parS* is called the partition complex. *parS* is a 34 bp sequence containing the IHF-binding site flanked on either side by sequences called *boxA* and *boxB* that are bound by ParB.

IHF is the integration host factor, named for the role in which it was first discovered (forming a structure that is involved in the integration of phage lambda DNA into the

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host chromosome). IHF is a heterodimer with has the capacity to form a large structure in which DNA is wrapped on the surface. The role of IHF is to bend the DNA so that ParB can bind simultaneously to the separated *boxA* and *boxB* sites, as indicated in **Figure 13.37**. Complex formation is initiated when *parS* is bound by a heterodimer of IHF together with a dimer of ParB (3088). This enables further dimers of ParB to bind cooperatively. The interaction of ParA with the partition complex structure is essential but transient.

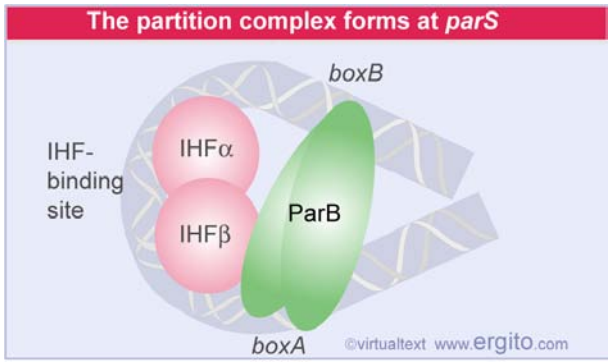

Figure 13.37 The partition complex is formed when IHF binds to DNA at *parS* and bends it so that ParB can bind to sites on either side. The complex is initiated by a heterodimer of IHF and a dimer of ParB, and then more ParB dimers bind.

The protein-DNA complex that assembles on IHF during phage lambda integration binds two DNA molecules to enable them to recombine (see *Molecular Biology 4.15.19 Lambda recombination occurs in an intasome*). The role of the partition complex is different: to ensure that two DNA molecules segregate apart from one another. We do not know yet how the formation of the individual complex accomplishes this task. One possibility is that it attaches the DNA to some physical site – for example, on the membrane – and then the sites of attachment are segregated by growth of the septum.

Proteins related to ParA and ParB are found in several bacteria. In *B. subtilis*, they are called Soj and SpoOJ, respectively. Mutations in these loci prevent sporulation, because of a failure to segregate one daughter chromosome into the forespore (see **Figure 9.42**). In sporulating cells, SpoOJ localizes at the pole and may be responsible for localizing the origin there. SpoOJ binds to a sequence that is present in multiple copies, dispersed over ~20% of the chromosome in the vicinity of the origin. It is possible that SpoOJ binds both old and newly synthesized origins, maintaining a status equivalent to chromosome pairing, until the chromosomes are segregated to the opposite poles. In *C. crescentus*, ParA and ParB localize to the poles of the bacterium, and ParB binds sequences close to the origin, thus localizing the origin to the pole. These results suggest that a specific apparatus is responsible for localizing the origin to the pole. The next stage of the analysis will be to identify the cellular components with which this apparatus interacts (547).

The importance to the plasmid of ensuring that all daughter cells gain replica plasmids is emphasized by the existence of multiple, independent systems in individual plasmids that ensure proper partition. **Addiction systems**, operating on the basis that "we hang together or we hang separately," ensure that a bacterium carrying a plasmid can survive only so long as it retains the plasmid. There are several ways

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to ensure that a cell dies if it is "cured" of a plasmid, all sharing the principle illustrated in **Figure 13.38** that the plasmid produces both a poison and an antidote. The poison is a killer substance that is relatively stable, whereas the antidote consists of a substance that blocks killer action, but is relatively short lived. When the plasmid is lost, the antidote decays, and then the killer substance causes death of the cell. So bacteria that lose the plasmid inevitably die, and the population is condemned to retain the plasmid indefinitely. These systems take various forms. One specified by the F plasmid consists of killer and blocking proteins. The plasmid R1 has a killer that is the mRNA for a toxic protein, while the antidote is a small antisense RNA that prevents expression of the mRNA.

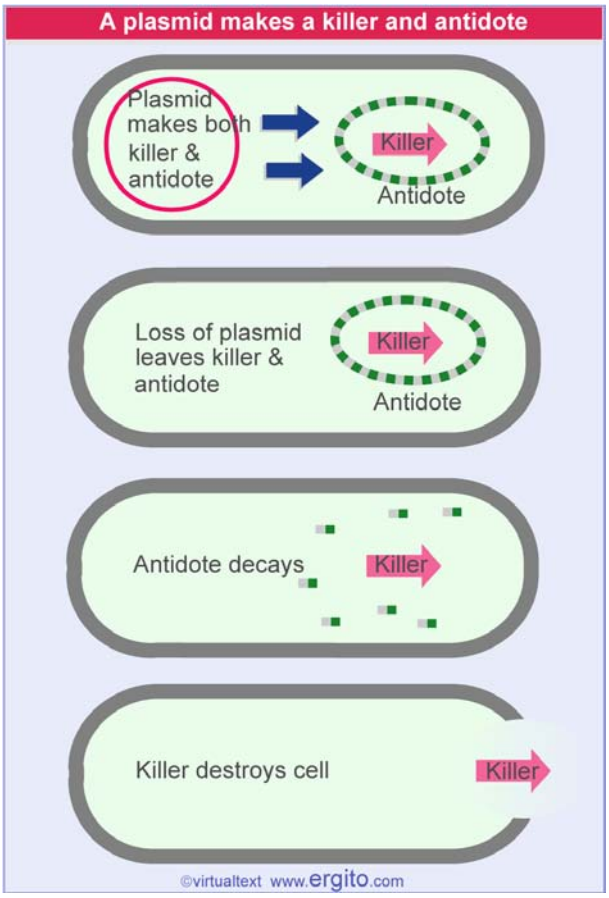

Figure 13.38 Plasmids may ensure that bacteria cannot live without them by synthesizing a long-lived killer and a short-lived antidote.

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THE REPLICON

4.13.22 Plasmid incompatibility is determined by the replicon

Key Terms

A **compatibility group** of plasmids contains members unable to coexist in the same bacterial cell.

Key Concepts

- Plasmids in a single compatibility group have origins that are regulated by a common control system.

The phenomenon of plasmid incompatibility is related to the regulation of plasmid copy number and segregation. A **compatibility group** is defined as a set of plasmids whose members are unable to coexist in the same bacterial cell. The reason for their incompatibility is that they cannot be distinguished from one another at some stage that is essential for plasmid maintenance. DNA replication and segregation are stages at which this may apply (for review see 107; 109).

The negative control model for plasmid incompatibility follows the idea that copy number control is achieved by synthesizing a repressor that measures the concentration of origins. (Formally this is the same as the titration model for regulating replication of the bacterial chromosome.)

The introduction of a new origin in the form of a second plasmid of the same compatibility group mimics the result of replication of the resident plasmid; two origins now are present. So any further replication is prevented until after the two plasmids have been segregated to different cells to create the correct prereplication copy number as illustrated in **Figure 13.39**.

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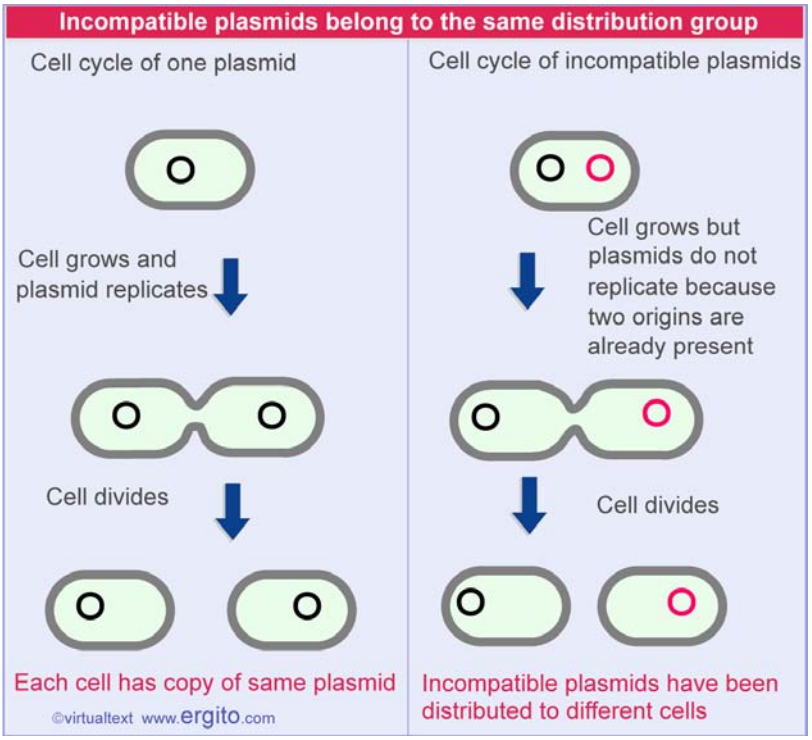

Figure 13.39 Two plasmids are incompatible (they belong to the same compatibility group) if their origins cannot be distinguished at the stage of initiation. The same model could apply to segregation.

A similar effect would be produced if the system for segregating the products to daughter cells could not distinguish between two plasmids. For example, if two plasmids have the same *cis*-acting partition sites, competition between them would ensure that they would be segregated to different cells, and therefore could not survive in the same line.

The presence of a member of one compatibility group does not directly affect the survival of a plasmid belonging to a different group. Only one replicon of a given compatibility group (of a single-copy plasmid) can be maintained in the bacterium, but it does not interact with replicons of other compatibility groups.

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THE REPLICON

4.13.23 The ColE1 compatibility system is controlled by an RNA regulator

Key Terms

A **countertranscript** is an RNA molecule that prevents an RNA primer from initiating transcription by base pairing with the primer.

Key Concepts

- Replication of ColE1 requires transcription to pass through the origin, where the transcript is cleaved by RNAaseH to generate a primer end.
- The regulator RNA I is a short antisense RNA that pairs with the transcript and prevents the cleavage that generates the priming end.
- The Rom protein enhances pairing between RNA I and the transcript.

The best characterized copy number and incompatibility system is that of the plasmid ColE1, a multicopy plasmid that is maintained at a steady level of ~20 copies per *E. coli* cell. The system for maintaining the copy number depends on the mechanism for initiating replication at the ColE1 origin, as illustrated in **Figure 13.40**.

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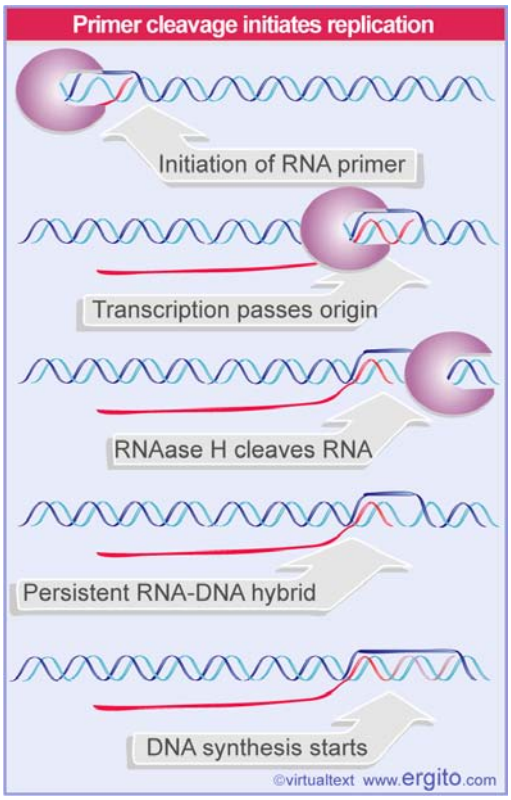

Figure 13.40 Replication of ColE1 DNA is initiated by cleaving the primer RNA to generate a 3′-OH end. The primer forms a persistent hybrid in the origin region.

Replication starts with the transcription of an RNA that initiates 555 bp upstream of the origin. Transcription continues through the origin. The enzyme RNAase H (whose name reflects its specificity for a substrate of RNA hybridized with DNA) cleaves the transcript at the origin. This generates a 3′–OH end that is used as the "primer" at which DNA synthesis is initiated (the use of primers is discussed in more detail in *Molecular Biology 4.14.8 Priming is required to start DNA synthesis*). The primer RNA forms a persistent hybrid with the DNA. Pairing between the RNA and DNA occurs just upstream of the origin (around position -20) and also farther upstream (around position -265).

Two regulatory systems exert their effects on the RNA primer. One involves synthesis of an RNA complementary to the primer; the other involves a protein coded by a nearby locus.

The regulatory species RNA I is a molecule of ~108 bases, coded by the opposite strand from that specifying primer RNA. The relationship between the primer RNA and RNA I is illustrated in **Figure 13.41**. The RNA I molecule is initiated within the primer region and terminates close to the site where the primer RNA initiates. So RNA I is complementary to the 5′–terminal region of the primer RNA. Base pairing between the two RNAs controls the availability of the primer RNA to initiate a cycle of replication (551; 552).

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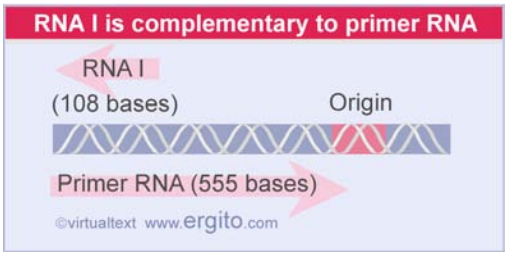

Figure 13.41 The sequence of RNA I is complementary to the 5′ region of primer RNA.

An RNA molecule such as RNA I that functions by virtue of its complementarity with another RNA coded in the same region is called a **countertranscript**. This type of mechanism, of course, is another example of the use of antisense RNA (see *Molecular Biology 3.11.19 Small RNA molecules can regulate translation*).

Mutations that reduce or eliminate incompatibility between plasmids can be obtained by selecting plasmids of the same group for their ability to coexist. Incompatibility mutations in ColE1 map in the region of overlap between RNA I and primer RNA. Because this region is represented in two different RNAs, either or both might be involved in the effect.

When RNA I is added to a system for replicating ColE1 DNA *in vitro*, it inhibits the formation of active primer RNA. But the presence of RNA I does not inhibit the initiation or elongation of primer RNA synthesis. This suggests that RNA I prevents RNAase H from generating the 3′ end of the primer RNA. The basis for this effect lies in base pairing between RNA I and primer RNA.

Both RNA molecules have the same potential secondary structure in this region, with three duplex hairpins terminating in single-stranded loops. Mutations reducing incompatibility are located in these loops, which suggests that the initial step in base pairing between RNA I and primer RNA is contact between the unpaired loops.

How does pairing with RNA I prevent cleavage to form primer RNA? A model is illustrated in **Figure 13.42**. In the absence of RNA I, the primer RNA forms its own secondary structure (involving loops and stems). But when RNA I is present, the two molecules pair, and become completely double-stranded for the entire length of RNA I. The new secondary structure prevents the formation of the primer, probably by affecting the ability of the RNA to form the persistent hybrid.

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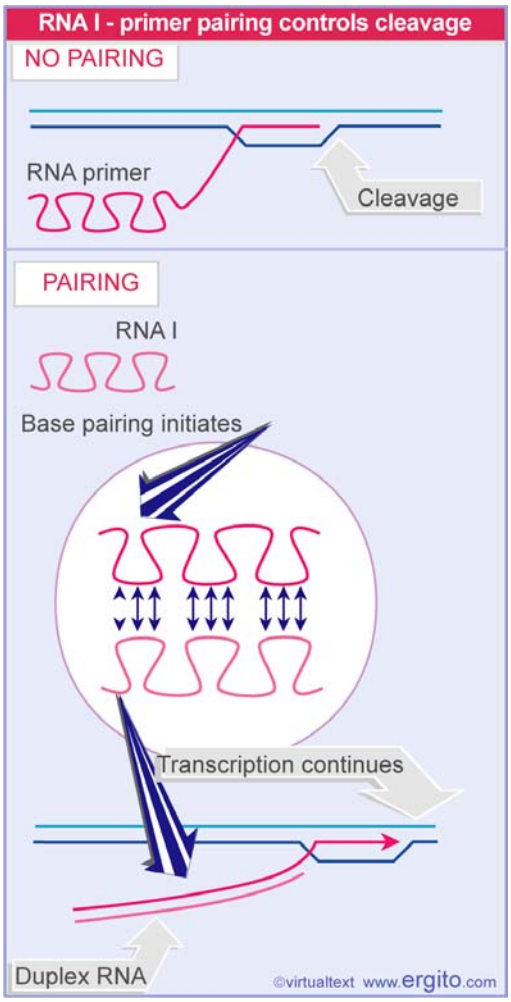

Figure 13.42 Base pairing with RNA I may change the secondary structure of the primer RNA sequence and thus prevent cleavage from generating a 3′-OH end.

The model resembles the mechanism involved in attenuation of transcription, in which the alternative pairings of an RNA sequence permit or prevent formation of the secondary structure needed for termination by RNA polymerase (see *Molecular Biology 3.11.14 Alternative secondary structures control attenuation*). The action of RNA I is exercised by its ability to affect distant regions of the primer precursor.

Formally, the model is equivalent to postulating a control circuit involving two RNA species. A large RNA primer precursor is a positive regulator, needed to initiate replication. The small RNA I is a negative regulator, able to inhibit the action of the positive regulator.

In its ability to act on any plasmid present in the cell, RNA I provides a repressor that prevents newly introduced DNA from functioning, analogous to the role of the lambda lysogenic repressor (see *Molecular Biology 3.12.10 The repressor and its operators define the immunity region*). Instead of a repressor protein that binds the new DNA, an RNA binds the newly synthesized precursor to the RNA primer.

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Binding between RNA I and primer RNA can be influenced by the Rom protein, coded by a gene located downstream of the origin. Rom enhances binding between RNA I and primer RNA transcripts of >200 bases. The result is to inhibit formation of the primer.

How do mutations in the RNAs affect incompatibility? **Figure 13.43** shows the situation when a cell contains two types of RNA I/primer RNA sequence. The RNA I and primer RNA made from each type of genome can interact, but RNA I from one genome does not interact with primer RNA from the other genome. This situation would arise when a mutation in the region that is common to RNA I and primer RNA occurred at a location that is involved in the base pairing between them. Each RNA I would continue to pair with the primer RNA coded by the same plasmid, but might be unable to pair with the primer RNA coded by the other plasmid. This would cause the original and the mutant plasmids to behave as members of different compatibility groups.

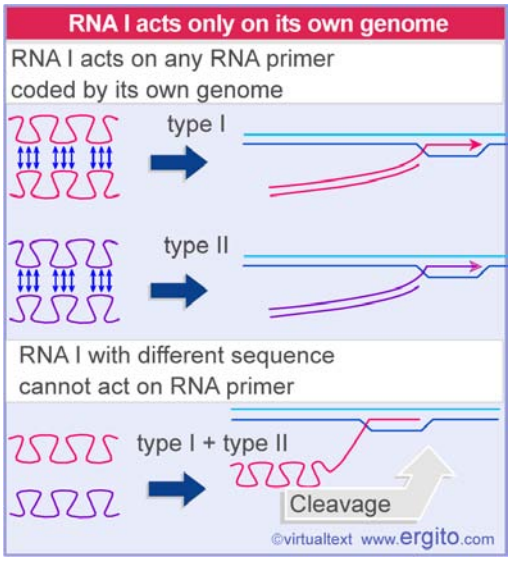

Figure 13.43 Mutations in the region coding for RNA I and the primer precursor need not affect their ability to pair; but they may prevent pairing with the complementary RNA coded by a different plasmid.

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THE REPLICON

4.13.24 How do mitochondria replicate and segregate?

Key Concepts

- mtDNA replication and segregation to daughter mitochondria is stochastic.
- Mitochondrial segregation to daughter cells is also stochastic.

Mitochondria must be duplicated during the cell cycle and segregated to the daughter cells. We understand some of the mechanics of this process, but not its regulation.

At each stage in the duplication of mitochondria – DNA replication, DNA segregation to duplicate mitochondria, organelle segregation to daughter cells – the process appears to be stochastic, governed by a random distribution of each copy (for review see 2288). The theory of distribution in this case is analogous that of multicopy bacterial plasmids, with the same conclusion that >10 copies are required to ensure that each daughter gains at least one copy (see *Molecular Biology 4.13.21 Single-copy plasmids have a partitioning system*). When there are mtDNAs with allelic variations (either because of inheritance from different parents or because of mutation), the stochastic distribution may generate cells that have only one of the alleles.

Replication of mtDNA may be stochastic because there is no control over which particular copies are replicated, so that in any cycle some mtDNA molecules may replicate more times than others. The total number of copies of the genome may be controlled by titrating mass in a way similar to bacteria (see *Molecular Biology 4.13.14 Replication is connected to the cell cycle*).

A mitochondrion divides by developing a ring around the organelle that constricts to pinch it into two halves. The mechanism is similar in principle to that involved in bacterial division. The apparatus that is used in plant cell mitochondria is similar to bacteria and uses a homologue of the bacterial protein FtsZ (see *Molecular Biology 4.13.17 FtsZ is necessary for septum formation*). The molecular apparatus is different in animal cell mitochondria, and uses the protein dynamin that is involved in formation of membranous vesicles (see *Molecular Biology 6.27.5 Different types of coated vesicles exist in each pathway*). An individual organelle may have more than one copy of its genome.

We do not know whether there is a partitioning mechanism for segregating mtDNA molecules within the mitochondrion, or whether they are simply inherited by daughter mitochondria according to which half of the mitochondrion they happen to lie in. **Figure 13.44** shows that the combination of replication and segregation mechanisms can result in a stochastic assignment of DNA to each of the copies, that is, so that the distribution of mitochondrial genomes to daughter mitochondria does not depend on their parental origins.

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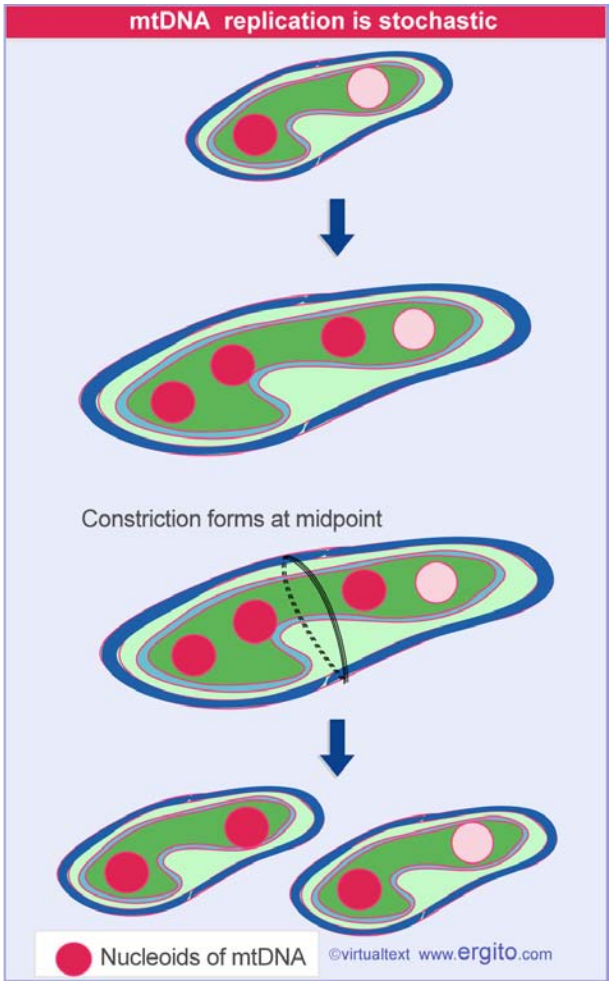

Figure 13.44 Mitochondrial DNA replicates by increasing the number of genomes in proportion to mitochondrial mass, but without ensuring that each genome replicates the same number of times. This can lead to changes in the representation of alleles in the daughter mitochondria.

The assignment of mitochondria to daughter cells at mitosis also appears to be random. Indeed, it was the observation of somatic variation in plants that first suggested the existence of genes that could be lost from one of the daughter cells because they were not inherited according to Mendel's laws (see **Figure 3.37**).

In some situations a mitochondrion has both paternal and maternal alleles. This has two requirements: that both parents provide alleles to the zygote (which of course is not the case when there is maternal inheritance; see *Molecular Biology 1.3.19 Organelles have DNA*); and that the parental alleles are found in the same mitochondrion. For this to happen, parental mitochondria must have fused.

The size of the individual mitochondrion may not be precisely defined. Indeed, there is a continuing question as to whether an individual mitochondrion represents a unique and discrete copy of the organelle or whether it is in a dynamic flux in which it can fuse with other mitochondria. We know that mitochondria can fuse in yeast, because recombination between mtDNAs can occur after two haploid yeast strains

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have mated to produce a diploid strain. This implies that the two mtDNAs must have been exposed to one another in the same mitochondrial compartment. Attempts have been made to test for the occurrence of similar events in animal cells by looking for complementation between alleles after two cells have been fused, but the results are not clear.

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THE REPLICON

4.13.25 Summary

The entire chromosome is replicated once for every cell division cycle. Initiation of replication commits the cell to a cycle of division; completion of replication may provide a trigger for the actual division process. The bacterial chromosome consists of a single replicon, but a eukaryotic chromosome is divided into many replicons that function over the protracted period of S phase. The problem of replicating the ends of a linear replicon is solved in a variety of ways, most often by converting the replicon to a circular form. Some viruses have special proteins that recognize ends. Eukaryotic chromosomes encounter the problem at their terminal replicons.

Eukaryotic replication is (at least) an order of magnitude slower than bacterial replication. Origins sponsor bidirectional replication, and are probably used in a fixed order during S phase. The only eukaryotic origins identified at the sequence level are those of *S. cerevisiae*, which have a core consensus sequence consisting of 11 base pairs, mostly A·T.

The minimal *E. coli* origin consists of ~245 bp and initiates bidirectional replication. Any DNA molecule with this sequence can replicate in *E. coli*. Two replication forks leave the origin and move around the chromosome, apparently until they meet, although *ter* sequences that would cause the forks to terminate after meeting have been identified. Transcription units are organized so that transcription usually proceeds in the same direction as replication.

The rolling circle is an alternative form of replication for circular DNA molecules in which an origin is nicked to provide a priming end. One strand of DNA is synthesized from this end, displacing the original partner strand, which is extruded as a tail. Multiple genomes can be produced by continuing revolutions of the circle.

Rolling circles are used to replicate some phages. The A protein that nicks the ϕX174 origin has the unusual property of *cis*-action. It acts only on the DNA from which it was synthesized. It remains attached to the displaced strand until an entire strand has been synthesized, and then nicks the origin again, releasing the displaced strand and starting another cycle of replication.

Rolling circles also are involved in bacterial conjugation, when an F plasmid is transferred from a donor to a recipient cell, following the initiation of contact between the cells by means of the F-pili. A free F plasmid infects new cells by this means; an integrated F factor creates an Hfr strain that may transfer chromosomal DNA. In the case of conjugation, replication is used to synthesize complements to the single strand remaining in the donor and to the single strand transferred to the recipient, but does not provide the motive power.

A fixed time of 40 minutes is required to replicate the *E. coli* chromosome and a further 20 minutes is required before the cell can divide. When cells divide more rapidly than every 60 minutes, a replication cycle is initiated before the end of the preceding division cycle. This generates multiforked chromosomes. The initiation

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event depends on titration of cell mass, probably by accumulating an initiator protein. Initiation may occur at the cell membrane, since the origin is associated with the membrane for a short period after initiation.

The septum that divides the cell grows at a location defined by the pre-existing periseptal annulus; a locus of three genes (*minCDE*) codes for products that regulate whether the midcell periseptal annulus or the polar sites derived from previous annuli are used for septum formation. Absence of septum formation generates multinucleated filaments; excess of septum formation generates anucleate minicells.

Many transmembrane proteins interact to form the septum. ZipA is located in the inner bacterial membrane and binds to FtsZ, which is a tubulin-like protein that can polymerize into a filamentous structure called a Z-ring. FtsA is a cytosolic protein that binds to FtsZ. Several other *fts* products, all transmembrane proteins, join the Z-ring in an ordered process that generates a septal ring. The last proteins to bind are the SEDS protein FtsW and the transpeptidase ftsI (PBP3), which together function to produce the peptidoglycans of the septum. Chloroplasts use a related division mechanism that has an FtsZ-like protein, but mitochondria use a different process in which the membrane is constricted by a dynamin-like protein.

Plasmids and bacteria have site-specific recombination systems that regenerate pairs of monomers by resolving dimers created by general recombination. The Xer system acts on a target sequence located in the terminus region of the chromosome. The system is active only in the presence of the FtsK protein of the septum, which may ensure that it acts only when a dimer needs to be resolved.

Partitioning involves the interaction of the ParB protein with the *parS* target site to build a structure that includes the IHF protein. This partition complex ensures that replica chromosomes segregate into different daughter cells. The mechanism of segregation may involve movement of DNA, possibly by the action of MukB in condensing chromosomes into masses at different locations as they emerge from replication.

Plasmids have a variety of systems that ensure or assist partition, and an individual plasmid may carry systems of several types. The copy number of a plasmid describes whether it is present at the same level as the bacterial chromosome (one per unit cell) or in greater numbers. Plasmid incompatibility can be a consequence of the mechanisms involved in either replication or partition (for single-copy plasmids). Two plasmids that share the same control system for replication are incompatible because the number of replication events ensures that there is only one plasmid for each bacterial genome.

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