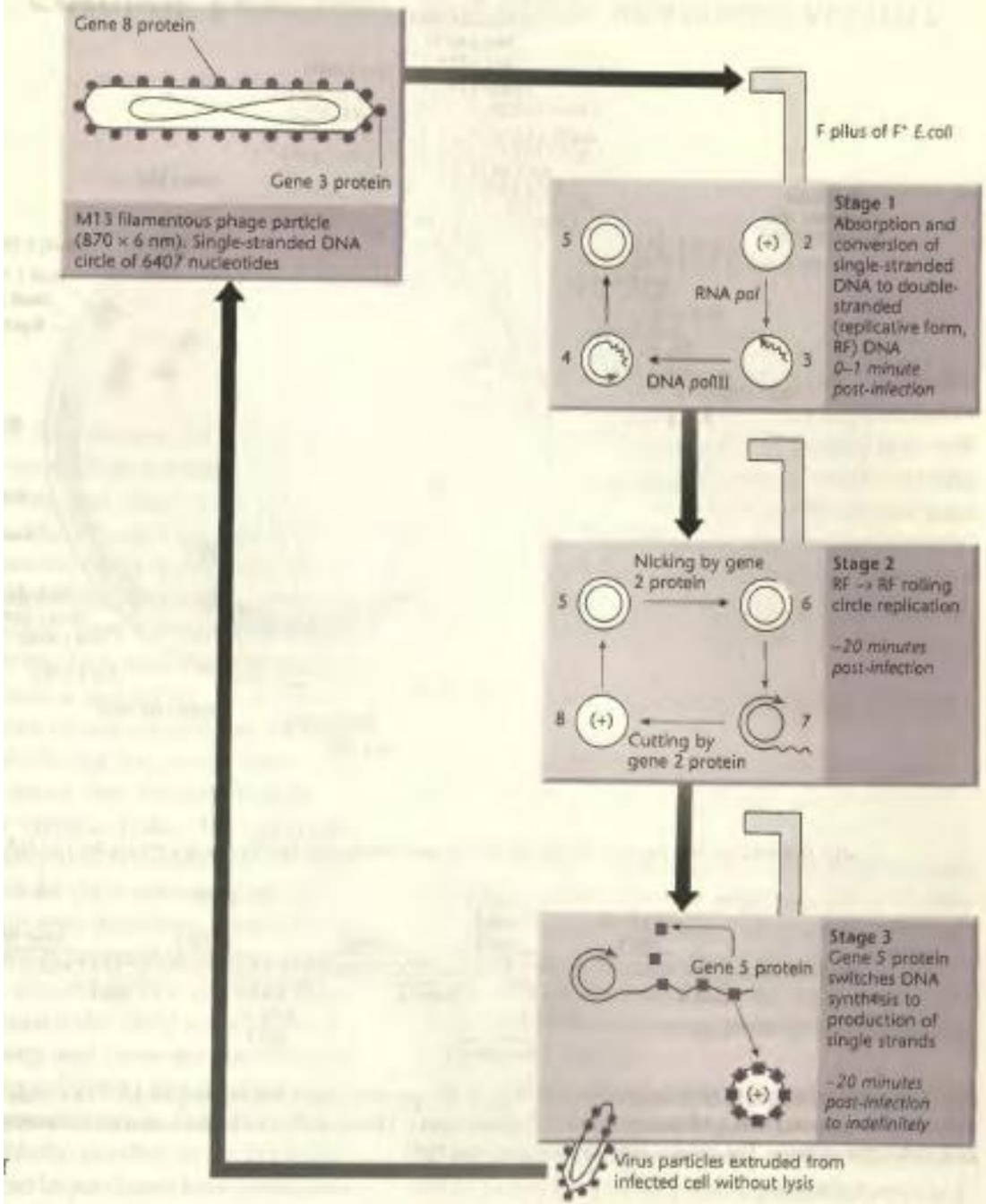

Gene 8 protein
Gene 3 protein
M13 filamentous phage particle (870 × 6 nm). Single-stranded DNA circle of 6407 nucleotides
F pilus of F+ E.coli
Stage 1
Absorption and conversion of single-stranded DNA to double-stranded (replicative form, RF) DNA
0–1 minute post-infection
RNA pol
DNA pol III
Stage 2
RF → RF rolling circle replication
~20 minutes post-infection
Nicking by gene 2 protein
Cutting by gene 2 protein
Stage 3
Gene 5 protein switches DNA synthesis to production of single strands
~20 minutes post-infection to indefinitely
Gene 5 protein
Virus particles extruded from infected cell without lysis

M13 adsorption and injection

M13 adsorbs to the tip of the F pilus, a hair-like structure on the surface of some bacteria. It can only infect bacteria that carry an F or F-like conjugative plasmid that encodes the proteins that make up the F pilus.

For the filamentous phage, it is known that infection is initiated by the binding of gpIII to the tip of the F pilus.

Protection of the M13 genome

The M13 DNA that ends up in the cytoplasm is a circular single-stranded DNA molecule. The strand present in phage particle is known as the plus or + strand.

After entry into the cytoplasm, the + strand DNA is immediately coated with an *E. coli single stranded* DNA binding protein known as **SSB**. The SSB coating protects the DNA from degradation.

M13 DNA replication

The M13 plus strand is converted to a double-stranded molecule immediately upon entry into *E. coli.*

Synthesis of the complementary strand is carried out entirely by *E. coli's DNA synthesis machinery.*

The complementary strand is called the minus or—strand.

Only the minus strand is used as the template for mRNA synthesis and ultimately it is the template for the translation of the encoded M13 gene products.

The SSB that coats the plus strand upon entry of the DNA into the *E. coli cytoplasm* fails to bind to ~60 nucleotides of the molecule.

These nucleotides form a hairpin loop that is protected from nuclease degradation.

M13 gpIII from the phage is found associated with the hairpin loop.

The hairpin loop is recognized by *E.coli RNA polymerase as a DNA replication origin and is used to initiate transcription of* a short RNA primer.

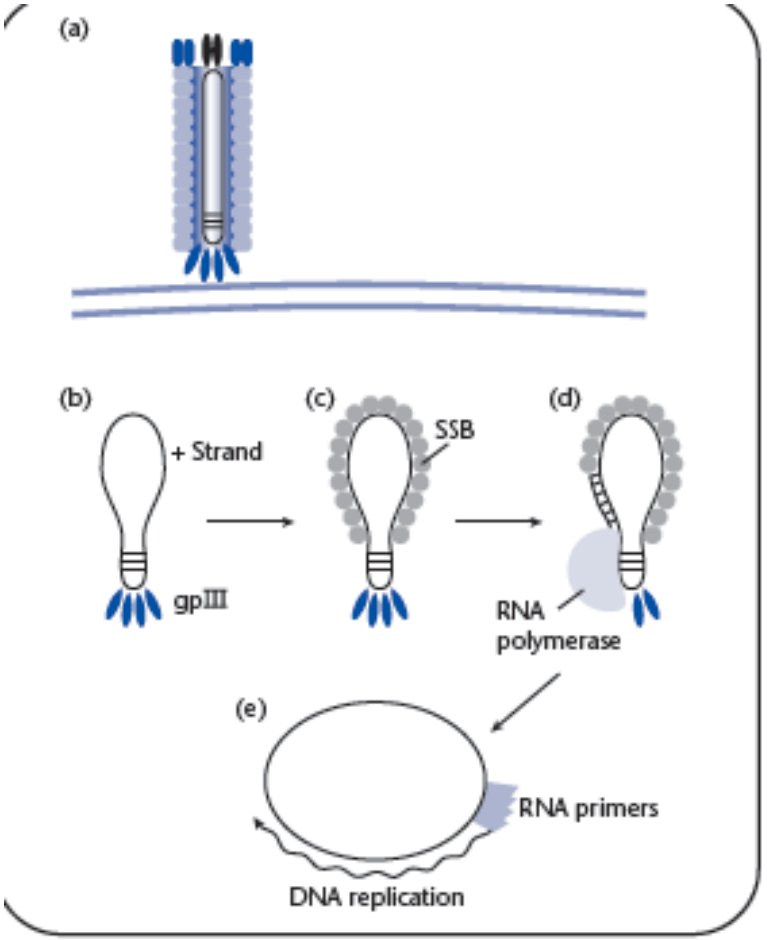
The RNA primer is extended by *E. coli DNA polymerase* III to create the minus strand.

The RNA primer is eventually removed by the exonuclease activities of *E. coli DNA polymerase I.*

The gap is filled in by the 5' to 3' polymerizing activity of the same DNA polymerase.

E. coli ligase *forms the final phosphodiester bond* resulting in a covalently closed double-stranded circular M13 chromosome.

The double-stranded form of M13 chromosome is called the replicative form (RF) DNA.



The RF form is replicated by rolling circle replication similar to the mechanism used by the lambda chromosome.

The M13 gene II encoded protein is an endonuclease that nicks the plus strand of the RF DNA at a specific place to initiate the replication process for M13 RF DNA.

Approximately 100 copies of M13 RF DNA are made.

While the M13 chromosome is being replicated, the genes encoding the coat proteins are being transcribed and translated.

When M13 gpV protein accumulates to sufficient levels, a switch from synthesizing RF DNA to synthesizing the plus strand occurs.

GpV blocks the synthesis of the minus strand, presumably by displacing SSB on the plus strand and preventing the plus strand from being used as a template.

The plus strand is circularized.

M13 phage production and release from the cell

M13 phage particles are assembled and released from *E. coli cells through a process that does not involve lysing E. coli or disrupting cell division* (*Fig. 7.13*).

The gpV coated plus strand makes contact with the bacterial inner membrane.

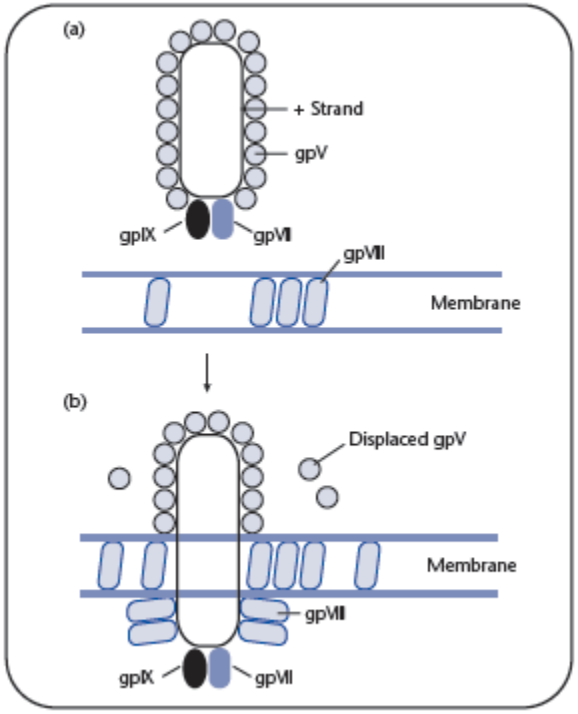
This interaction requires a specific packaging sequence on the DNA and **gpVII and gpIX**.

The protein-coated DNA traverses the membrane and gpV is replaced by gpVIII in the process .

GpVIII is found in the membrane.

When the last of the phage particle crosses the membrane**, gpIII and gpVI** are added.

M13 phage are continually released from actively growing infected *E. coli.*



M13 infection and replication

M13 is a filamentous *bacteriophage* which infects *E. coli* host.

The M13 genome has the following characteristics:

Circular *single-stranded* DNA
6400 base pairs long
The genome codes for a total of 10 genes (named using Roman numerals I through X)

Gene VIII codes for the major structural protein of the bacteriophage particles
Gene III codes for the minor coat protein

The gene VIII protein forms a tubular array of approx. 2,700 identical subunits surrounding the viral genome

Approximately five to eight copies of the gene III protein are located at the ends of the filamentous phage (i.e. genome plus gene VIII assembly)

Allows binding to bacterial "sex" pilus

Infection

Single strand genome (designated '+' strand) attached to pilus enters host cell

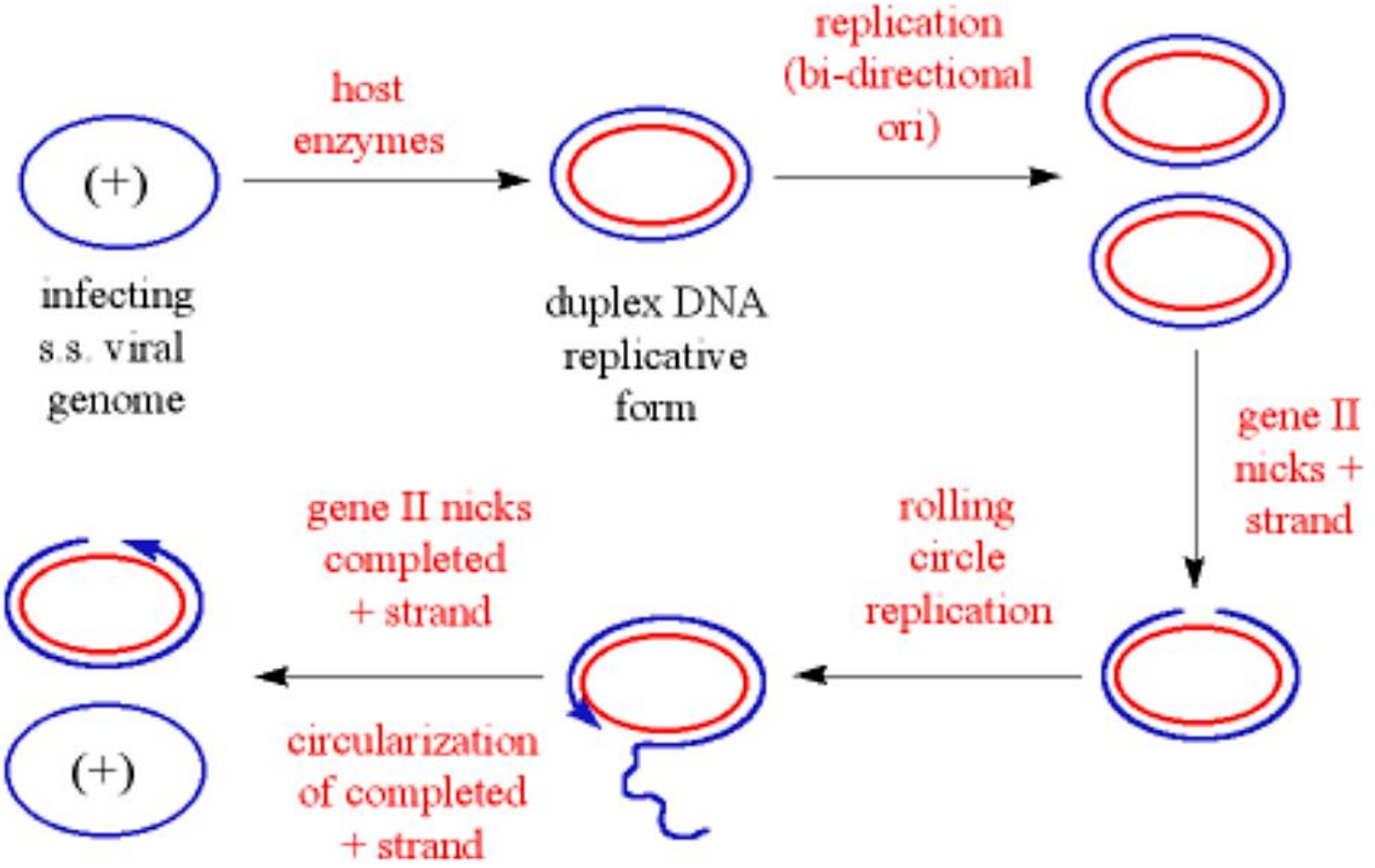
- Major coat protein (gene VIII) stripped off
- Minor coat protein (gene III) remains attached

Host components convert single strand (+) genome to double stranded circular DNA (called the replicative or "RF" form)

Transcription begins

- Series of promoters
  - Provides a gradient of transcription such that gene nearest the two transcription terminators are transcribed the most
- Two terminators
  - One at the end of gene VIII
  - One at the end of gene IV

Transcription of all 10 genes proceeds in same direction


(+)
infecting
s.s. viral
genome
host
enzymes
duplex DNA
replicative
form
replication
(bi-directional
ori)
gene II
nicks +
strand
rolling
circle
replication
gene II nicks
completed
+ strand
circularization
of completed
+ strand
(+)

Development of M13 into a cloning vector

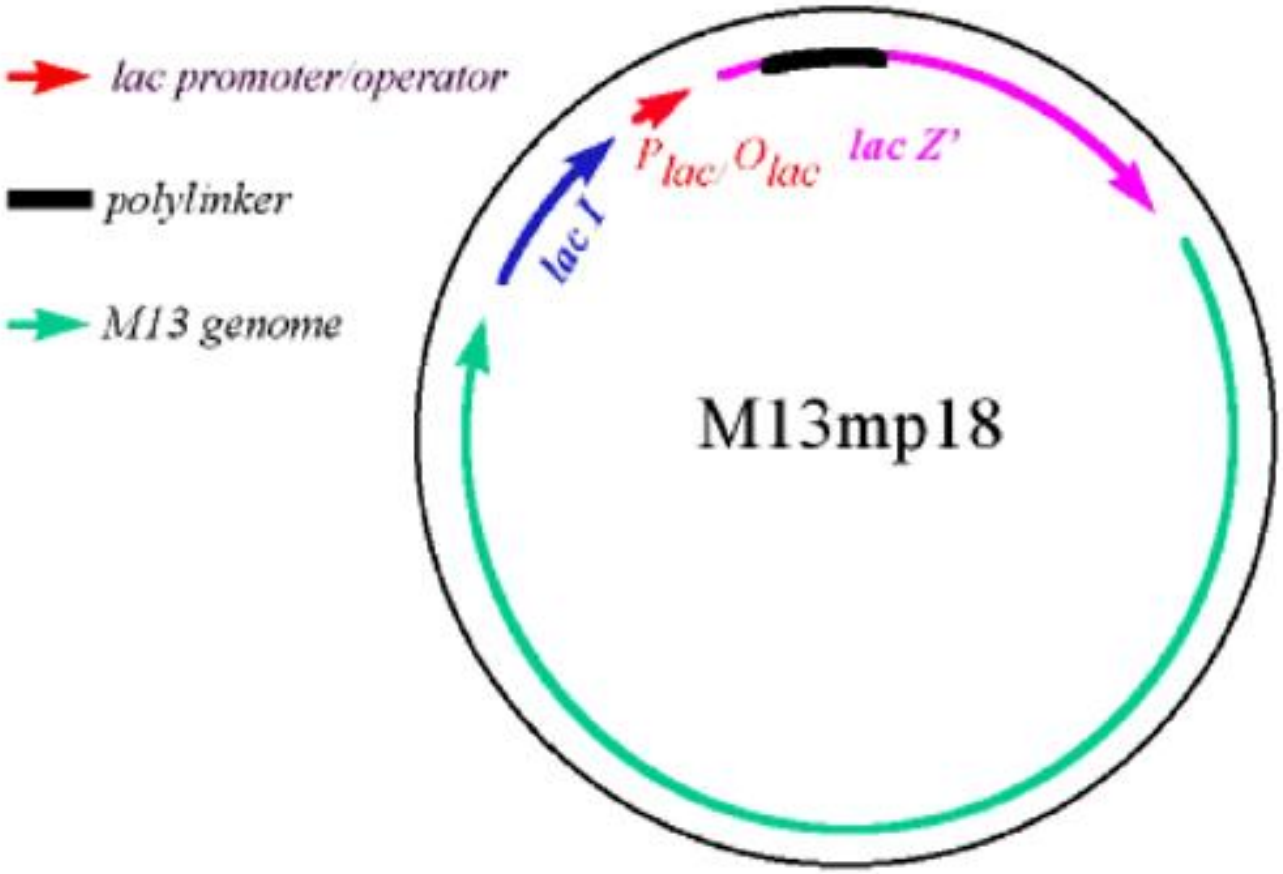
M13 was developed into a useful cloning vector by inserting the following elements into the genome:

a gene for the *lac* repressor (*lac I*) protein to allow regulation of the *lac* promoter

the operator-proximal region of the *lac Z* gene (to allow for a-complementation in a host with operator-proximal deletion of the *lac Z* gene).

a *lac* promoter upstream of the *lac Z* gene

a polylinker (multiple cloning site) region inserted several codons into the *lac Z* gene


lac promoter/operator
polylinker
M13 genome
lac I
P_{lac}/O_{lac}
lac Z'
M13mp18

Several features of M13 make this phage attractive as the basis for a cloning vector.

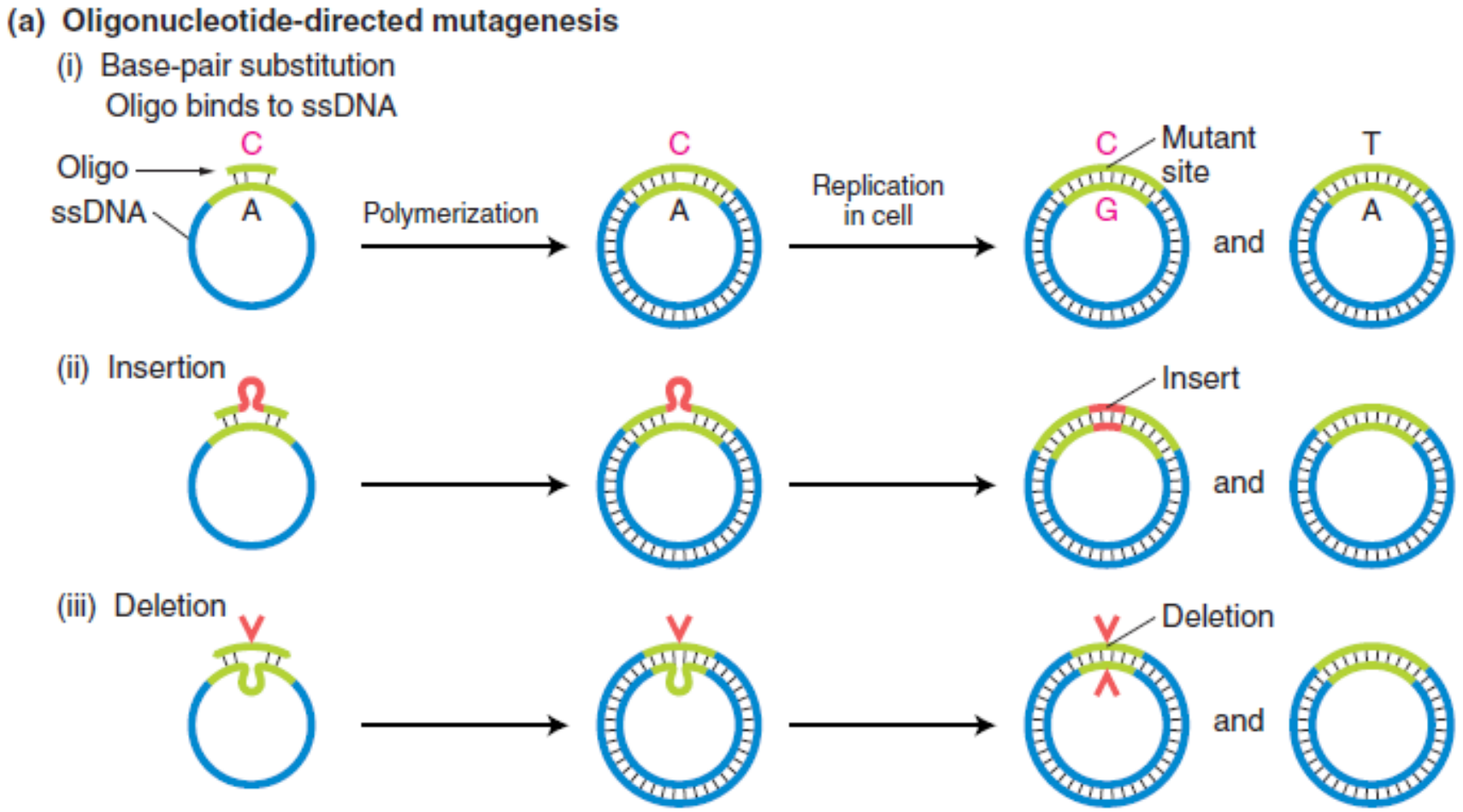
The genome is less than 10 kb in size, well within the range desirable for a potential vector.

In addition, the double-stranded replicative form (RF) of the M13 genome behaves very much like a plasmid, and can be treated as such for experimental purposes.

It is easily prepared from a culture of infected E. coli cells and can be reintroduced by transfection.

Most importantly, genes cloned with an M13- based vec tor can be obtained in the form of single- stranded DNA.

Single stranded version of cloned genes are useful for several techniques, notably DNA sequencing and in vitro mutagenesis.



This method can create mutations at any specific site in a gene that has been cloned and sequenced. The mutation must be introduced into a transgene borne on a vector.

In one protocol, the gene of interest is inserted into a single-stranded bacteriophage vector, such as the phage M13. A synthetic oligonucleotide containing the desired mutation is designed. This oligonucleotide is allowed to hybridize to the complementary site in the gene of interest residing in the vector. The oligonucleotide then serves as a primer for the in vitro synthesis of the complementary strand of the M13 vector

Disadvantages of bacteriophage M13 vec tors:

The following are the disadvantages of bacteriophage M13 vectors:

1. Gene of interest more than 2kb cannot be cloned.

2. It has low yield of DNA.

3. The phage produce many toxins in high concentration.

The basics of adsorption are that a specific structure on the surface of the phage interacts with a specific structure on the surface of the bacterium.

It binds to an outer membrane protein called **LamB** via a protein that resides at the tip of the **Λ** tail called the J protein.

LamB normally functions in the binding and uptake of the sugars maltose and maltodextrin.

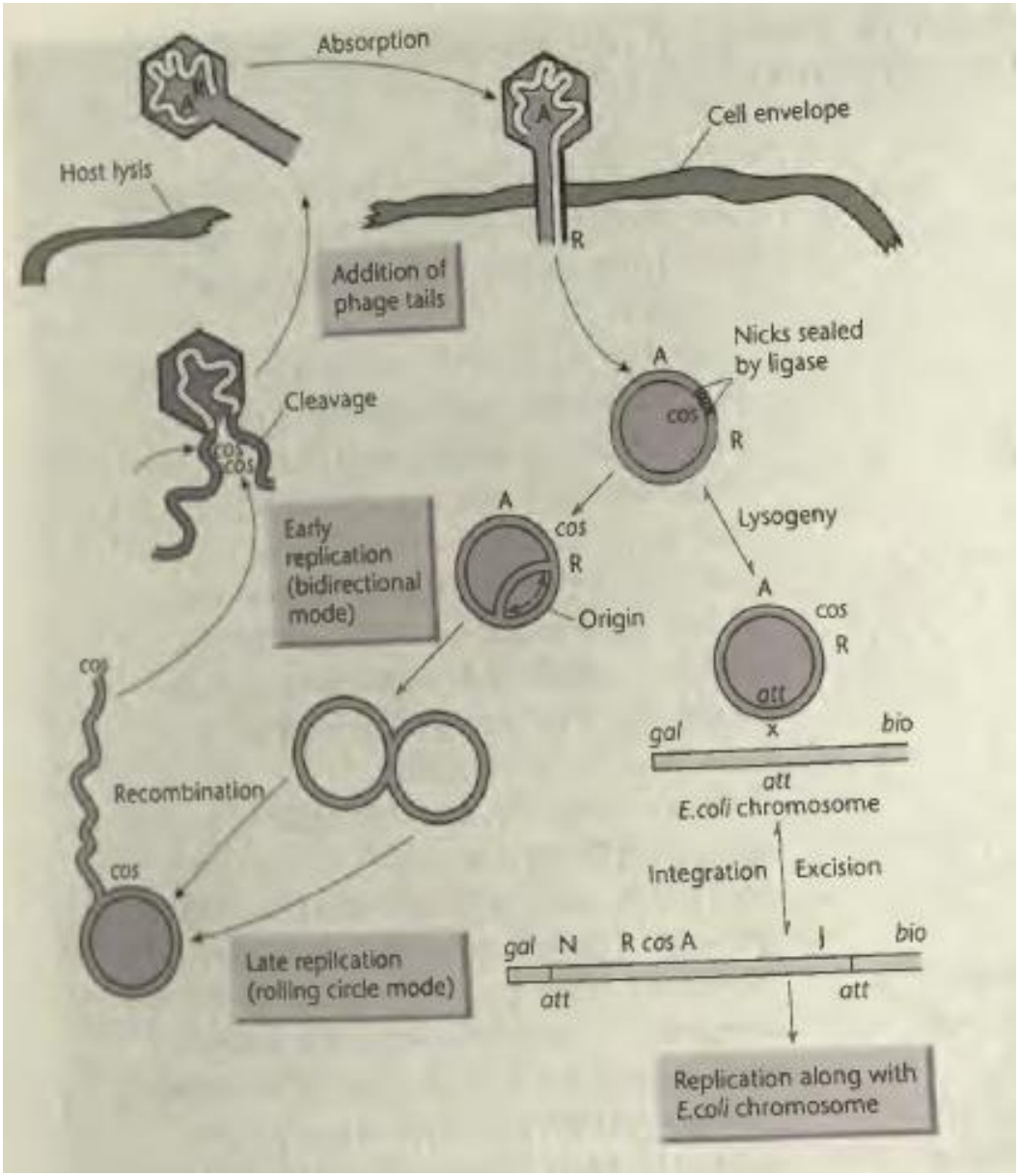
Protecting the λ genome in the bacterial cytoplasm

What protection the phage genome needs in the cytoplasm depends on the physical state of the injected nucleic acid.

Λ contains a linear double-stranded DNA molecule in its capsid.

In the bacterial cytoplasm, dsDNA molecules are subject to degradation by exonucleases that need a free end to digest the DNA.

The first event that happens to newly injected **λ** DNA is that the DNA circularizes to prevent it from being degraded.



After being injected into the host cell, linear double-stranded (ds) lambda DNA cir-cu-la-rizes via its cohesive ends (*cos* sites). Circular dsDNA then replicates a few times in the theta-mode (θ) before swit-ch-ing to the sigma-mode (σ).

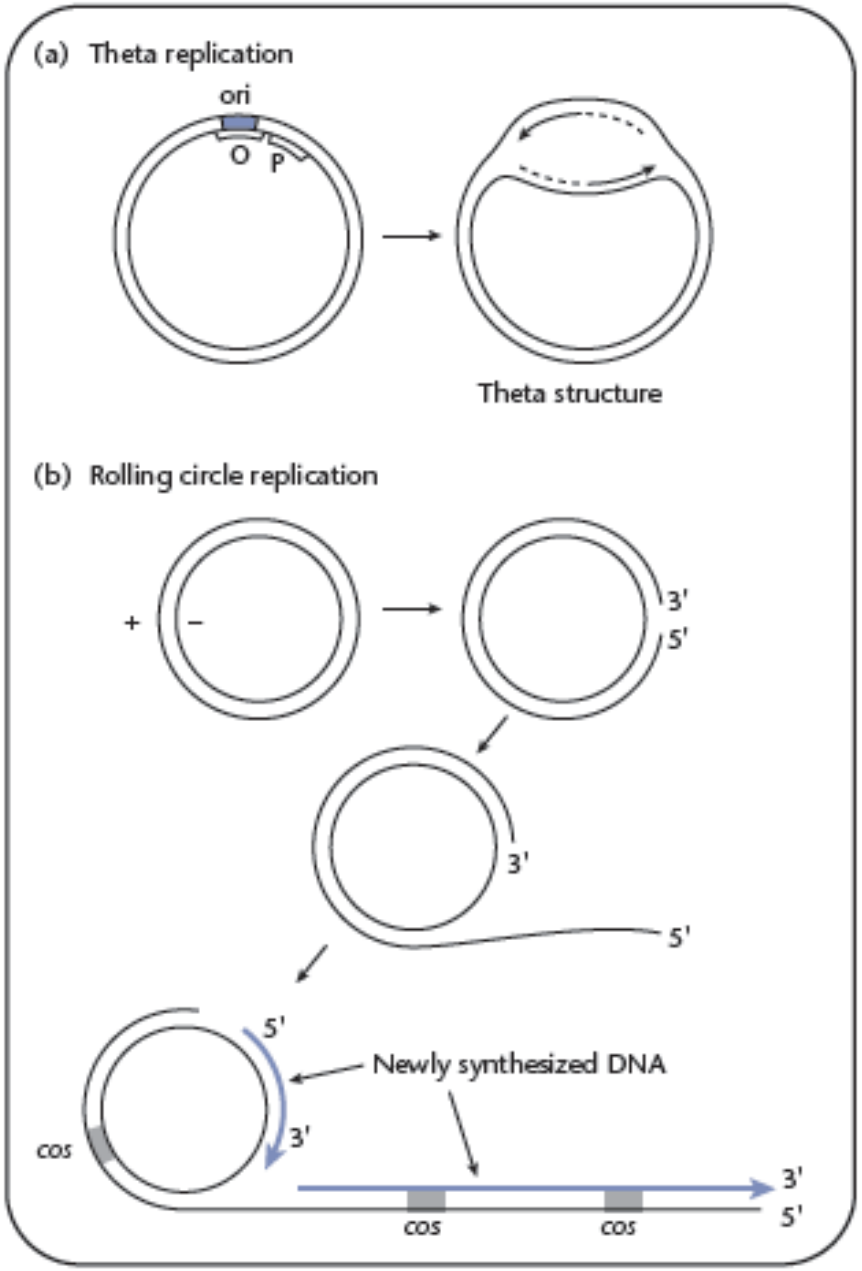
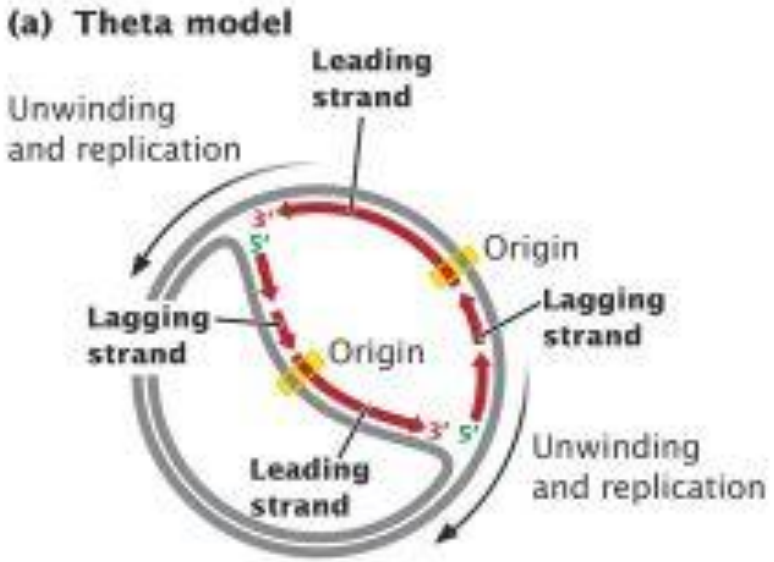
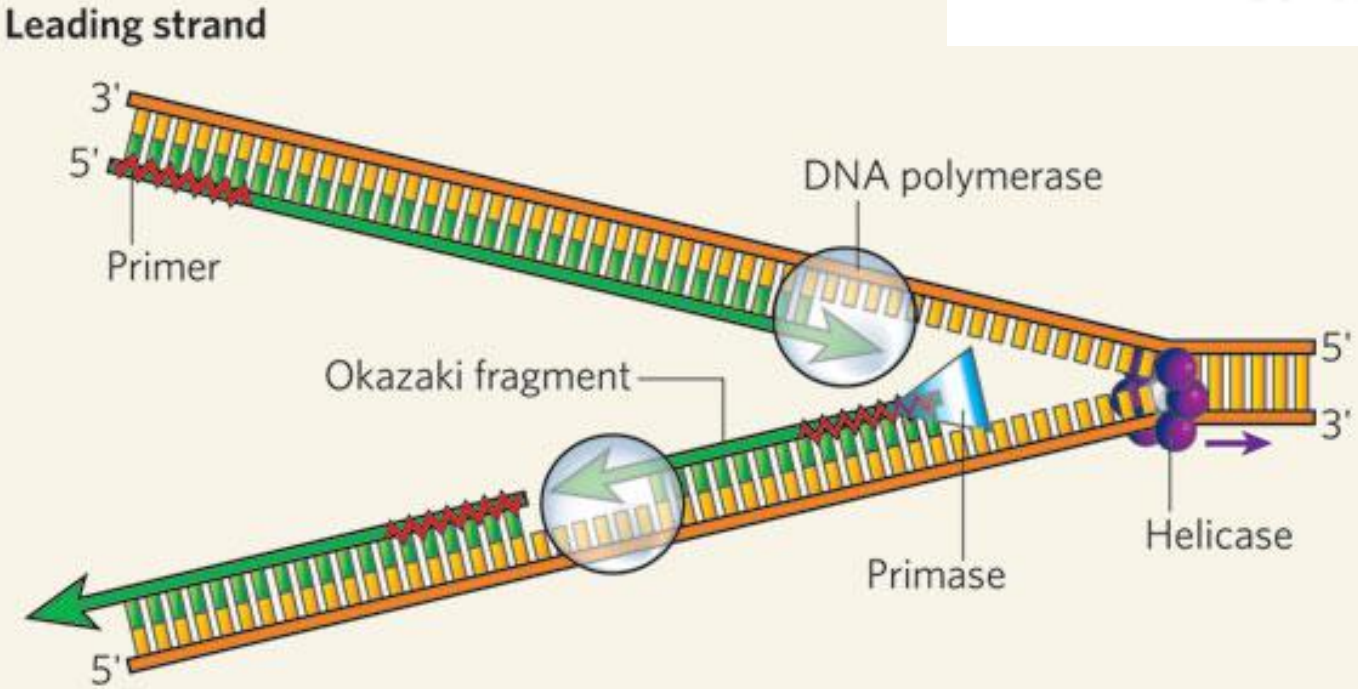


Fig. 7.9 λ has two modes of DNA replication: theta replication (a) and rolling circle replication (b). Theta replication occurs early in infection and rolling circle replication occurs late in infection. Rolling circle replication produces concatamers for packaging into phage heads.


(a) Theta model
Leading strand
Unwinding and replication
3'
5'
Origin
Lagging strand
Lagging strand
Origin
3'
5'
Leading strand
Unwinding and replication


Leading strand
3'
5'
Primer
DNA polymerase
Okazaki fragment
5'
3'
Helicase
Primase
5'
Lagging strand

λ has a specific site on its DNA, termed the ***cos site****, which it uses to circularize the* DNA.

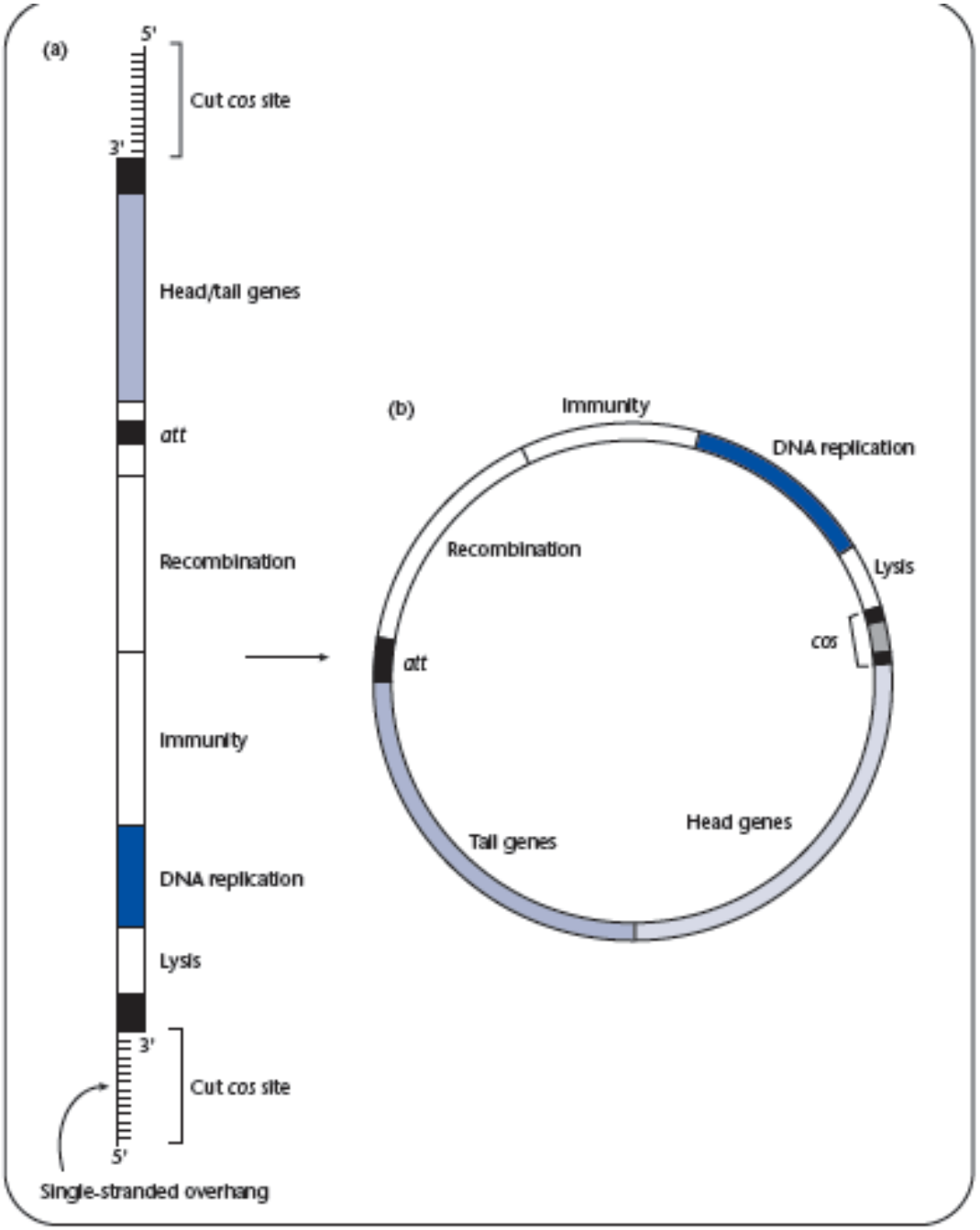
The *cos site is a 22 bp sequence that is cut asymmetrically when the* **λ** DNA is packaged. The cut *cos site has a 12 bp overhang.*

There is one cut cos site at the left end of the **λ** has a specific site on its DNA, termed the *cos site, which it uses to circularize the* DNA.

When the **λ** DNA is injected into the cytoplasm, the cut *cos sites at* either of the linear **λ** genome anneal.

A host enzyme, DNA ligase, seals the nicks at either end of the *cos site generating a covalently closed, circular* **λ** *genome.*

The host encoded enzyme, DNA gyrase, supercoils the l molecule.


(a)
5'
3'
Cut cos site
Head/tail genes
att
Recombination
Immunity
DNA replication
Lysis
3'
5'
Cut cos site
Single-stranded overhang
(b)
Immunity
DNA replication
Recombination
Lysis
cos
att
Tail genes
Head genes

Bacteriophage vectors

Different classes of bacteriophage vectors can carry different sizes of donor DNA insert.

A given bacteriophage can harbor a standard amount of DNA as an insert "packaged" inside the phage particle.
Bacteriophage λis a genetically complex but very extensivelystudied virus ofE. *coli*

Bacteriophage (lambda) is an effective cloning vector for double-stranded DNA inserts as long as about 23 kb.

Lambda phage heads can package DNA molecules no larger than about 50 kb in length (the size of a normal chromosome).

The central part of the phage genome is not required for replication or packaging of DNA molecules in *E. coli and so can be cut out by using restriction* enzymes and discarded.

The deleted central part is then replaced by inserts of donor DNA.

Bacteriophage *vectors* that can be readily cleaved into three pieces, two of which contain essential genes but which together are only about 30,000 bp long.

The third piece, "filler" DNA, is discarded when the vector is to be used for cloning, and additional DNA is inserted between the two essential segments to generate ligated DNA molecules long enough to produce viable phage particles.

In effect, the packaging mechanism *selects for recombinant viral* DNAs.

Bacteriophage *vectors permit the cloning of DNA* fragments of up to 23,000 bp.

Once the bacteriophage *fragments are ligated to foreign DNA fragments of suitable* size, the resulting recombinant DNAs can be packaged into phage particles by adding them to crude bacterial cell extracts that contain all the proteins needed to assemble a complete phage.

This is called **in vitro packaging. All viable phage particles will** contain a foreign DNA fragment.
The subsequent transmission of the recombinant DNA into *E. coli cells is* highly efficient.

- Mutant phages designed for cloning have been constructed.

- An especially useful one called gt- contains only two *EcoRI cleavage sites* instead of the five normally present.

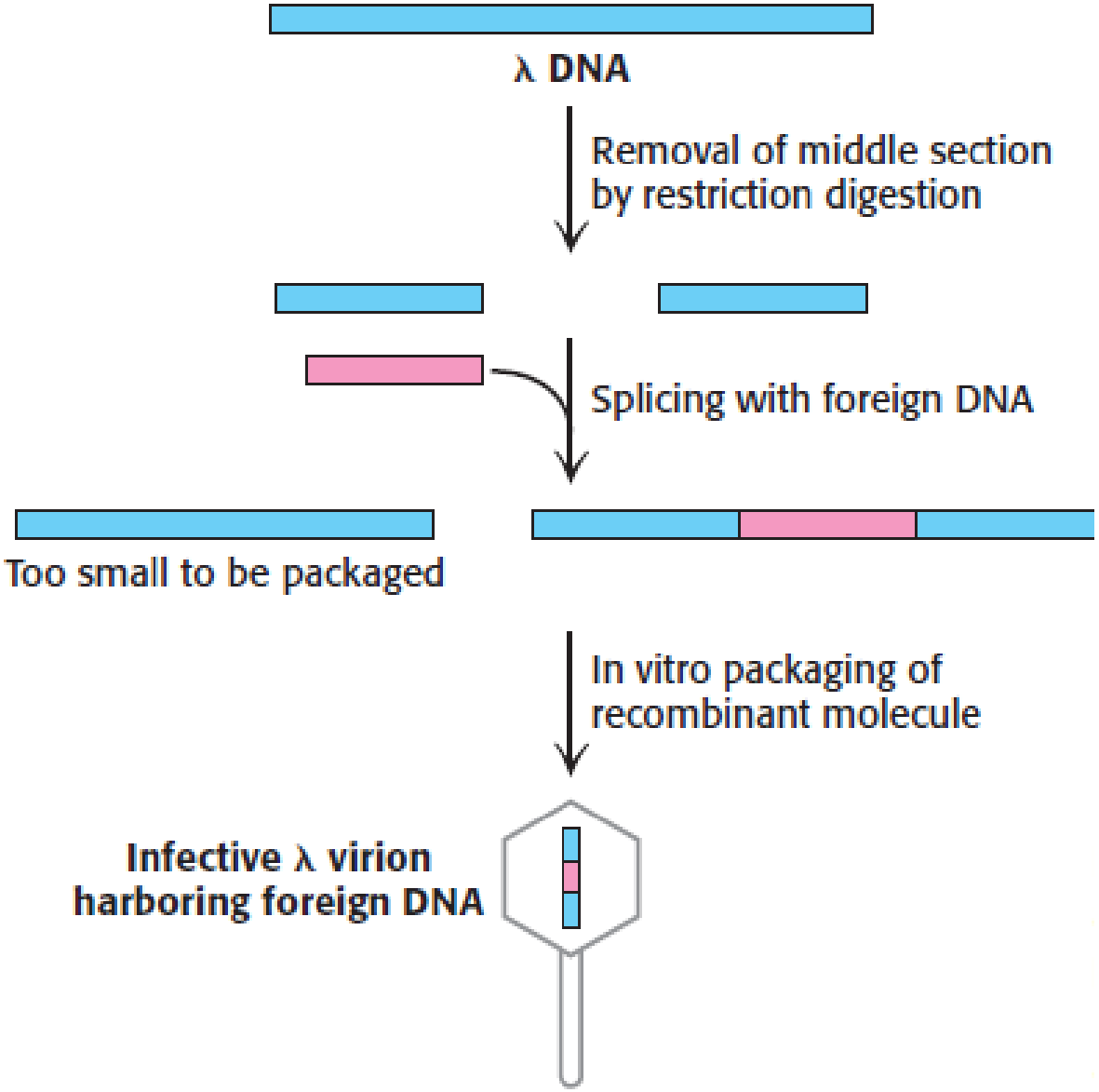
After cleavage, the middle segment of this DNA molecule can be removed.

The two remaining pieces of DNA (called arms) have a combined length equal to 72% of a normal genome length.

This amount of DNA is too little to be packaged into a particle, because only DNA measuring from 75% to 105% of a normal genome in length can be readily packaged.

However, a suitably long DNA insert (such as 10 kb) between the two ends of DNA enables such a recombinant DNA molecule (93% of normal length) to be packaged.

Nearly all infectious particles formed in this way will contain an inserted piece of foreign DNA.


λ DNA
Removal of middle section by restriction digestion
Splicing with foreign DNA
Too small to be packaged
In vitro packaging of recombinant molecule
Infective λ virion harboring foreign DNA