Biochem 717
Gene Cloning

Prof Amer Jamil

Dept of Biochemistry

University of Agriculture

Faisalabad

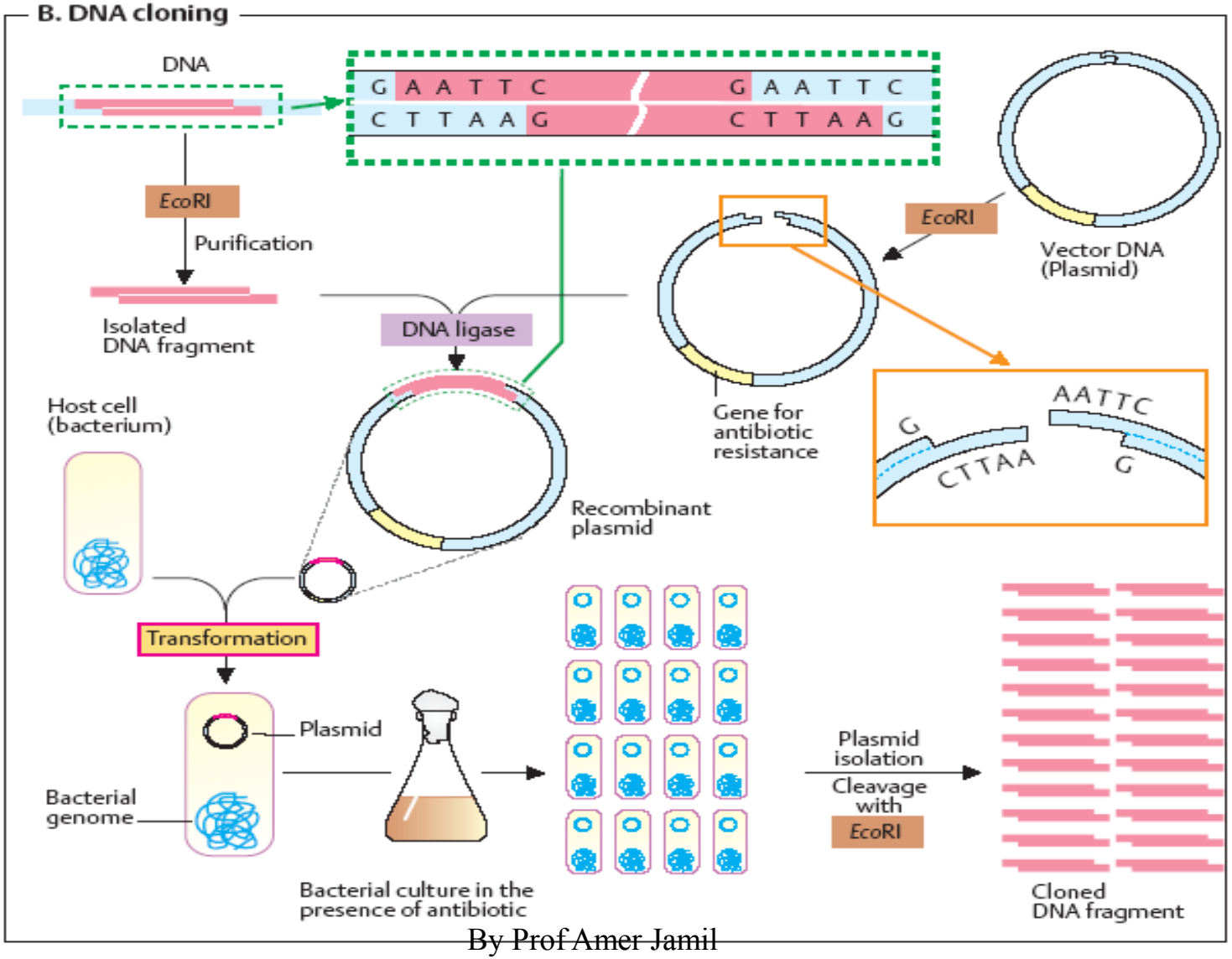
How to construct a recombinant DNA molecule?

- DNA isolation
- Cutting of DNA molecule with the help of restriction enzymes
- Transfer of DNA molecule into a suitable vector

With the help of DNA ligases

Transformation of recombinant molecule into suitable host like *E. coli*

- Production of large number of copies of the recombinant molecule in the host
- Checking the gene expression


B. DNA cloning
DNA
G A A T T C
C T T A A G
G A A T T C
C T T A A G
EcoRI
Purification
Isolated DNA fragment
DNA ligase
EcoRI
Vector DNA (Plasmid)
Gene for antibiotic resistance
AATTC
G
CTTAA
G
Host cell (bacterium)
Recombinant plasmid
Transformation
Plasmid
Bacterial genome
Bacterial culture in the presence of antibiotic
Plasmid isolation
Cleavage with
EcoRI
Cloned DNA fragment

Overexpression of proteins

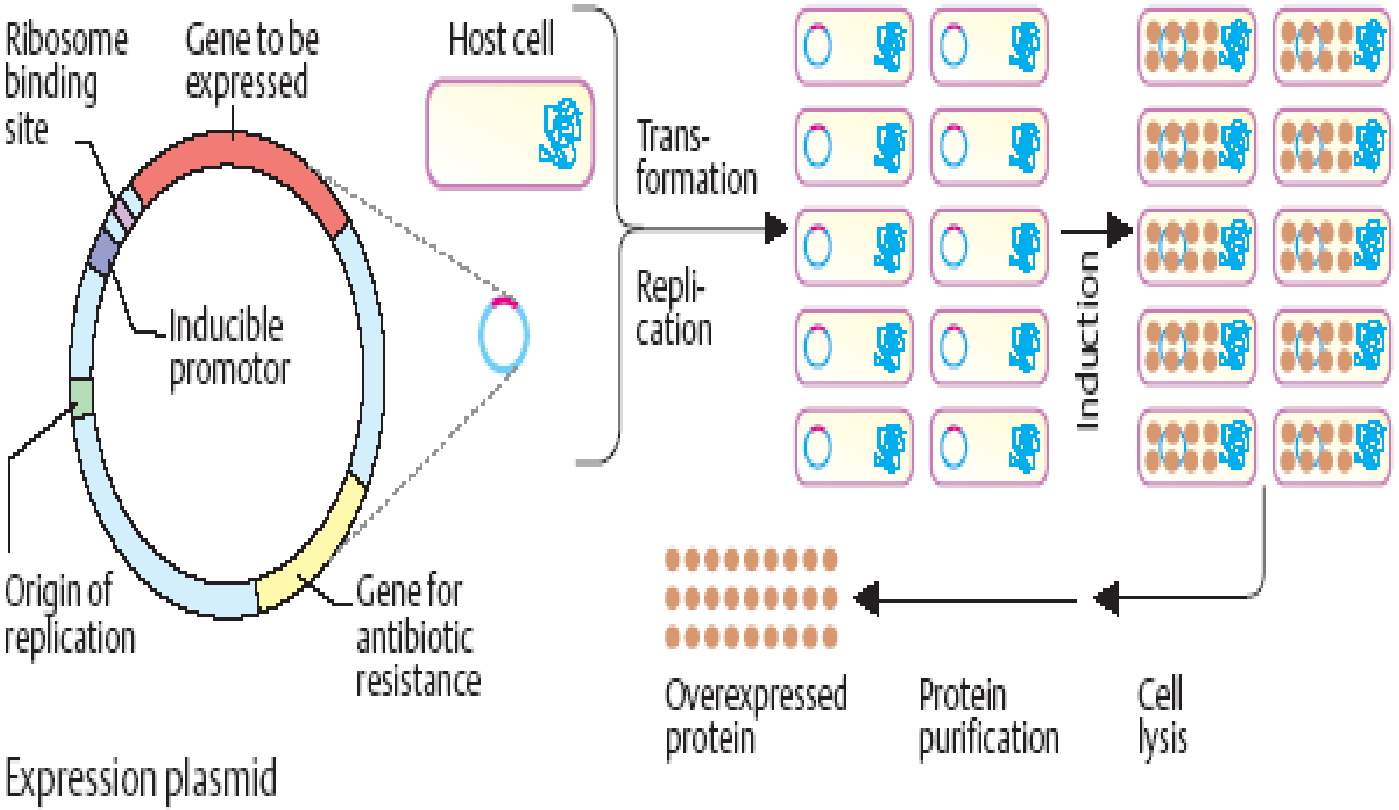


TABLE 9–1 Some Enzymes Used in Recombinant DNA Technology

Enzyme(s)	Function
Type II restriction endonucleases	Cleave DNAs at specific base sequences
DNA ligase	Joins two DNA molecules or fragments
DNA polymerase I (*E. coli*)	Fills gaps in duplexes by stepwise addition of nucleotides to 3′ ends
Reverse transcriptase	Makes a DNA copy of an RNA molecule
Polynucleotide kinase	Adds a phosphate to the 5′-OH end of a polynucleotide to label it or permit ligation
Terminal transferase	Adds homopolymer tails to the 3′-OH ends of a linear duplex
Exonuclease III	Removes nucleotide residues from the 3′ ends of a DNA strand
Bacteriophage λ exonuclease	Removes nucleotides from the 5′ ends of a duplex to expose single-stranded 3′ ends
Alkaline phosphatase	Removes terminal phosphates from either the 5′ or 3′ end (or both)

Table 9-1
Lehninger Principles of Biochemistry, Fifth Edition

Restriction endonucleases

- **TYPE I: Restrict away from recognition site**
- **TYPE II: Restrict within recognition site**

- **TYPE III: Restrict away from recognition site**

Type II Restriction endonucleases

- Also called restriction enzymes
- Occur naturally in bacteria
- Hundreds are purified and available commercially
- Named for bacterial genus, species, strain, and type

Example: *Eco*RI

Genus: *Escherichia*
Species: *coli*
Strain: R

Restriction Endonuclease Specificity

Restriction endonucleases recognize a specific DNA sequence, cutting ONLY at that sequence

- They recognize 4-bp, 6-bp, 8-bp palindromic sequences
- The frequency of cuts lessens as the recognition sequence is longer
- They cut DNA reproducibly in the same place

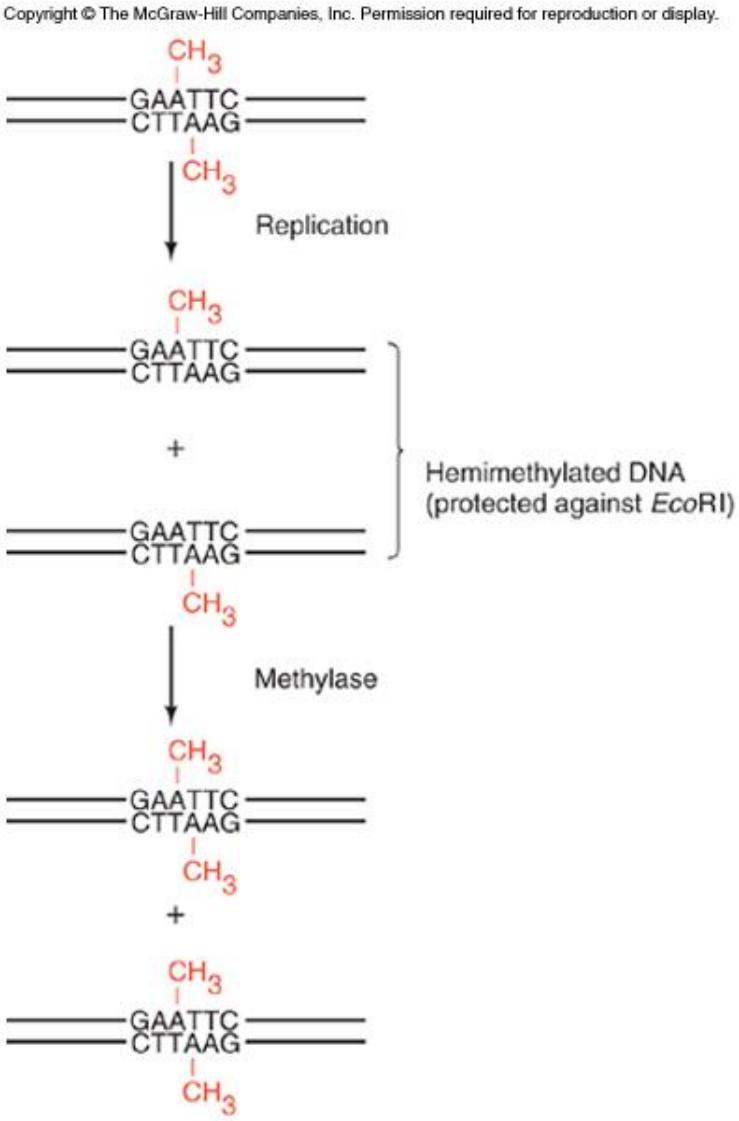
Table 4.1 Recognition Sequences and Cutting Sites of Selected Restriction Endonucleases

Enzyme	Recognition Sequence*
*Alu*I	A G ↓ C T
*Bam*HI	G ↓ G A T C C
*Bgl*II	A ↓ G A T C T
*Cla*I	A T ↓ C G A T
*Eco*RI	G ↓ A A T T C
*Hae*III	G G ↓ C C
*Hin*dII	G T Py ↓ Pu A C
*Hin*dIII	A ↓ A G C T T
*Hpa*II	C ↓ C G G
*Kpn*I	G G T A C ↓ C
*Mbo*I	↓ G A T C
*Pst*I	C T G C A ↓ G
*Pvu*I	C G A T ↓ C G
*Sal*I	G ↓ T C G A C
*Sma*I	C C C ↓ G G G
*Xma*I	C ↓ C C G G G
*Not*I	G C ↓ G G C C G C

*Only one DNA strand, written 5′→3′ left to right is presented, but restriction endonucleases actually cut double-stranded DNA as illustrated in the text for *Eco*RI. The cutting site for each enzyme is represented by an arrow.

Restriction-Modification System

- What prevents these enzymes from cutting up the host DNA?
  - They are paired with methylases
  - Theses enzymes recognize, methylate the same site
- Together they are called a restriction-modification system, R-M system
- Methylation protects DNA, after replication the parental strand is already methylated



- Star activity
- Buffer systems
- Enzyme activity
- Isoschizomers
- dam/dcm sensitivity

Cutting and Joining DNA Fragments

Restriction Enzyme: cleaves DNA at specific sequences

DNA Ligase: joins DNA fragments by sealing nicks in backbone

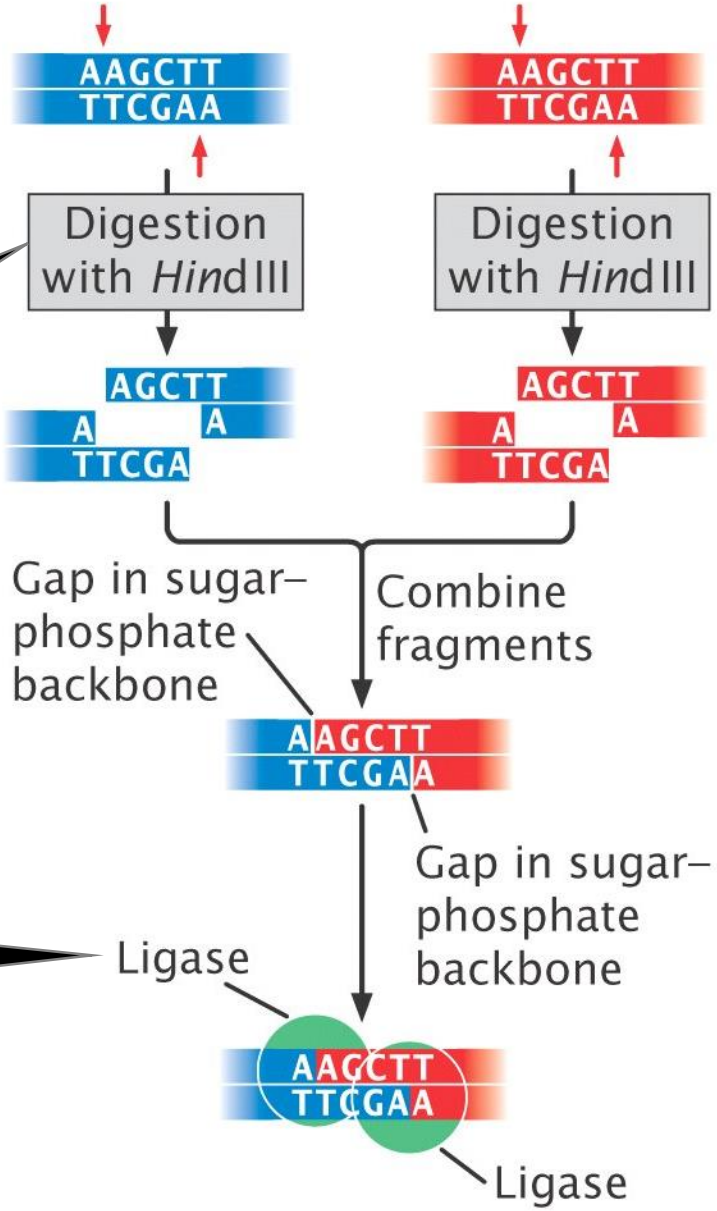
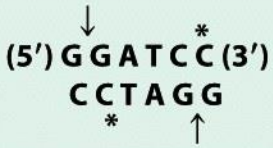
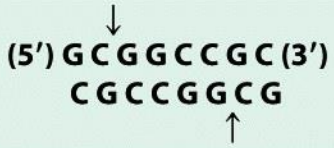
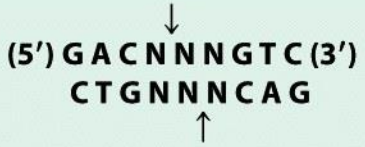


TABLE 9–2 Recognition Sequences for Some Type II Restriction Endonucleases

***Bam*HI**		***Hind*III**	
***Cla*I**		***Not*I**	
***Eco*RI**		***Pst*I**	
***Eco*RV**		***Pvu*II**	
***Hae*III**		***Tth*111I**	

Arrows indicate the phosphodiester bonds cleaved by each restriction endonuclease. Asterisks indicate bases that are methylated by the corresponding methylase (where known). N denotes any base. Note that the name of each enzyme consists of a three-letter abbreviation (in italics) of the bacterial species from which it is derived, sometimes followed by a strain designation and Roman numerals to distinguish different restriction endonucleases isolated from the same bacterial species. Thus *Bam*HI is the first (I) restriction endonuclease characterized from B*acillus* amy*loliquefaciens,* strain H.

Table 9-2

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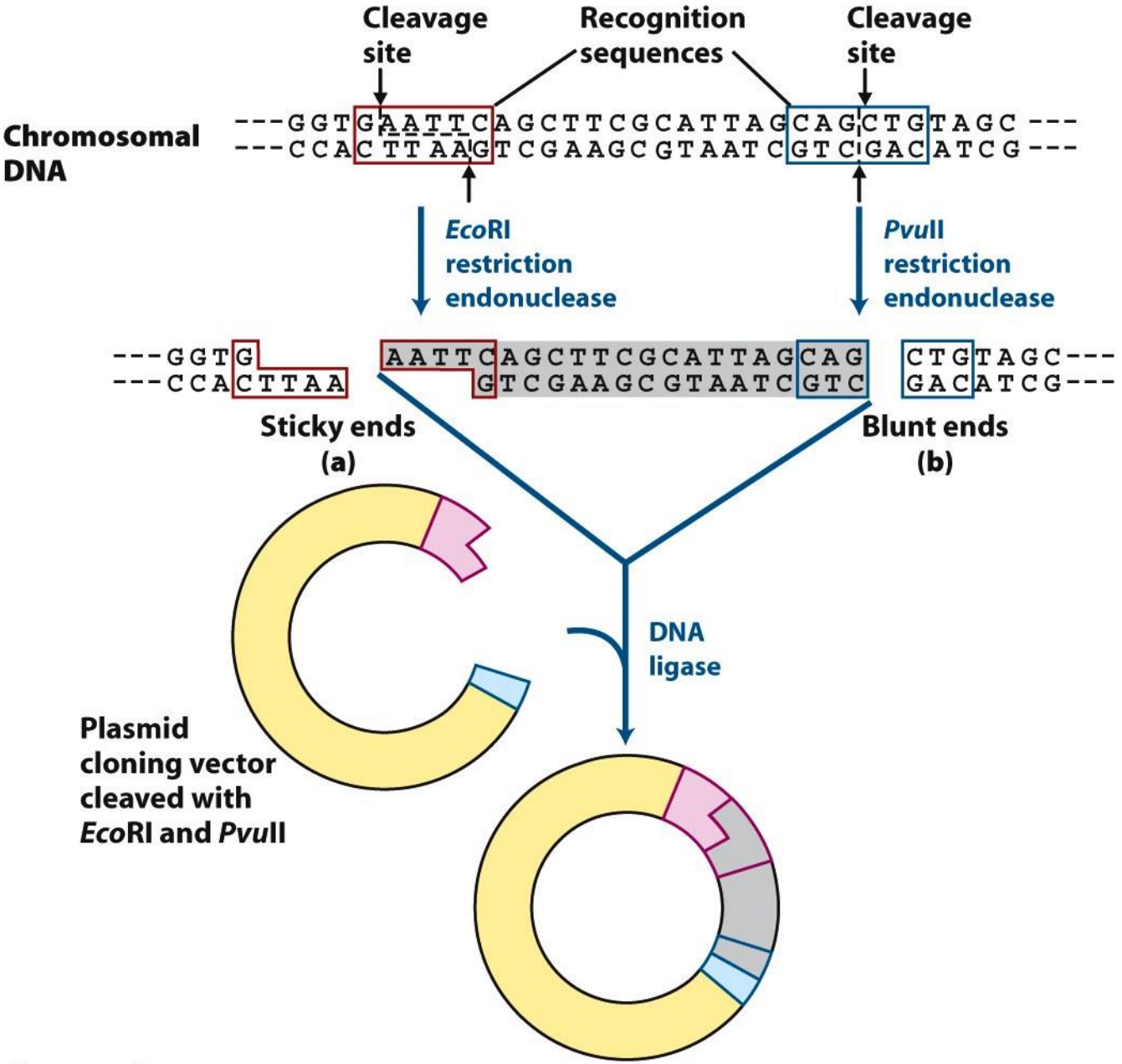

Figure 9-2ab

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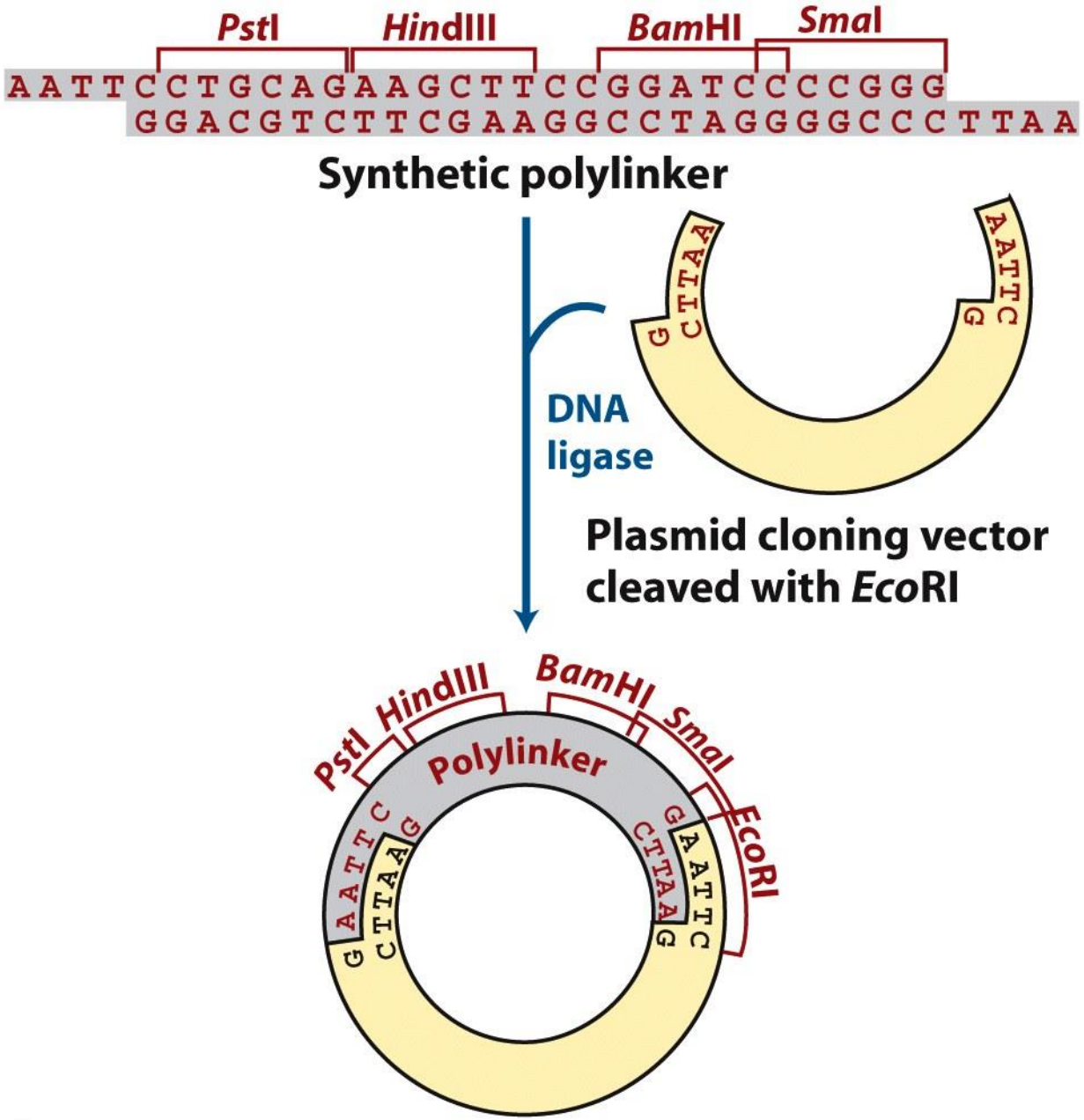

Figure 9-2c

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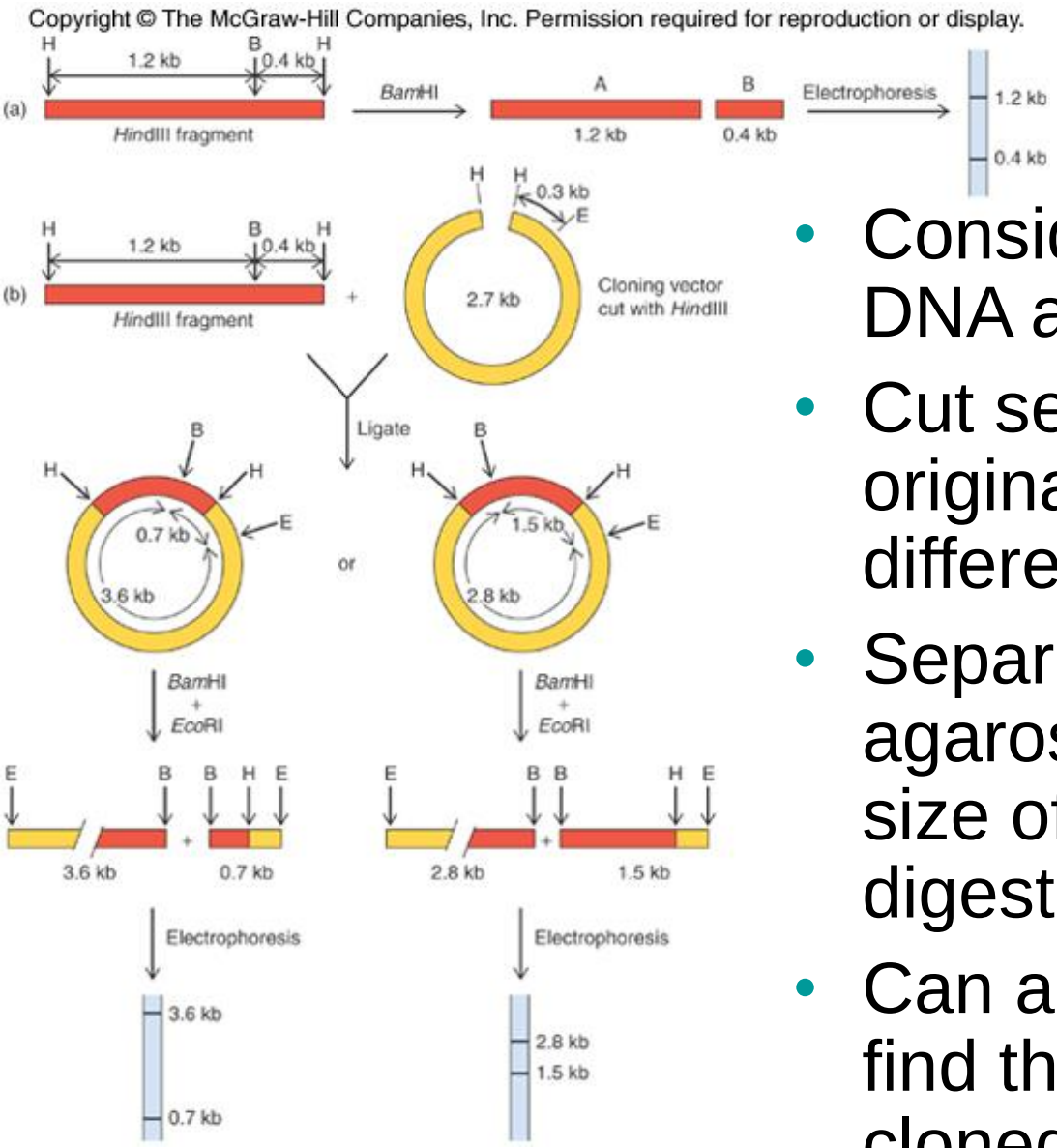
Terminal Transferase

- cDNAs don't have the sticky ends of genomic DNA cleaved with restriction enzymes
- Blunt ends will ligate, but is inefficient
- Generate sticky ends using enzyme terminal deoxynucleotidyl transferase (TdT), terminal transferase with one dNTP
  - If use dCTP with the enzyme
  - dCMPs are added one at a time to 3' ends of the cDNA
  - Same technique adds oligo(dG) ends to vector
  - Generate ligation product ready for transformation

Restriction Mapping

- Prior to the start of large-scale sequencing preliminary work is done to locate landmarks
  - A map based on physical characteristics is called a physical map
  - If restriction sites are the only map features then a restriction map has been prepared

Restriction Map Example



- Consider a 1.6 kb piece of DNA as an example
- Cut separate samples of the original 1.6 kb fragment with different restriction enzymes
- Separate the digests on an agarose gel to determine the size of pieces from each digest
- Can also use same digest to find the orientation of an insert cloned into a vector

About vectors!

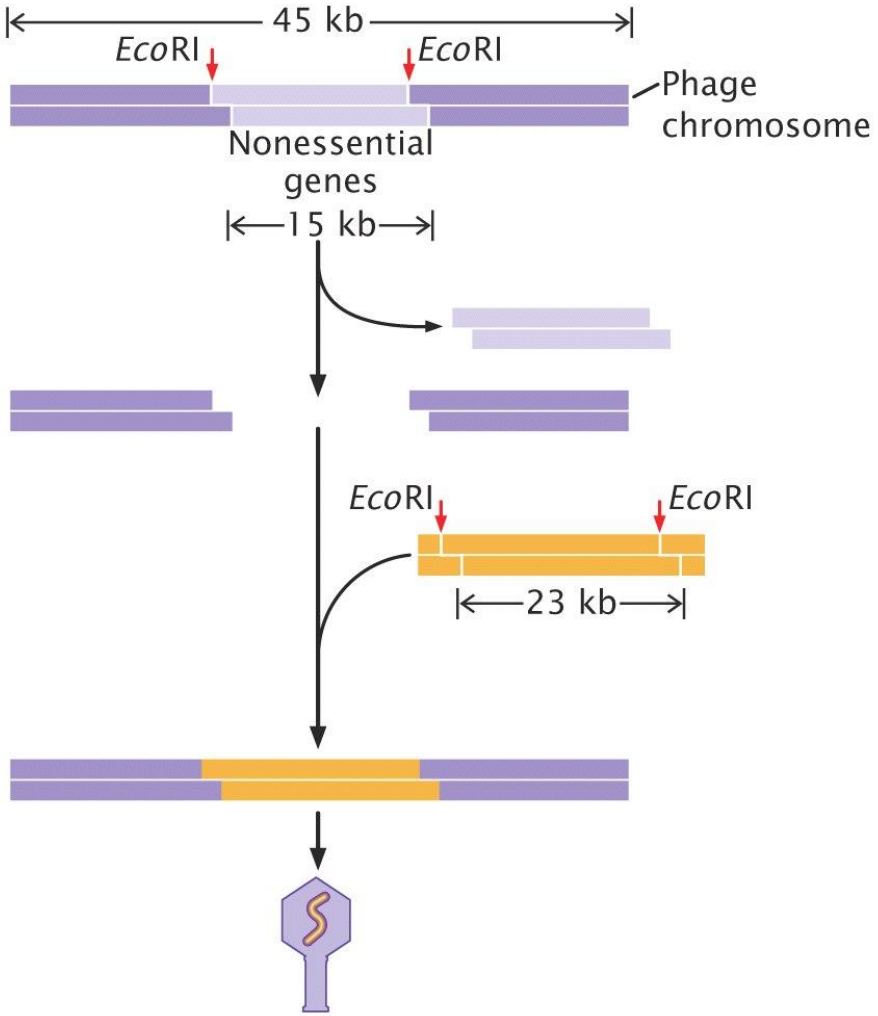
- **They have selectable markers, origin of replication and increased copy numbers**
- **They can carry different sizes of DNA molecules**
- **Cosmids carry large sized DNA molecules**
- **YAC carry even larger pieces of DNA**

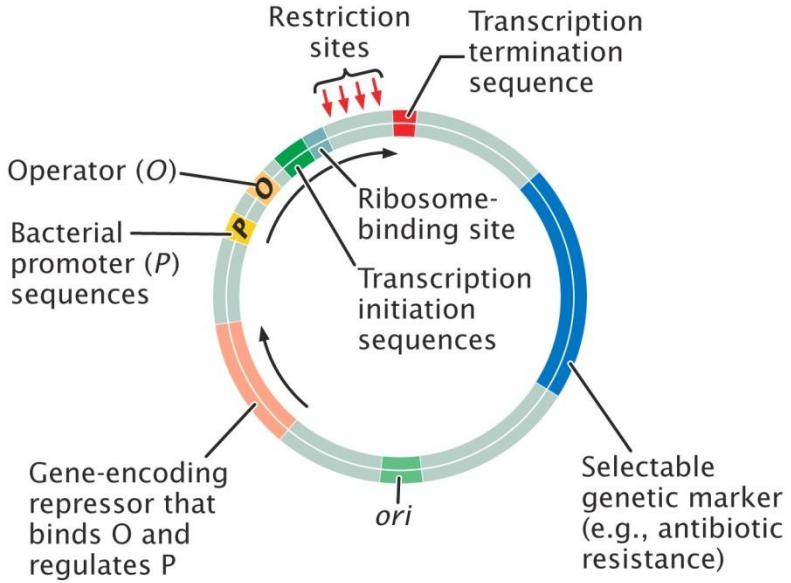
Commonly used vectors

- **pUC18 (Expression vector)**
- **pBR322 (cloning vector)**
- **M13 (sequencing vector)**
- **Lambda vector**
- ***Agrobacterium tumefaciencs* (used for plants)**
- **YAC**

Cloning Vectors

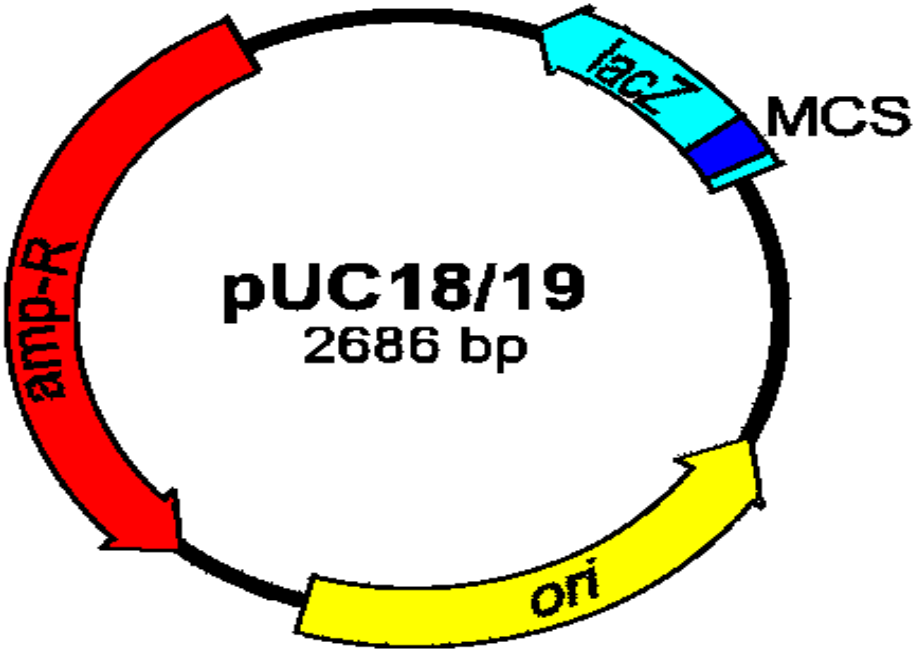
- For Bacterial Hosts
  - Bacteriophage
  - Cosmid
  - Expression Vector

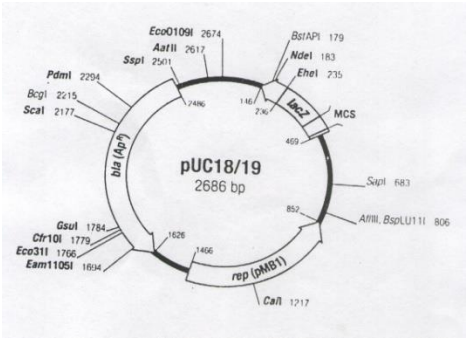




Plasmids As Vectors

- pBR plasmids were developed early but are rarely used today
- pUC series is similar to pBR
  - 40% of the DNA has been deleted
  - Cloning sites are clustered together into one area called the multiple cloning site (MCS)
  - MCS allows one to cut the vector and foreign gene with two different restriction enzymes and use a directional cloning technique to know the orientation of the insert





Useful Plasmid Features

- Relaxed Replication
- Selectable Markers
- Streamlined
- Polylinker or MCS
- Identification of Recombinants
- most derived from pUC or pBR322

Multiple Cloning Site:

```
|SacI|  |ScII|          |XbaI||SpeI||BamH||SmaI||PstI||EcRI||EcRV||HIII||ClaI|   |SalI||XhoI|        |KpnI|
GAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACC
CTCGAGGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAAGCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGG
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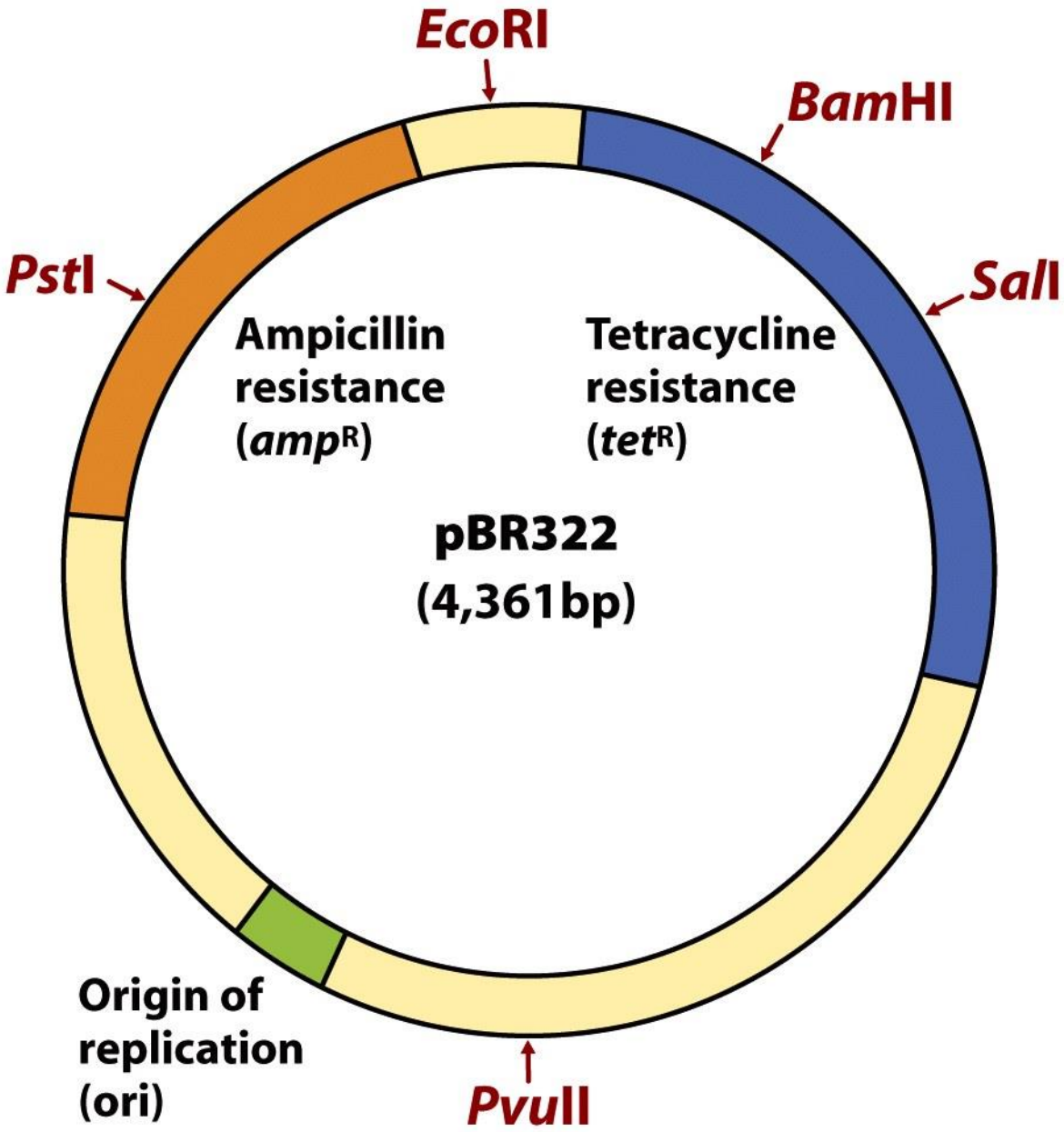

Figure 9-3
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Screening: antibiotics and β-galactosidase

Screening capabilities within plasmids:

- Antibiotic resistance genes (i.e., ampicillin resistance gene) allow for the selection of bacteria that have received a copy of the vector
- Multiple cloning site inserted into the gene *lac*Z' coding for the enzyme β-galactosidase
  - Clones with foreign DNA in the MCS disrupt the ability of the cells to make β-galactosidase
  - Plate on media with a β-galactosidase indicator (X-gal) and clones with intact β-galactosidase enzyme will produce blue colonies
  - Colorless colonies should contain the plasmid with foreign DNA compared to blue colonies that do not contain the plasmid with DNA

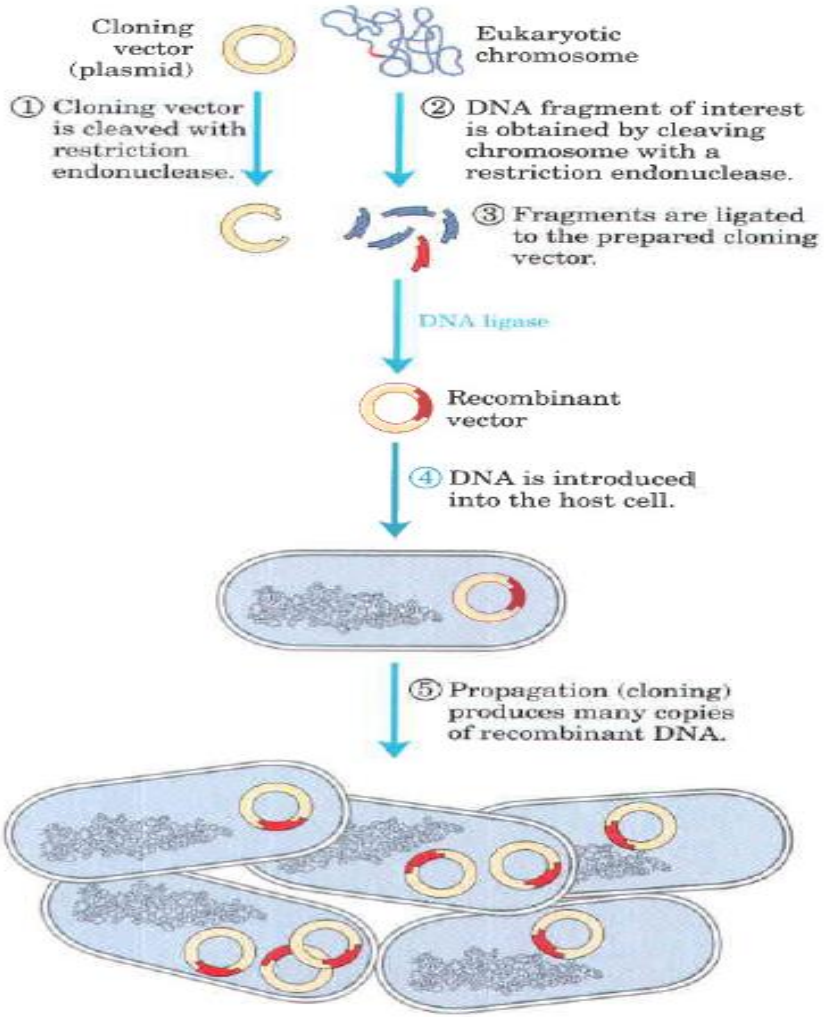

FIGURE 9–1 Schematic illustration of DNA cloning. A cloning vector

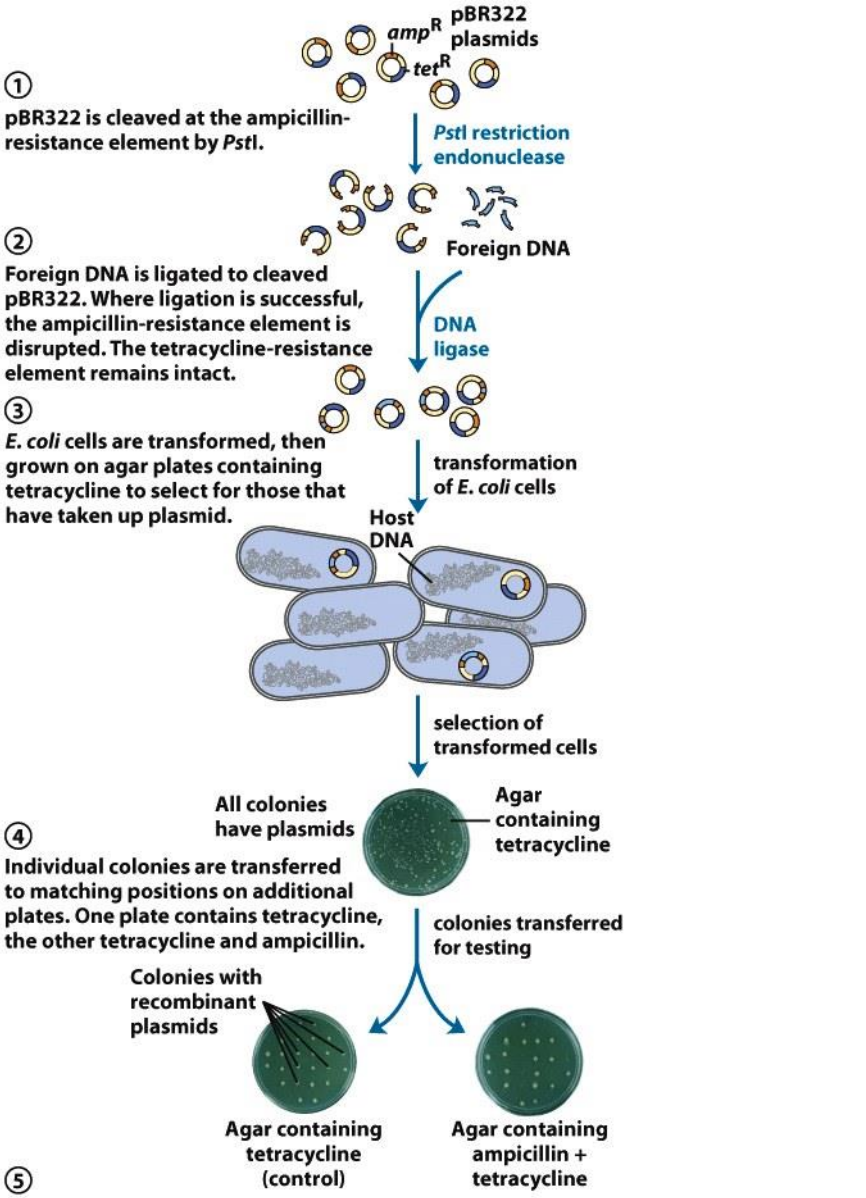

Figure 9-4

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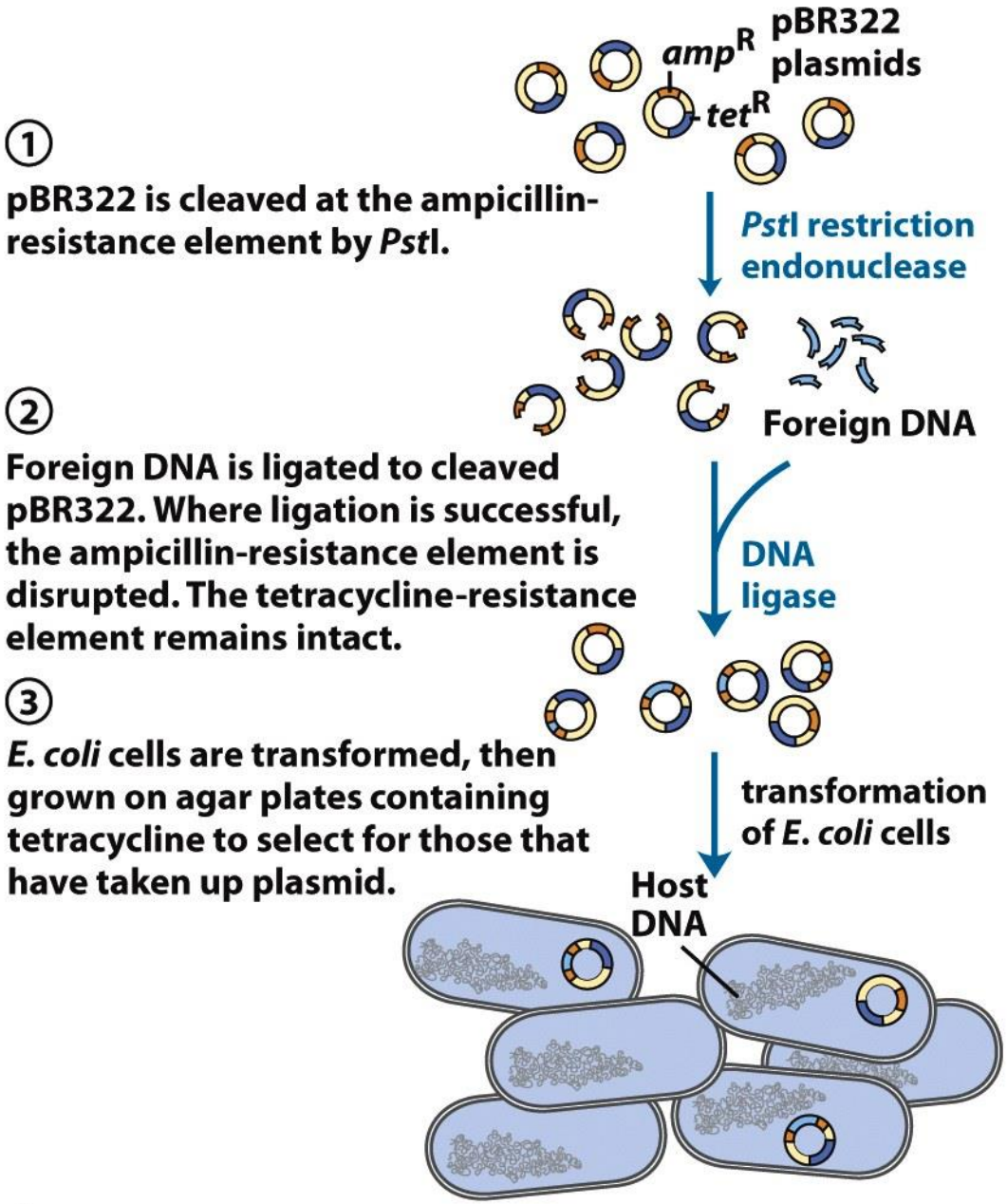

Figure 9-4 part 1

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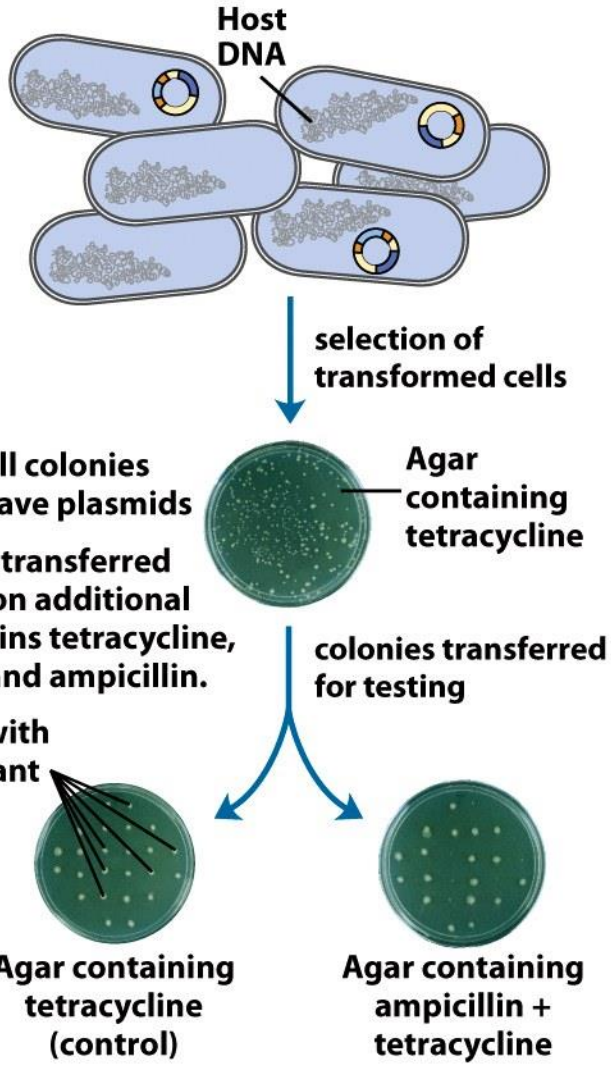

Figure 9-4 part 2

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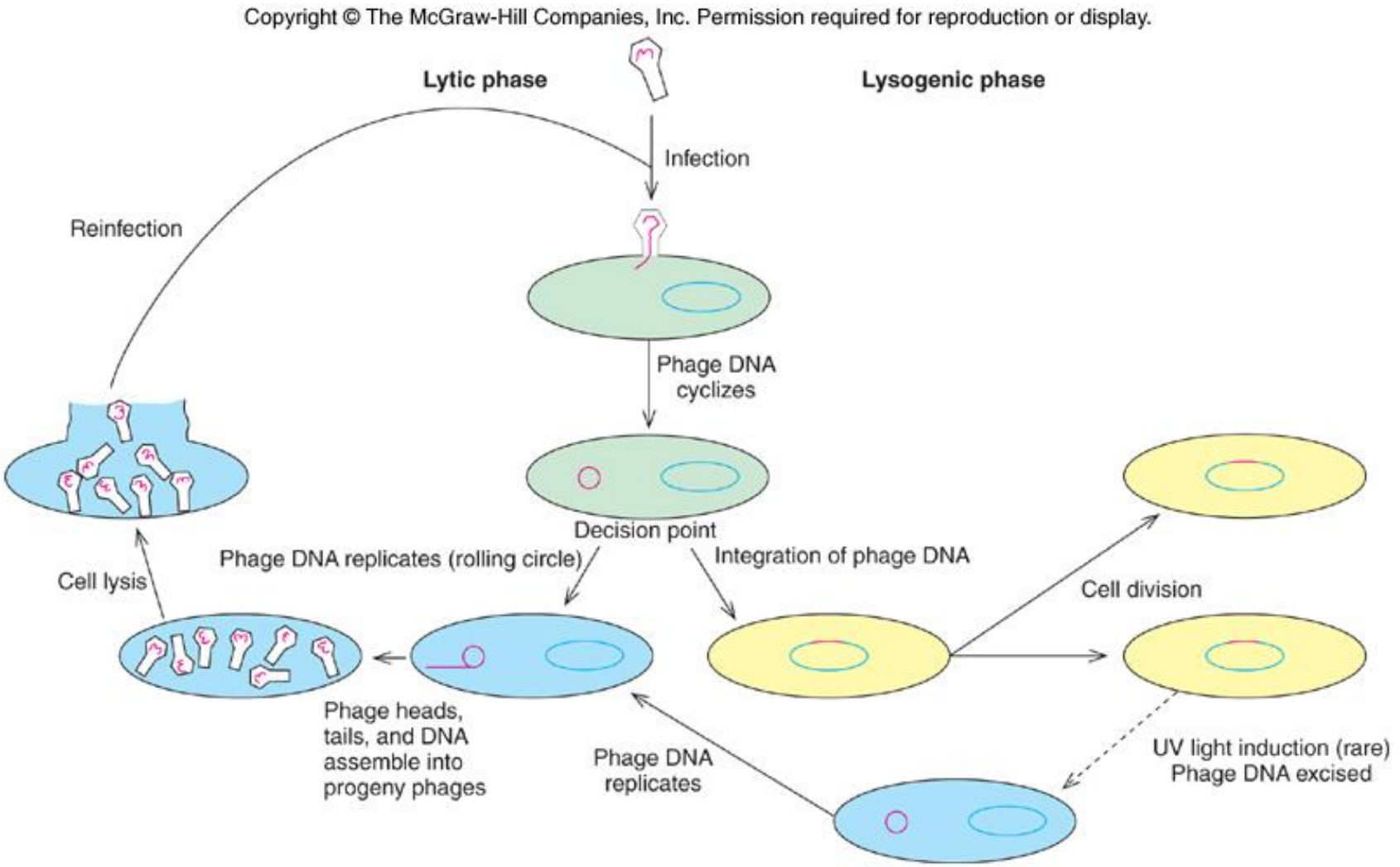
Phages As Vectors

- Bacteriophages are natural vectors that transduce bacterial DNA from one cell to another
- Phage vectors infect cells much more efficiently than plasmids transform cells
- Clones are not colonies of cells using phage vectors, but rather plaques, a clearing of the bacterial lawn due to phage killing the bacteria in that area

λ Phage Vectors

- First phage vectors were constructed by Fred Blattner and colleagues
  - Modifications included removal of the middle region and retention of the genes needed for phage replication
  - Could replace removed phage genes with foreign DNA
- Advantage: Phage vectors can receive larger amounts of foreign DNA (up to 20kb of DNA)
  - Traditional plasmid vectors take much less
- Phage vectors require a minimum size foreign DNA piece (12 kb) inserted to package into a phage particle

Two Paths of Phage Reproduction



Lysogenic Mode

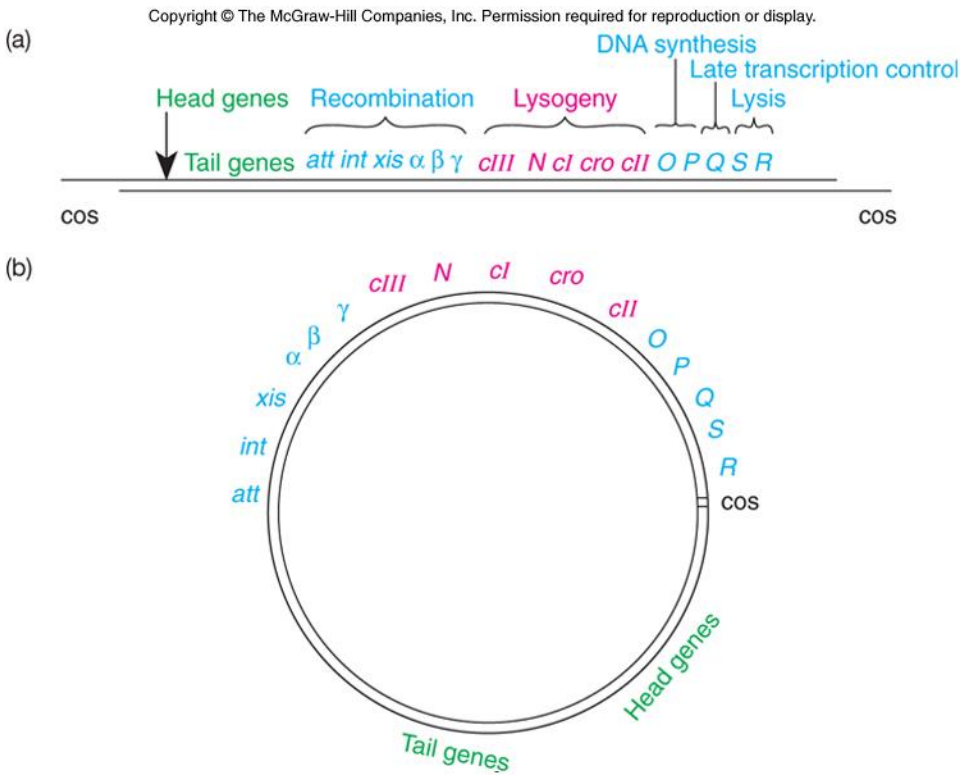
- A 27-kD phage protein (λ repressor, CI) appears and binds to 2 phage operator regions
- CI shuts down transcription of all genes except for *cI*, gene for λ repressor itself
- When lysogeny is established the phage DNA integrates into the bacterial genome
- A bacterium harboring integrated phage DNA is called a lysogen and the integrated DNA is called a prophage
- The phage DNA in the lysogen replicates along with the host DNA

Lytic Reproduction of Phage λ

- Lytic reproduction cycle of phage λ has 3 phases of transcription:
  - Immediate early
  - Delayed early
  - Late
- Genes of these phases are arranged sequentially on the phage DNA

Genetic Map of Phage λ

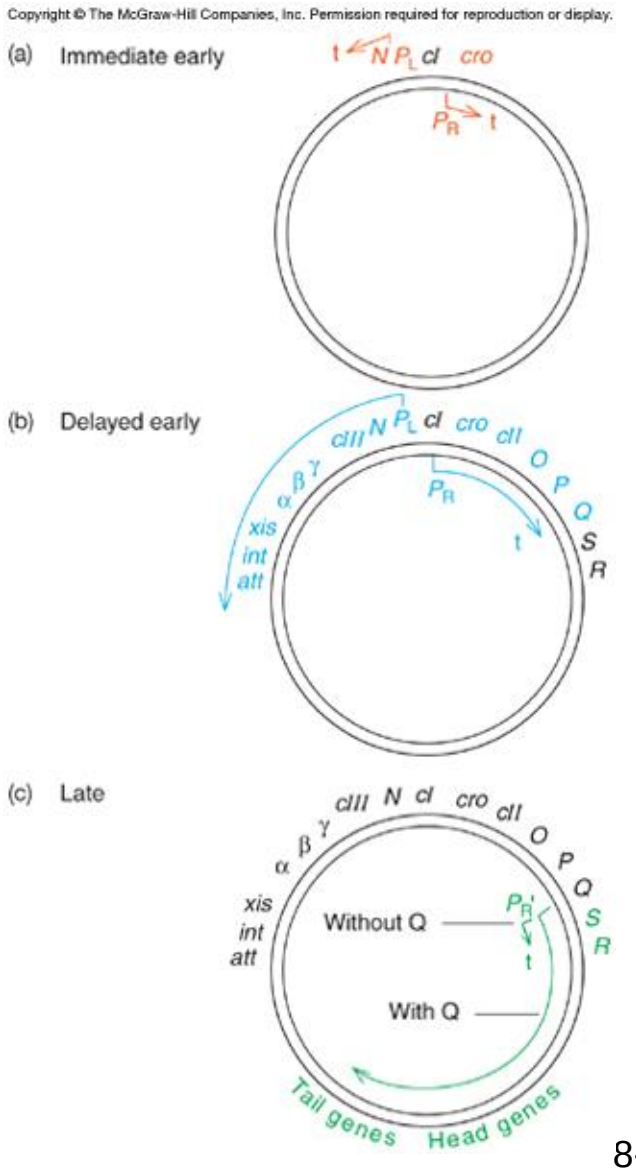
- DNA exists in linear form in the phage
- After infection of host begins the phage DNA circularizes
- This is possible as the linear form has sticky ends
- Gene transcription is controlled by transcriptional switches



Antitermination and Transcription

One of 2 immediate early genes is *cro*

- *cro* codes for a repressor of *cI* gene that allows lytic cycle to continue
- Other immediate early gene is *N* coding for N, an antiterminator

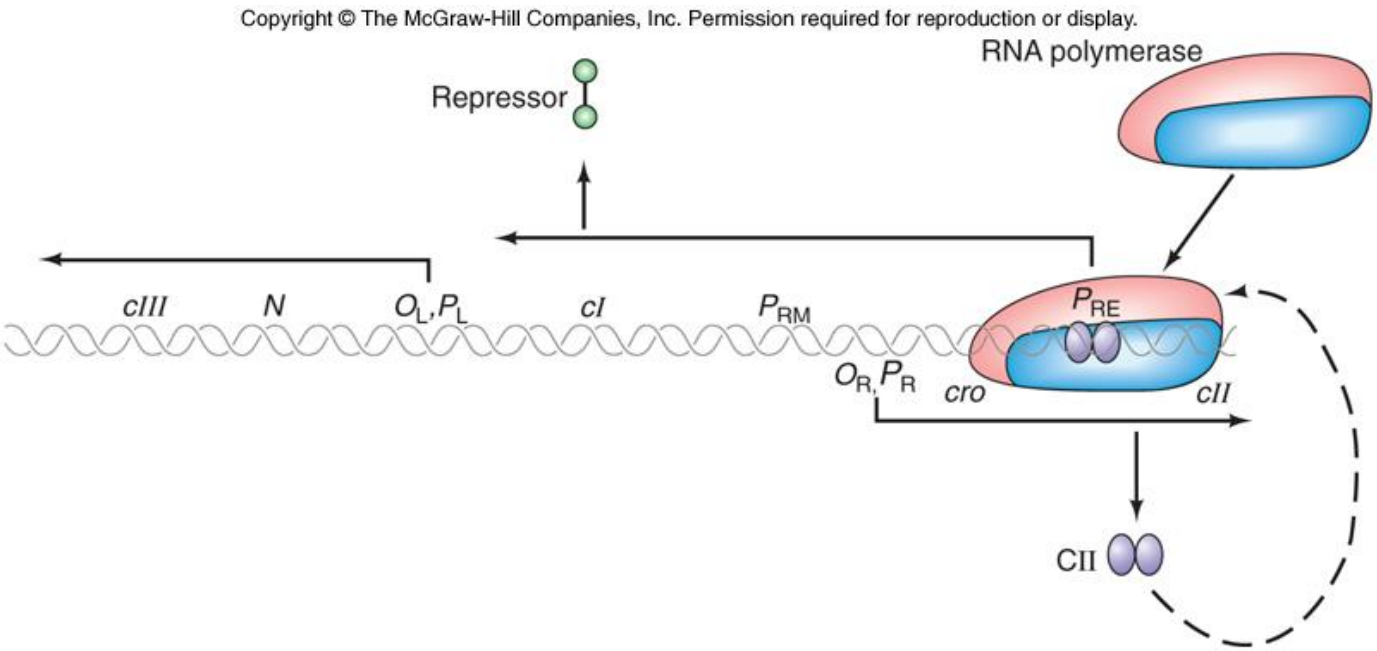


Establishing Lysogeny

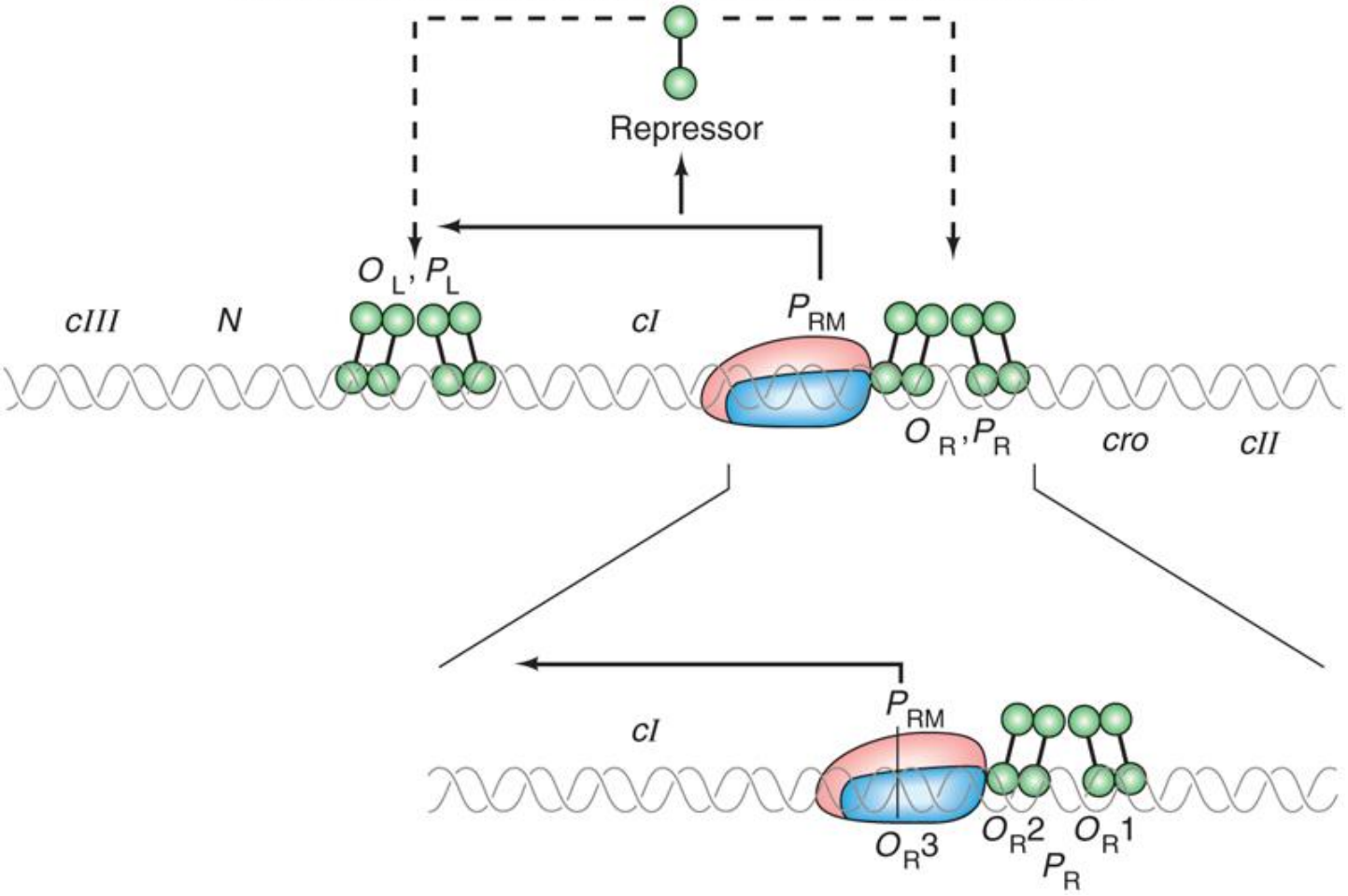
- Phage establish lysogeny by:
  - Causing production of repressor to bind to early operators
  - Preventing further early RNA synthesis
- Delayed early gene products are used
  - Integration into the host genome
  - Products of *c*II and *c*III allow transcription of the *c*I gene and production of λ repressor
- Promoter to establish lysogeny is P_{RE}

Model of Establishing Lysogeny

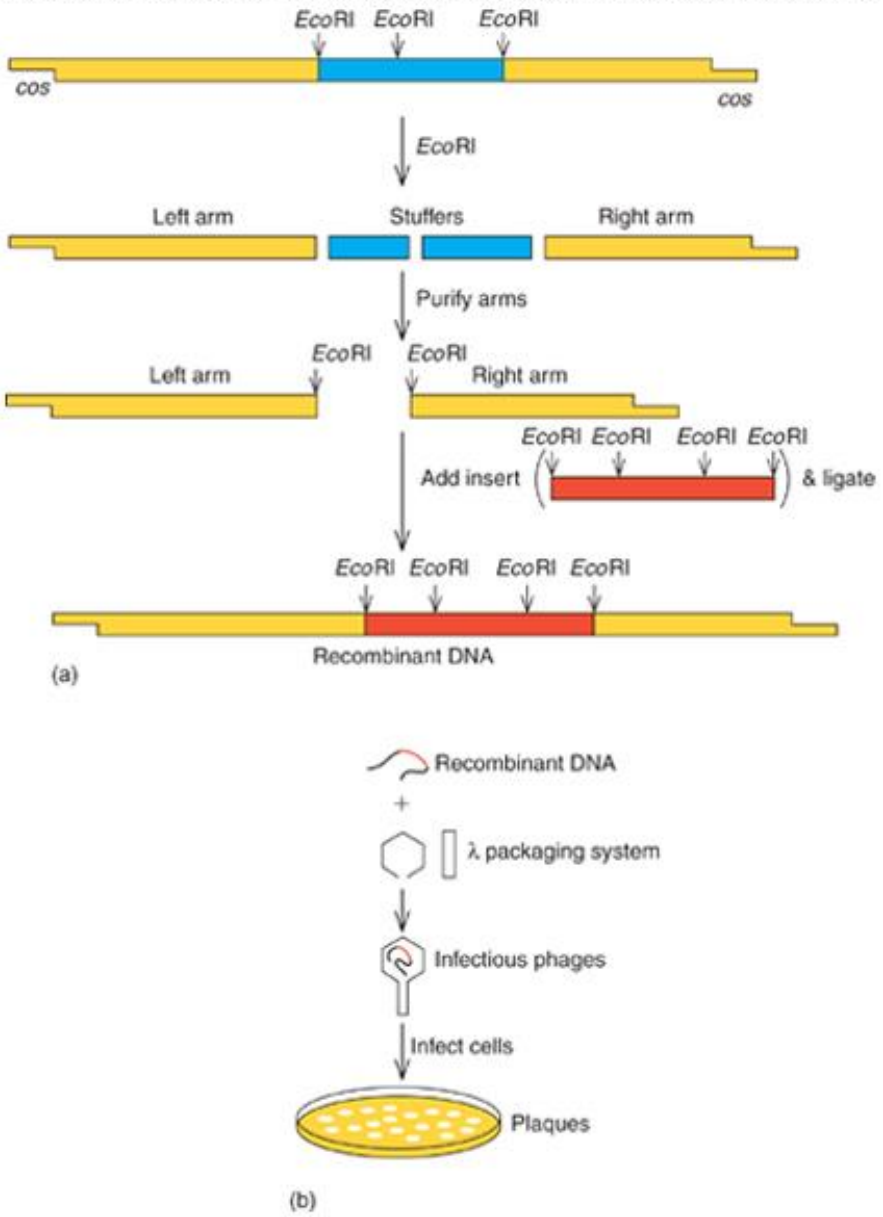
- Delayed early transcription from P_R produces *cII* mRNA translated to CII
- CII allows RNA polymerase to bind to P_{RE} and transcribe the *cI* gene, resulting in repression



Maintaining Lysogeny



Cloning Using a Phage Vector



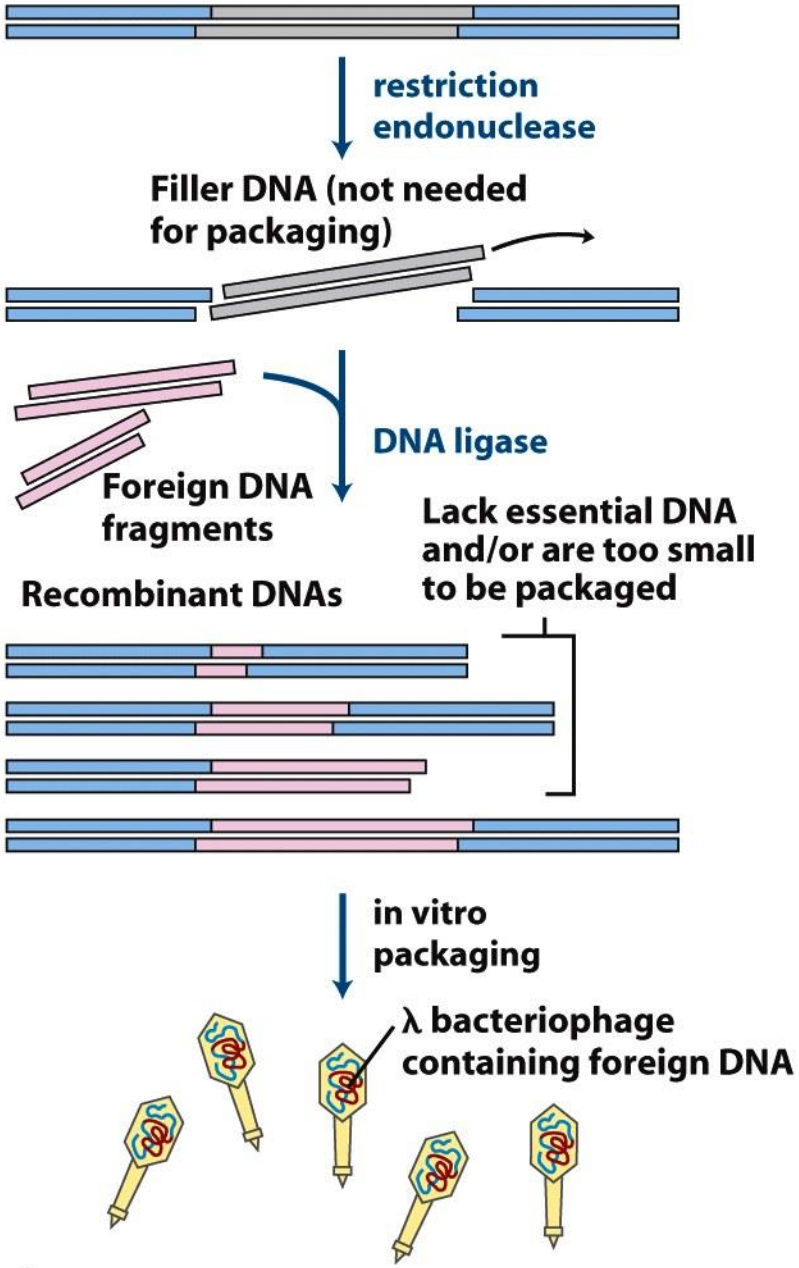


Figure 9-5

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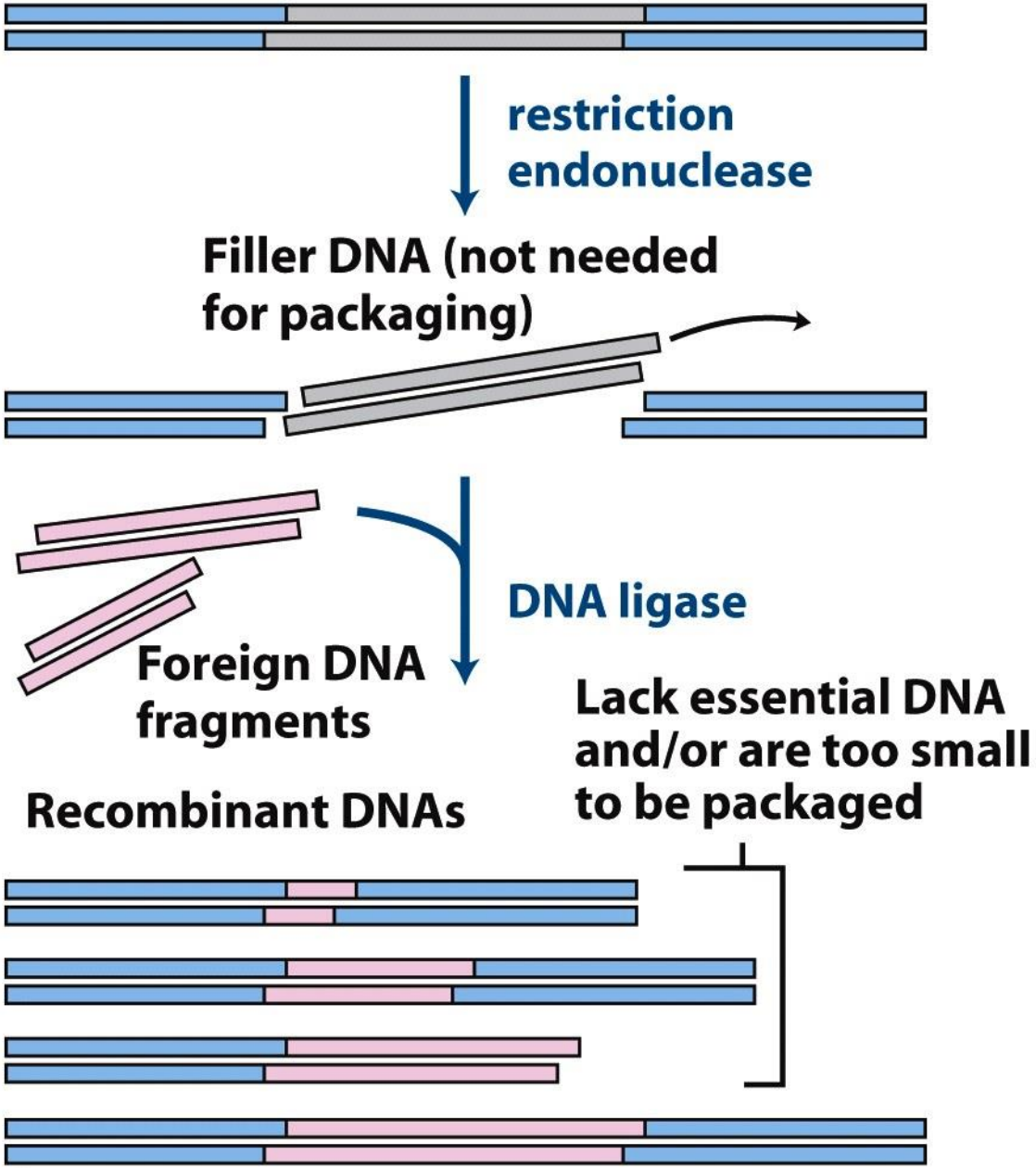

Figure 9-5 part 1

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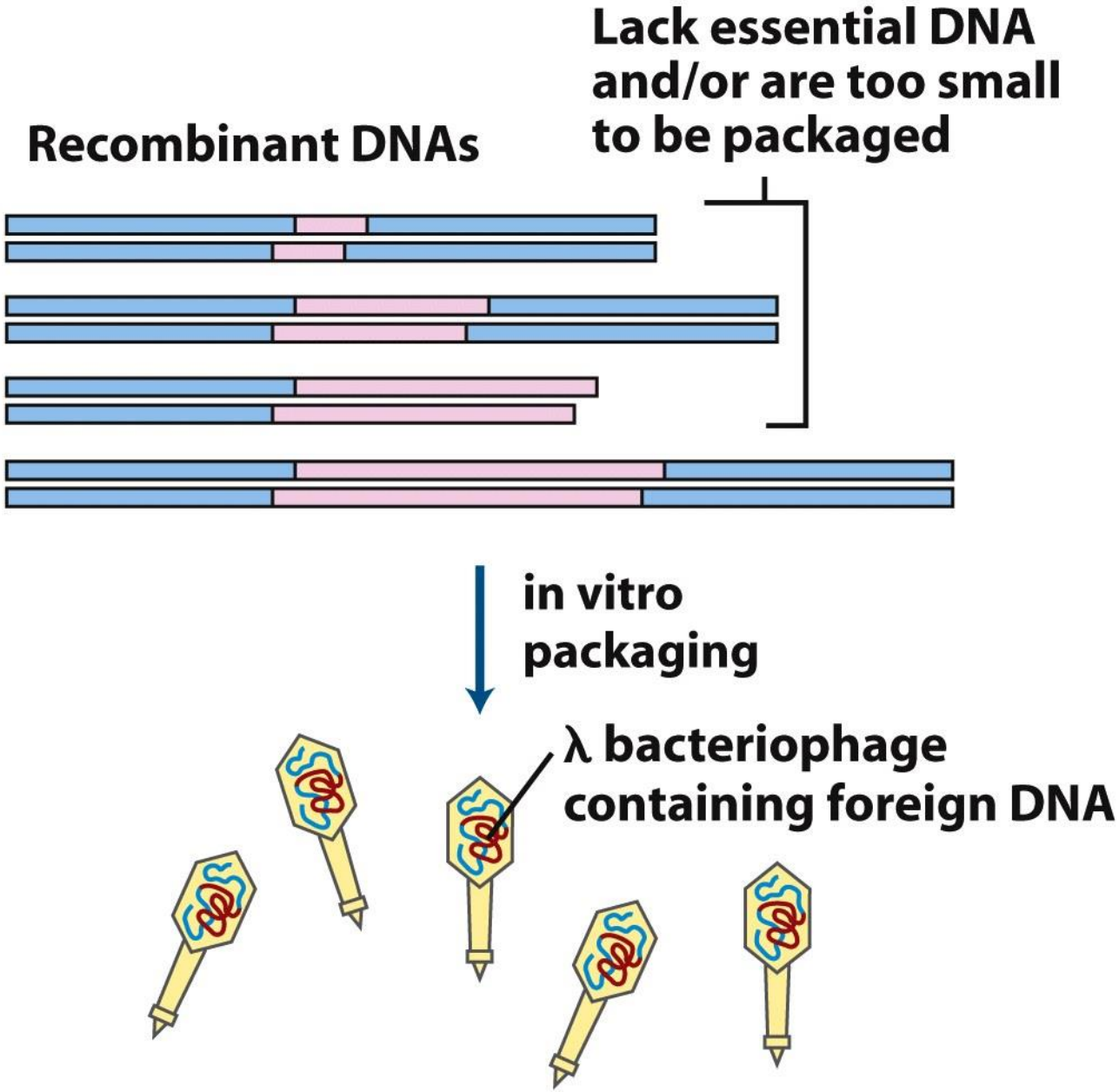

Figure 9-5 part 2
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hfl^{-} selection

- HflA decreases cII stability….hence lytic mode
- *hflA*$^{-}$ strain…..more suppressor….No plaques
- cI within MCS……
  - With insert…… lytic
  - Without insert….lysogenic (in *hflA*$^{-}$ strain)

Cosmids

Cosmids are designed for cloning large DNA fragments

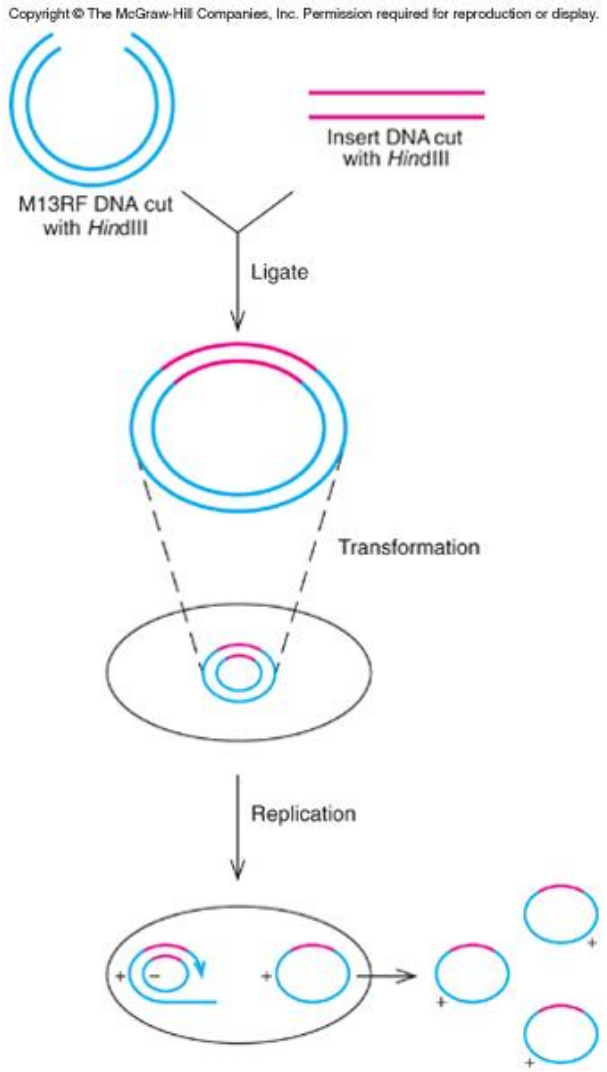
- Behave both as plasmid and phage and contain
  - *cos* sites, cohesive ends of phage DNA that allow the DNA to be packaged into a λ phage head
  - Plasmid origin of replication permitting replication as plasmid in bacteria
- Nearly all λ genome removed so there is room for large inserts (40-50 kb)
- Very little phage DNA yields them unable to replicate, but they are infectious and carry their recombinant DNA into bacterial cells

M13 Phage Vectors

- Long, thin, filamentous phage
- Contains:
  - Gene fragment with β-galactosidase
  - Multiple cloning site like the pUC family
- Advantage
  - This phage's genome is single-stranded DNA
  - Fragments cloned into it will be recovered in single-stranded form

M13 Cloning to Recover Single-stranded DNA Product

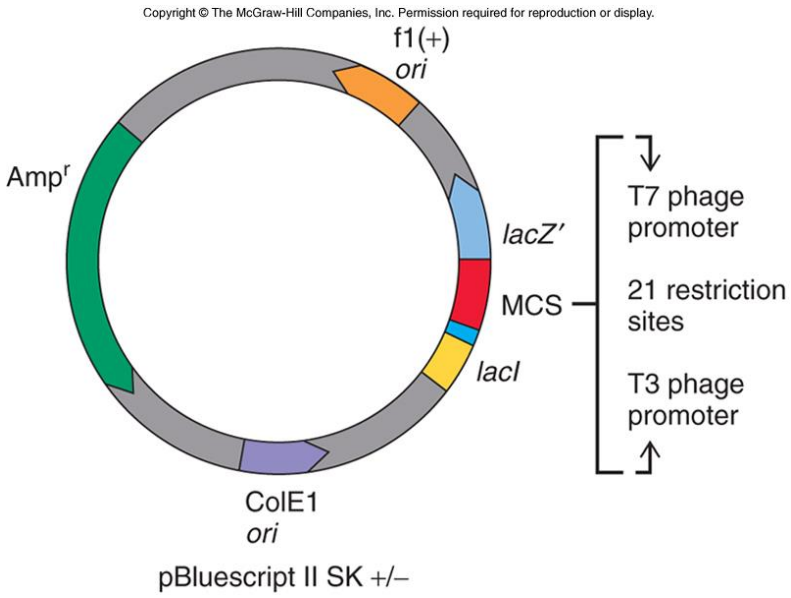
- After infecting *E. coli* cells, single-stranded phage DNA is converted to double-stranded replicative form (RF)
- Use the replicative form for cloning foreign DNA into MCS
- Recombinant DNA infects host cells resulting in single-stranded recombinant DNA
- Phage particles, containing single-stranded phage DNA is secreted from transformed cells and can be collected from media



Phagemids

Phagemids are also vectors

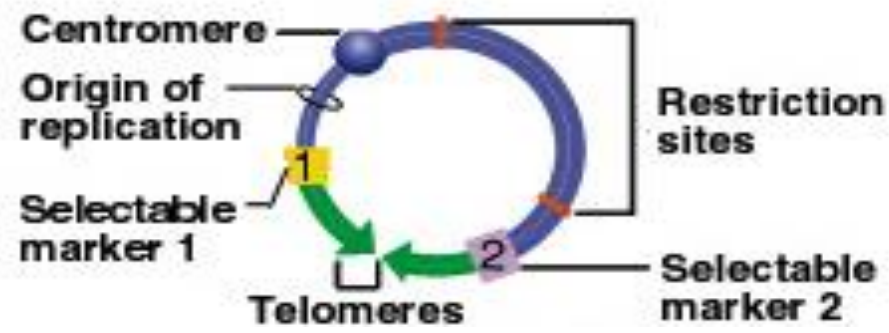
- Like cosmids have aspects of both phages and plasmids
- Has MCS inserted into *lacZ'* gene to screen blue/ white colonies
- Has origin of replication of single-stranded phage f1 to permit recovery of single-stranded recombinant DNA
- MCS has 2 phage RNA polymerase promoters, 1 on each side of MCS

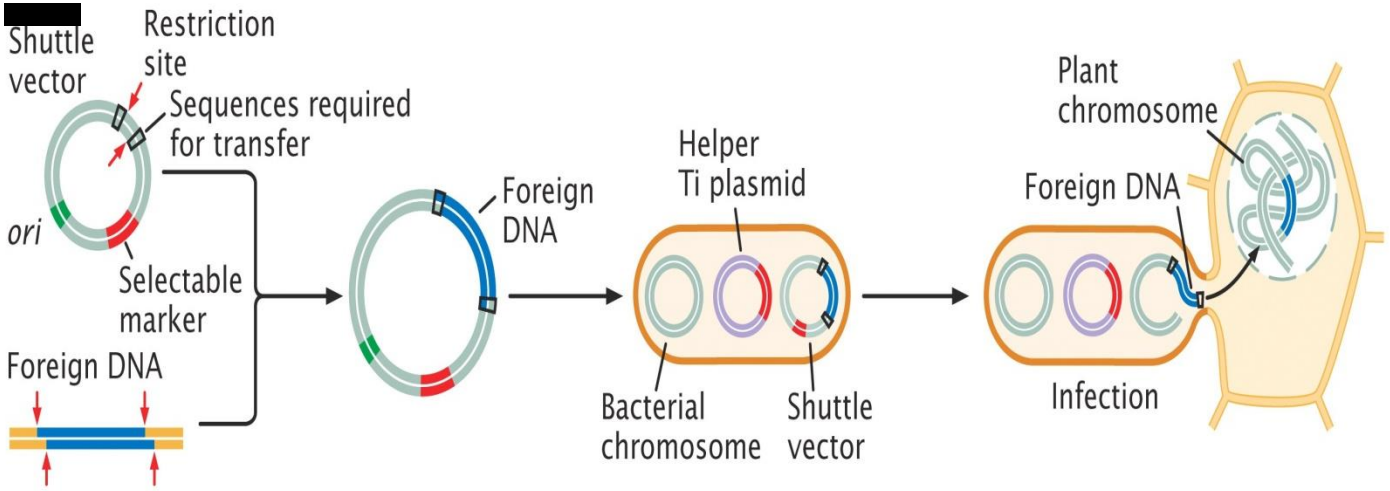


Eukaryotic Vectors and Very High Capacity Vectors

- There are vectors designed for cloning genes into eukaryotic cells
- Other vectors are based on the Ti plasmid to carry genes into plant cells
- Yeast artificial chromosomes (YAC) and bacterial artificial chromosomes (BAC) are used for cloning huge pieces of DNA

Ti plasmid and YAC





Vector	Insert (kb)	Host	Copy No.	
P1	70-100	*E. coli*	1	Bacteriophage P1
PAC	130-150	*E. coli*	1	P1 artificial chromosome
BAC	120-300	*E. coli*	1	
YAC	250-400	Yeast	1	