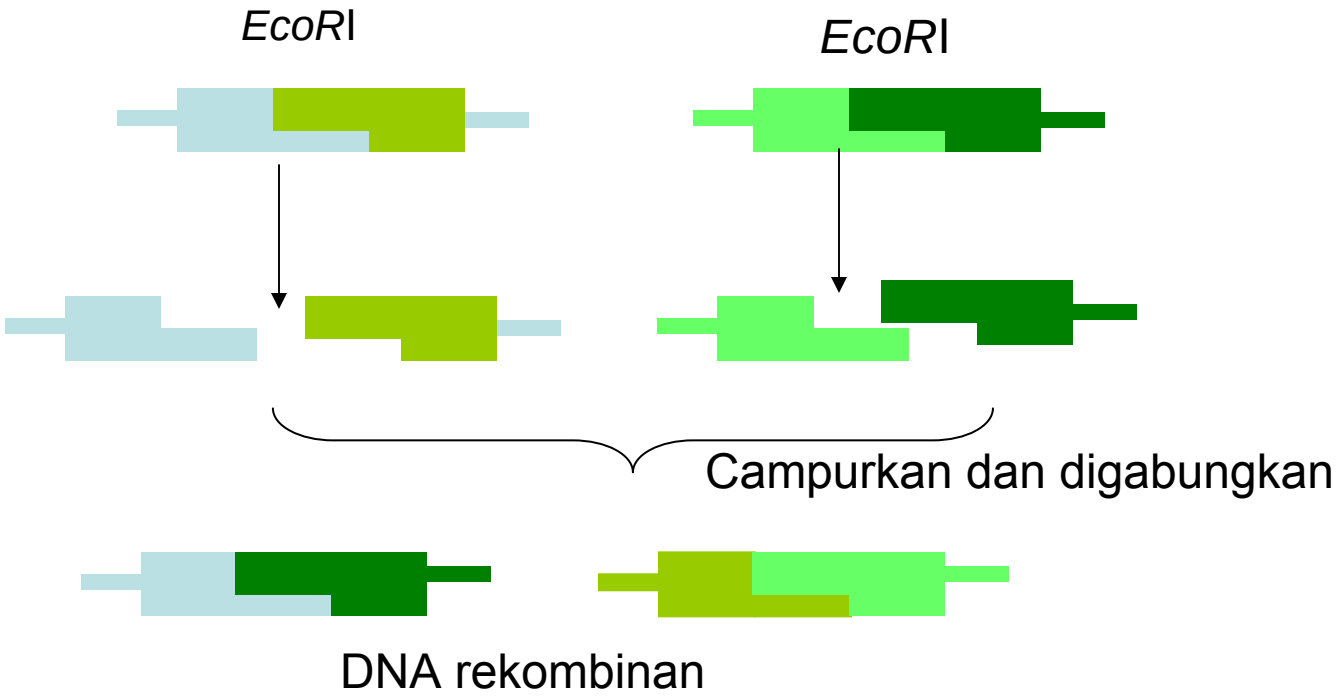
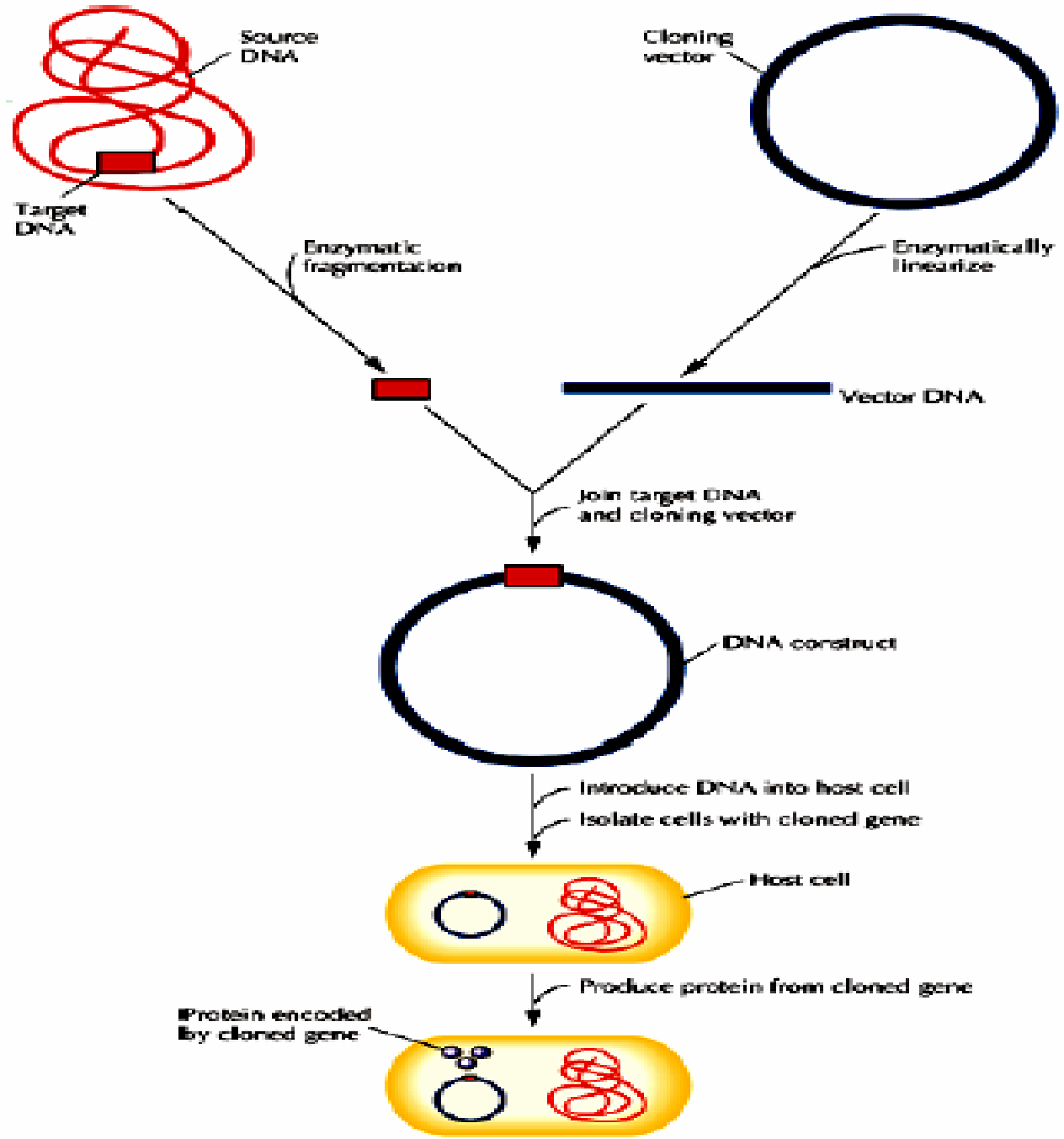
Teknologi DNA Rekombinan

Utut Widyastuti
Departemen Biologi-FMIPA IPB dan
Pusat Penelitian Sumberdaya Hayati dan Bioteknologi IPB

- DNA rekombinan– teknik yang memungkinkan penggabungan 2 molekul DNA yang berbeda, memperbanyaknya, dan memodifikasi.
- Isolasi potongan DNA diluar genomnya dan memperbanyaknya diluar selnya.
- Komponen yang terlibat: enzim restriksi endonuklease dan DNA ligase




Source DNA
Target DNA
Cloning vector
Enzymatic fragmentation
Enzymatically linearize
Vector DNA
Join target DNA and cloning vector
DNA construct
Introduce DNA into host cell
Isolate cells with cloned gene
Host cell
Produce protein from cloned gene
Protein encoded by cloned gene

Langkah dalam Rekayasa Genetika

1. Isolasi gen target
2. Isolasi DNA plasmid
3. Manipulasi urutan DNA
   a. Pemotongan- Enzim Restriksi
   b. Penggabungan- DNA ligasi
4. Transformasi ke bakteri
5. Seleksi bakteri rekombinan

How does one isolate a gene for an inherited disorder?

There are three options:

- **Start with a candidate protein**

- **Start with a candidate mRNA**

- **Direct positional cloning**

DNA

All three options require the cloning of DNA.

Manipulasi urutan DNA

Enzim Restriksi

Enzim restriksi- enzim yang akan spesifik memotong DNA beradasarkan urutan tertentu, endonuclease

"blunt end (ujung tumpul)" vs. "sticky end (ujung lancip)"

Pemotongan terbatas pada sekuen yang spesifik, 4-6 bp dan palindrom

Terdapat lebih 800 enzim restriksi yang telah diketahui

A. Origin and function

- Bacterial origin = enzymes that cleave foreign DNA
- Named after the organism from which they were derived
  - EcoRI from *Escherichia coli*
  - BamHI from *Bacillus amyloliquefaciens*
- Protect bacteria from bacteriophage infection
  - Restricts viral replication
- Bacterium protects it's own DNA by methylating those specific sequence motifs

C. Classes

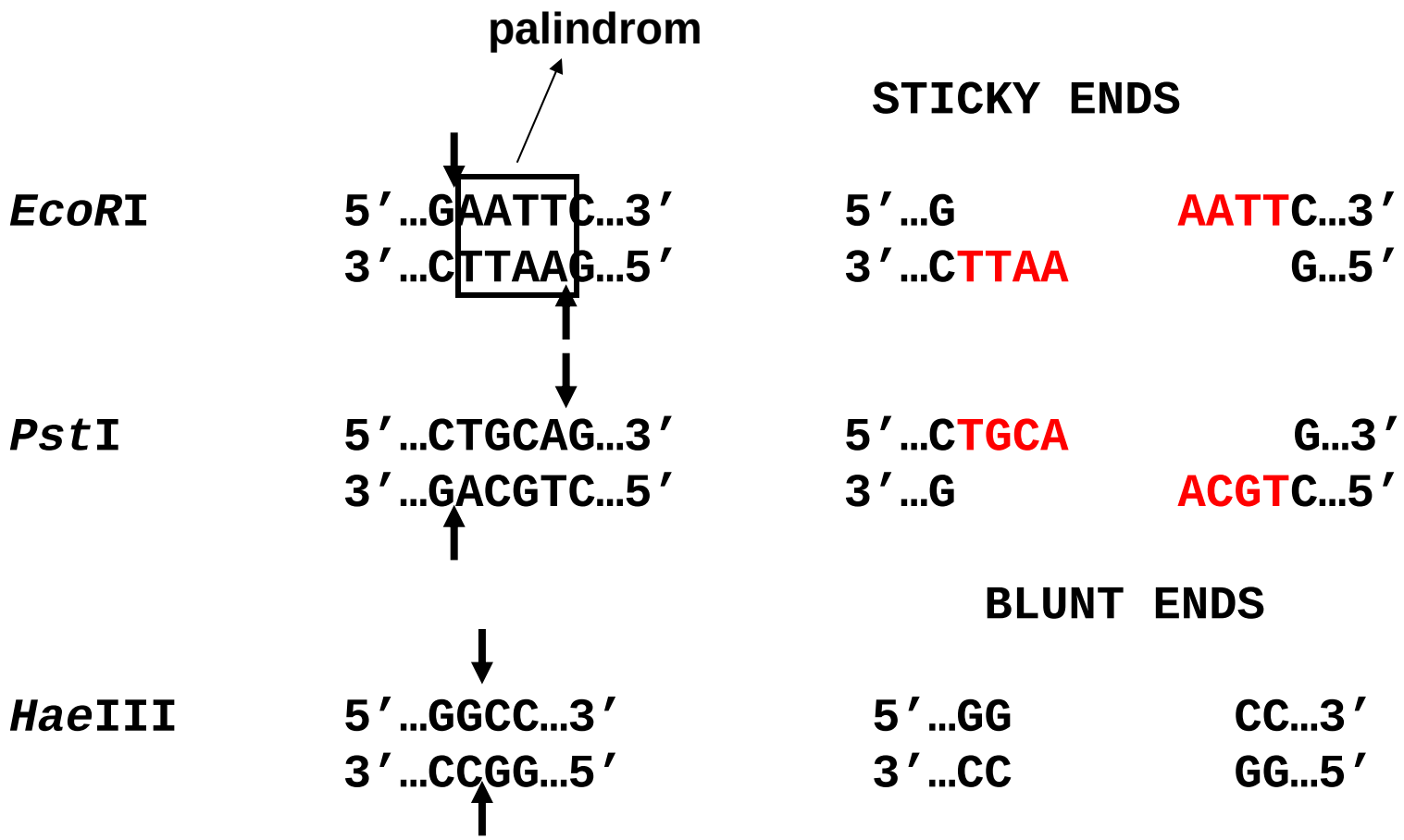
- Type I
  - Cuts the DNA on both strands but at a non-specific location at varying distances from the particular sequence that is recognized by the restriction enzyme
  - Therefore random/imprecise cuts
  - Not very useful for rDNA applications

- Type II
  - Cuts both strands of DNA within the particular sequence recognized by the restriction enzyme
  - Used widely for molecular biology procedures
  - DNA sequence = symmetrical

Type III- multisubunit, endonuclease and methylase about 25 bp from recognition sequence

- Reads the same in the 5'→ 3' direction on both strands = Palindromic Sequence
- Some enzymes generate "blunt ends" (cut in middle)
- Others generate "sticky ends" (staggered cuts)
  - H-bonding possible with complementary tails
  - DNA ligase covalently links the two fragments together by forming phosphodiester bonds of the phosphate-sugar backbones

Produk yang dihasilkan oleh enzim restriksi

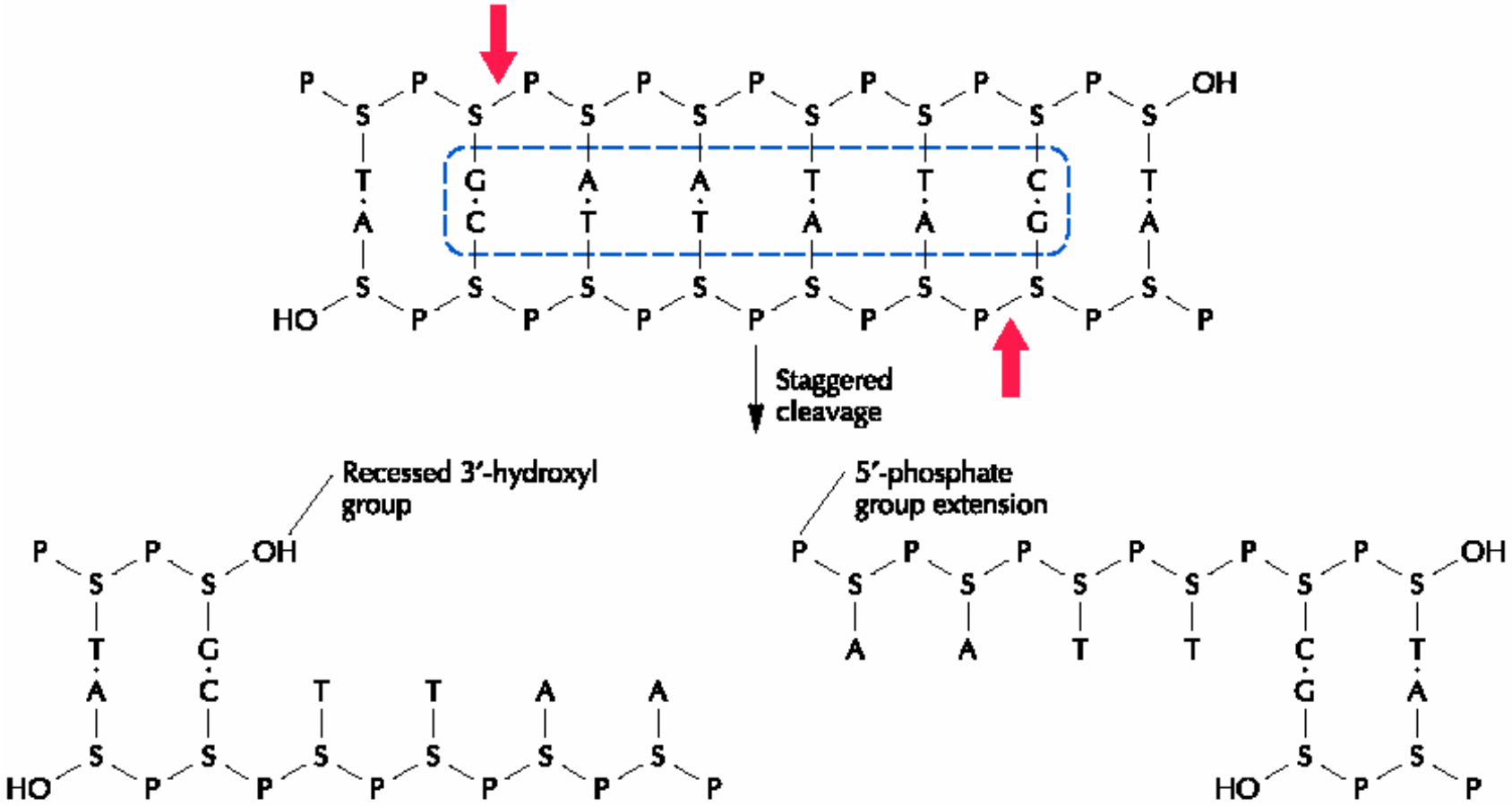


- One of the common features of most enzyme recognition sites is that they are palindromes. A palindrome is a sequence which is read the same on both strands in the 5' --> 3' direction.

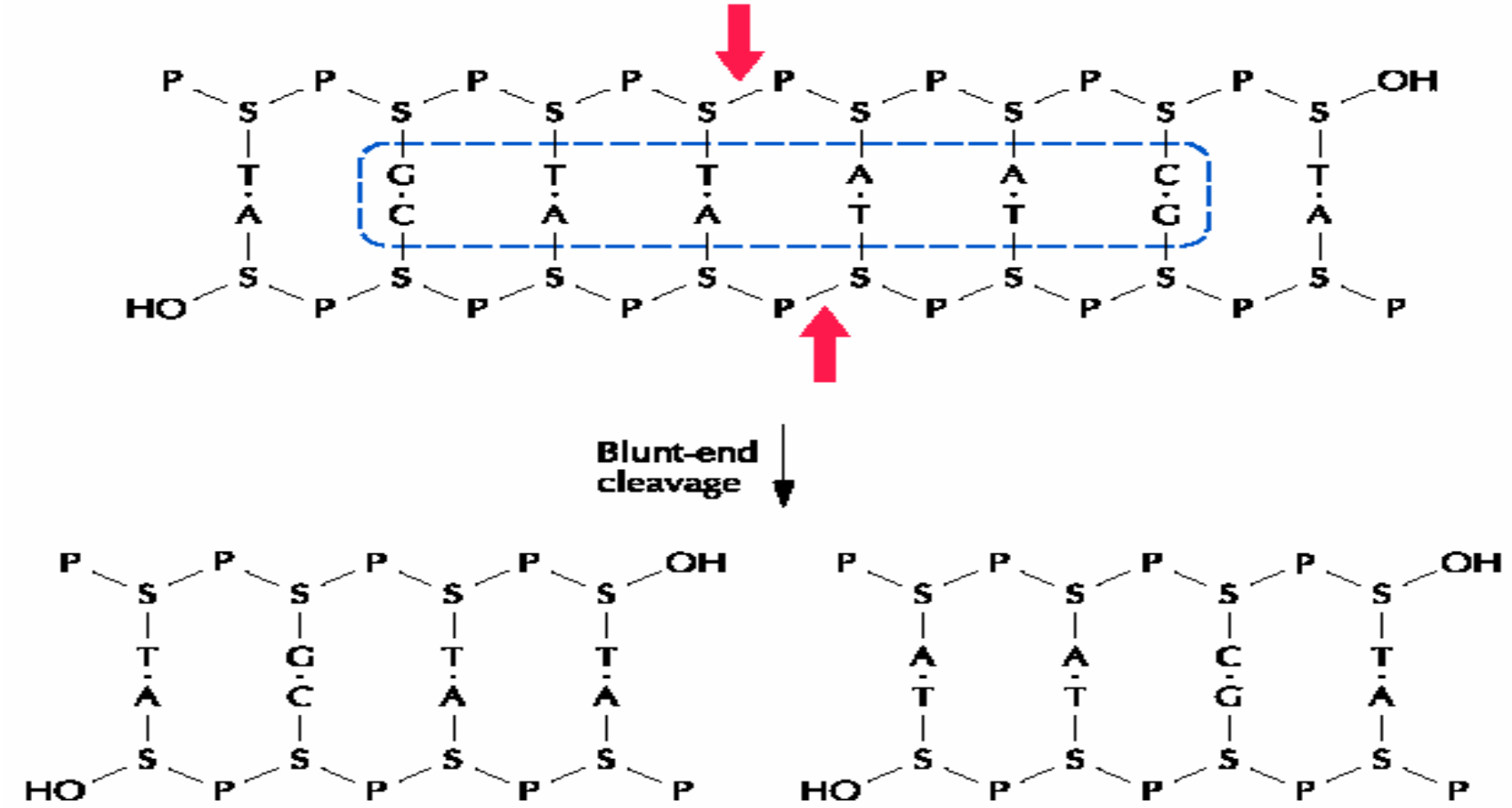
- For Res. Enzyme EcoRI 5’ GAATTC 3’
- 3’ CTTAAG 5’
-
- This type of cut is called **staggered**, because it results in fragments with single-stranded ends. The single-stranded ends are said to be **sticky** because they are able to bind to a **complementary** single-stranded region
- According to the pattern of cleavage Res. Enzymes results in 3 types of DNA fragments:
- 5’ overhangs 5’ G.----------3’
- 3’ CTTAAG 5’
-

- 3’ overhangs 5’ GAATTC 3’
- 3’ C---------- 5’ these make “sticky ends”
- Blunt ends 5’ GTT….3’
- 3’ CAA…..5’
- Different Res. Enzymes recognize and cleave different # of bases
- Eg. 4 base cutters, 5,6,7 etc ex. EcoRI is a 6 base cutter
- How often will a RE cut a random DNA fragment?
- A 4 base cutter 1/4x1/4x1/4x1/4 = 1/256 bp of DNA (one in every 256 base pairs of DNA)
- The general rule is $(1/4)^n$ where n is the # of nucleotide bases in the restriction site.
- These sticky ends can reanneal with complementary single stranded tails on other DNA fragmets. If mixed under the proper conditions, DNA fragments from two sources form recombinant molecules and DNA ligase links the two fragments.

DNA Cleavage By Restriction Endonucleases



DNA Cleavage By Restriction Endonucleases (2)

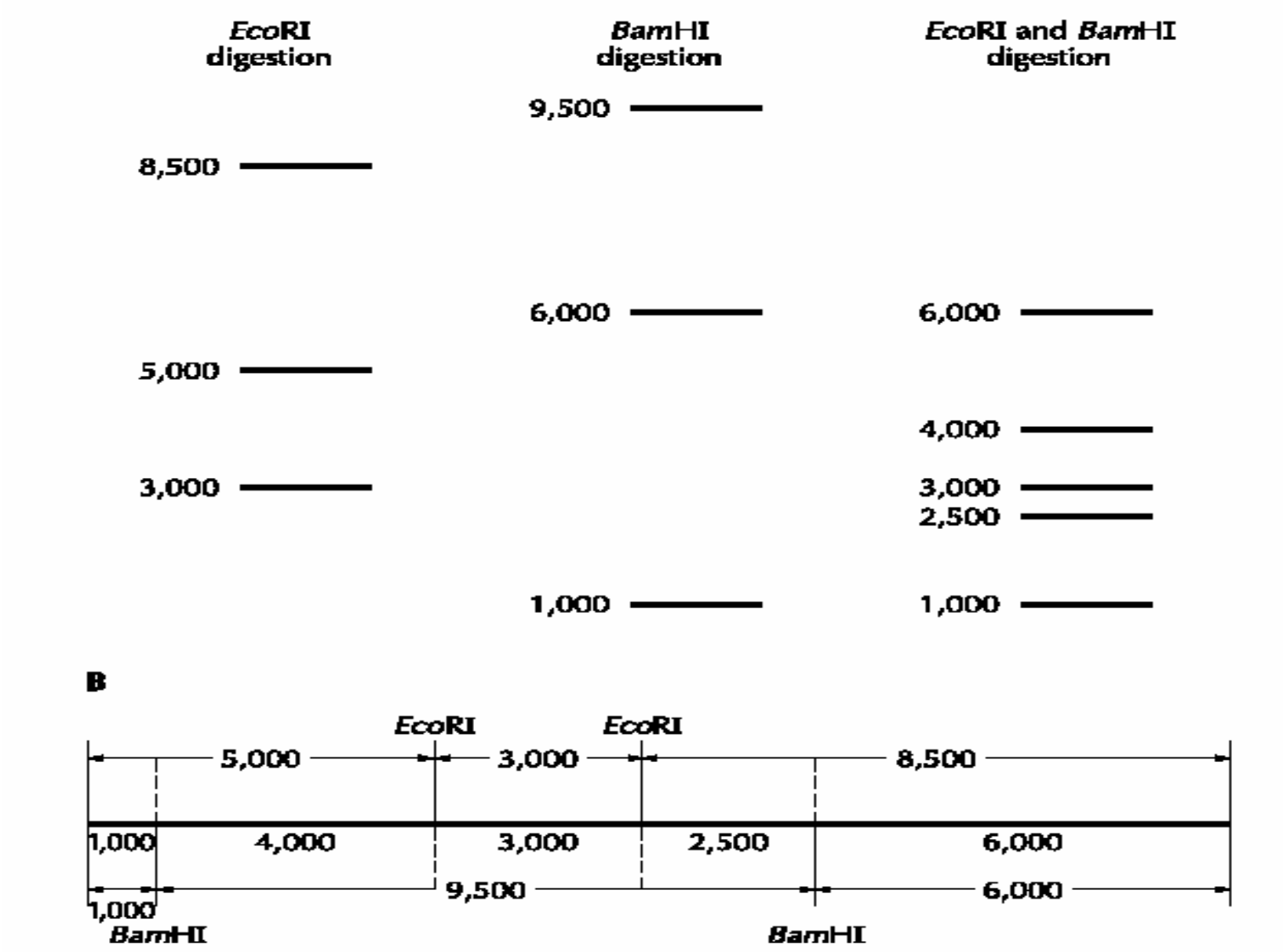


Recognition Sequences of Restriction Endonucleases

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

Arrows denote cleavage sites.

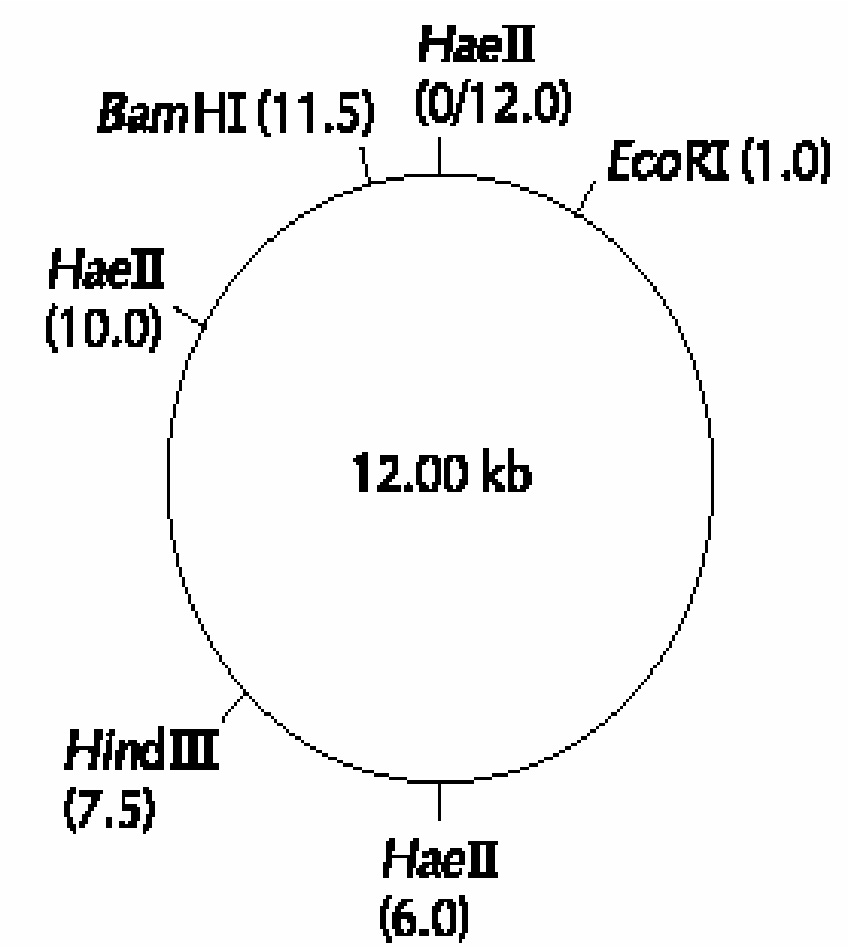
Restriction Mapping



DNA fragment sizes (in kilobase pairs) after single and double restriction endonucleases digestions of a plasmid

EcoRI	*BamHI*	*HindIII*	*HaeII*	*EcoRI* + *HaeII*	*BamHI* + *HaeII*	*HindIII* + *HaeII*	*EcoRI* + *HindIII*	*EcoRI* + *BamHI*	*BamHI* + *HindIII*
12.0	12.0	12.0	6.0	5.0	6.0	6.0	6.5	10.5	8.0
			4.0	4.0	4.0	2.5	5.5	1.5	4.0
			2.0	2.0	1.5	2.0			
				1.0	0.5	1.5			

Restriction Endonuclease Cleavage Map



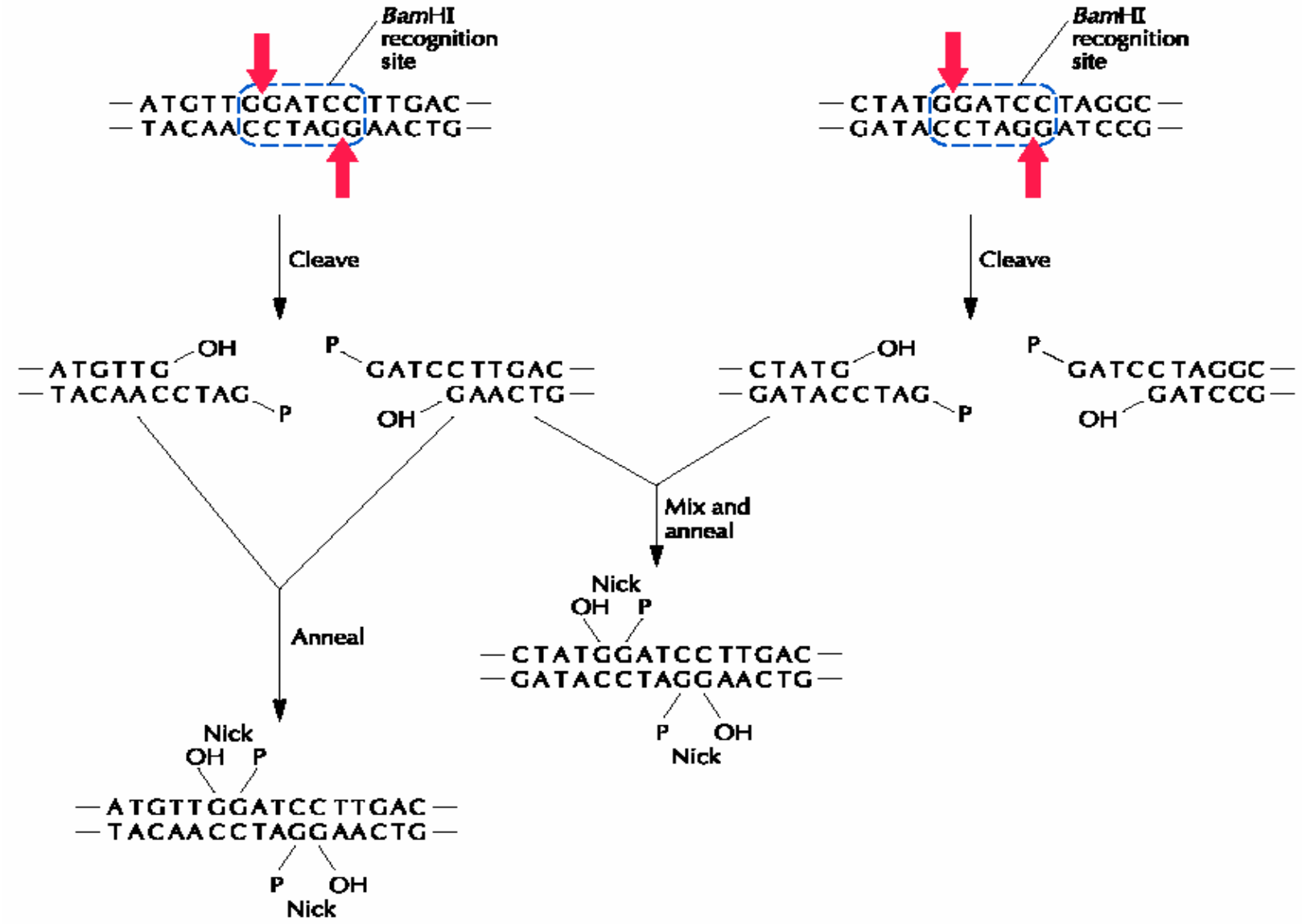
- Created from single and multiple enzyme digestions
- Useful markers for noting gene locations and subcloning strategies

Enzymes Used In Recombinant DNA Protocols

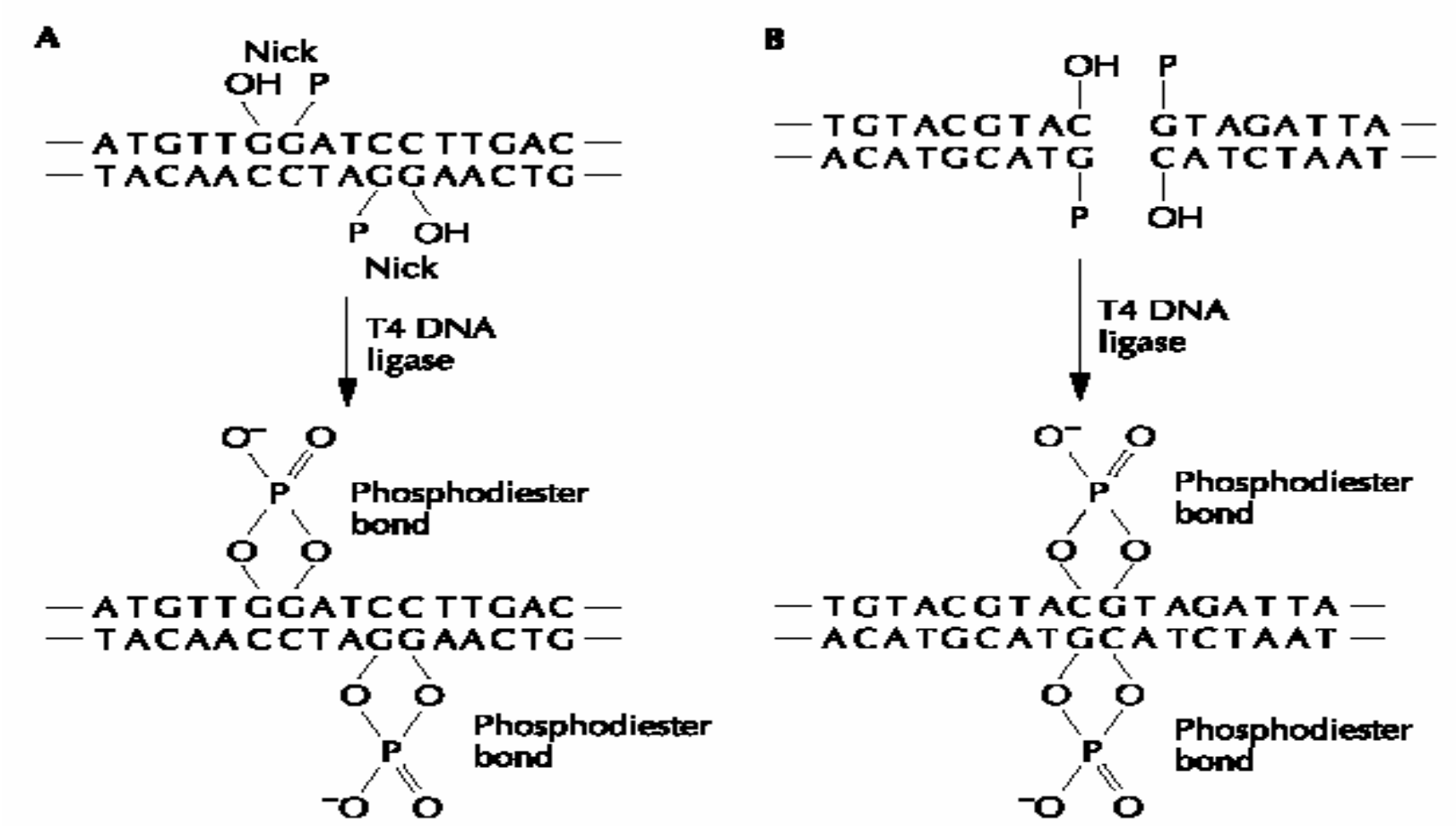
Enzyme	Activity
Alkaline phosphatase	Removes 5′ phosphate groups of DNA molecules; BAP is more stable but less active than CIP.
DNase I	Degrades double-stranded DNA by hydrolyzing internal phosphodiester linkages
E. coli exonuclease III	Sequentially removes nucleotides from 3′ OH ends of DNA molecules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

BAP, bacterial alkaline phosphatase; CIP, calf intestinal alkaline phosphatase.

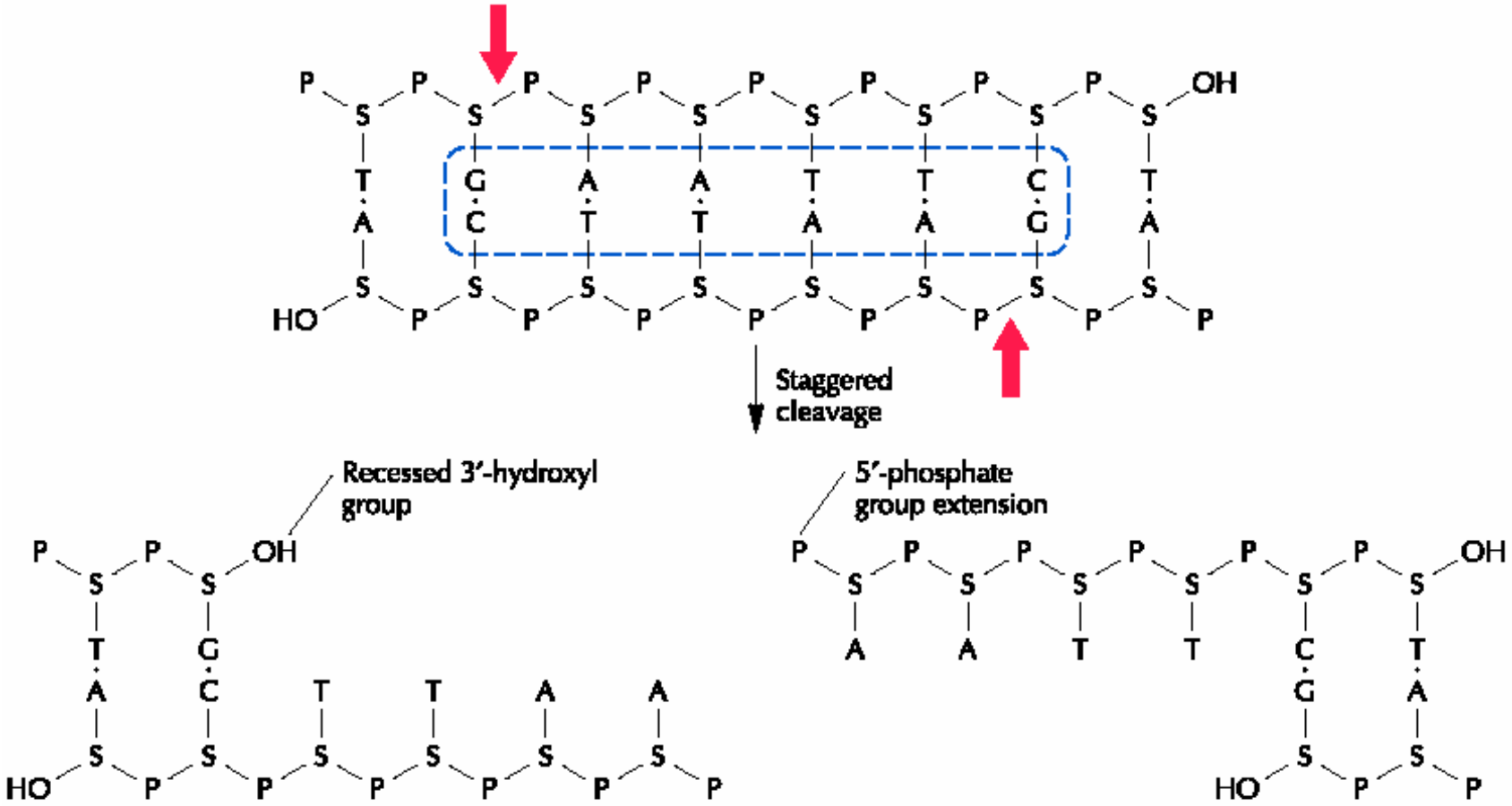
Annealing of Complementary “Sticky” Ends



T4 DNA Ligase Action



DNA Cleavage By Restriction Endonucleases



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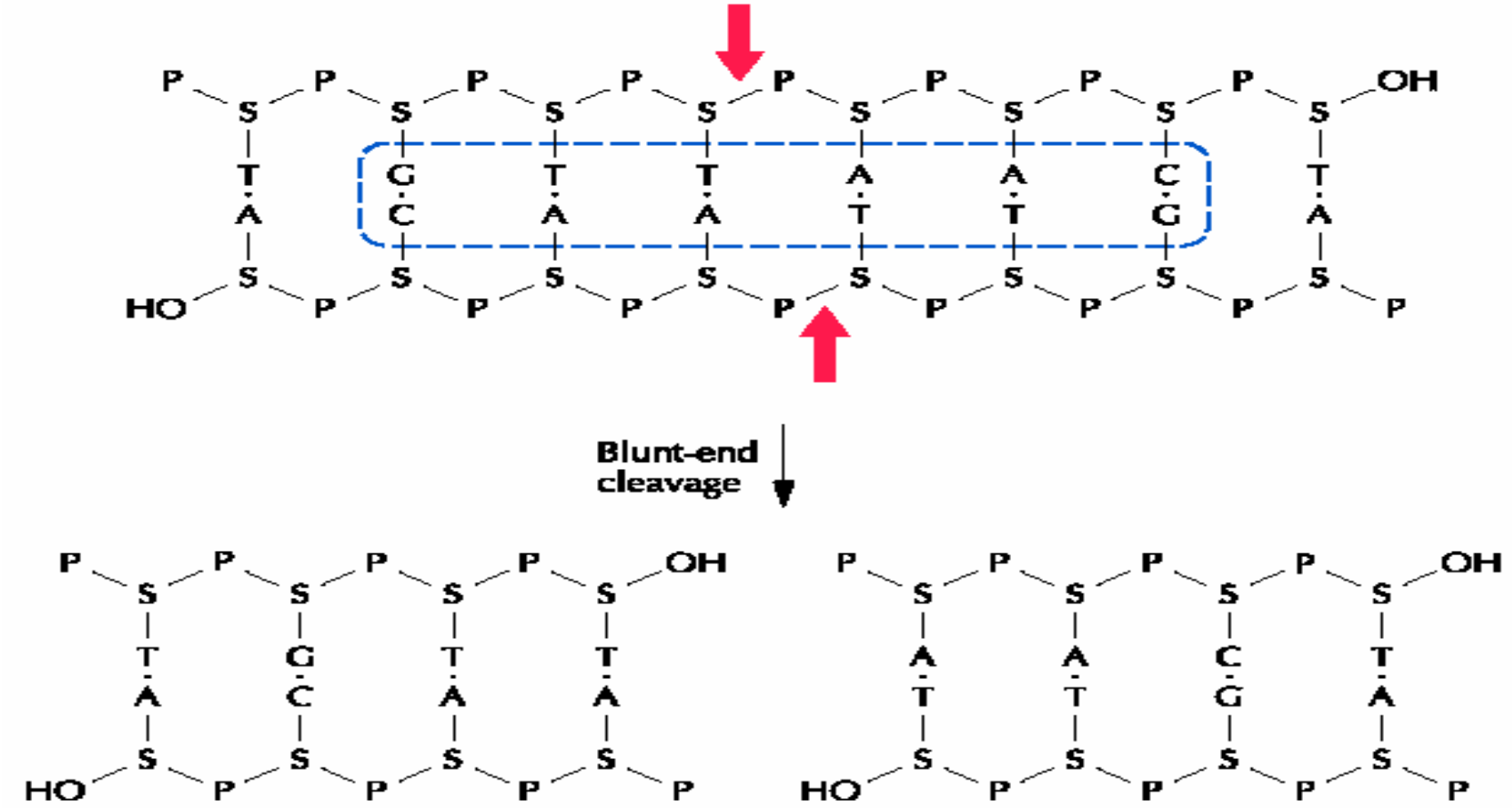

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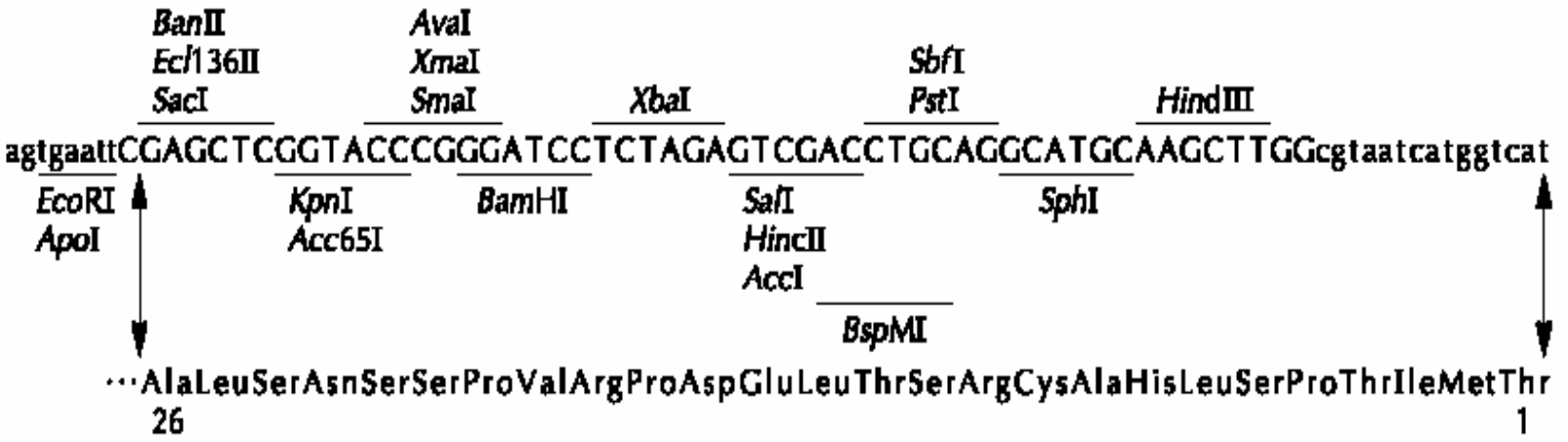
*Not*I	G↓C—G—G—C—C—G—C C—G—C—C—G—G—C↑G	5′ phosphate extension

Arrows denote cleavage sites.

Multiple Cloning Sites

- Synthetic oligonucleotide construction
- “Polymer of cutting sites”
- Can be included in reporter gene coding sequence (e.g. lacZ’)



Antibiotics Commonly Used as Selective Agents

Antibiotic	Description
Ampicillin (Ap, Amp)	Inhibits cell wall formation; inactivated by β-lactamase
Hygromycin B (HygB)	Blocks translocation from amino acyl site to peptidyl site; inactivated by a phosphotransferase
Kanamycin (Km, Kan)	Binds to 30S subunit and prevents translocation from aminoacyl-tRNA site to peptidyl site; inactivated by a phosphotransferase
Neomycin (Nm, Neo)	Binds to 30S subunit and inhibits protein synthesis; inactivated by a phosphotransferase
Streptomycin (Sm, Str)	Blocks protein initiation complex formation and causes misreading during translation; inactivated by a phosphotransferase
Tetracycline (Tc, Tet)	Prevents binding of aminoacyl-tRNA to 30S ribosomal subunit; resistance gene encodes an inner cell membrane protein that passes the antibiotic out of the cell and blocks the passage of the antibiotic through the cell wall

Transformasi ke bakteri

Bacterial transformation

Introduction of DNA into bacteria
Spontaneous uptake – low probability
E. coli – cells treated with $CaCl_2$
Less than 1 of 10^3 cells acquire a plasmid

Transformasi dengan $CaCl_2$

- Sel kompeten dan DNA diinkubasi bersama di $CaCl_2$ pada 0^oC, kemudian di *heat shock* pada 42^oC
- Hanya sedikit DNA yang dapat diambil

Prokaryote Advantages

1. Grow fast
2. Manipulation easier
3. Eukaryotic technology still somewhat embryonic

Prokaryote Disadvantages

1. Can’t splice out introns
2. Introns are needed for good expression
3. Size of DNA that can be put into bacteria is limited
4. Prokaryotes don’t glycosylate proteins

Seleksi bakteri pembawa DNA rekombinan

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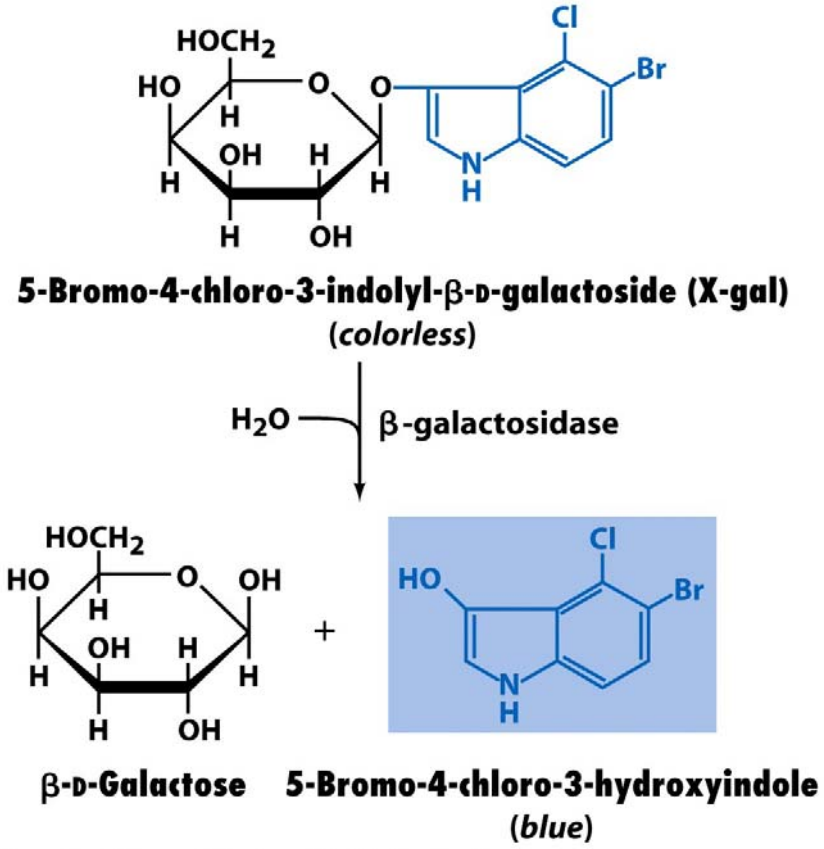
Selection of transformed cells:

resistance to antibiotics

using chromogenic substances

Antibiotics: molecules produced by microorganism that kill other microorganism

peniciline, tetracycline, ciplroflaxine – inhibits gyrase in the complex with DNA – inhibits DNA replication



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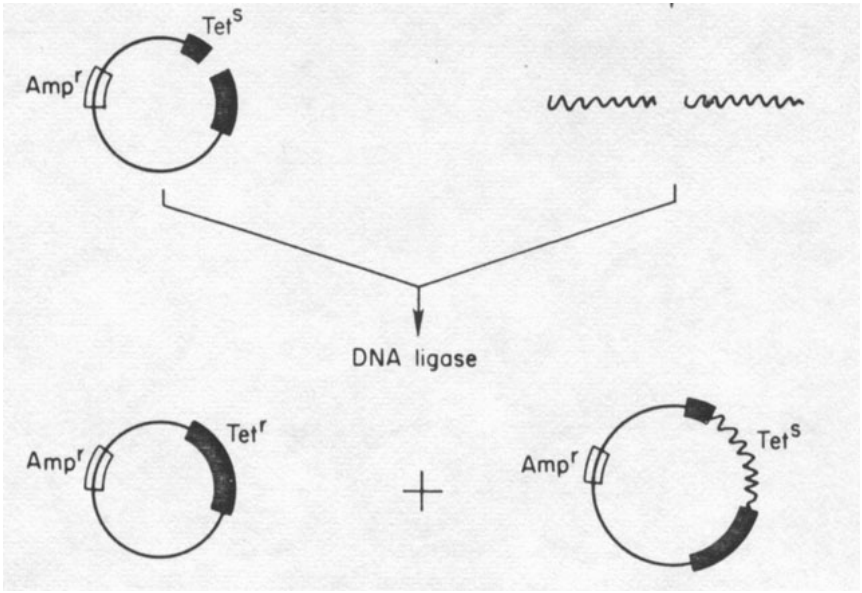
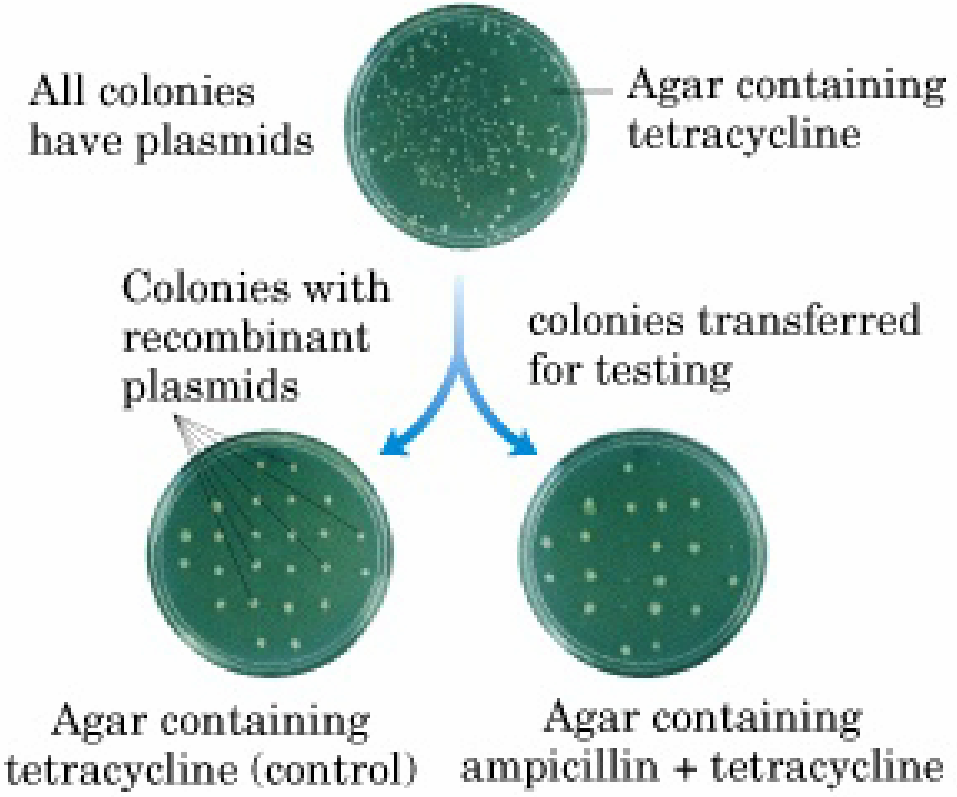
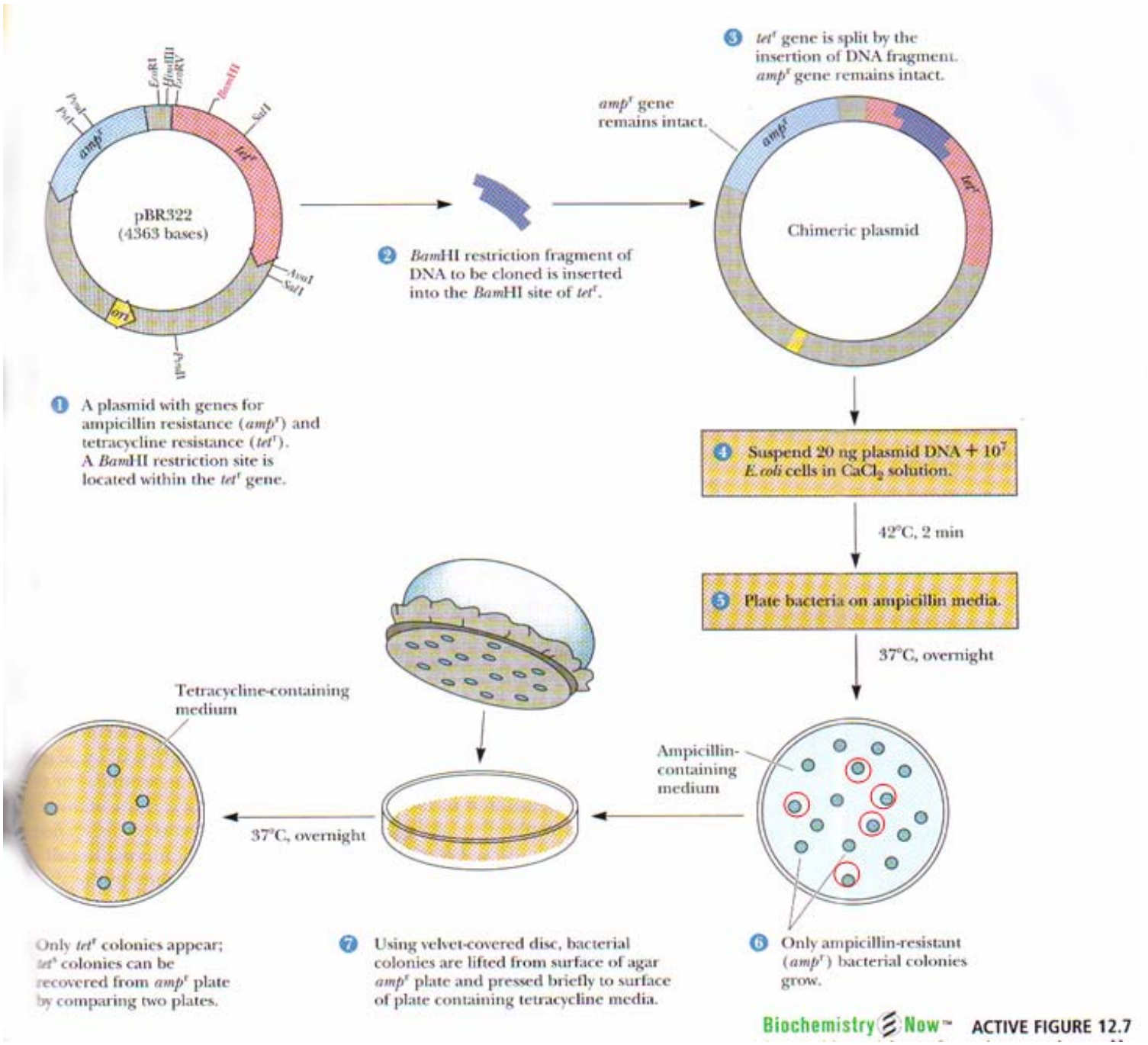
Antibiotic Resistance Genes are a Part of Many (Constructed) Plasmids

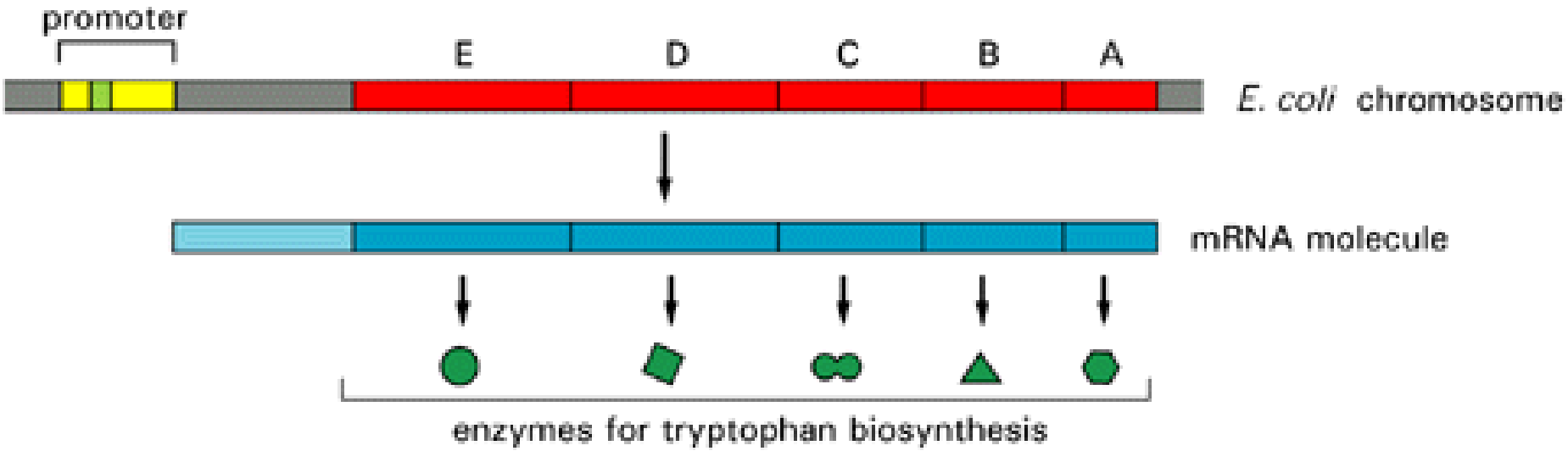




- Follow with replicate plating of transformants on Amp and Tet



Biochemistry Now™ ACTIVE FIGURE 12.7

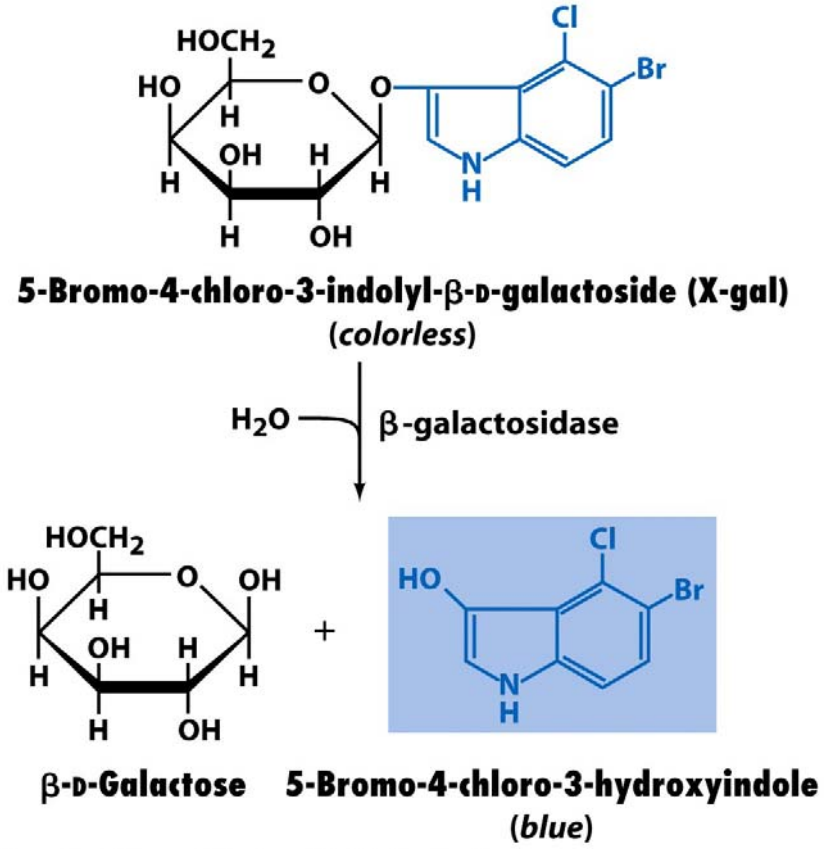


Operon:Section of DNA in which two or more related genes lie adjacent to one another and are transcribed from a single promoter into polycistronic mRNA.

Chromogenic substances:

Blue-White Screening Promega Corp; Madison, WI

- pGEM-3Z, e.g.
  - Amp^r
  - *lac*Z
  - polycloning site in *lac*Z gene
  - T7 promoter one side, SP6 other



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III. Vectors for Gene Cloning

A. Requirements of a vector to serve as a carrier molecule

- The choice of a vector depends on the design of the experimental system and how the cloned gene will be screened or utilized subsequently
- Most vectors contain a prokaryotic origin of replication allowing maintenance in bacterial cells.

- Some vectors contain an additional eukaryotic origin of replication allowing autonomous, episomal replication in eukaryotic cells.
- Multiple unique cloning sites are often included for versatility and easier library construction.

- Antibiotic resistance genes and/or other selectable markers enable identification of cells that have acquired the vector construct.
- Some vectors contain inducible or tissue-specific promoters permitting controlled expression of introduced genes in transfected cells or transgenic animals.

- Modern vectors contain multi-functional elements designed to permit a combination of cloning, DNA sequencing, in vitro mutagenesis and transcription and episomal replication.

B. Main types of vectors

- Plasmid, bacteriophage, cosmid, bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC), yeast 2 micron plasmid, retrovirus, baculovirus vector……

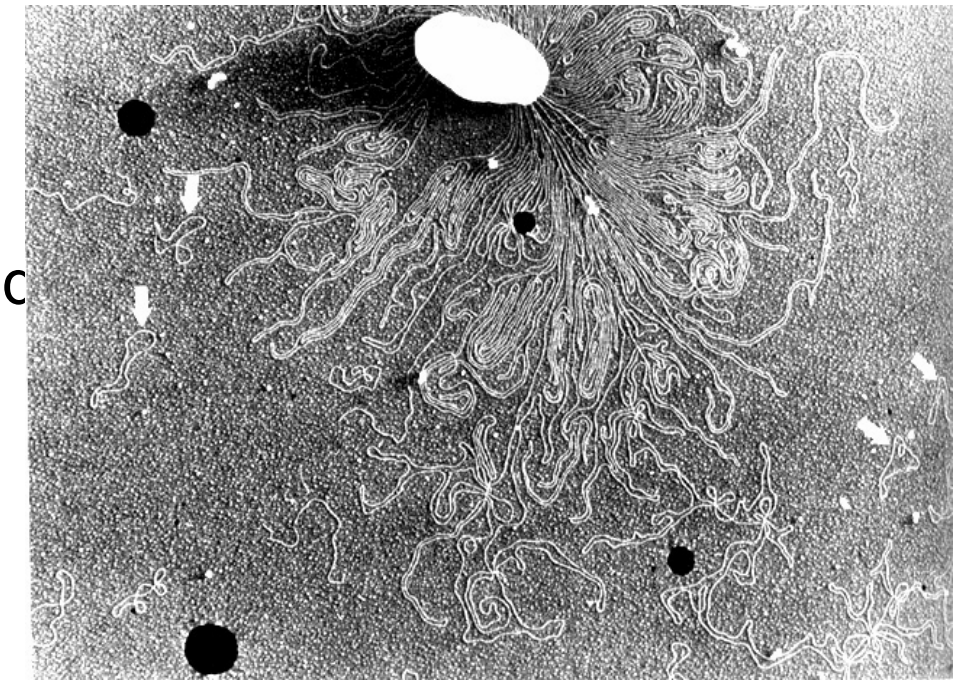
Plasmids

Plasmid- small, circular, extrachromosomal DNA which replicates independently of host chromosomal DNA.

Most (experimental) derived from a single clinical specimen in 1974

Low copy # *vs*. high copy number

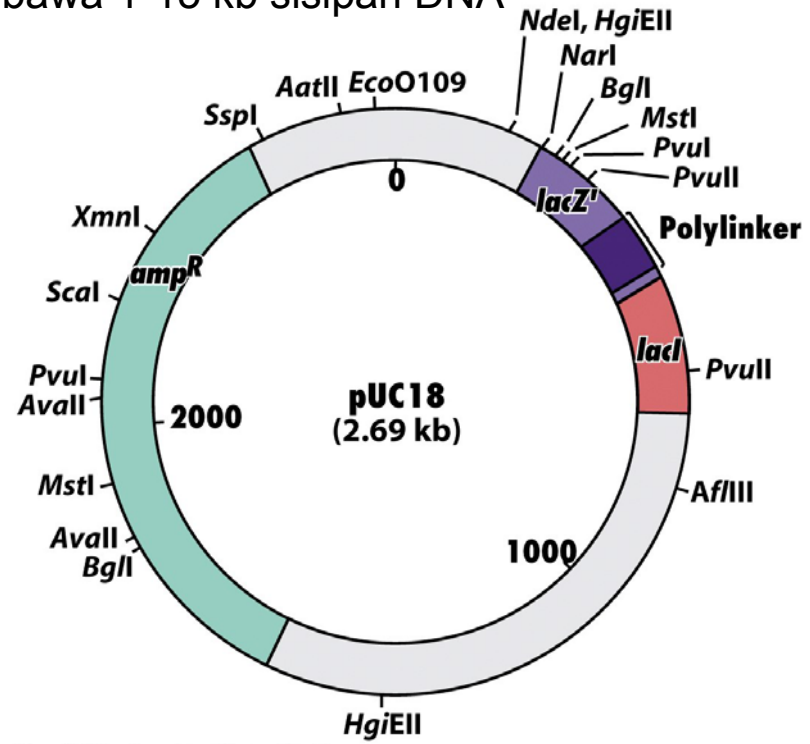
Incompatible plasmids



- Komponen DNA rekombinan: Vektor

Perbanyakan potongan DNA dapat dilakukan di dalam sel dengan bantuan vektor untuk kloning, antara lain: plasmid atau bacteriophages

– Plasmid Molekul DNA berukuran kecil dan berbentuk sirkular yang terdapat pada bakteri atau yeast. Bisa membawa 1-15 kb sisipan DNA



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Syarat suatu plasmid sebagai vektor:
Berukuran kecil
- Susunan DNA diketahui
- Harus mempunyai jumlah kopi yang banyak di dalam sel inang
- Memiliki titik Ori
- Memiliki marker seleksi
- Memiliki marker seleksi kedua yang berguna untuk tanda bila plasmid disisipkan gen asing.
- Memiliki situs restriksi yang unik sebagai tanda untuk menyisipkan gen asing.

LacZ encodes β-galactosidase
LacI – encodes factor controling transcription of lacZ

Cloning Vectors

1. Plasmids- 5,000 to 400,000 bp
   useful for putting 0.01-10 kb in
2. Bacteriophages-virus that infects bacteria
   useful for putting 10-20 kb in
3. Cosmids- artificially generated
   useful for putting 20-50 kb in
4. YACs- yeast artificial chromosomes
   useful for putting 500 kb
5. Other, newer exist

C. Choice of vector

- Depends on nature of protocol or experiment
- Type of host cell to accommodate rDNA
  - Prokaryotic
  - Eukaryotic

D. Plasmid vector

- Covalently closed, circular, double stranded DNA molecules that occur naturally and replicate extrachromosomally in bacteria
- Many confer drug resistance to bacterial strains
- Origin of replication present (ORI)

- Examples
  - pBR322
    - One of the original plasmids used
    - Two selectable markers (Amp and Tet resistance)
    - Several unique restriction sites scattered throughout plasmid (some lie within antibiotic resistance genes = means of screening for inserts)
    - ColE1 ORI

- pUC18
  - Derivative of pBR322
  - Advantages over pBR322:
    - Smaller – so can accommodate larger DNA fragments during cloning (5-10kbp)
    - Higher copy # per cell (500 per cell = 5-10x more than pBR322)
    - Multiple cloning sites clustered in same location = “polylinker”

- Interruptable gene encoding for enzyme beta galactosidase (lacZ)
  - Polylinker resides in the middle
  - Enzyme activity can be used as marker for gene insertion
  - Disrupted gene = nonfunctional
  - Intact gene = functional
  - Media containing XGAL chromagenic substrate used (blue colonies = intact; white colonies = disrupted)
- Amp resistance gene still present (= beta lactamase), Tet resistance gene omitted

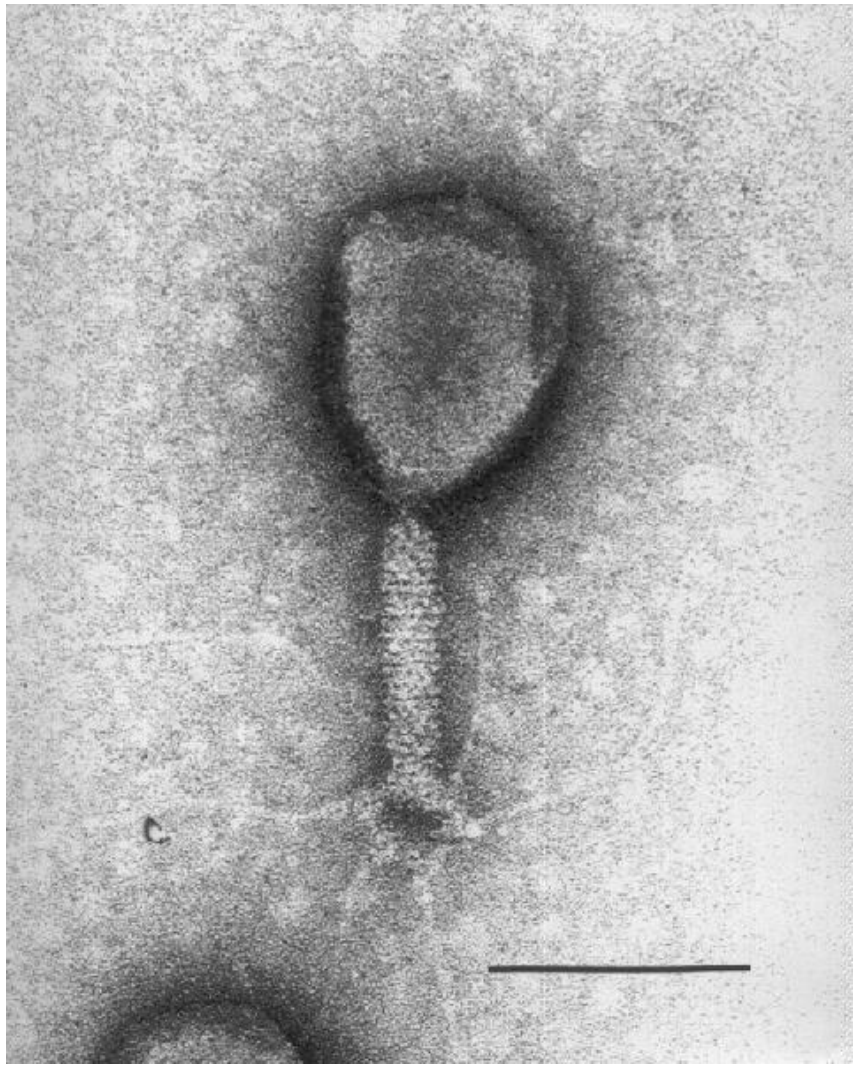
Bacteriophage

Bacteriophages are bacteria viruses.
When they infect their host they use host machinery to replicate their DNA.

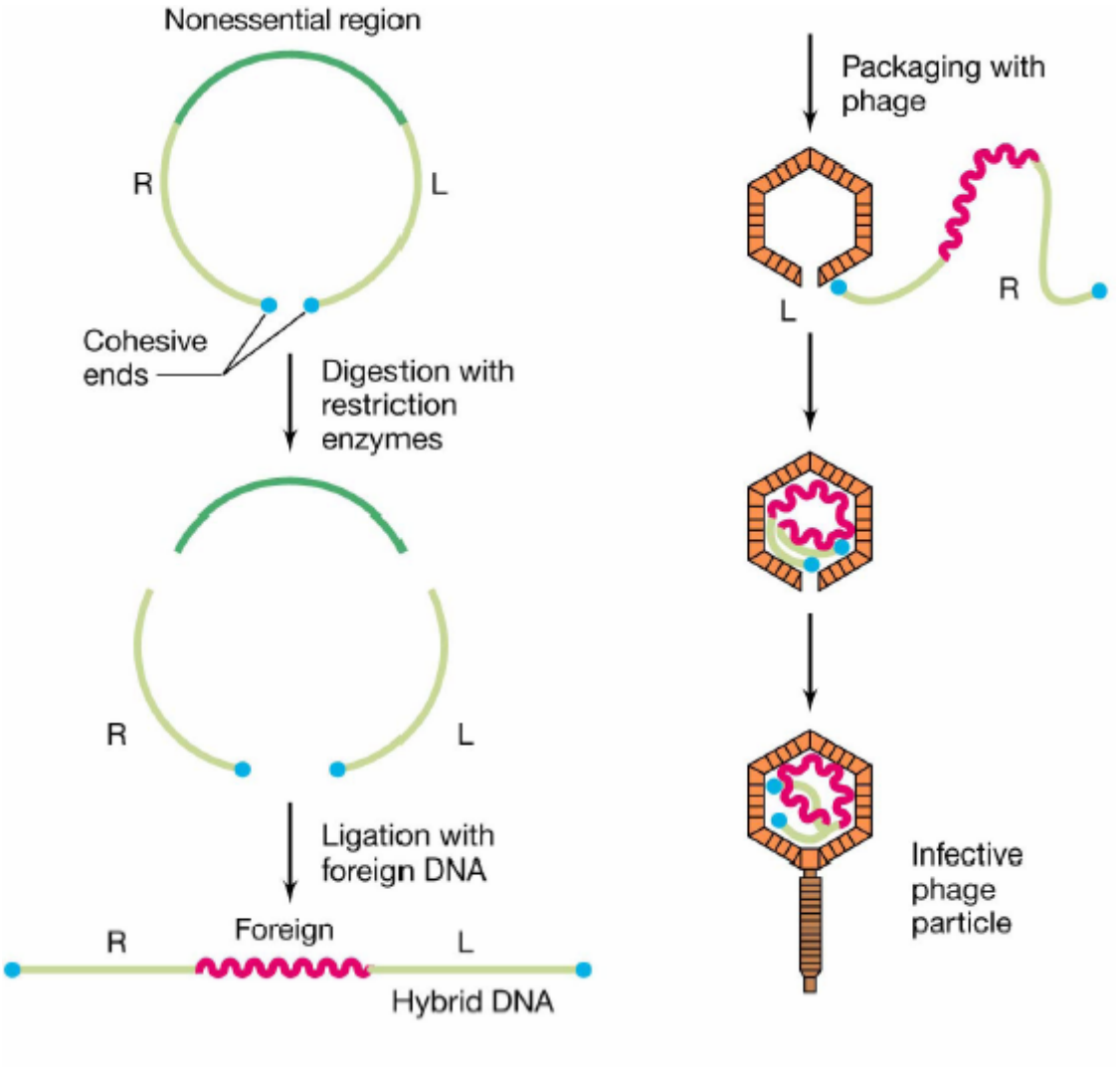
In bacteriophage vectors part of the phage DNA is replaced by the gene of interest (max. size is 15-25 kbp)

When this new recombinant phage DNA infects a host the gene of interest will be replicated.

commonly used phage vectors include M13 and λ.



Cloning DNA in bacteriophage λ



<u>Infection of bacterial cell:</u>

Tail sticks to the cell wall

DNA from head is squired into bacteria

Phage genes are transcribed by bacterial RNA polymerase

Corresponding mRNA is translated using bacterial machinery

Newly replicated phage DNA and head and tail protein ensemble spontaneously

Lyses of host bacteria results in formation of plagues.

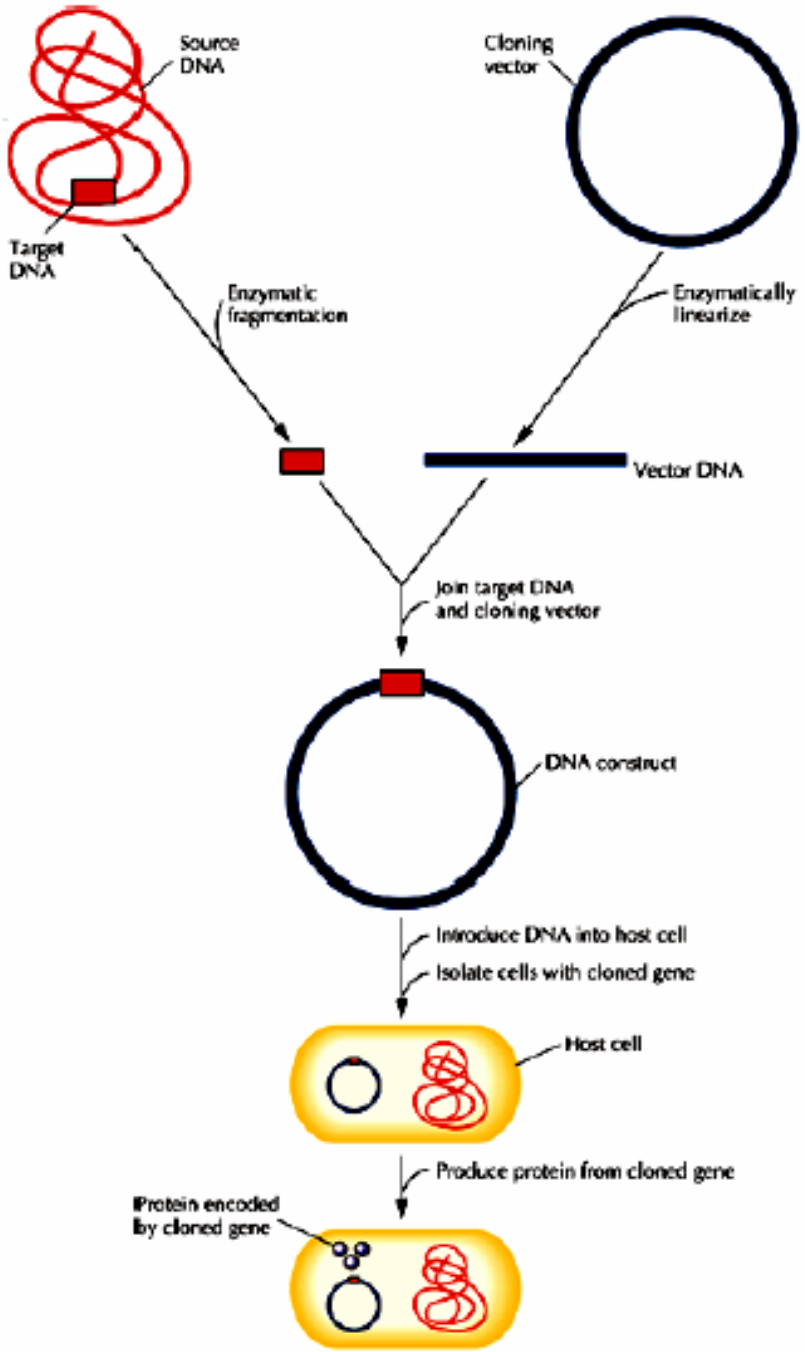
Other Important Vectors

1. Baculovirus- infects insect (Sf9, e.g.) cells
2. For plants, wound and infect with engineered *Agrobacterium tumefaciens*

Protein expression

- Gene is inserted into plasmid
- Plasmid is transformed into a host cell (E. coli)

-Cell culture is prepared
-Each cell contains several copies of the plasmid with gene
-Gene expression leads to the production of protein
-Protein level may reach
-30% of total cellular protein
-Isolation of protein



- Application of recombinant DNA technology
  - Antisense nucleic acids – complementary to mRNA = > block mRNA translation
  - Insertion of antisence nucleic acids helps to understand protein function in the cell
  - Synthetic DNA oligonucleotides have been produced to inhibit viral infection including HIV in cultured cell
  - Possible application: introduction of antisence HIV nucleic acids into bone marrow cell from AIDS patient

Transgenic animals:

Animals expressing a gene from another organism

Multiple copies gene is injected into fertilized egg.

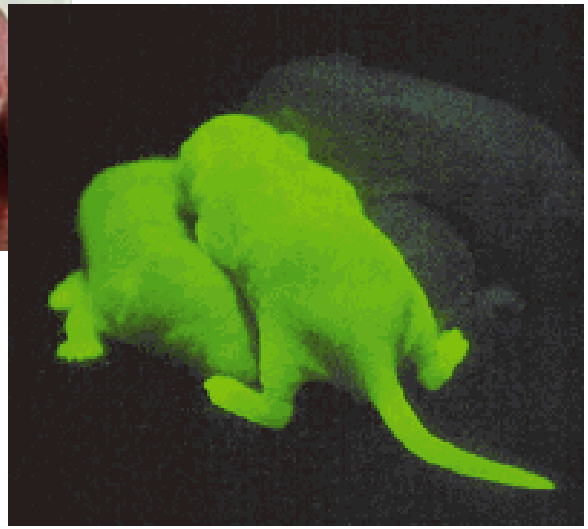
The foreign DNA inserts randomly into genome

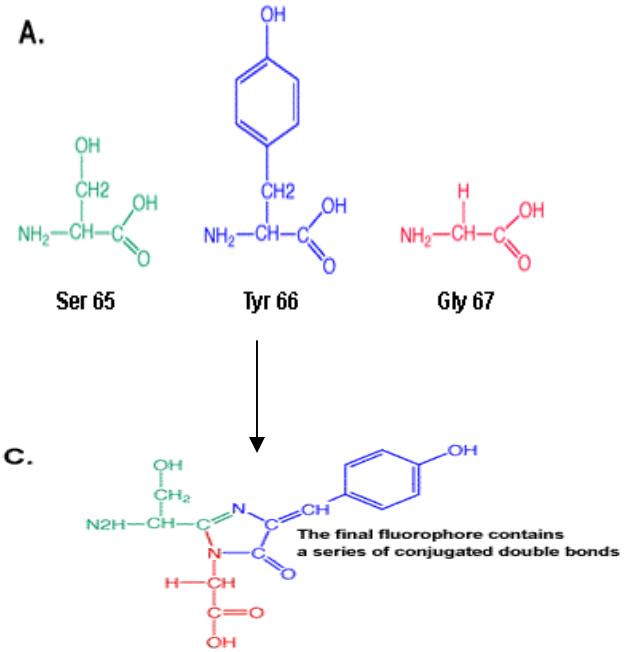
The fertilized egg is inserted into surrogate mother

Example of transgenic animals:
Protein with therapeutic value were produced in milk several animals:
Collagulation factors, fibrinogen (used for burns and after surgery), monoclonal antibodies, human hemoglobin and serum albumin etc.

Transgenic plants to increase crop yields, resistance to cold, insects etc.

Fluorescent animals





Aequorea victoria