Tools of recombinant DNA technology

Enzymes

1. Restriction enzymes

Methods for cutting DNA

- Chemical methods
- Mechanical shearing in a blender
- Restriction Endonuclease digestion
- sonication

Restriction and Modification systems: reported in several bacteria

- Function: Restriction systems checks the presence of foreign DNA and destroys it.
- recognize specific sequences in the incoming DNA and breaks into fragments.
- Cutting is either at specific sites or random.

Discovery

- Bacteriophages: have a single host they can infect, or a small number of related bacteria: which is the '**host range**'

- This effect is seen in efficiency of plating, i.e. the number of plaques formed in plating tests gets reduced.

- Observation: In the 50's and 60's occasionally a virus could become active in a new strain but would no longer be effective in the original host

Discovery

- This change in host range seemed to be associated with specific bases in the **viral DNA being methylated:**
- **This is called modification : a change conferred by the host**

Methylases

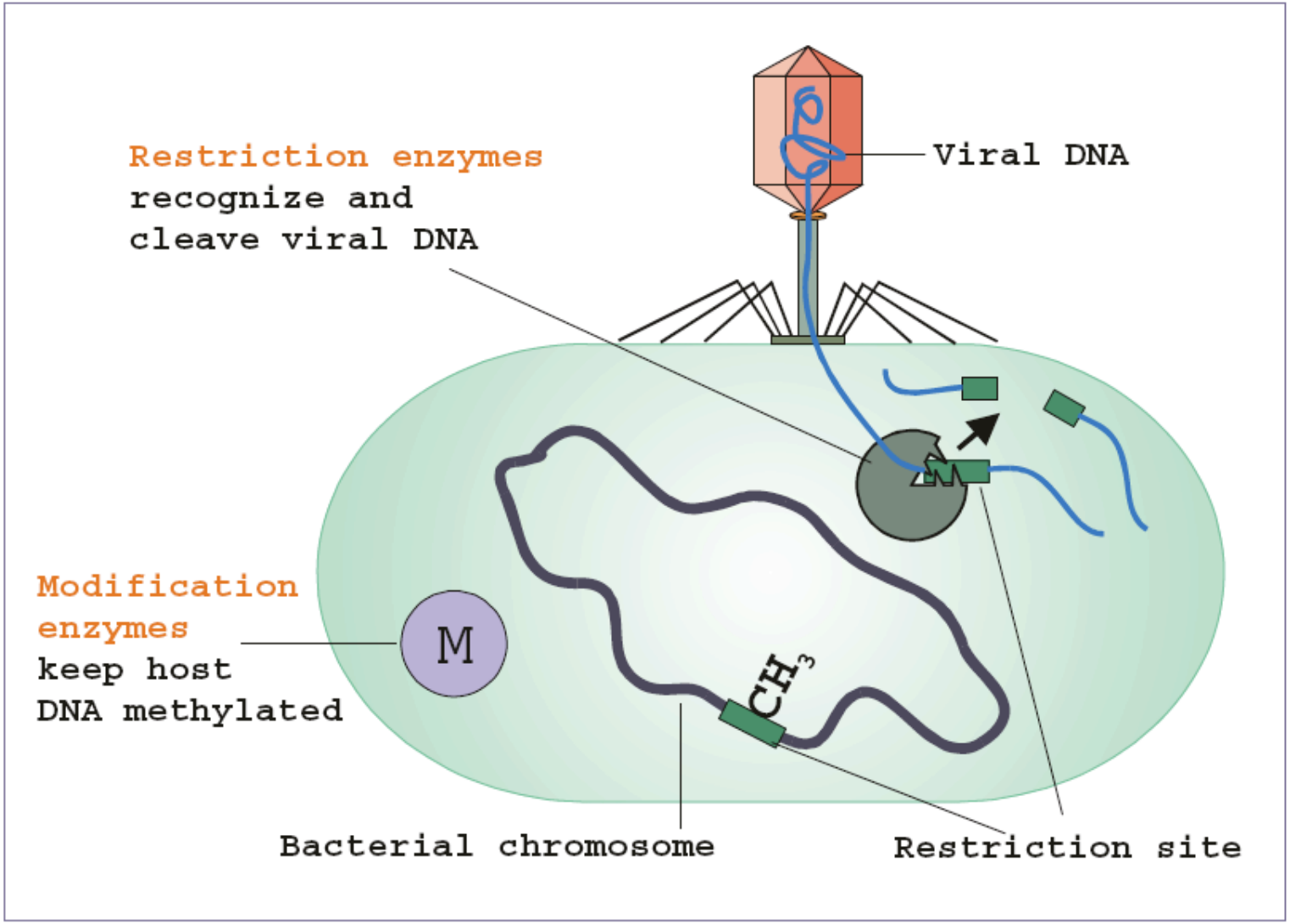
- 1969 **Werner Arber and Stewart Linn** discovered that most bacteria contained a class of enzymes which modify the bases in DNA termed **methylases.**
- These enzymes recognize a specific sequence of DNA and add a **methyl group onto certain bases, most often an adenine or cytosine.**
- The presence of these methyl groups on the viral DNA seemed to be the cause of their ability to grow in some strains of bacteria, but not others.

methylation effect:

- This ability to grow was because the methyl groups were protecting the viral DNA from an unusual type of enzyme.
- DNA that was methylated was no longer recognized by the DNA breaking enzyme, and was safe from attack.
- This enzyme , a nuclease therefore limited or restricted, the number of bacteria that made up the host range; they became known as '**host range restriction endonucleases**" which quickly became **'restriction enzymes**'
- Hence if a bacterium is having a restriction enzyme, it also needs the **methylase (a modification enzyme)**, to distinguish its own DNA from foreign DNA, otherwise it will destroy its own DNA

Restriction Enzymes

Bacterial defense against viral infection by restriction-modification complexes



Identification of Restriction

- The phenomena of restriction and modification were first well illustrated in **bacteriophage λ on two *E. coli* host strains.** C and K

- Experiment----

a stock of phage λ, is made by growth upon *E. coli* strain C

stock is then titred upon *E. coli* C and E coli K

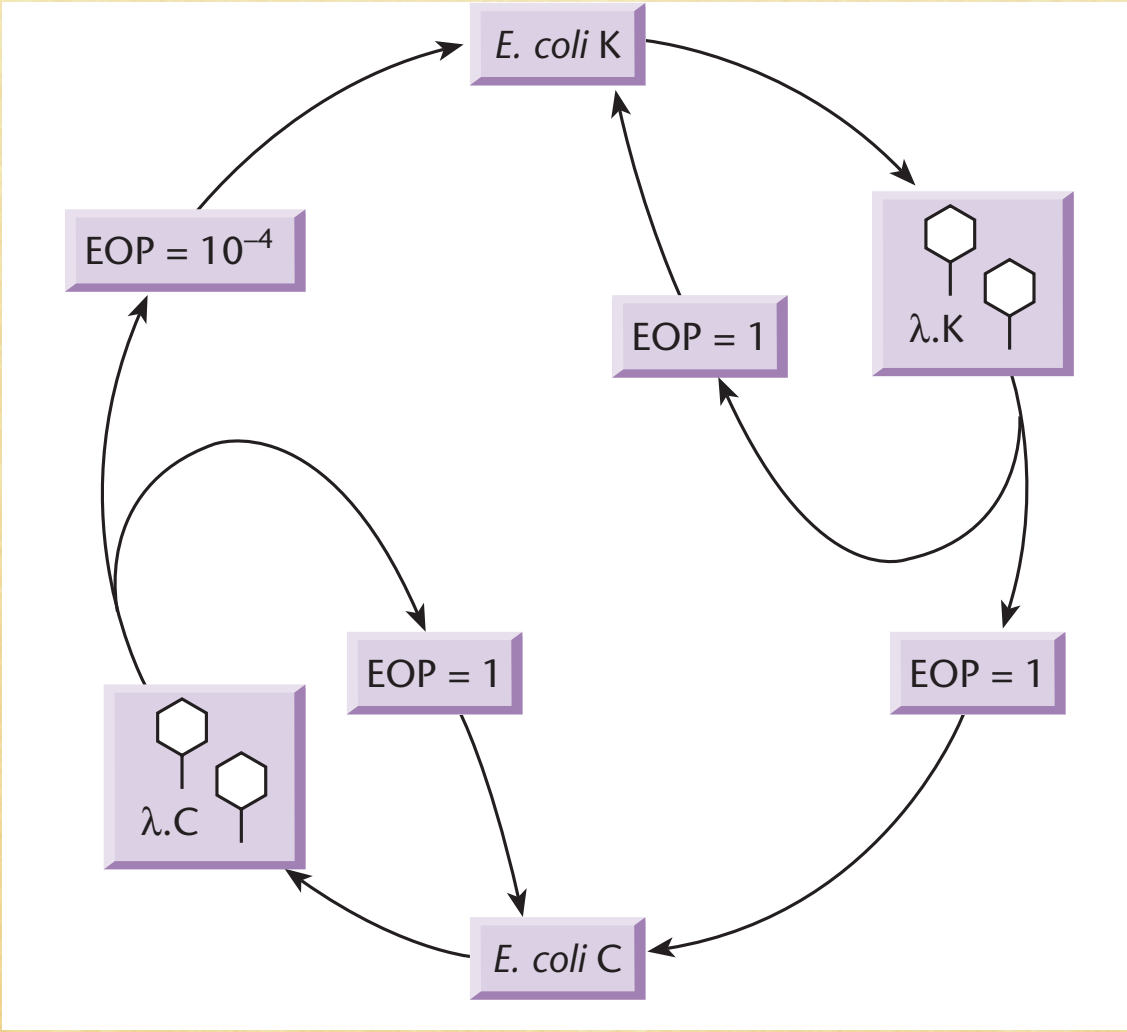
Titre is higher on *E.Coli* C than on K

Phage from *E.coli K* now replated on

E.coli K

They are no longer restricted gives high titre; but if they are first cycled through *E. coli* C they are once again restricted when plated upon *E. coli* K

Restriction-modification



Conclusion of experiment

- a non-heritable change is conferred upon the phage by the second host strain
- If the phage has survived one cycle of growth upon the restrictive host it can subsequently reinfect that host more efficiently;
- This is because their DNA has been replicated in the presence of the **host modifying methylase** which makes it like the host DNA, to get protection from the host restriction system.
- This process can also occur whenever DNA is transferred from one bacterial to another.

Discovery

- In 1970**, Hamilton O. Smith, Thomas Kelly and Kent Wilcox** isolated and characterized the first type II restriction enzyme, HindII, from the bacterium *Haemophilus influenzae.*

- **Daniel Nathans and Kathleen Danna** showed that cleavage of SV40 DNA by restriction enzymes yielded specific fragments which can be separated using polyacrylamide gel electrophoresis, thus showing that restriction enzymes cut at specific sites and can be used for mapping of the DNA.

- For their work in the discovery and characterization of restriction enzymes, the 1978 Nobel Prize for Physiology or Medicine was awarded to Arber, Nathans, and Smith.

Naming restriction enzymes
Smith and Nathans (1973)

Derivation of the *Eco*RI name

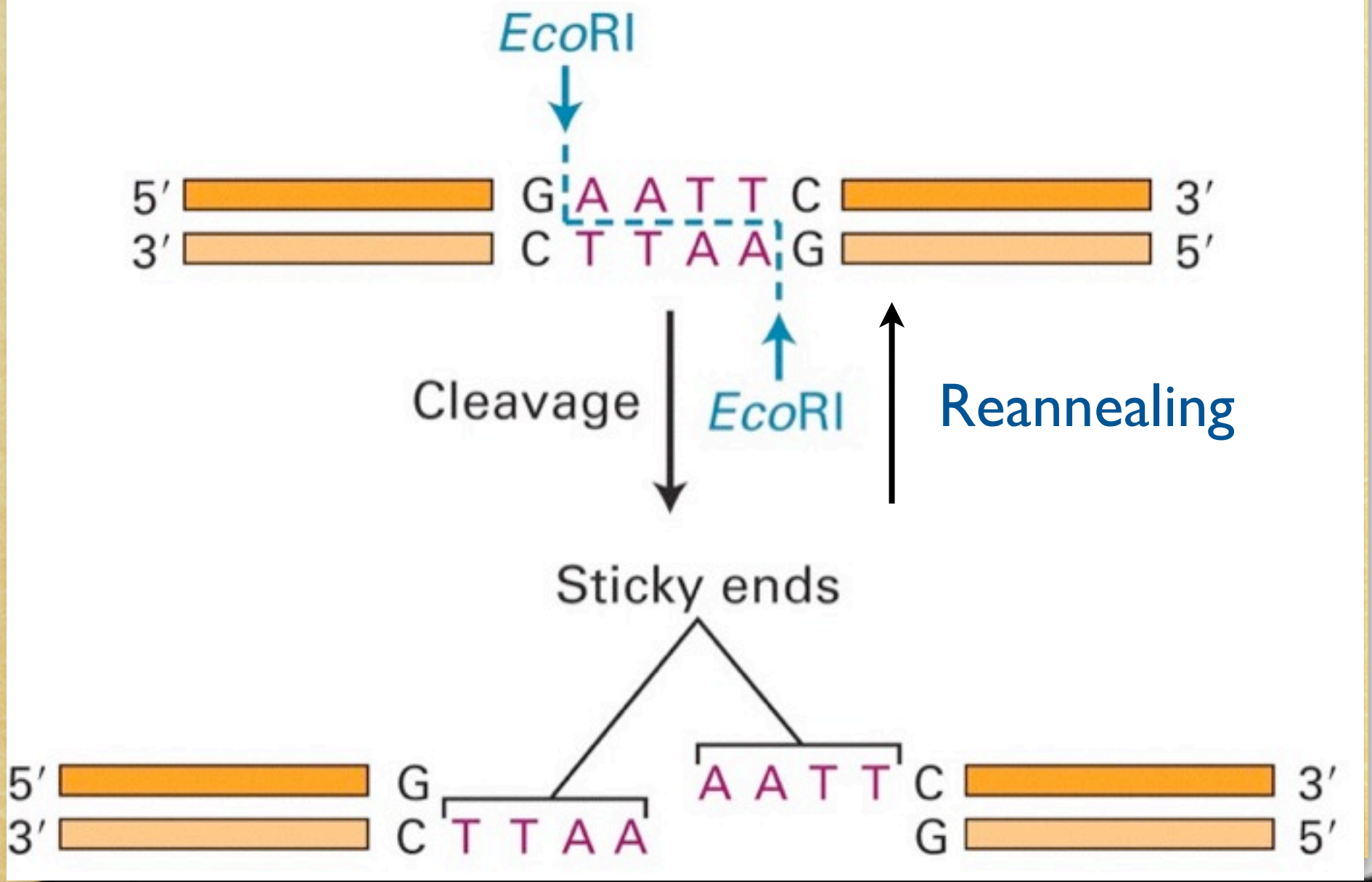
Abbreviation	**Meaning**	**Description**
E	*Escherichia*	genus
co	*coli*	specific epithet
R	RY13	strain
I	First identified in the bacterium	order of identification

- Usually the name is taken from the first letter of genus name and first two letters of species names of the bacterium that makes the enzyme
- *Bacillus globigii*---Bgl
- *Moraxella bovis*---Mbo
- If there are more than one restriction-modification systems. They are then numbered in order of discovery.
- So HpaI, HpaII *etc.*

Recognition sites

- May be between 4, 6, 8, 10,12 bases long. Enzymes are often termed ‘four cutters’,‘six cutters’ etc. based on the length of the site
- Sequence is Usually palindromic: enzyme attaches to BOTH strands and cuts them
- In some cases not every base is constant in recognition site
- The cut site may be centered or off-centered:The former produce what are termed **‘blunt ends’** and the enzymes are termed **‘blunt cutters’**
- Most produce a **‘staggered cut’** that generates **‘sticky ends’**

Sticky ends


EcoRI
5′
3′
G A A T T C
C T T A A G
3′
5′
Cleavage
EcoRI
Reannealing
Sticky ends
5′
3′
G
C T T A A
A A T T C
G
3′
5′

Types of Restriction enzymes

Type I

- These were the first to be discovered
- One enzyme with 3 different subunits which are **coded separately**
  - **recognition,**
  - **cleavage(nuclease), and**
  - **methylation(methylase)**
- The binding is at a specific recognition site (not symmetrical), which can be methylated and then the DNA loops back. The cut site may be as far as 1000 bases from the recognition site.
- Each is used just once, then inactivated.

Type I

- *E.g E. coli* K12 system. The active enzyme consists of two restriction subunits, two modification (methylation) subunits, and one recognition subunit. These subunits are the products of the *hsd*R, *hsd*M, and *hsd*S genes.
- The methylation and cutting reactions both require ATP and *S*-adenosyl methionine(SAM) as cofactors.
- type I systems are of little value for gene manipulation

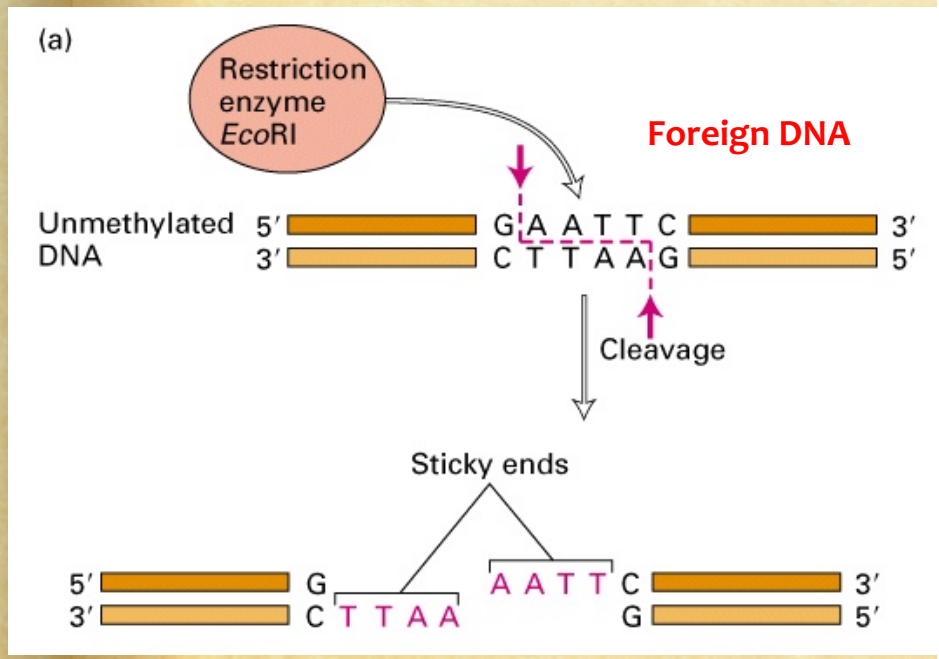
Type II

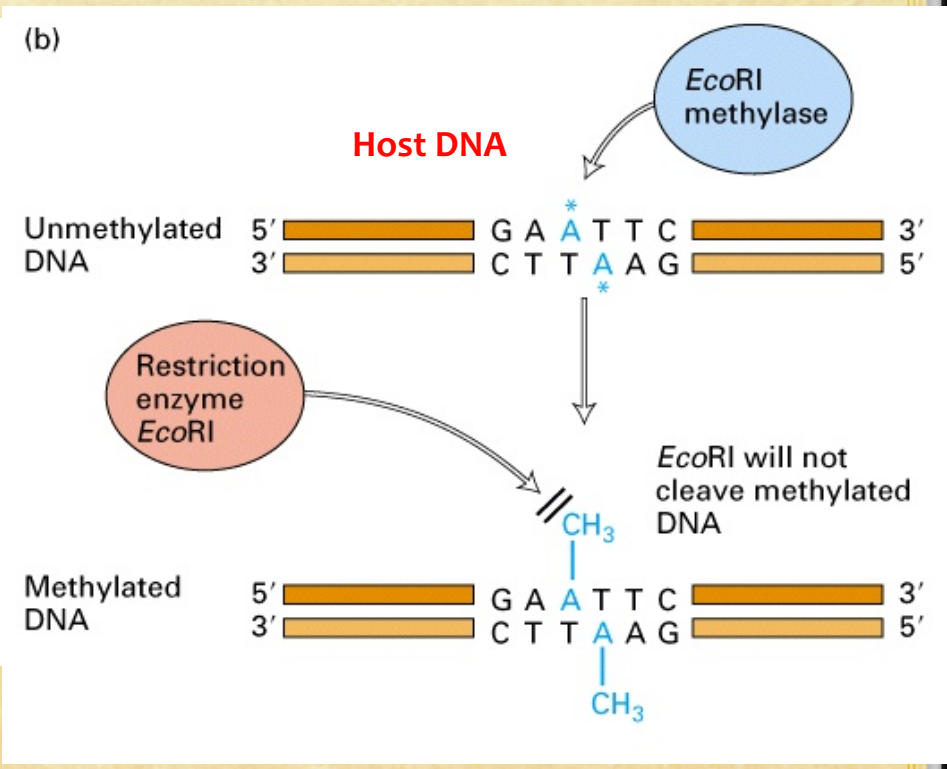
- These are the ones that are used in most recombinant DNA experiments
- **The nuclease and methylase are separate enzymes/molecules, but recognize the same target base sequence.**
- The target sequence,is symmetrical
- The nuclease will not attach if the site is methylated(modified)
- The cut site is within the recognition site: therefore a given enzyme will always cut in the same way, unlike Type I nucleases
- The two enzymes either cleave or modify the recognition sequence
- restriction activities do not require cofactors such as ATP or SAM

e.g.*Eco*RI, *Bam*HI, *Hind*III

A: Restriction Endonucleases (enzymes that cleave DNA at specific sites)

The Restriction-Modification System of Bacteria





Part I: Restriction

→ **Bacteria produce restriction enzymes that digest foreign (viral DNA)**

Part II: Modification

→ **Bacteria methylate their DNA to protect it from digestion**

Type III

- One enzyme with two different subunits,
  - one is for recognition and modification
  - Other is for cleavage.
- Recognizes and methylates same sequence but cleaves 24–26 bp away
- Type III enzymes have symmetrical recognition sequences

Type IIs

- Two different enzymes
- recognition sequence is asymmetric.
- Cleavage occurs on one side of recognition sequence up to 20 bp away

There are Three Types of Restriction Endonucleases

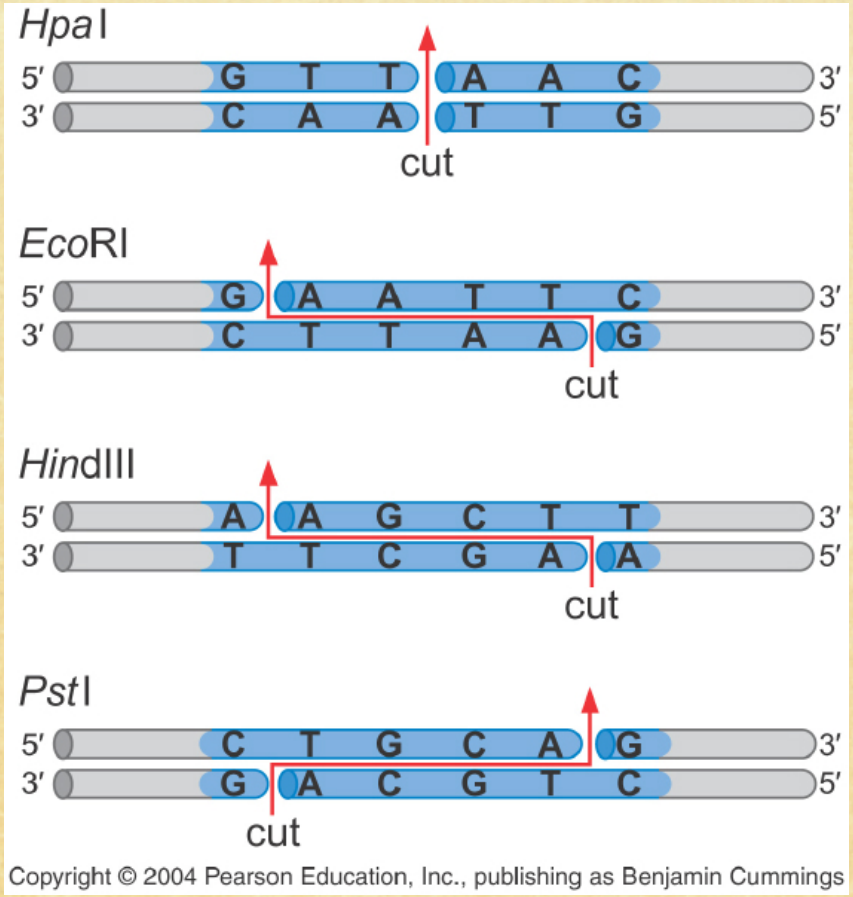
Properties of Restriction Endonucleases	Type I	Type II	Type III
Example	*Eco*B	*Eco*RI	*Eco*PI
Recognition site	$TGAN_8TGCT$	GAATTC	AGACC
Cleavage site	About 1 kb away from recognition site	Between G and A (both strands)	24–26 base pairs 3′ to recognition site
Methylation site	$TGAN_8TGCT$ (m) / $ACTN_8ACGA$ (m)	GAATTC (m) / CTTAAG (m)	AGACC (m) (only one strand methylated)
Nuclease and methylase in one enzyme?	Yes	No	Yes
Requirements for cleavage	ATP, Mg^{2+}, *S*-AdoMet	Mg^{2+} or Mn^{2+}	Mg^{2+}, *S*-AdoMet
Requirements for methylation	ATP, Mg^{2+}, *S*-AdoMet	*S*-AdoMet	Mg^{2+}, *S*-AdoMet

Type II Restriction Enzymes are the Most Useful for Cloning

1) They recognize palindromic sequences → They make double-stranded cuts

2) They cut at the site of recognition → They generate predictable ends

Examples of Type II Restriction Endonucleases



← Blunt ends

Cohesive "sticky" ends

Q: How Frequently Will a Restriction Enzyme Cut DNA?

A: It Depends of the Length of the Recognition Sequence

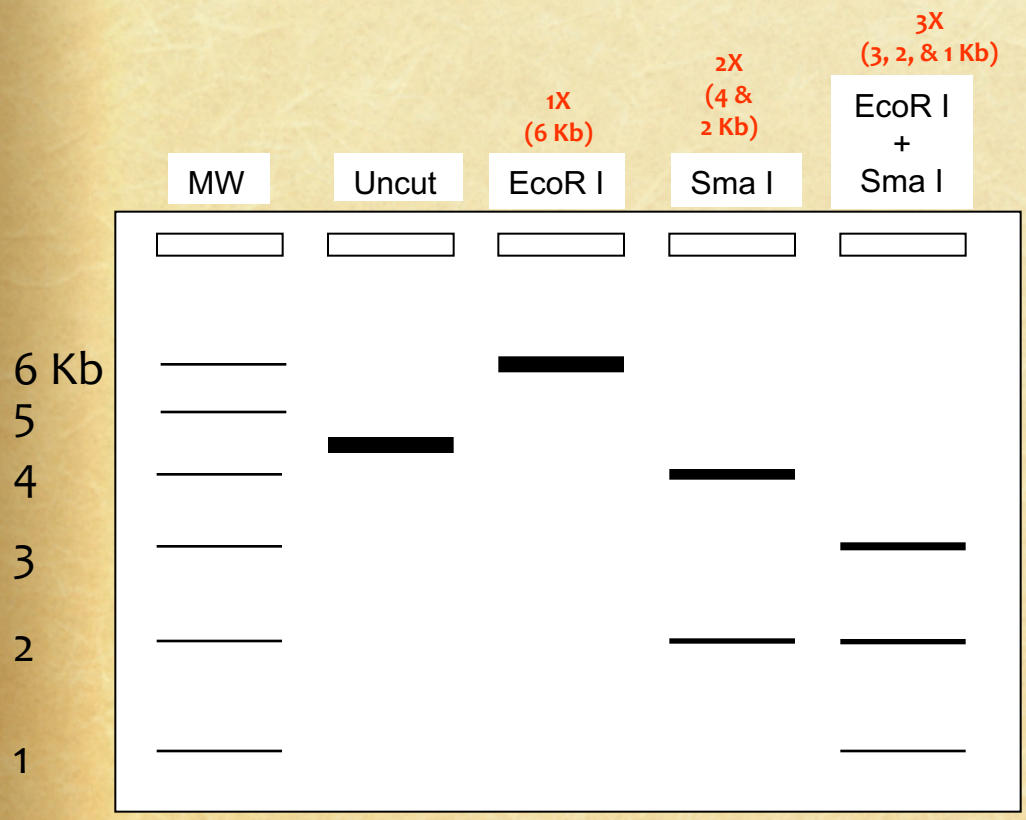
A four-base cutter:
(*Sau*3A) GATC

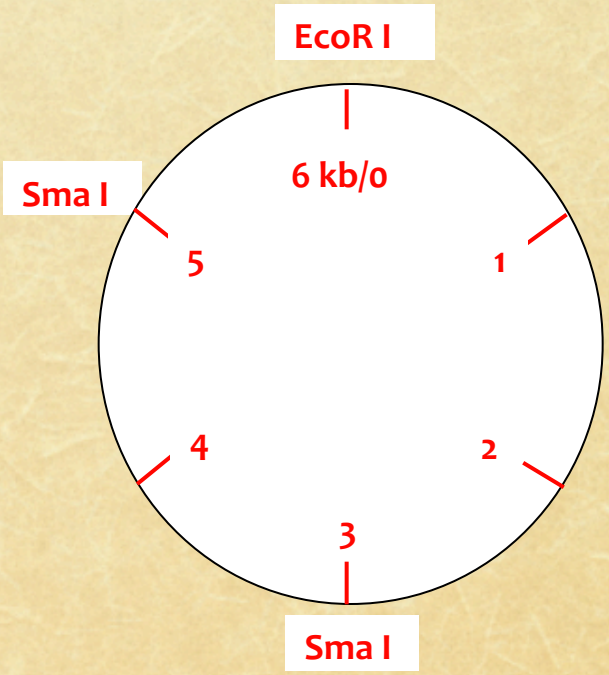
1/4 X 1/4 X 1/4 X 1/4 = 1/256 bp

A six-base cutter:
(*Bam*HI) GGATCC

$(1/4)^6 = 1/4096$ bp

Restriction Mapping Problem #1





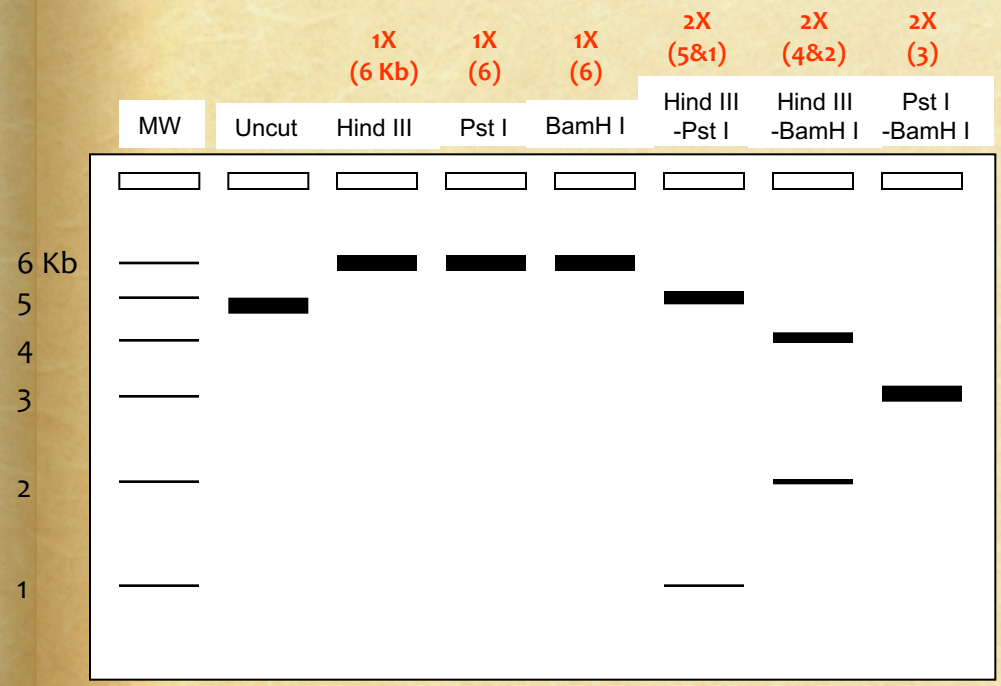
1) How big is the plasmid?

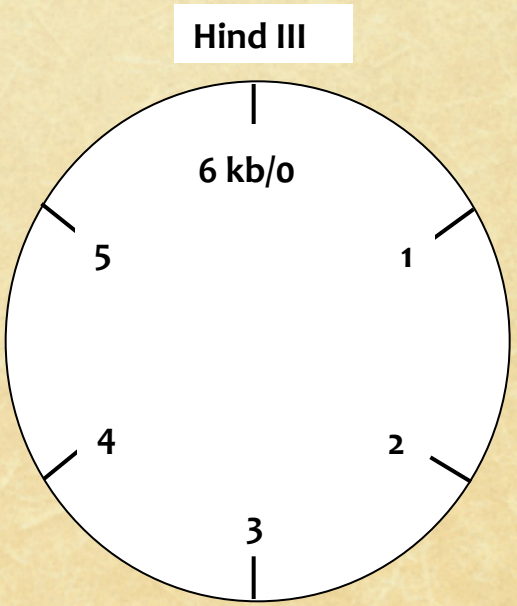
2) How many times was the plasmid cut? What are the sizes of each band?

3) Do the band sizes add up the total plasmid length?
(Are the fragments in equal stochiometric amounts?)

4) In double digests, which band(s) gets cut by the other enzyme?

Restriction Mapping Problem #2





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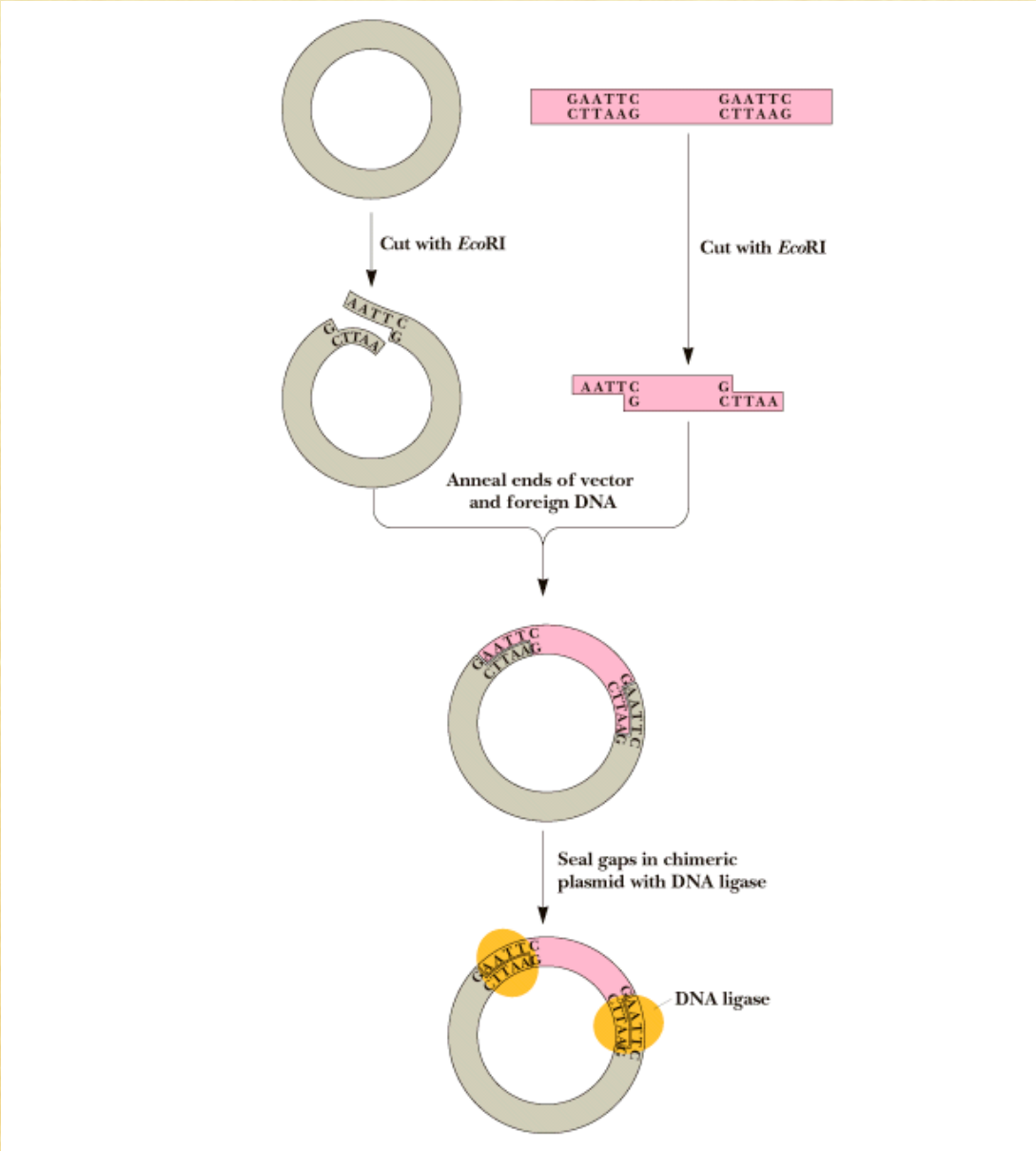
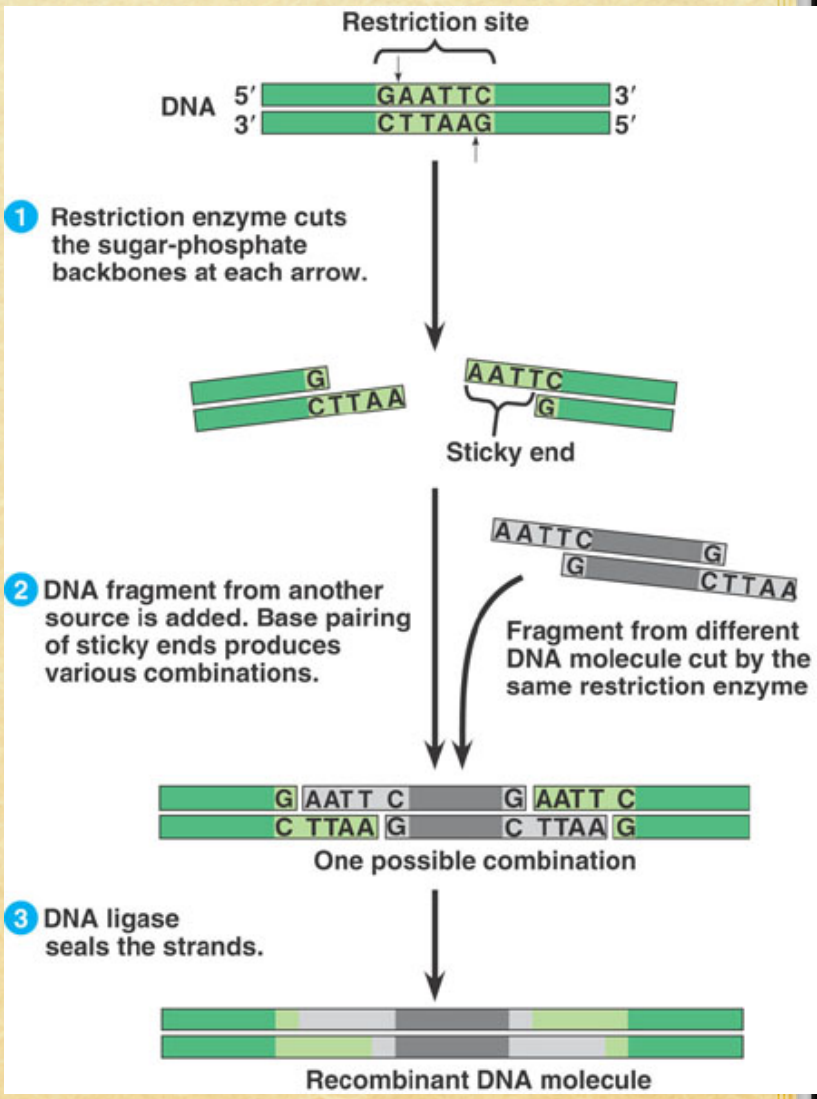
An Example of a Sticky End Ligation



*DNA ligase can also religate (reseal) a cut plasmid (ligate without the insert)!

Recombinant DNA

- Recombinant DNA is constructed using restriction enzymes
- **Important:** In order to join 2 pieces of DNA together they have to be cut by the same restriction enzyme
  - Why?
  - Otherwise, the sticky ends won't match– DNA can't bind together



Examples of Restriction Enzymes

Enzyme	Organism source	Recognized Sequence
EcoRI	*Escherichia coli*	5' **GA**ATTC 3' 3' CTTA**AG** 5'
TaqI	*Thermus aquaticus*	5' **TC**GA 3' 3' AG**CT** 5'
HindIII	*Haemophilus influenzae*	5'**AA**GCTT 3' 3'TTCG**AA** 5'
BamHI	*Bacillus amyloliquefaciens*	5' **GG**ATCC 3' 3' CCTA**GG** 5'
AluI	*Arthrobacter luteus*	5' A**GC**T 3' 3' T**CG**A 5'

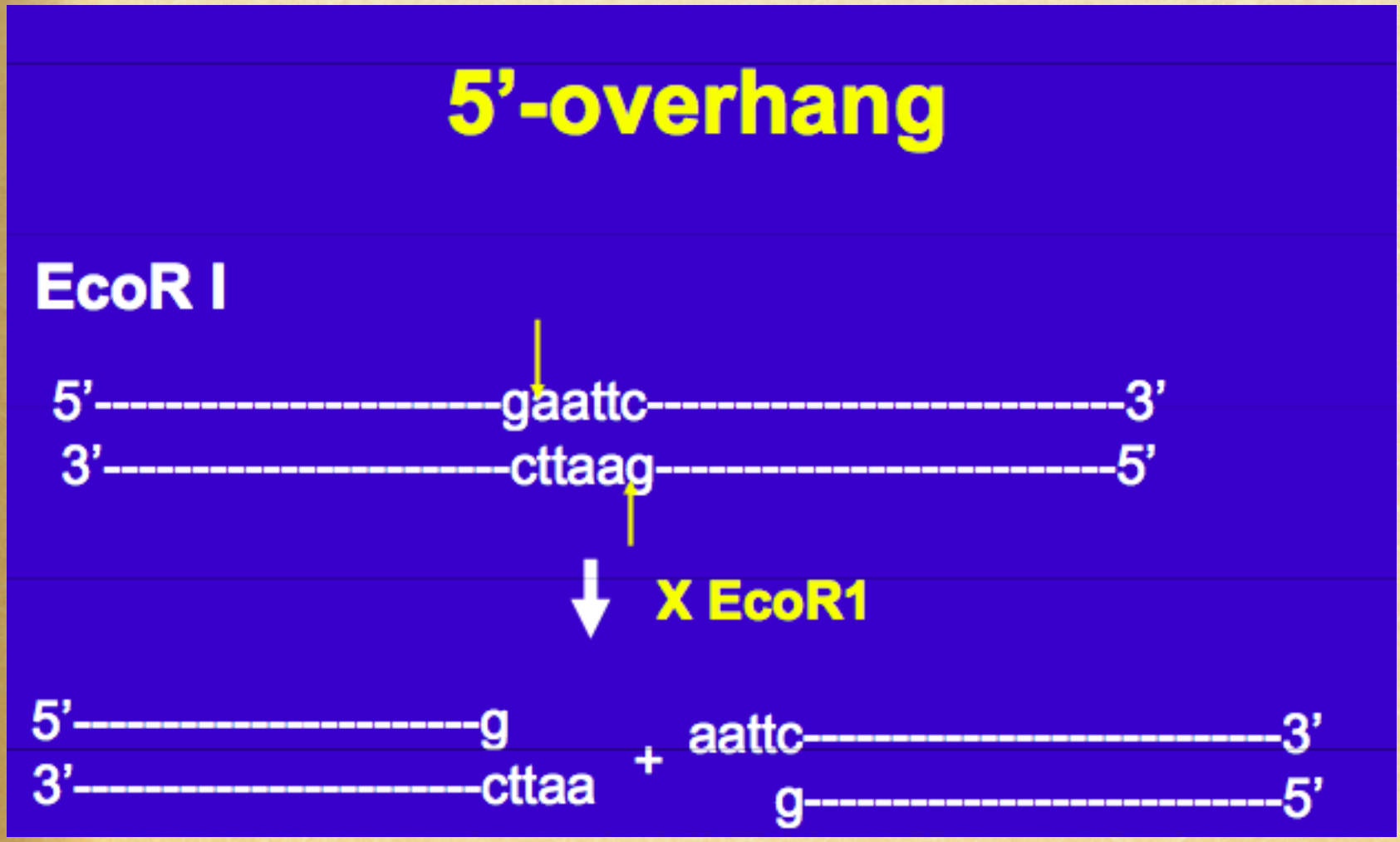
Restriction Analysis

- Using restriction enzymes to find out information about a piece of DNA
- We can use restriction enzymes to find out
  - The size of a plasmid
  - If there are any restriction sites for a particular enzyme on a piece of DNA (*ex. EcoRI*)
  - How many restriction sites for a particular enzyme
  - Where the restriction sites are located

Three types of ends produced by type II restriction enzymes

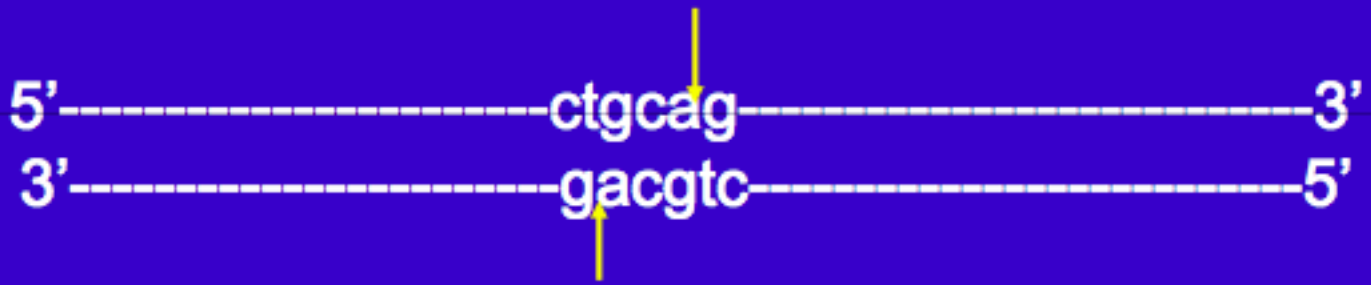
- 3'-overhang (protruding)
- 5'-overhang
- Blunt end

5’-overhang

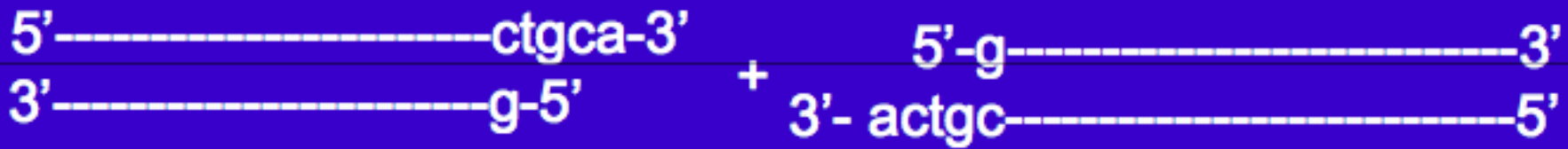


3’-overhang

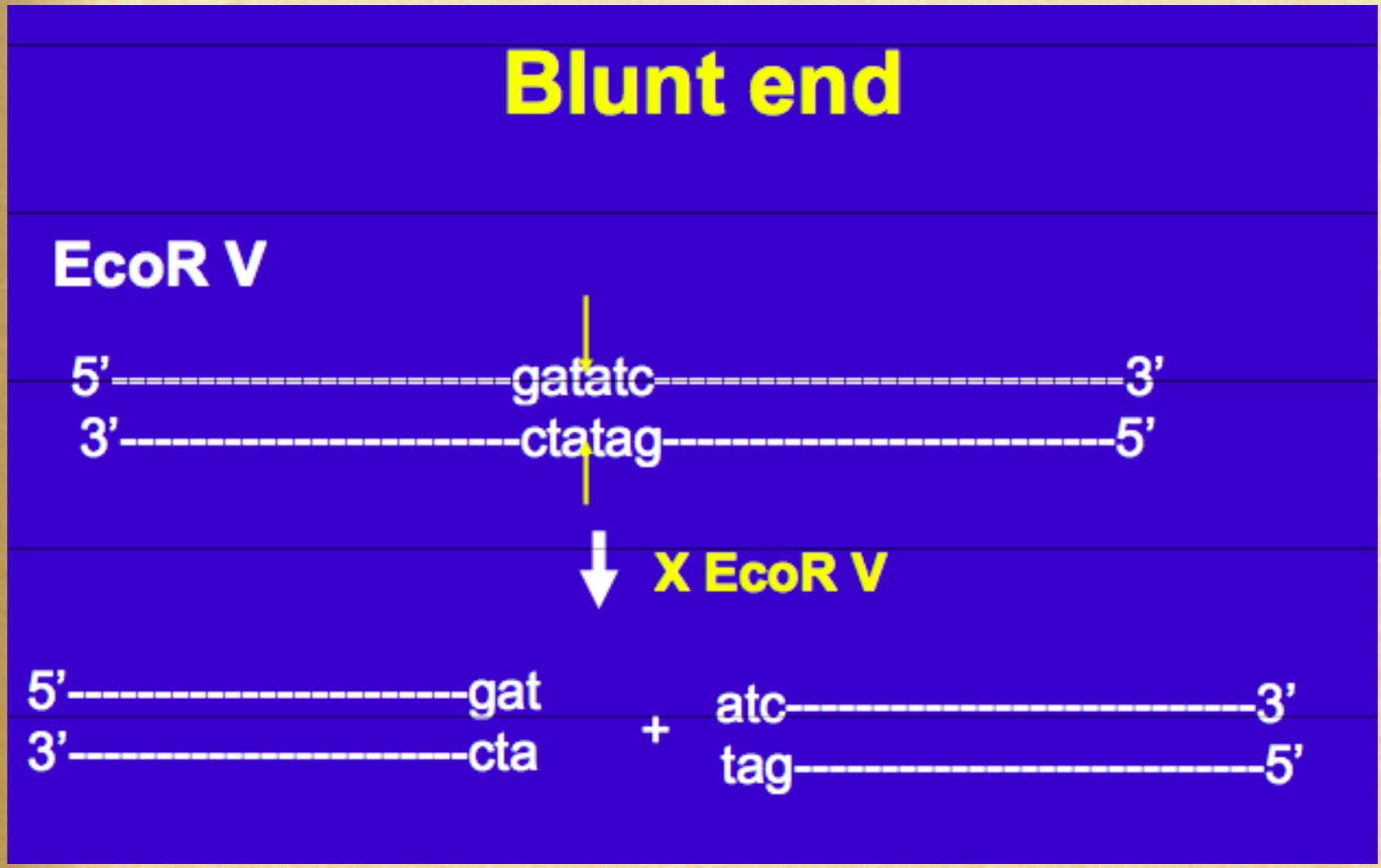
Pst I:



X PstI



Blunt end



- Over 10,000 bacteria species have been screened for restriction enzymes
- Over 2,500 restriction enzymes have been found
- Over 250 distinct specificities
- Occasionally enzymes with novel DNA sequence specificities are still found while most now prove to be duplicates (isoschizomers) of already discovered specificities.

Isoschizomers

- Pairs of restriction enzymes isolated from different bacteria which have same recognition sequence.
- SphI (CGTAC/G) and BbuI (CGTAC/G) are isoschizomers of each other
- The first enzyme discovered which recognizes a given sequence is known as the **prototype**; all subsequently isolated enzymes with same recognition sequence are isoschizomers
- Isoschizomers are isolated from different strains of bacteria and therefore may require different reaction conditions

Neoschizomers

- enzymes that recognizes the same sequence but cuts it differently is neoschizomer.

Neoschizomers are a specific type (subset) of isoschizomer.

For example,

SmaI (CCC/GGG)

XmaI (C/CCGGG)

are neoschizomers of each other.

Star activity of restriction enzyme

- Under extreme conditions e.g. elevated pH or low ionic strength, restriction endonucleases are capable of cleaving sequences which are similar but not identical to their defined recognition sequence.

- This altered specificity is known as ***star* activity**.

Star activity of restriction enzyme

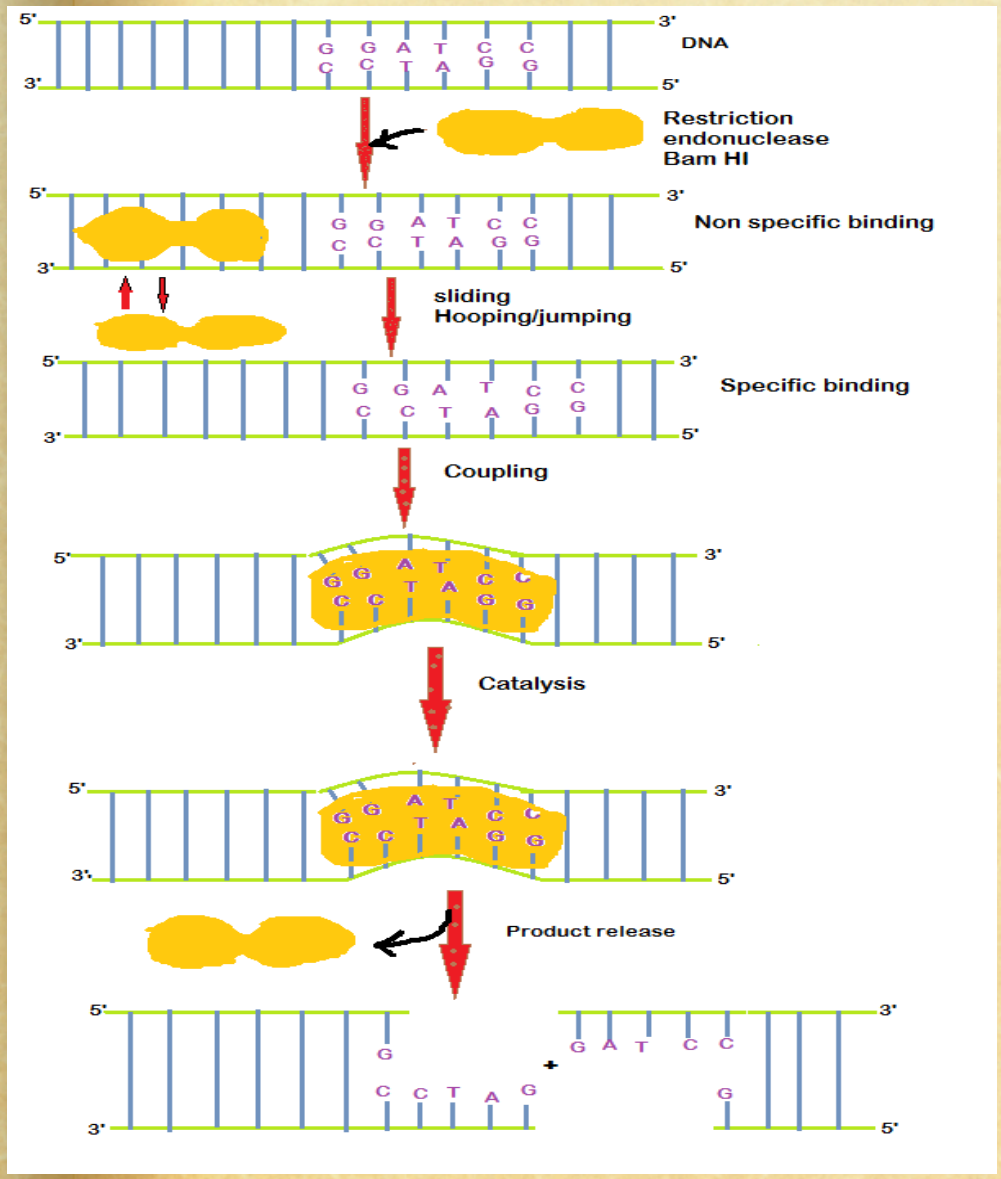
- Changes are base substitutions and truncation of the number of bases in the recognition sequence.
  - For example, *Eco*RI* (*Eco*RI star activity) cleaves the sequence N/AATTN, where N is any base, whereas *Eco*RI cleaves the sequence GAATTC.

Avoiding star activity

- Use the optimal buffer system recommended in the pack insert.
- Use the optimal amount of enzyme recommended.
- Make sure that the DNA preparation is free of organic solvents and contaminating salts that have been used during isolation and/or purification of DNA.

- **The steps involved in DNA binding and cleavage by a type II restriction endonuclease:**
  - Non-specific contact with DNA
  - Bind of enzyme to DNA as dimmer.
  - The target site is then located by a combination of linear diffusion or “sliding” of the enzyme along the DNA over short distances, and hopping/jumping over longer distances.

- Once the target restriction site is located, the recognition process (coupling) triggers large conformational changes of the enzyme and the DNA, which leads to activation of the catalytic center.
- • Catalysis results in hydrolysis of phosphodiester bond and product release.

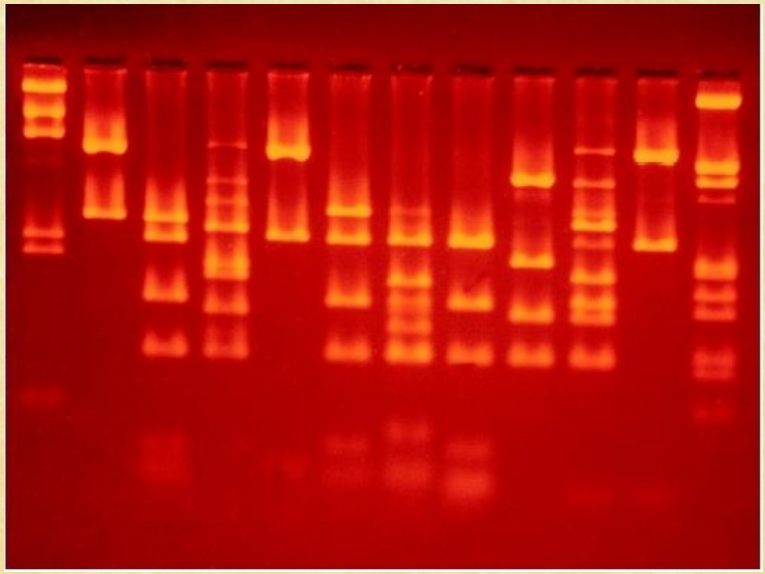


The two dimers are shown in brown, the DNA backbone is in green and the bases in gray. *BamH*I becomes progressively more closed around the DNA as it goes from the nonspecific to specific DNA binding mode.

Fig 2-1.4.2: Structures of free, nonspecific, and specific DNA-bound forms of BamHI.

Applications:

- In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.
- They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.
- Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restriction site present in the allele.
- Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.



Cleaving a single piece of DNA with multiple restriction enzymes creates a "DNA fingerprint." The pattern of fragments can be compared to similar DNA from another source treated with the same enzymes, to determine if the two are identical or different. https://www.youtube.com/watch?v=GsWo8dCivWs