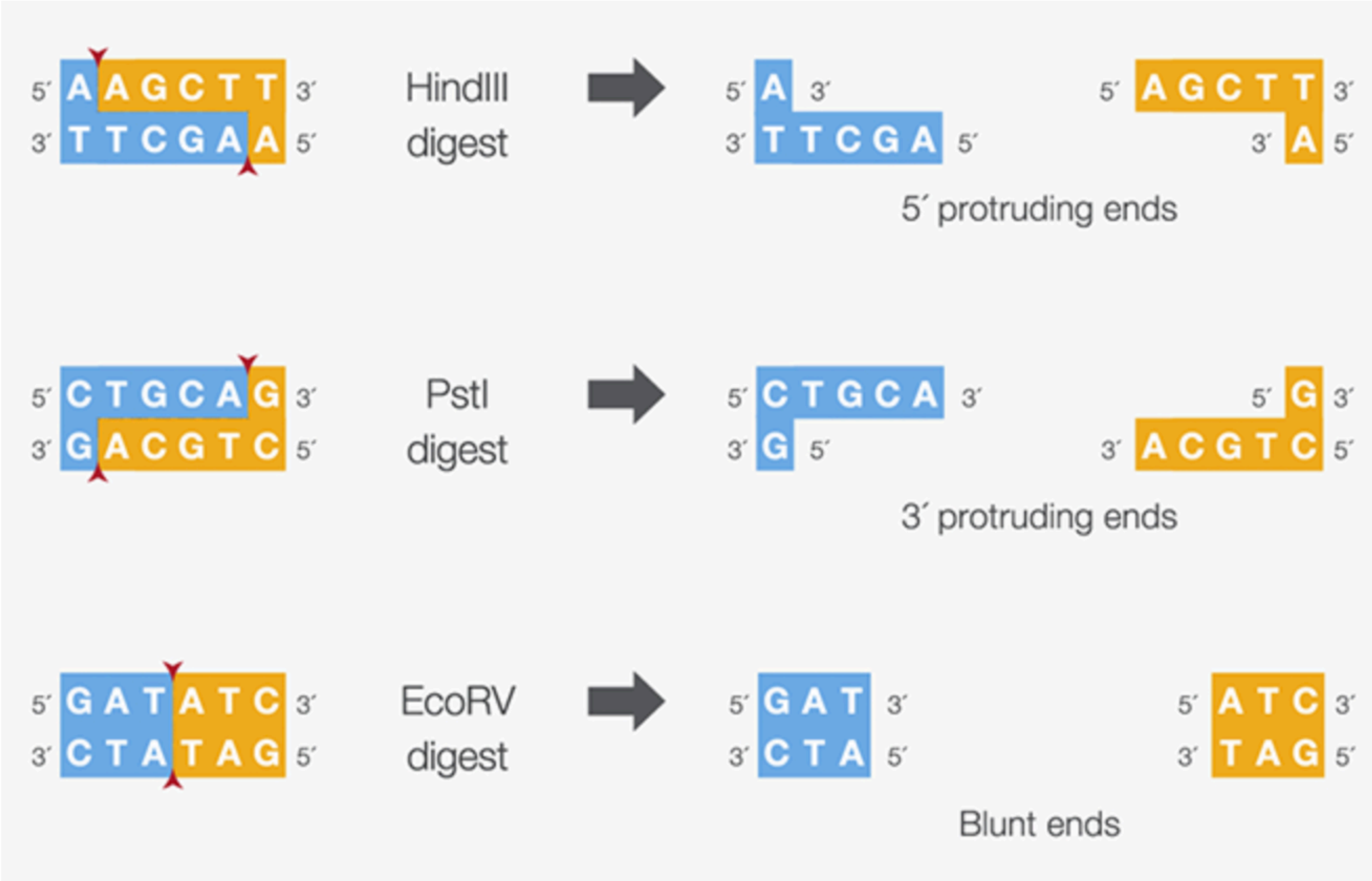
Endonucelasi di restrizione (=enzimi di restrizione)
Restriction endonucleases (=restriction enzymes)



Endonucelasi di restricion (=enzimi di restrizione)
Restriction endonucleases (=restriction enzymes)

- Enzymes that cut the double helix of DNA at defined seqeunces
- 3000 different restriction enzymes exist
- 500 restriction enzymes for the use in the laboratory
- Encoded by prokaryotes (bacteria)

Note:
Endonucleases cut DNA at internal position
Exonucleases cut DNA starting at termini
DNAase: cuts DNA
RNase: cuts RNA

Discovery of restriction endonucleases

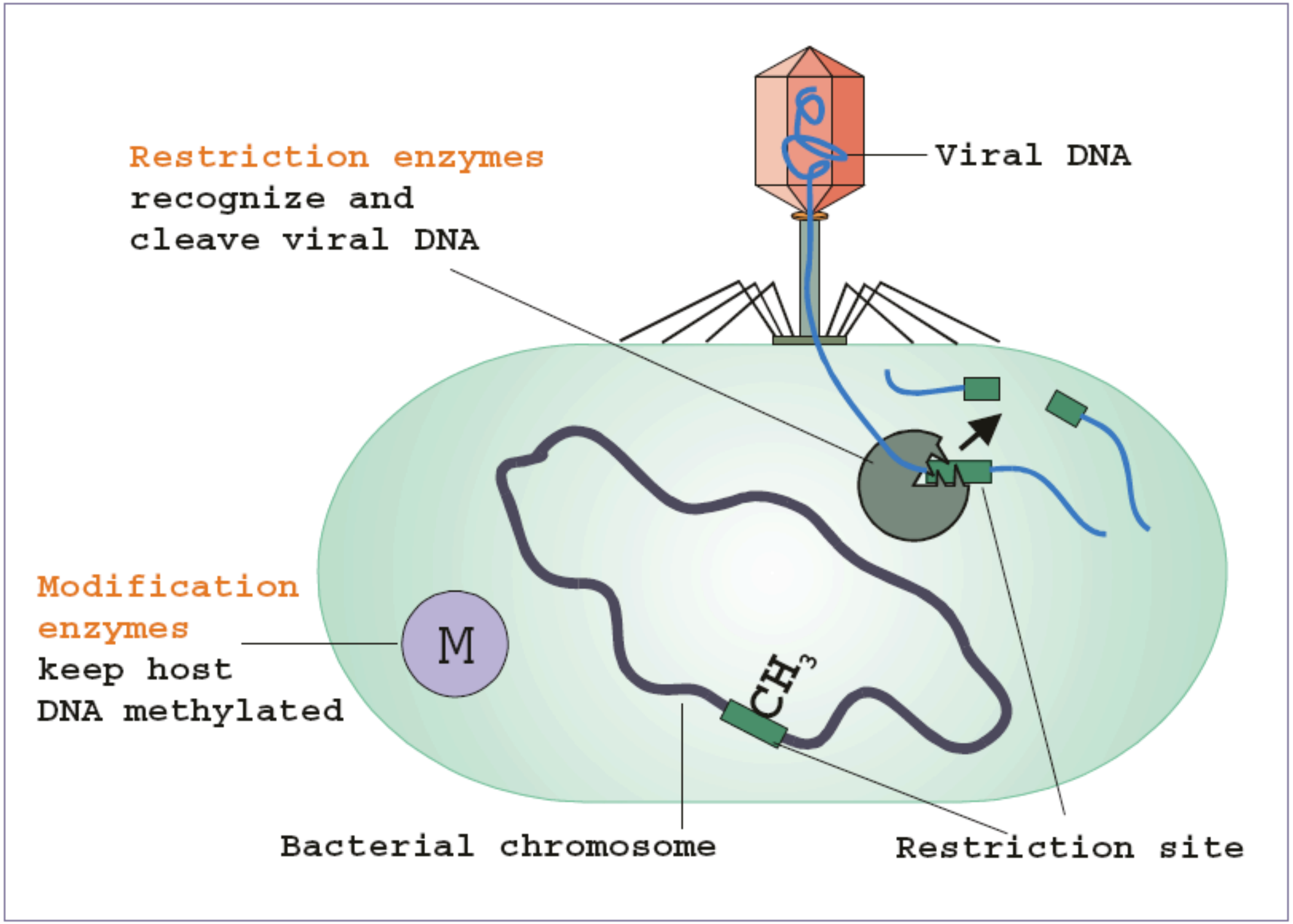
- Arbor and Dussoix in 1962 discovered that certain bacteria contain Endonucleases which have the ability to cleave DNA.
- In 1970 Smith and colleagues purified and characterized the cleavage site of a Restriction Enzyme.
- Werner Arbor, Hamilton Smith and Daniel Nathans shared the 1978 Nobel prize for Medicine and Physiology for their discovery of Restriction Enzymes.

Restriction Enzymes

- *Also known as restriction endonucleases*
- *Scan the DNA sequence*
- *Find a very specific set of nucleotides*
- *Make a specific cut (with defined termini or blunt)*
- *Used to construct recombinant DNA plasmids*

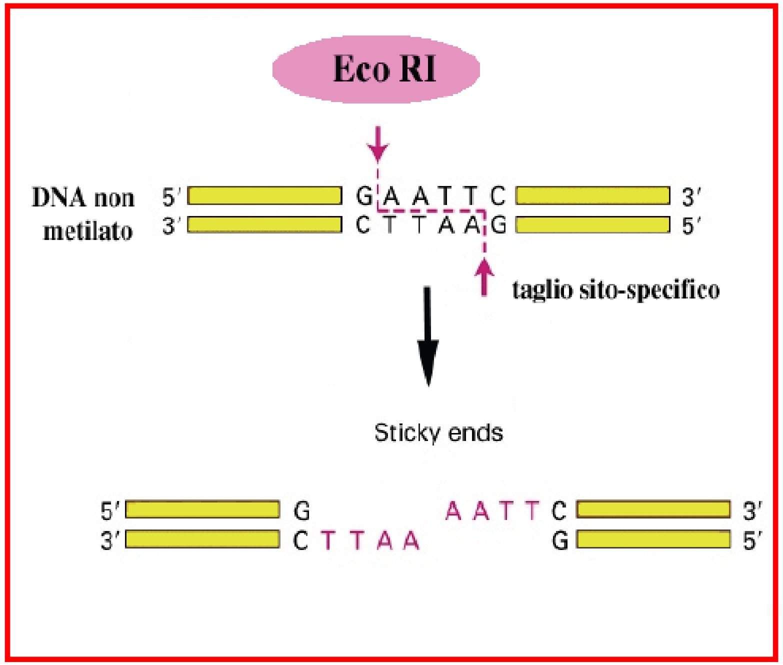
The origin of restriction endonucleases

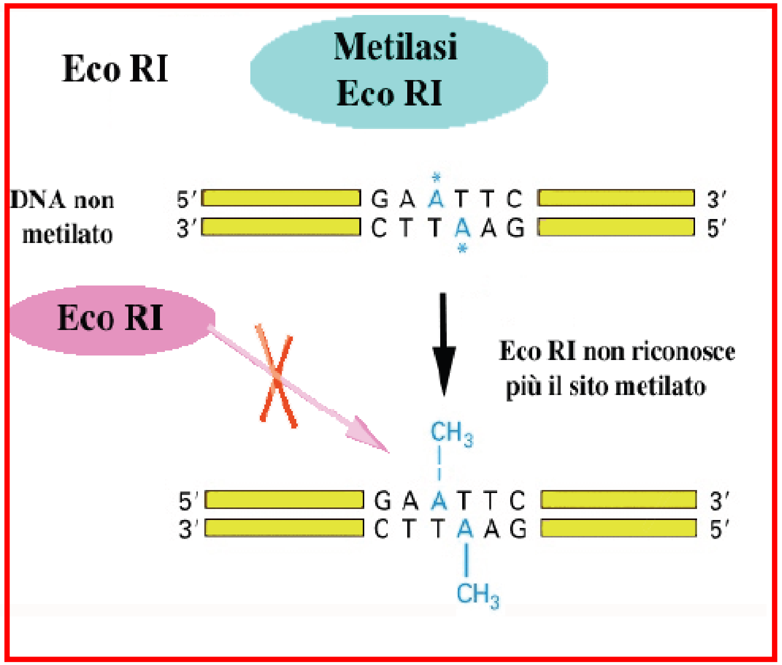
Bacterial defense against viral infection by restriction-modification complexes



The origin of restriction endonucleases

Bacteria contain coupled DNA metylation – restriction endonucelase systems





Bacterial DNA methylases can exclusively methylate **A or C**, when present in correct seqeunce context.

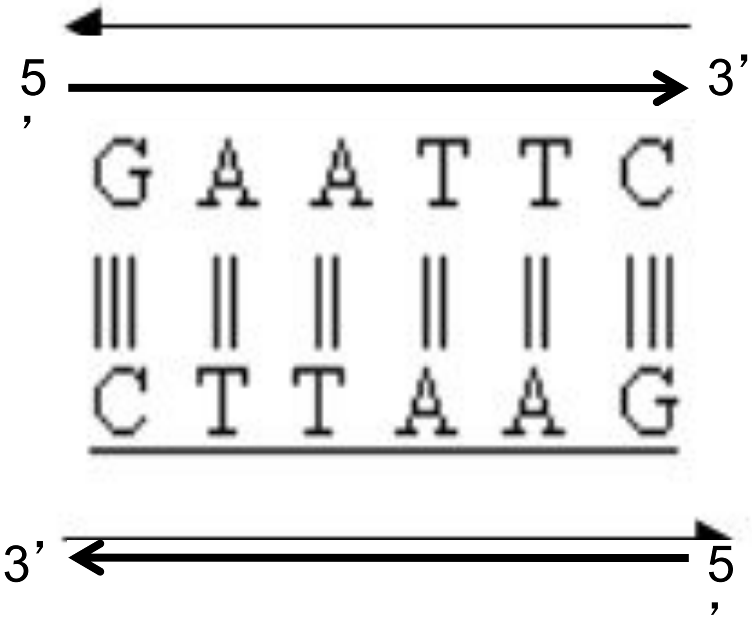
Fage DNA or **plasmid DNA** with unmethylated or methylated by other DNA methylation system enters bacteria. EcoRI expressed by recipient bacteria cuts at all GAATTC sites in the foreign DNA

EcoRI methylase methylates host DNA at defined seqeunce: GAATTC EcoRI restriction endonuclease expressed by bacteria can cut only un-methylated DNA. DNA integroty is maintained

Types of Restriction Enzymes

	Cleavage site	Location of methylase	Examples
Type I	Random Around 1000bp away from recognition site	Endonuclease and methylase located on a **single protein** molecule	EcoK I EcoA I CfrA I
Type II	Specific Within the recognition site	Endonuclease and methylase are **separate entities**	EcoR I BamH I Hind III
Type III	Random 24-26 bp away from recognition site	Endonuclease and methylase located on a **single protein molecule**	EcoP I Hinf III EcoP15 I

Restriction enzymes typically recognize palindromic sequences on double stranded DNA



Genetic palindromes are similar to verbal palindromes. A palindromic sequence in DNA is one in which the 5' to 3' base pair sequence is identical on both strands.

(typically 4 or 6 nucleotide palindromes)

4 nucleotide palindromes: 1 cut every 256 nucleotides (4^4).
6 nuceotide palindromes: 1 cut every 4.096 nucleotides (4^6).

Enzyme	Target sequence	Cleavage
EcoRI	5' GAATTC 3' 3' CTTAAG 5'	5' G AATTC 3' 3' CTTAA G 5'
EcoRV	5' GATATC 3' 3' CTATAG 5'	5' GAT ATC 3' 3' CTA TAG 5'
HaeIII	5' GGCC 3' 3' CCGG 5'	5' GG CC 3' 3' CC GG 5'
HindIII	5' AAGCTT 3' 3' TTCGAA 5'	5' A AGCTT 3' 3' TTCGA A 5'
PpuMI	5' RGGWCCY 3' 3' YCCWGGR 5'	5' RG GWCCY 3' 3' YCCWG GR 5'

Small number (PpuMI)

Single Letter Code List

- **B** = C or G or T
- **D** = A or G or T
- **H** = A or C or T
- **K** = G or T
- **M** = A or C
- **N** = A or C or G or T
- **R** = A or G
- **S** = C or G
- **V** = A or C or G
- **W** = A or T
- **Y** = C or T

Sistemi di restrizione-modificazione

Tipo I

•Un singolo enzima contiene attività di restrizione e di metilazione su subunità diverse

•Il taglio viene effettuato in modo non specifico lontano dalla sequenza di riconoscimento (da 100 fino a 1000 bp a valle)

• Mg^{2+}, ATP e S-adenosilmetionina come cofattori

Tipo II

•Due enzimi distinti per il taglio e la metilazione.

•Non richiedono cofattori se non **Mg2+**

•Riconoscono la stessa sequenza **palindromica** e agiscono al suo interno

Tipo III

•Caratteristiche analoghe a quelli di tipo I

•Riconosce e modifica una sequenza **palindromica** ma taglia a 25-27 bp di distanza

Tipo IIs

• *Due enzimi separati che riconoscono una sequenza non palindromica*

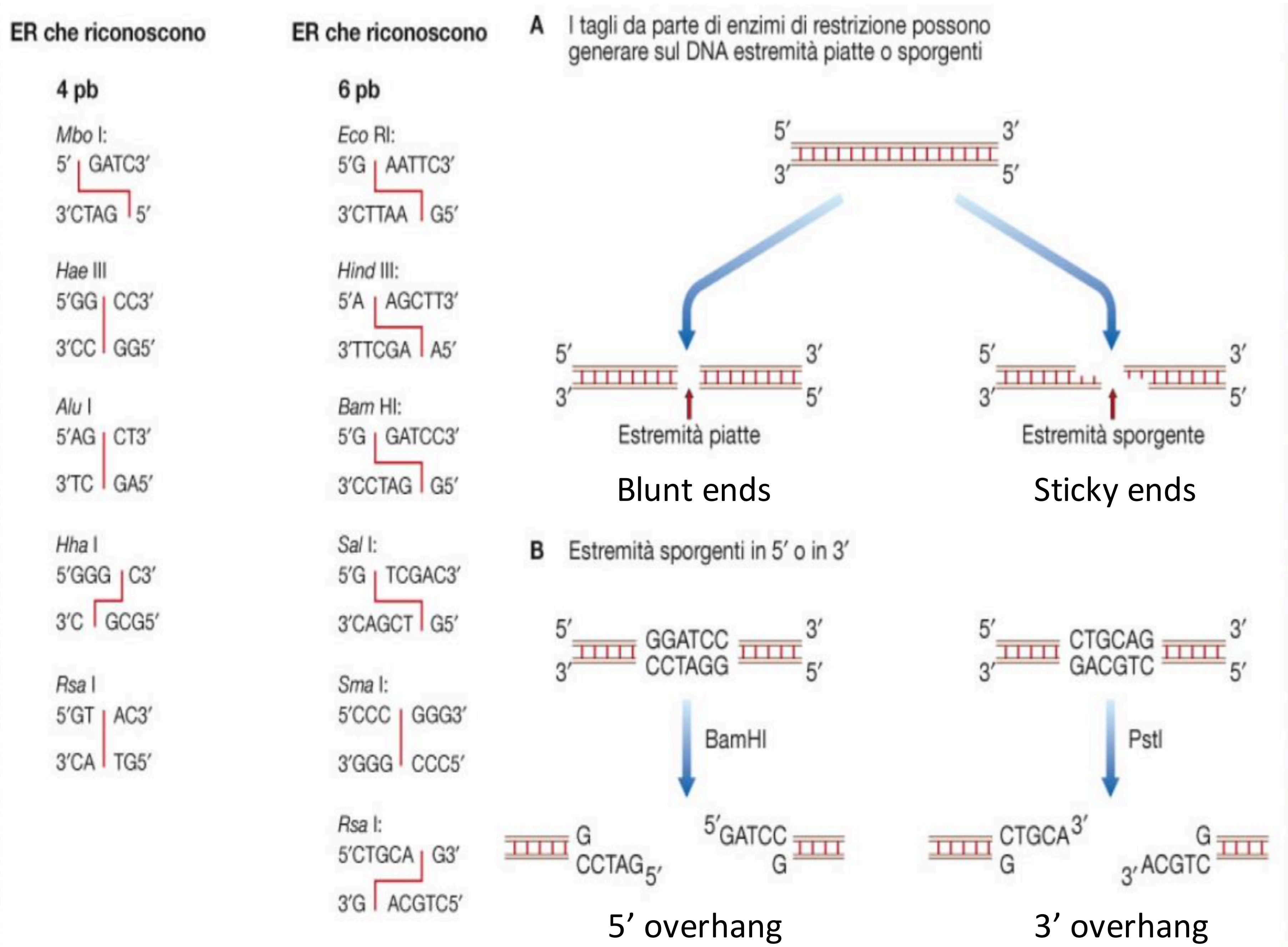
• *Tagliano su di un solo lato delle sequenza bersaglio entro 20 bp*

Nomenclature of restriction enzymes

1. Le prime tre lettere, scritte in corsivo, sono prese da genere e specie del batterio di origine.
2. Sierotipi differenti dello stesso organismo possono essere identificati da una quarta lettera minuscola (Es. *Hind*, *Hinf*).
3. Può seguire una lettera maiuscola o un numero, che identifica un ceppo particolare di quel batterio.
4. Un numero romano indica l'ordine di scoperta, qualora dallo stesso batterio vengano isolati enzimi diversi.

Enzima	Organismo di provenienza
*Sma*I	*Serratia marcescens*, 1° enzima
*Hae*III	*Haemophilus aegyptius*, 3° enzima
*Hind*II	*Haemophilus influenzae*, ceppo d, 2° enzima
*Hind*III	*Haemophilus influenzae*, ceppo d, 3° enzima
*Bam*HI	*Bacillus amyloliquefaciens*, ceppo H, 1° enzima

Types of DNA cuts by restriction endonucelases



Isoschizomers and Neochischizomers

- Restriction enzymes that have the same recognition sequence as well as the same cleavage site are **Isoschizomers.**

C G T A C|G
G|C A T G C
SphI

C G T A C|G
G|C A T G C
BbuI

- Restriction enzymes that have the same recognition sequence but cleave the DNA at a different site within that sequence are **Neochizomers.** Eg:SmaI and XmaI

C|C C G G G
G G G C C|C
Xma I

C C C|G G G
G G G|C C C
Sma I

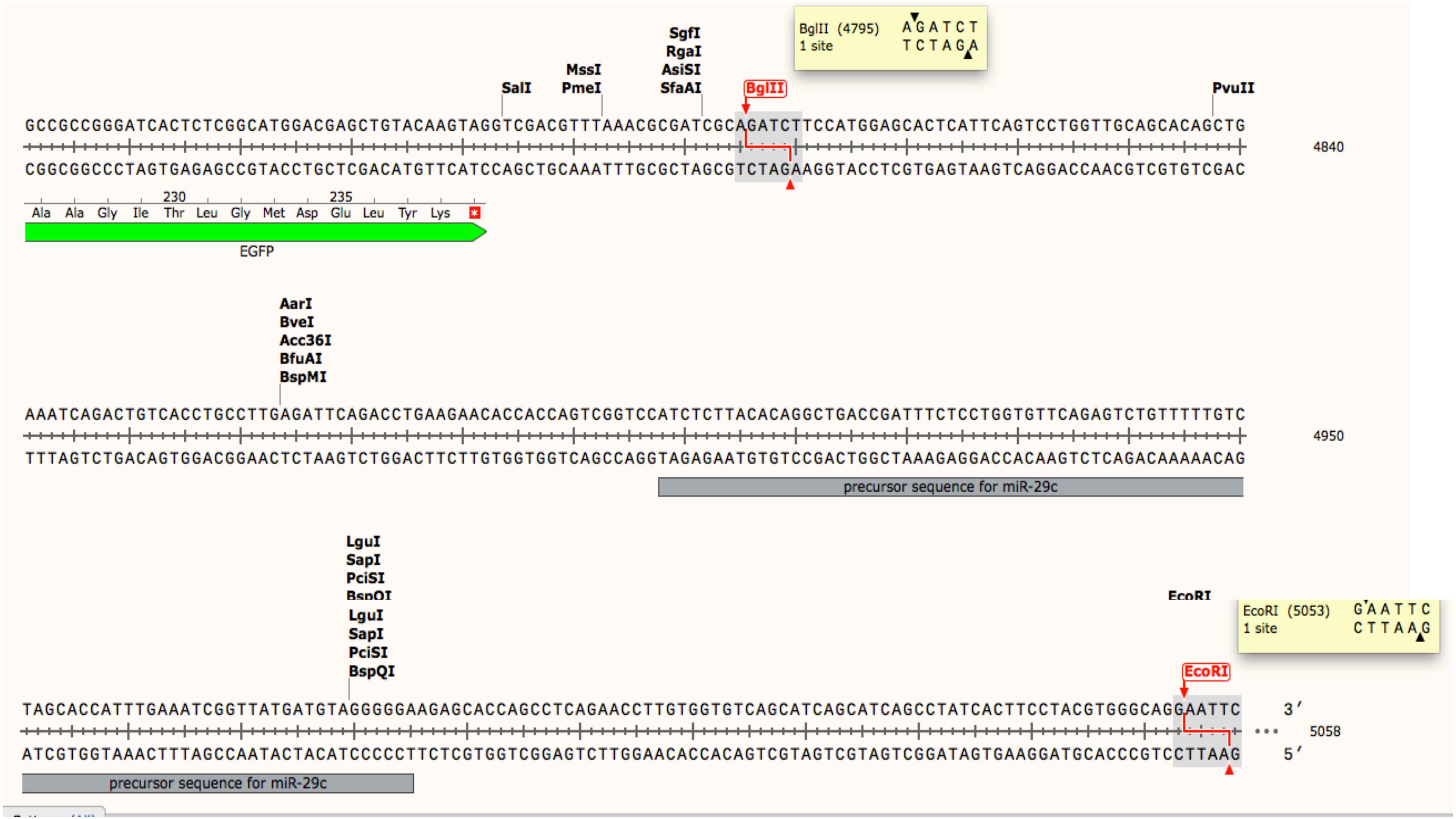
Isocaudomers

Isocaudomers are pairs of restriction enzymes that have **slightly different recognition sequences**, but upon cleavage of DNA, **generate identical overhanging termini sequences**. These sequences **can be ligated to one another**, but then form an asymmetrical sequence that **cannot be cleaved** by a restriction enzyme.

```
Mbo I
  N|GATCN
  NCTAG|N

BamH I
  G|GATCC
  CCTAG|G
```

Example of laboratory plasmid sequence

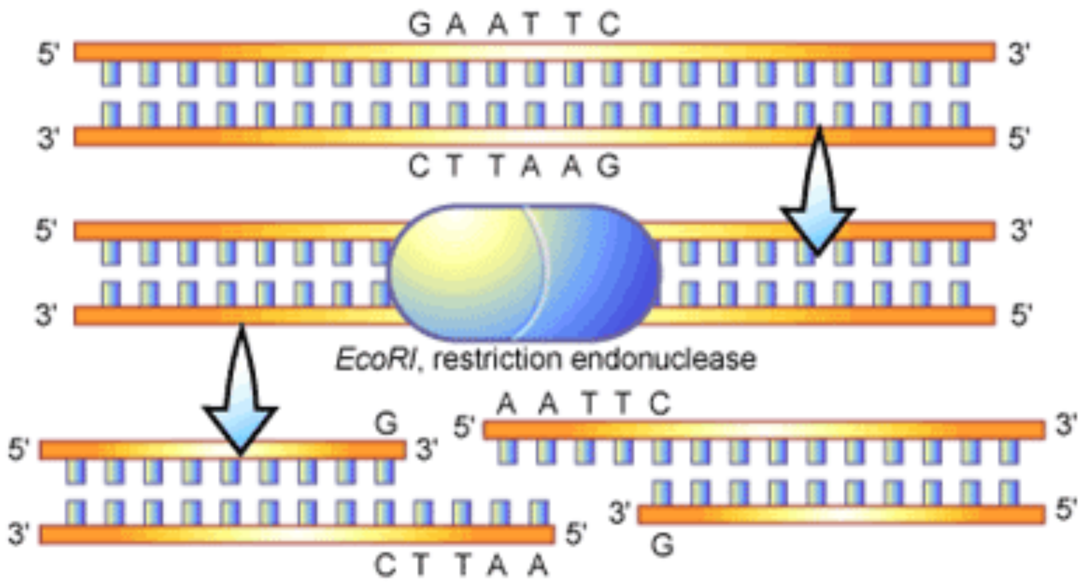


Mechanism of Action of restriction endonucleases

Restriction Endonuclease scan the length of the DNA , binds to the DNA molecule when it recognizes

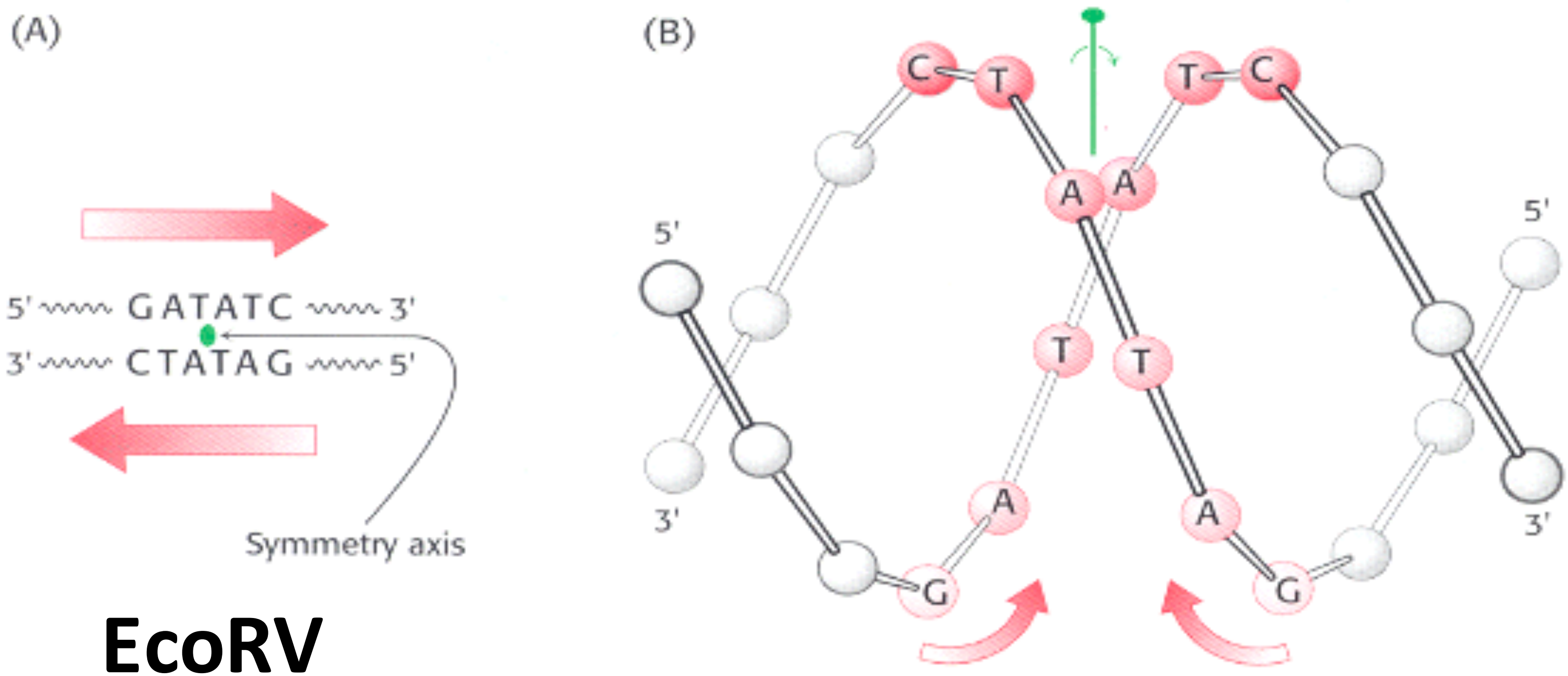
specific sequence and makes one cut in each of the sugar phosphate backbones of the double helix – by

hydrolyzing the phoshphodiester bond. Specifically, the bond between the 3’ O atom and the P atom is broken.



Mechanism of restriction endonucleases

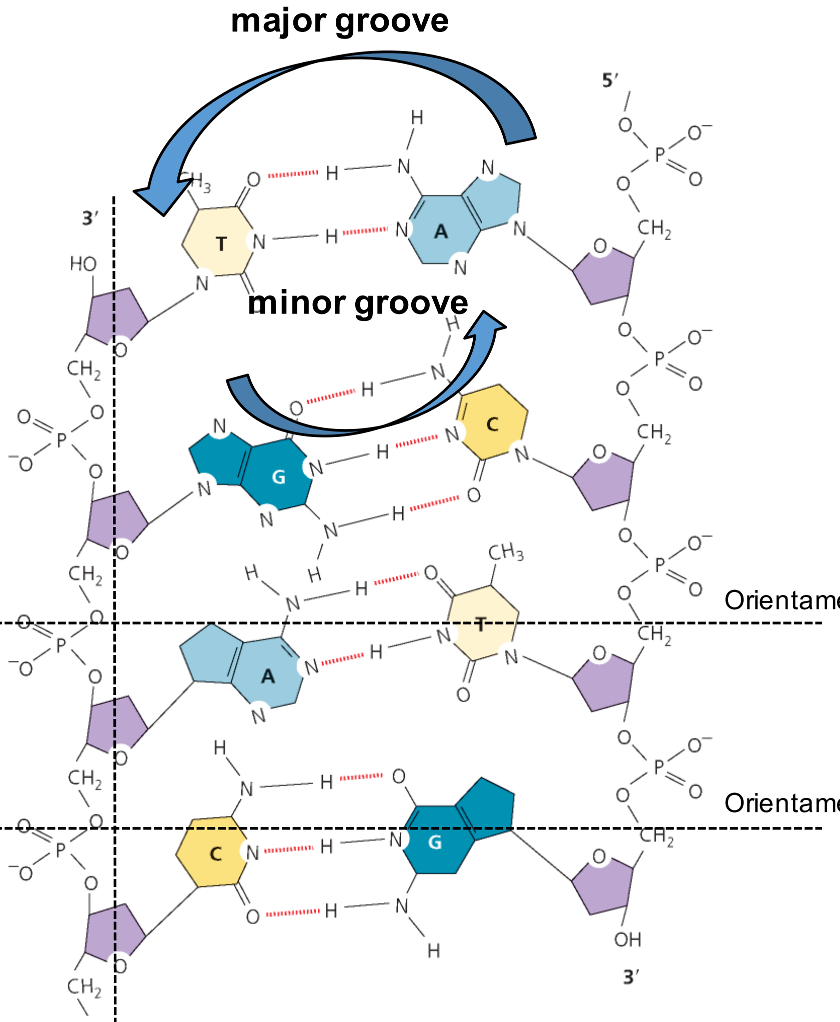
Recognition sites of most restriction enzymes have a twofold rotational symmetry



EcoRV

Restriction enzymes have corresponding symmetry to facilitate recognition and usually cleave the DNA on the axis of symmetry

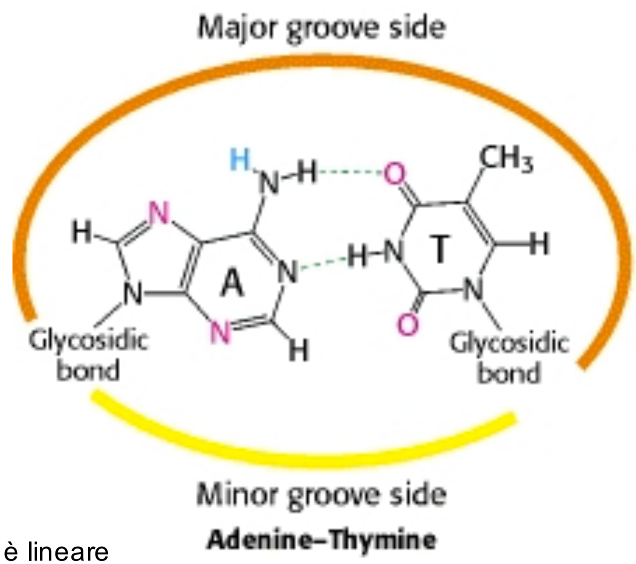
La doppia elica presenta un solco minore ed un solco maggiore (minor and major groove)

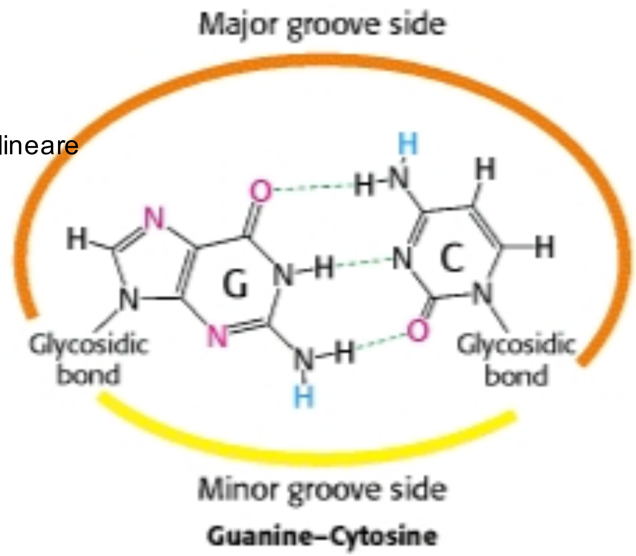


Orientamento non è lineare

Orientamento non è lineare

L’angolo fra i legami glicosidici è di circa **120°** (formerà il solco minore) o **240°** (formerà il solco maggiore)



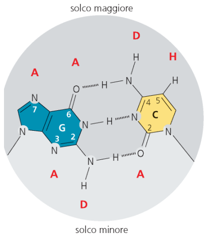


Restriction enzymes are DNA binding proteins that recoginice specific seqenced by interaction with chemical groups in major and minor groove

A: accettore legame idrogeno
D: donatore legame idrogeno
M: gruppo metilico
H: idrogeno non polare

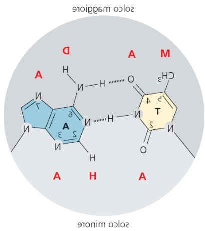
Queste codice formato da gruppi chimici posti al interno del solco maggiore/minore identificano in modo specifico le coppie di basi.

Le proteine possono riconoscere specifiche sequenze di DNA senza che sia necessario aprire o rompere la doppia helica !!!
Esempio:
→Fattori di trascrizione
→Elicasi, etc…
→Endonucleasi



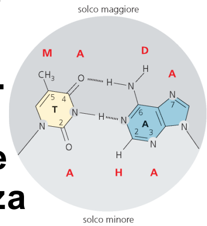
Solco maggiore: A-A-D-H

Solco minore: A-D-A



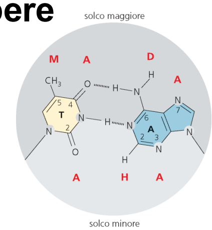
Solco maggiore: A-A-D-H

Solco minore: A-D-A



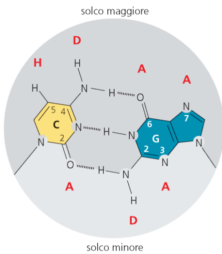
Solco maggiore: A-A-D-H

Solco minore: A-D-A



Solco maggiore: A-A-D-H

Solco minore: A-D-A



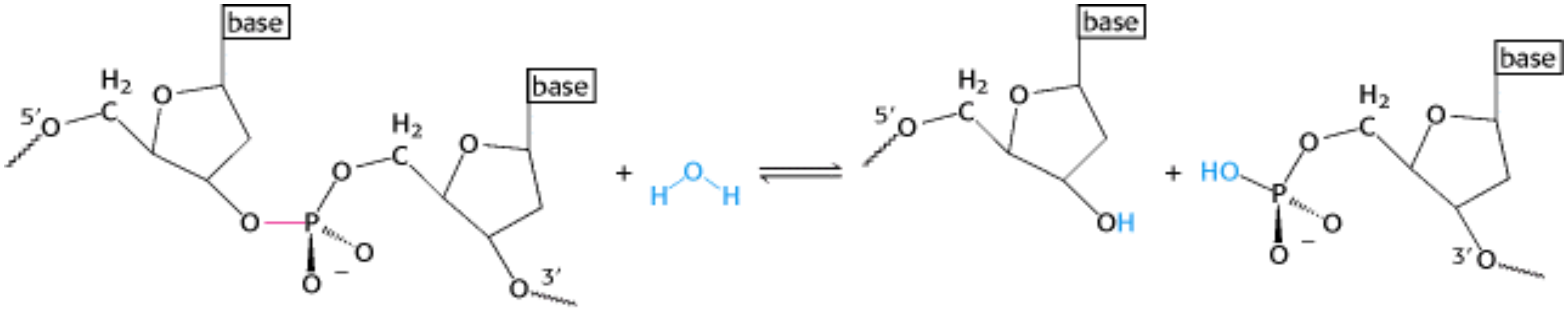
Solco maggiore H-D-A-A

Solco minore A-D-A

5’ 3’
G -- C
A -- T
T -- A
T -- A
C -- G
3’ 5’

EcoRI

Direct hydrolysis by nucleophilic attack at the phosphorous atom



3’OH and 5’ PO_4^{3-} is produced. Mg^{2+} is required for the catalytic activity of the enzyme.

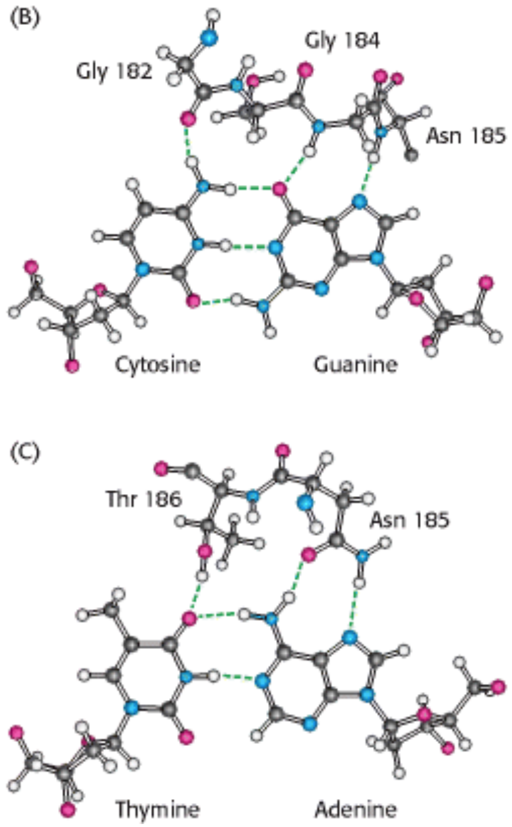
It holds the water molecule in a position where it can attack the phosphoryl group and also helps polarize the water molecule towards deprotonation .

Structure - Function of EcoR V endonuclease

5'...GAT | ATC...3'
3'...CTA | TAG...5'

- Consists of two subunits – dimers related by two fold rotational symmetry.
- Binds to the matching symmetry of the DNA molecule at the restriction site and produces a kink at the site.

Structure - Function of EcoR V endonuclease



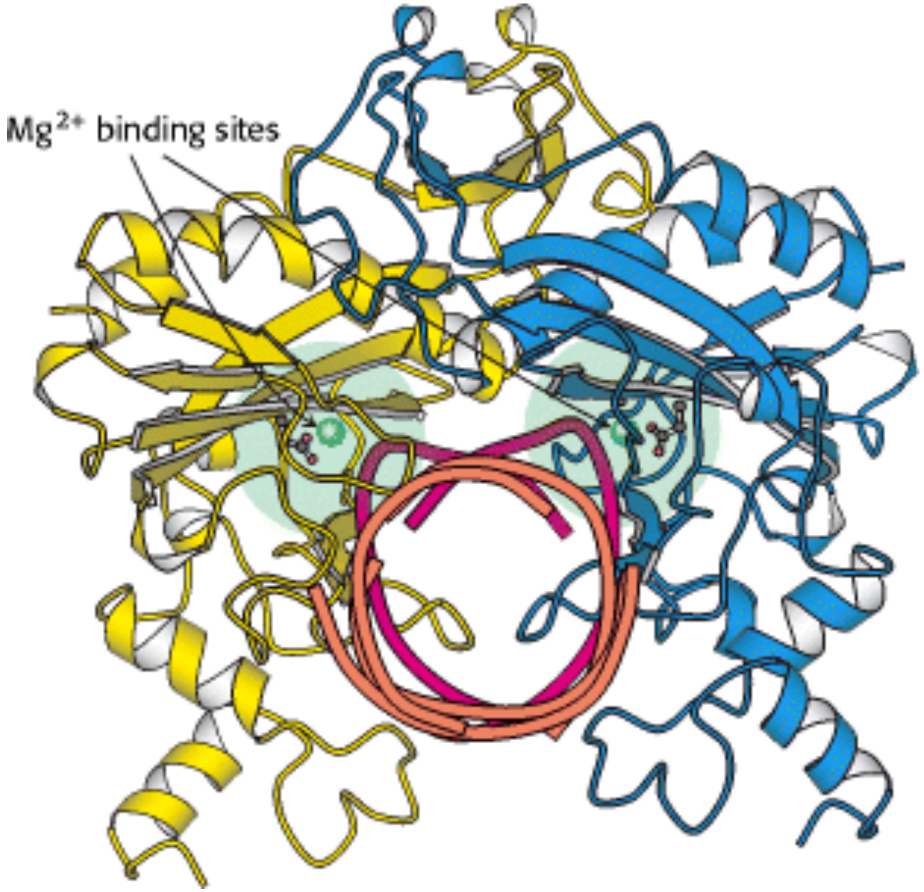
5'...GAT | ATC...3'
3'...CTA | TAG...5'

Hydrogen bonding interactions between EcoRV and its DNA substrate

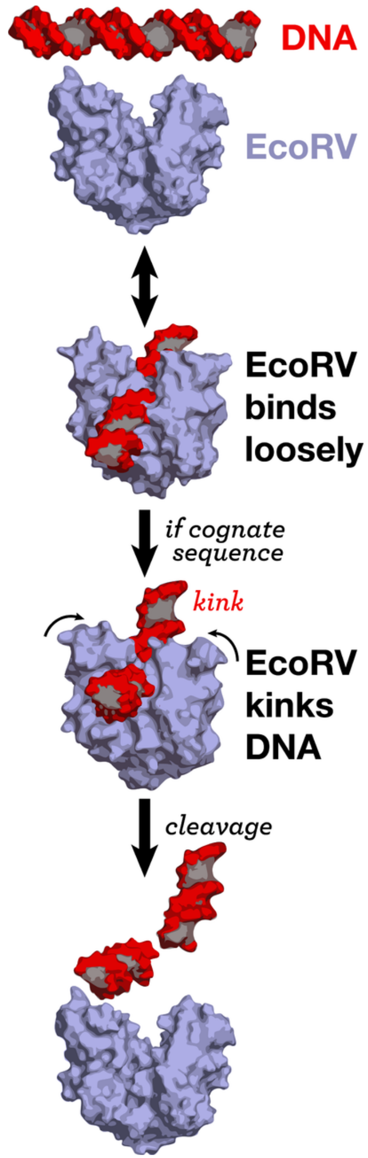
Like EcoRI, EcoRV forms a **homodimer in solution before binding** and acting on its recognition sequence. Initially the enzyme **binds weakly to a non-specific site** on the DNA. It randomly **walks along the molecule until the specific recognition site is found.**

EcoRV has a high specificity for its target DNA **sequence. Binding of the enzyme induces a conformational change in the DNA, bending it by about 50°**. DNA bending results in the **unstacking of the bases**, widening of the minor groove, and compression of the major groove. This brings the **phosphodiester linkage to be broken closer to the active site of the enzym**e, where it can be cleaved. Cleavage occurs within the recognition sequence, and **does not require ATP hydrolysis**. EcoRV is the **only type II restriction endonuclease known to cause a major protein-induced conformational change in the DNA**.

Structure - Function of EcoR V endonuclease



A comparison of cognate and non-specific DNA in the EcorV-DNA complex.



How to set up a restriction digest

Order of solution addition	Solution	Volume(μl)
1	Nuclease free water	23.5
2	10X Buffer K	5.0
4	100 μg BSA	0.5
5	Plasmid DNA	20.0
3	ScaI(20U/ μl)	1.0
Total Volume		50.0

ScaI buffer

100 mM NaCl
50 mM Tris-HCl
10 mM $MgCl_2$
1 mM DTT
(pH 7.9 @ 25°C)

- NaCl (or other salt) provides the correct ionic strength
- Tris-HCl provides the proper pH
- Mg^{2+} is an enzyme co-factor
- DDT is a reducing agent

Each enzyme has defined buffer composition

DTT commonly is used as redox reagent to prevent formation of disulfide bonds in cysteine-containing proteins. Such proteins require proper formation or absence disulfide bonds for exhibiting of specific activity. DTT helps to keep cysteine-containing proteins in active state.However, if protein doesn't contain cysteins, there is no need to use DTT for its activity.

How to set up a restriction digest

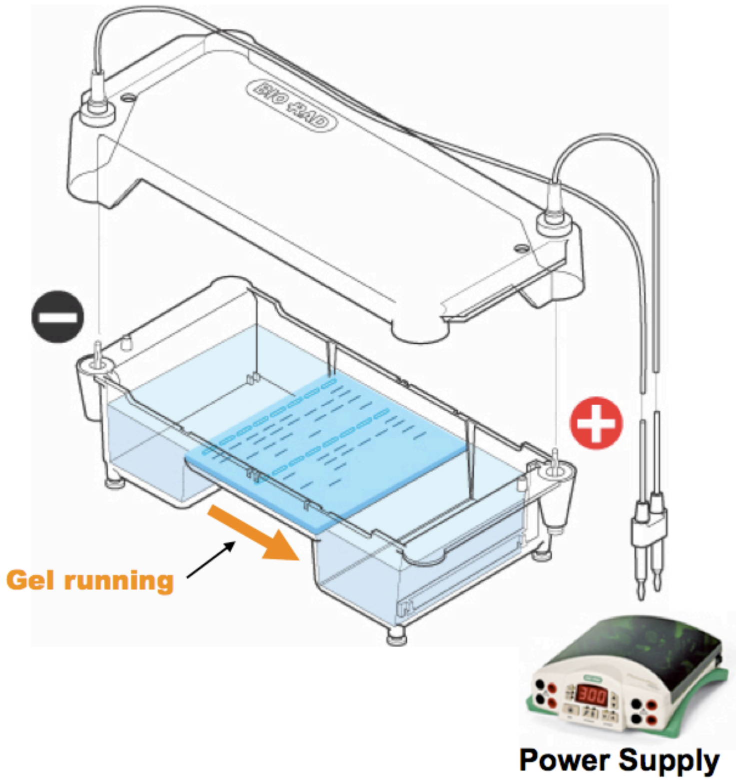
DNA
Digestion
Temperature

Normally at 37°C?
What happens if the temperature is too hot or cool?

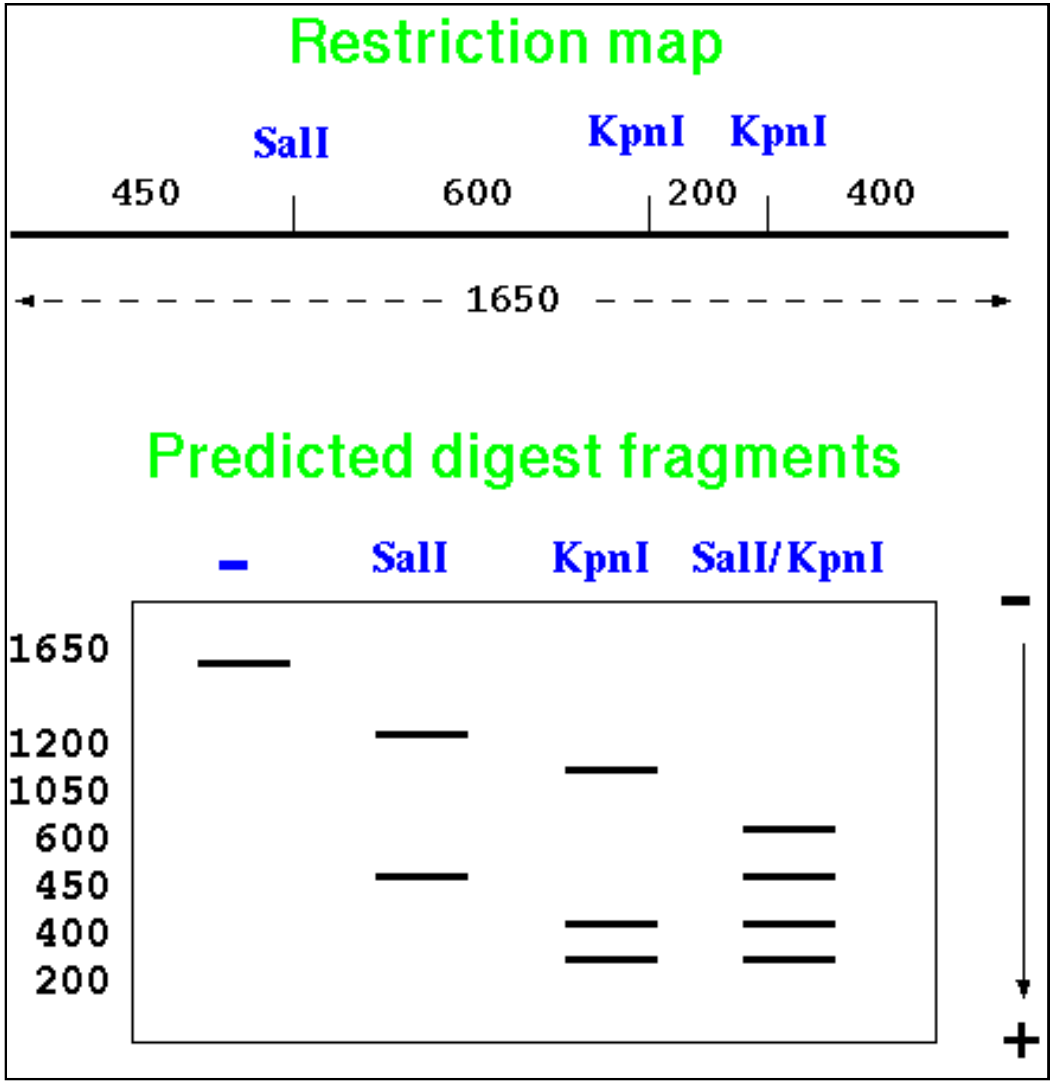
- *Too hot* = enzyme may be denatured (killed)
- *Too cool* = enzyme activity lowered, requiring longer digestion time

Agarose
Electrophoresis
Running

- **Agarose gel sieves DNA fragments according to size**
  - **Small fragments move farther than large fragments**



Restriction Digest Analysis



- Length=1650bp
- *SalI* yields two fragments (1200bp and 450bp)
- *KpnI* cuts at 2 sites giving 3 fragments
- *SalI* and *KpnI* cut 3X yielding 4 fragments