Genetic Engineering

L3 Biochemistry, Microbiology

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Genetic Engineering

Introduction:

- **Genetic** engineering is a set of techniques, part of molecular biology , aimed by using knowledge acquired in genetics to use, reproduce, or modify the genome of living beings.
- Its aim is to modify genotypes , and therefore phenotypes .
- The potential applications of genetic engineering are numerous:
  - In human health (correction of a gene carrying a deleterious mutation , production of therapeutic proteins , vaccines, etc.)
  - In biotechnological agriculture (development of new generations of genetically modified plants , etc.)
  - The development of tools for research (for example to explore the function of a gene).

Chapter I:

Enzymatic tools for genetic engineering

Enzymatic tools for genetic engineering

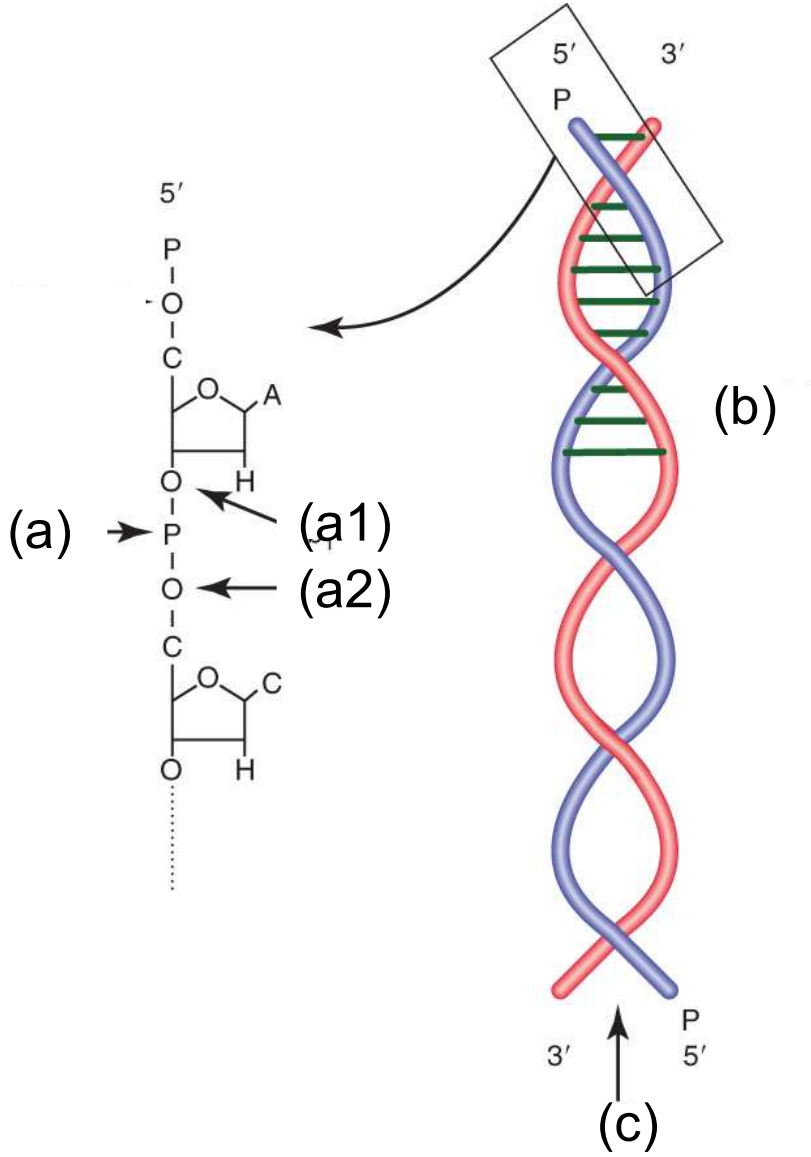
- These enzymes are proteins that serve as tools to manipulate nucleic acids.
- They are very present during the replication of genetic material, transcription, translation and even DNA repair.
- They represent a significant portion of the basic tools for molecular biologists and genetic engineers.

Enzymatic tools for genetic engineering

- Enzymes that cut nucleic acids
- Enzymes that ligate pieces of DNA together
- Enzymes that work on phosphate group
- Enzymes that copy nucleic acids
- Enzymes that modify DNA

Enzymes that cut nucleic acids : **Nucleases**

- Nucleases hydrolyze an ester bond within a phosphodiester bond.



a nuclease (a), an endonuclease (b), and an exonuclease (c)

Enzymes that cut nucleic acids : **Nucleases**

- **Endonucleases** : nucleases that cleave phosphodiester bonds in a nucleic acid chain.
    - They can be specific for single-stranded or double-stranded RNA or DNA.
- **Exonucleases** : nucleases that cleave phosphodiester bonds one at a time from the end of a polynucleotide chain.
    - They can be specific for the 5' or 3' end of DNA or RNA.

Enzymes that cut nucleic acids : **Nucleases**

- **Endonucleases**
  - Restriction Enzymes: three types
  - Deoxyrebinucleases (DNase)
    - DNase I
    - Nuclease S1 and Mung Bean Nuclease
  - Ribonucleases (RNase)
    - RNase A and RNase H…
- **Exonucleases**
  - Exonuclease III
  - λ phage exonuclease
  - Exonuclease Bal 31

Enzymes that cut nucleic acids : **Nucleases**

- Restriction enzymes:
  - Restriction enzymes are produced by bacteria and constitute a defense mechanism against infections by bacteriophages, viruses specific to bacteria.

Enzymes that cut nucleic acids : **Nucleases**

- Restriction enzymes:
  - Molecular scissors that cut double-stranded DNA molecules at specific points (4 to 8 bp) often unmethylated.
  - Found naturally in a wide variety of prokaryotes
  - An important tool for DNA manipulation.

Enzymes that cut nucleic acids : **Endonucleases**

- Types of restriction enzymes:

  - **Type I enzymes:**

    They recognize the restriction site on DNA and cut the molecule far beyond this site, approximately 1000 to 5000 nucleotides beyond.
    The cut is fairly random, making this enzyme useless in molecular biology.

  - **Type II enzymes:**

    They recognize the restriction site and cut at that location. Widely used in genetic engineering.

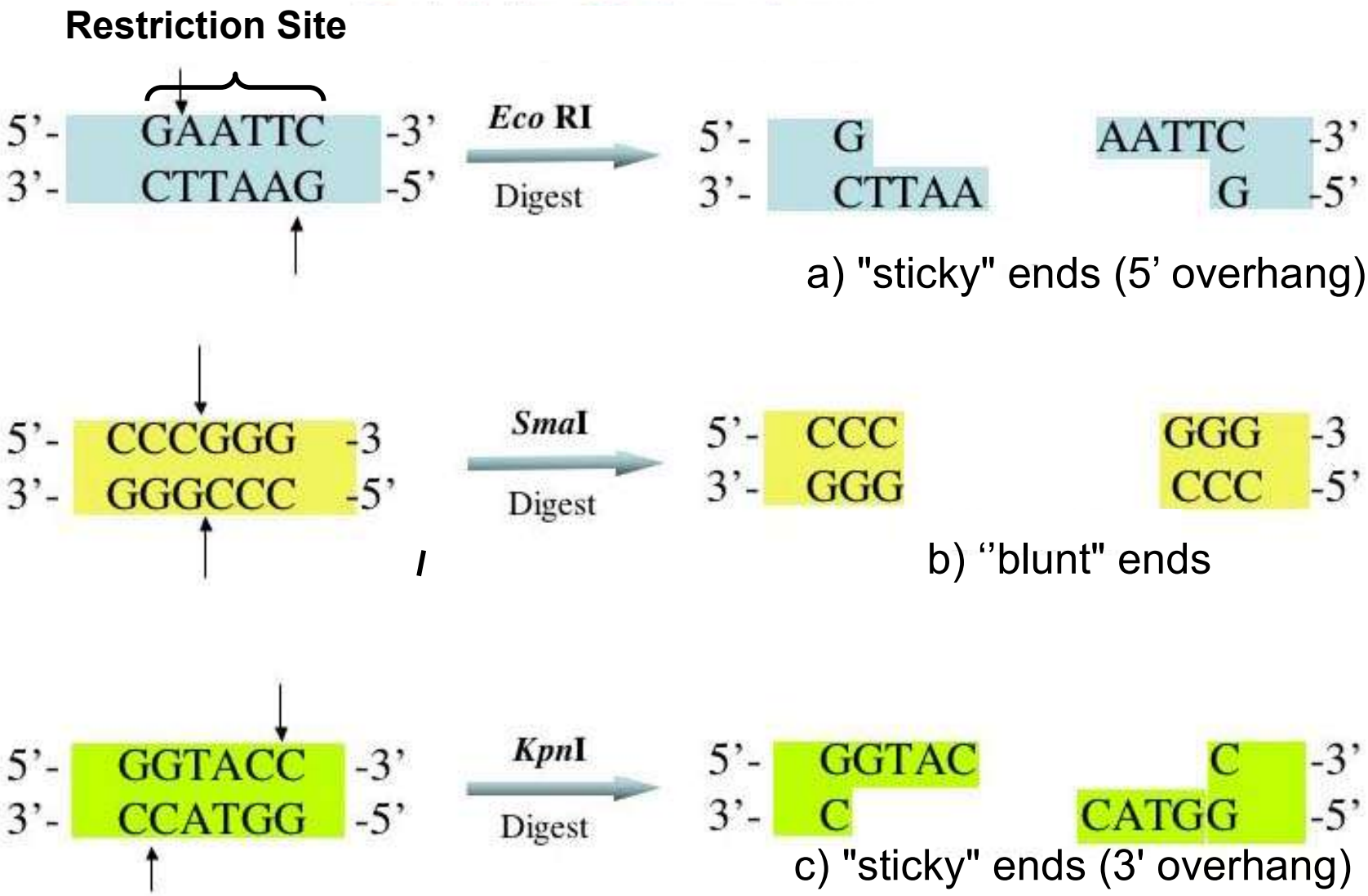
  - **Type III enzymes:**

    They recognize the restriction site and cut about twenty nucleotides further along.

Enzymes that cut nucleic acids : **Endonucleases**

- Restriction enzymes: Type II

Restriction enzyme breaks down the sugar-phosphate skeleton.



Enzymes that cut nucleic acids : **Endonucleases**

- Nomenclature of restriction enzymes:
  - Derived from the species or variety name of the bacterium that produces it. The initial of the genus name is written, followed by the two initials of the species name, one letter or number to designate the variety (or strain), and after a space, a Roman numeral to successively designate the different restriction enzymes obtained from the same strain.

Enzyme	Source	Site restriction	Cut	Extremities (after cutting)
Eco RI	*Escherichia coli*	5' GAATTC 3' CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'	"sticky" ends
BamHI	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'	"sticky" ends
HindIII	*Haemophilus influenzae*	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'	"sticky" ends
TaqI	*Thermus aquaticus*	5'TCGA 3'AGCT	5'---T CGA---3' 3'---AGC T---5'	"sticky" ends
NotI	*Nocardia otitidis*	5'GCGGCCGC 3'CGCCGGCG	5'--GC GGCCGC--3' 3'—CGCCGG CG--5'	"sticky" ends
AluI	*Arthrobacter luteus*	5'AGCT 3'TCGA	5'---AG CT---3' 3'---TC GA---5'	‘’blunt" ends
BglIII	*Bacillus globigii*	5'AGATCT 3'TCTAGA	5'---A GATCT---3' 3'---TCTAG A---5'	"sticky" ends
HaeIII	*Haemophilus aegyptius*	5'GGCC 3'CCGG	5'---GG CC---3' 3'---CC GG---5'	‘’blunt" ends
HhaI	*Haemophilus haemolyticus*	5'GCGC 3'CGCG	5'---GCG C---3' 3'---C GCG---5'	"sticky" ends
PstI	*Providencia stuartii*	5'CTGCAG 3'GACGTC	5'---CTGCA G---3' 3'---G ACGTC---5'	"sticky" ends
SmaI	*Serratia marcescens*	5'CCCGGG 3'GGGCCC	5'---CCC GGG---3' 3'---GGG CCC---5'	‘’blunt" ends

Enzymes that cut nucleic acids : **Endonucleases**

- **Use in RFLP**
  - Restriction fragment length analysis (RFLP) is one of the techniques for analyzing the primary DNA sequence in search of substitutions, insertions or deletions that modify the number of restriction sites and therefore the length of restriction fragments.

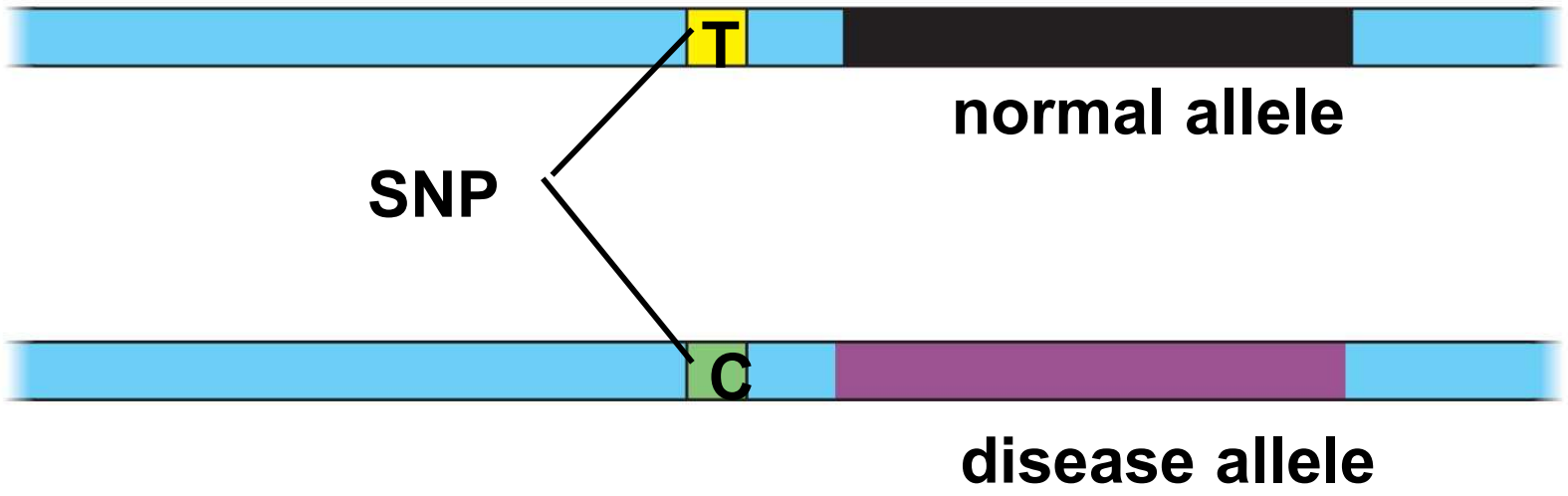
Enzymes that cut nucleic acids : **Endonucleases**

- **Use in RFLP** :
  - **polymorphisms (SNPs**) are useful genetic markers. They are sites consisting of a single base pair that vary within a population.
  - When a restriction enzyme is added, the SNPs give rise to DNA fragments of different lengths.
  - **A restriction map** can be generated using the overlaps between fragments generated by different restriction enzymes.

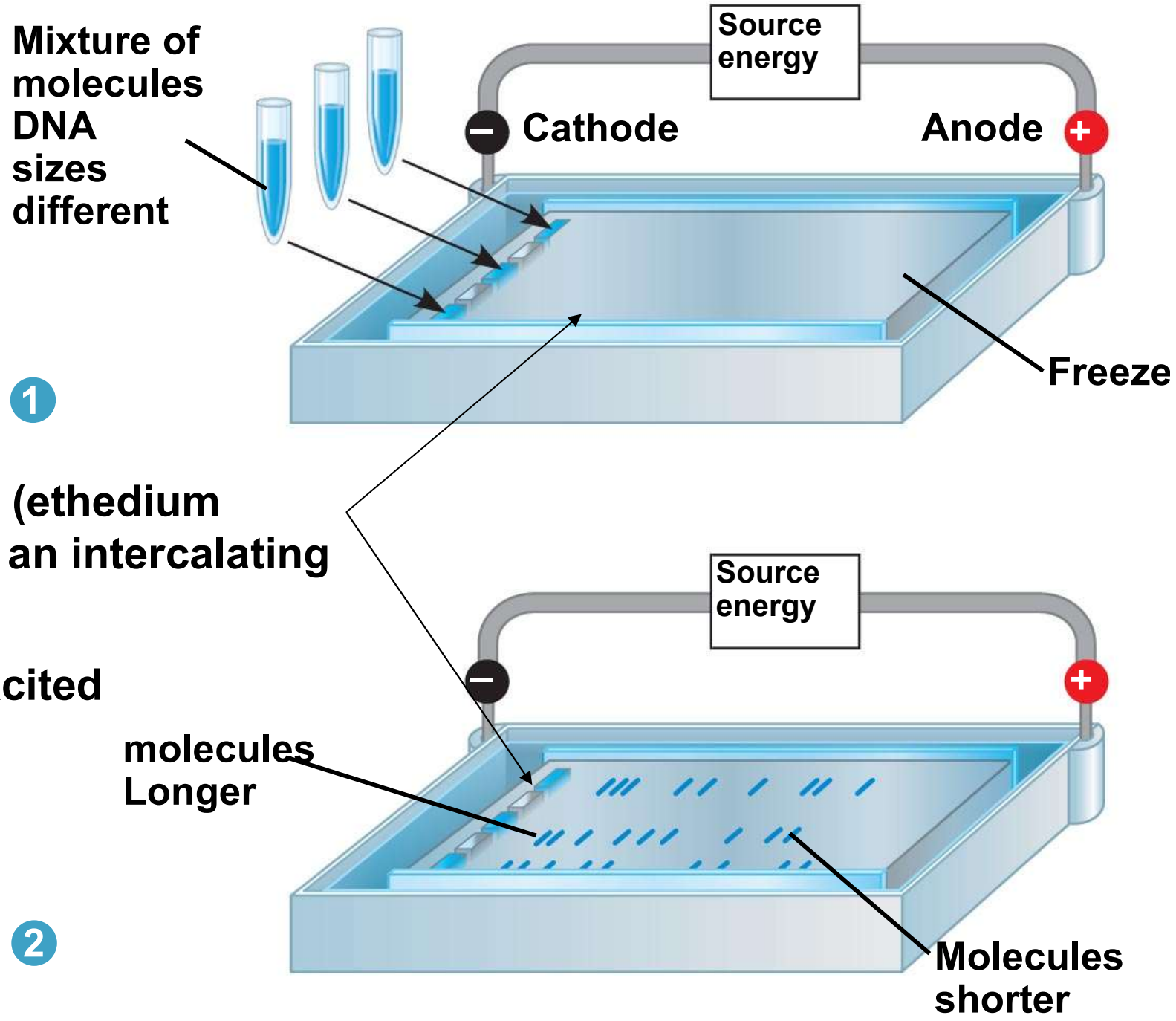
Enzymes that cut nucleic acids : **Endonucleases**

- **Use in RFLP** :
  - In restriction fragment analysis, DNA fragments produced by the digestion of a DNA molecule by a restriction enzyme are sorted by gel electrophoresis.
  - **Electrophoresis** uses a gel as a molecular sieve to separate negatively charged nucleic acids according to their sizes
  - An electric current is applied, causing the charged molecules to move through the gel.
  - The molecules are sorted into "bands" by their size.

DNA



TECHNICAL

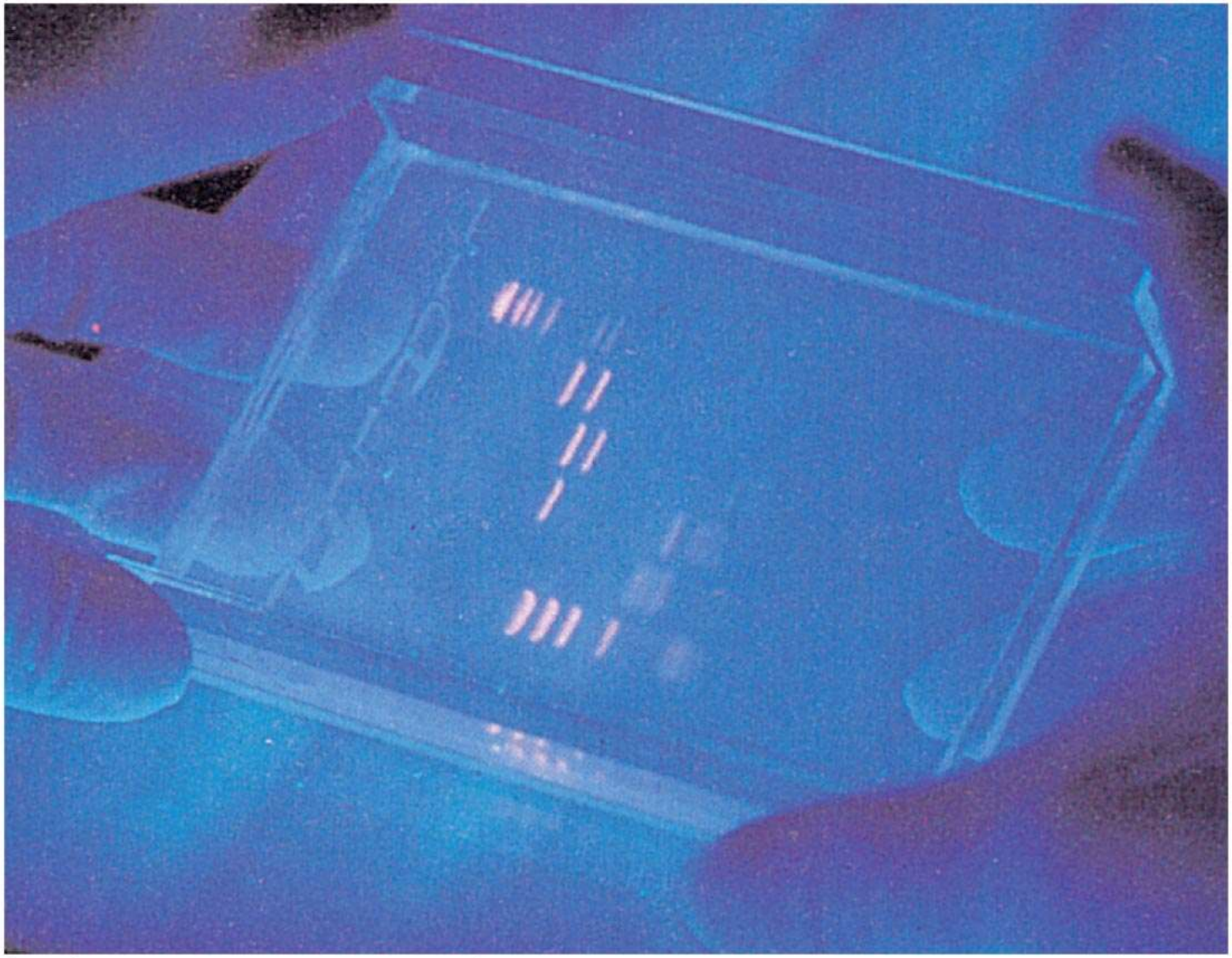


Agarose gel + BET (ethedium bromide), which is an intercalating agent

The BET can be excited

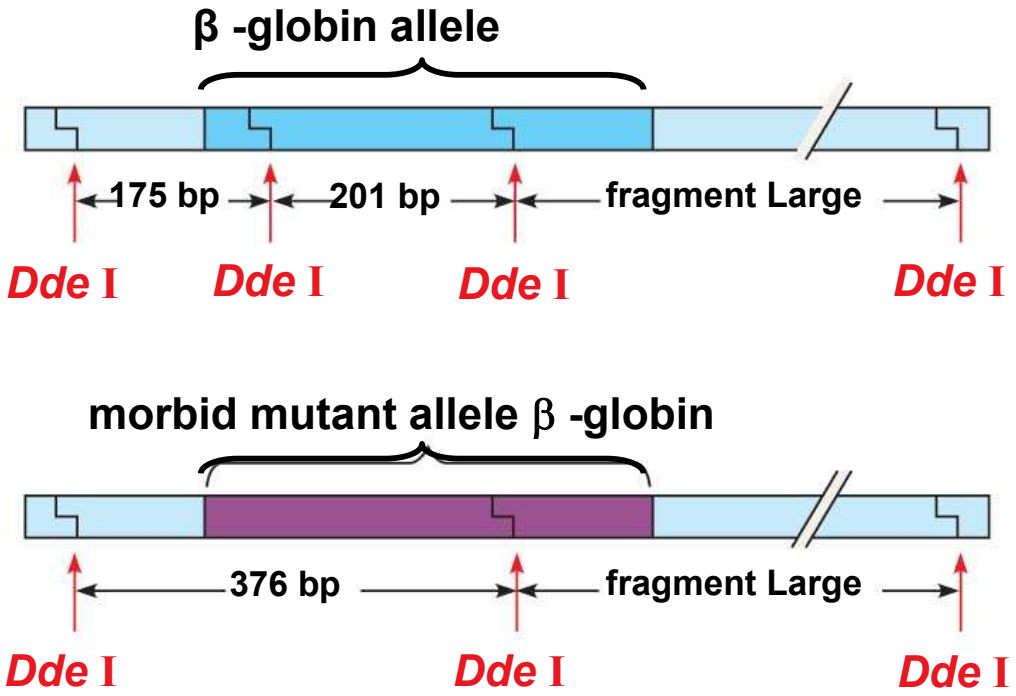
by UV

RESULTS

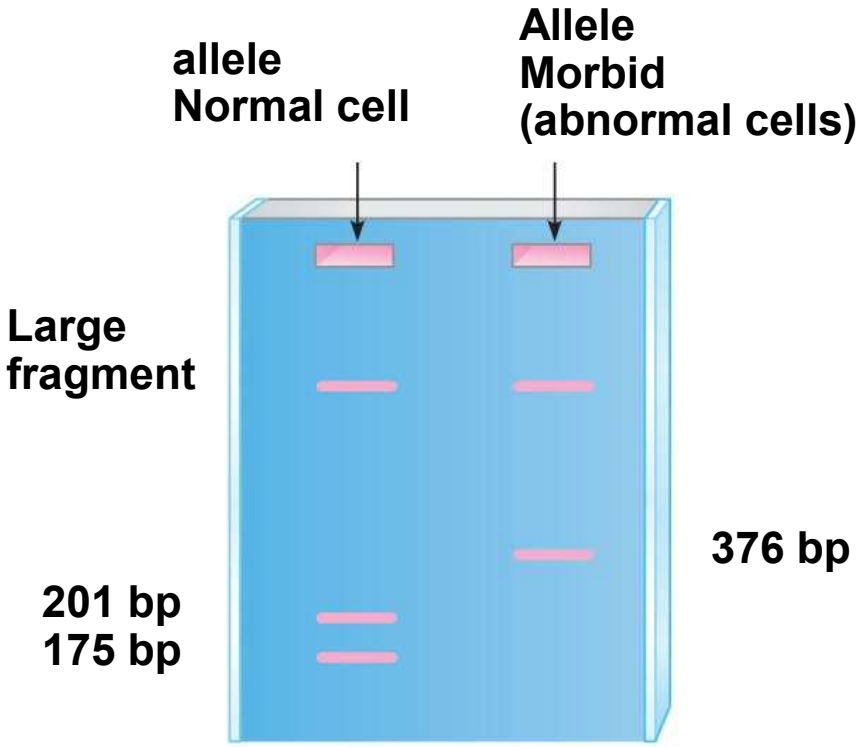


Development of the bands by excitation with UV (ultraviolet) rays

RFLP and Electrophoresis Product Detection: Example



(a) ***DdeI*** **enzyme restriction sites in the normal and morbid alleles of the β- globin gene**



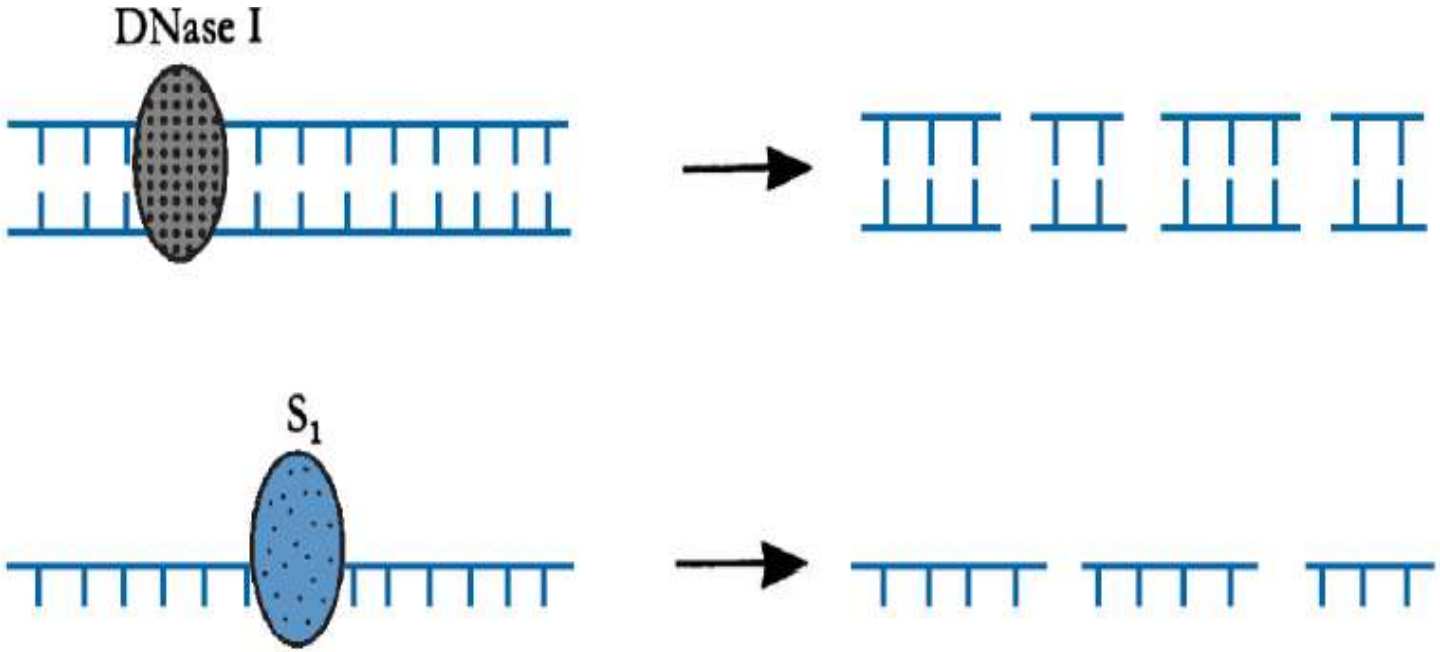
(b) Electrophoresis of restriction fragments of normal and diseased alleles

Enzymes that cut nucleic acids : **Endonucleases**

-Deoxyribonucleases (DNase)

-**DNase I** cuts either single-stranded or double-stranded DNA at essentially random sites.

-**S1 & Mung Bean nuclease** is specific to RNA or single-stranded DNA.



Enzymes that cut nucleic acids : **Exonucleases**

-Mung Bean nuclease

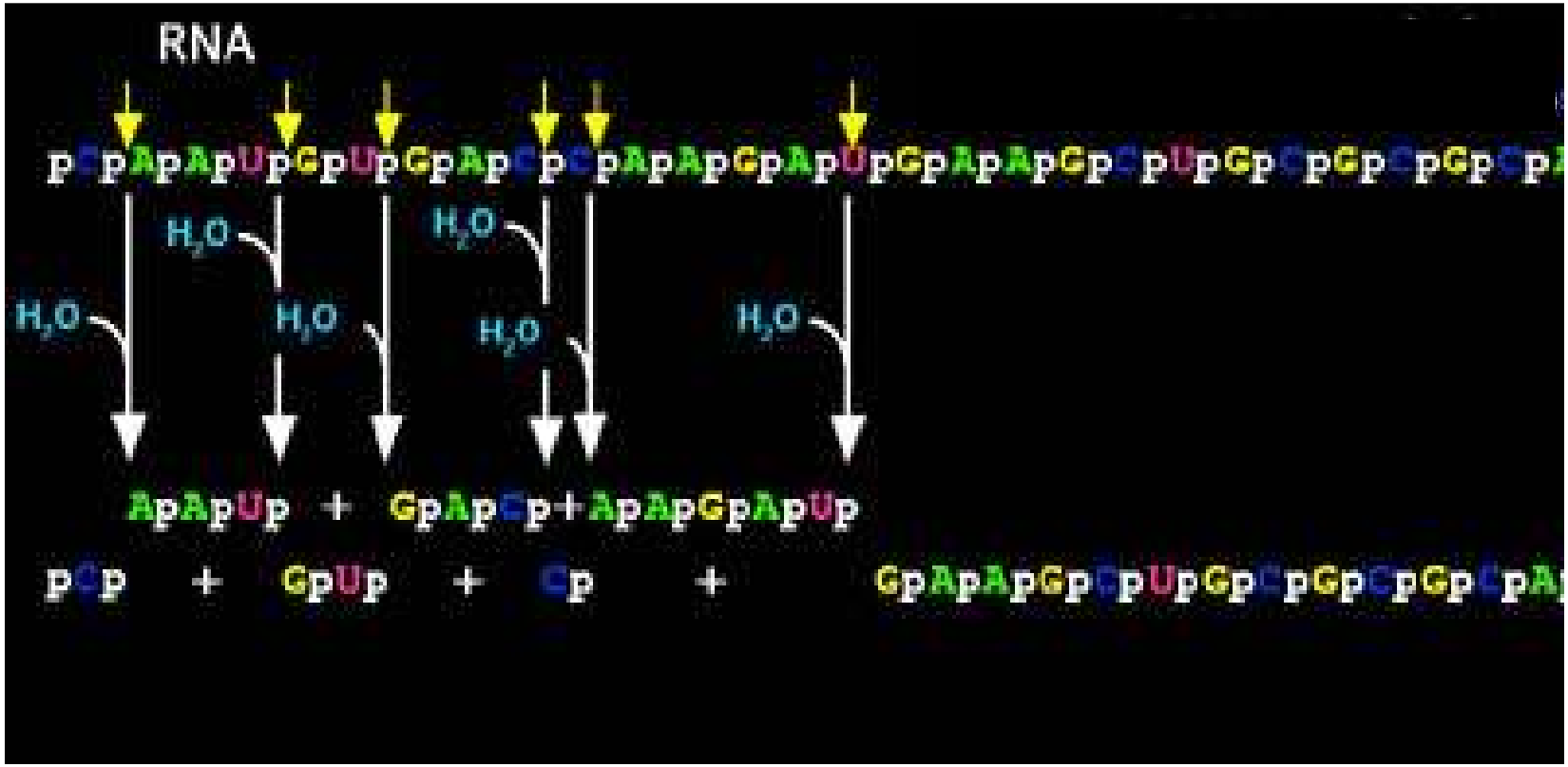
-It digests the single strand. However, if there is a "nick (gap)" on a double-stranded fragment, it will have difficulty digesting the phosphodiester bond located opposite the "nick" on the other strand.

-We will use it, for example, to make cohesive ends distinct.

Enzymes that cut nucleic acids : **Endonucleases**

-Ribonucleases (RNase)

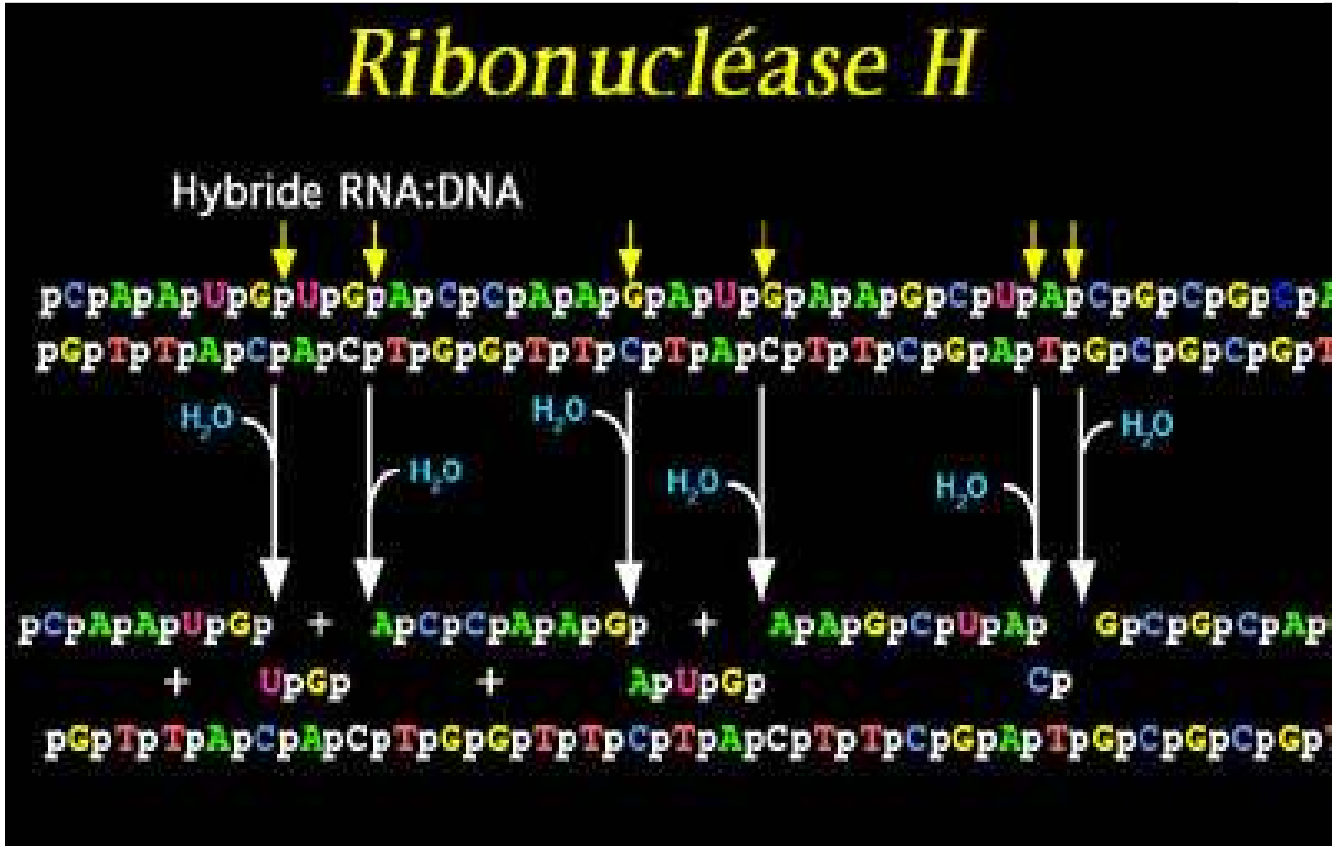
-**Ribonuclease A** is the enzyme of RNA digestion. It acts as an endonuclease, preferentially after pyrimidine nucleotides, by hydrolyzing the bond between the phosphate and the 5' carbon of the following nucleotide.



Enzymes that cut nucleic acids : **Endonucleases**

-Ribonucleases (RNase)

-**Ribonuclease H** is an enzyme that hydrolyzes the RNA strand into a double-stranded hybrid DNA:RNA. RNase H does not hydrolyze single-stranded or double-stranded RNA.



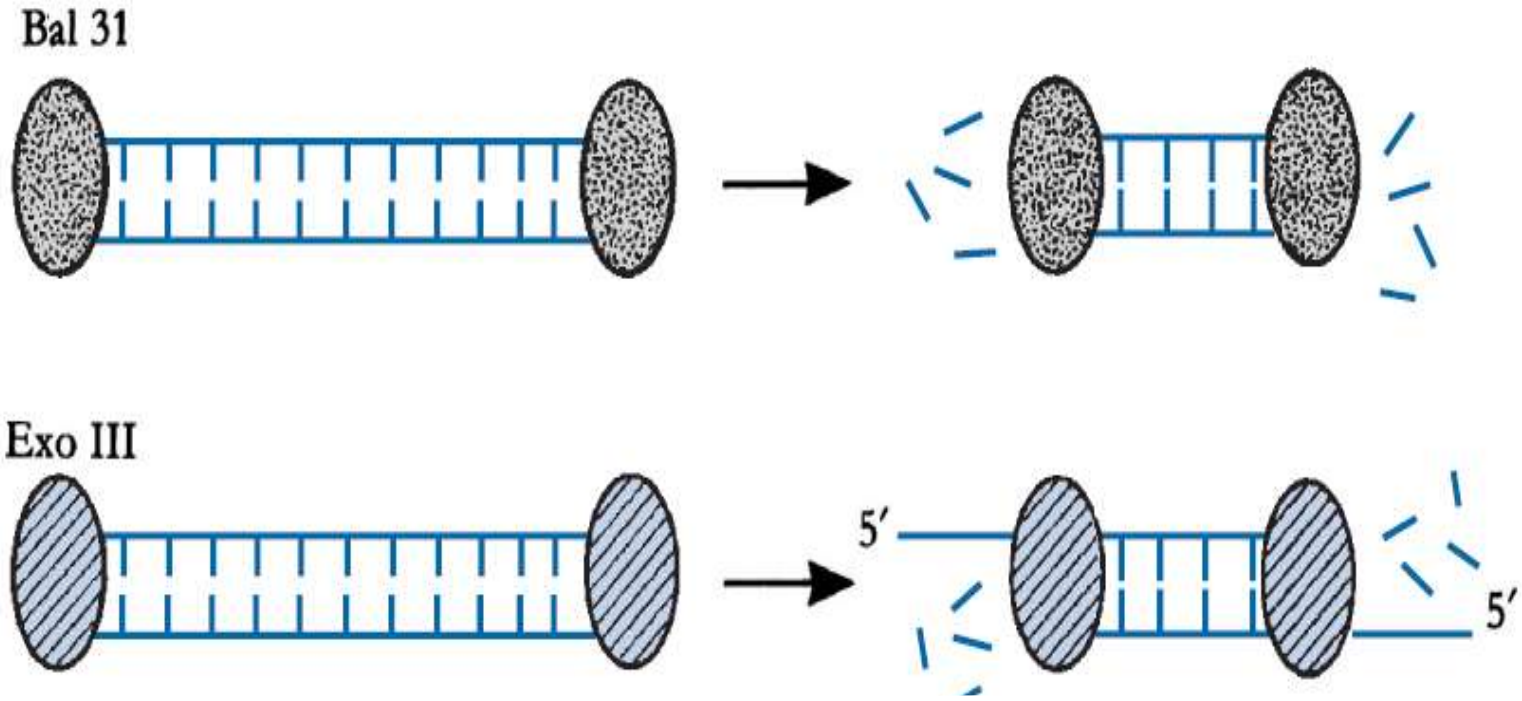
Enzymes that cut nucleic acids : **Exonucleases**

-A second group of nucleases that degrade DNA from the end of the molecule are known as **exonucleases** .

-Exp:

- Bal 31 exonuclease
- -Exonuclease III
- λ phage exonuclease

Enzymes that cut nucleic acids : **Exonucleases**



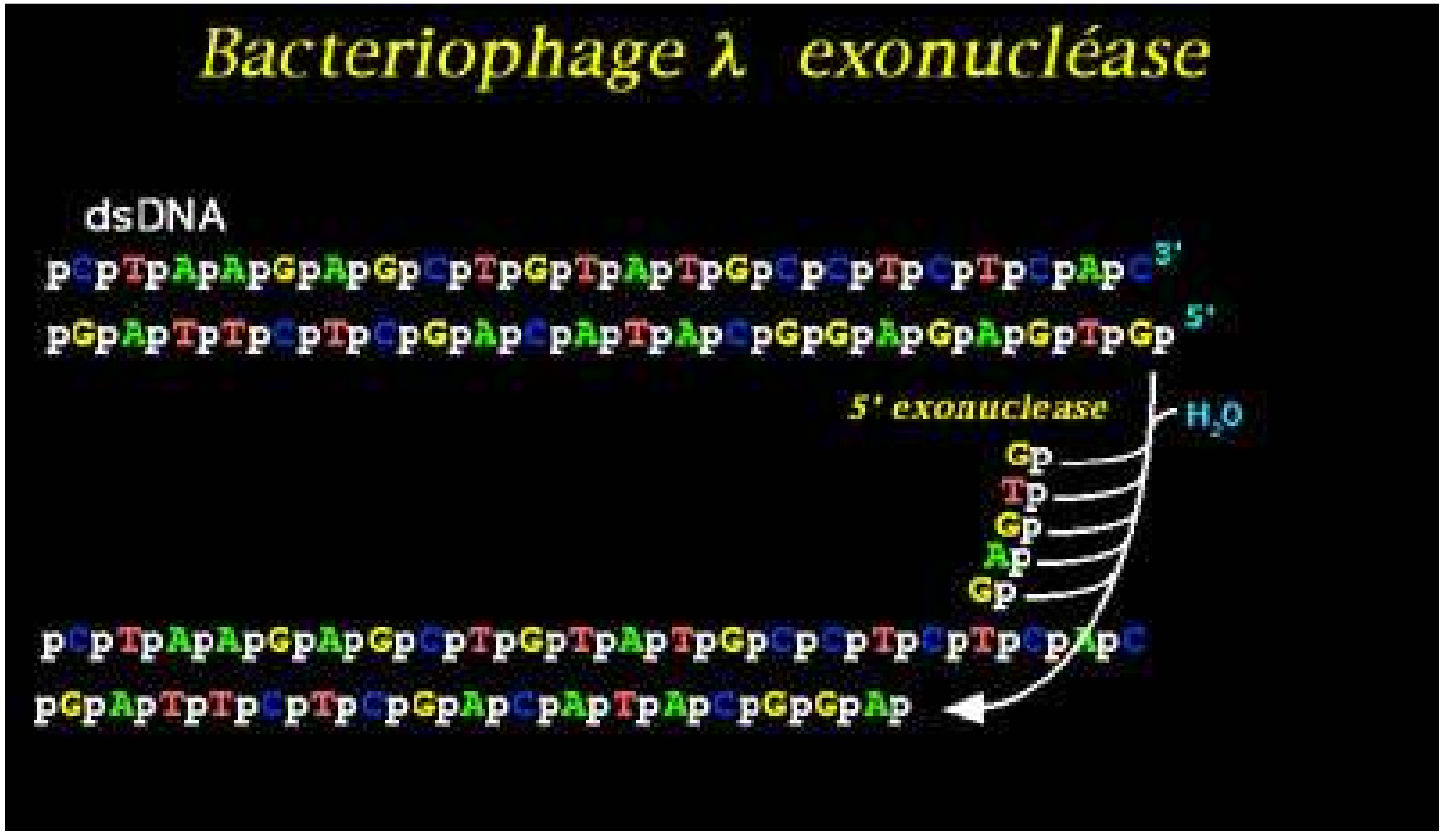
-Bal 31 exonuclease: exonuclease activity capable of simultaneously degrading the 3' and 5' terminals of double-stranded DNA

-Exonuclease III is a 3' exonuclease that generates molecules with 5' exiting ends.

Enzymes that cut nucleic acids : **Exonucleases**

-λ phage exonuclease

- Preferentially hydrolyzes the 5'-phosphate ends of double-stranded DNA

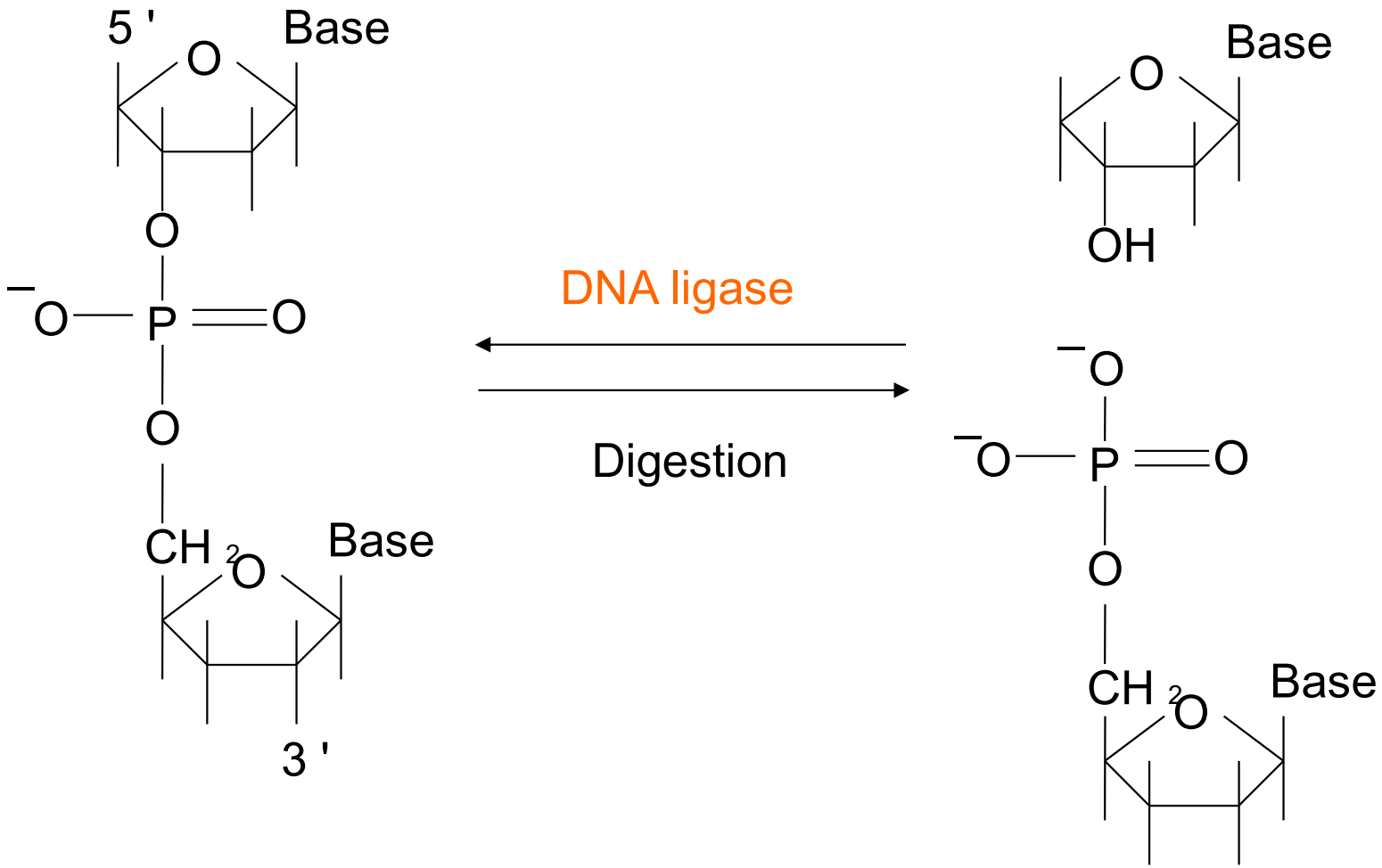


Enzymatic tools for genetic engineering

- Enzymes that cut nucleic acids
- Enzymes that stick pieces of DNA together
- Enzymes that work on phosphates
- Enzymes that copy nucleic acids
- Enzymes that modify DNA

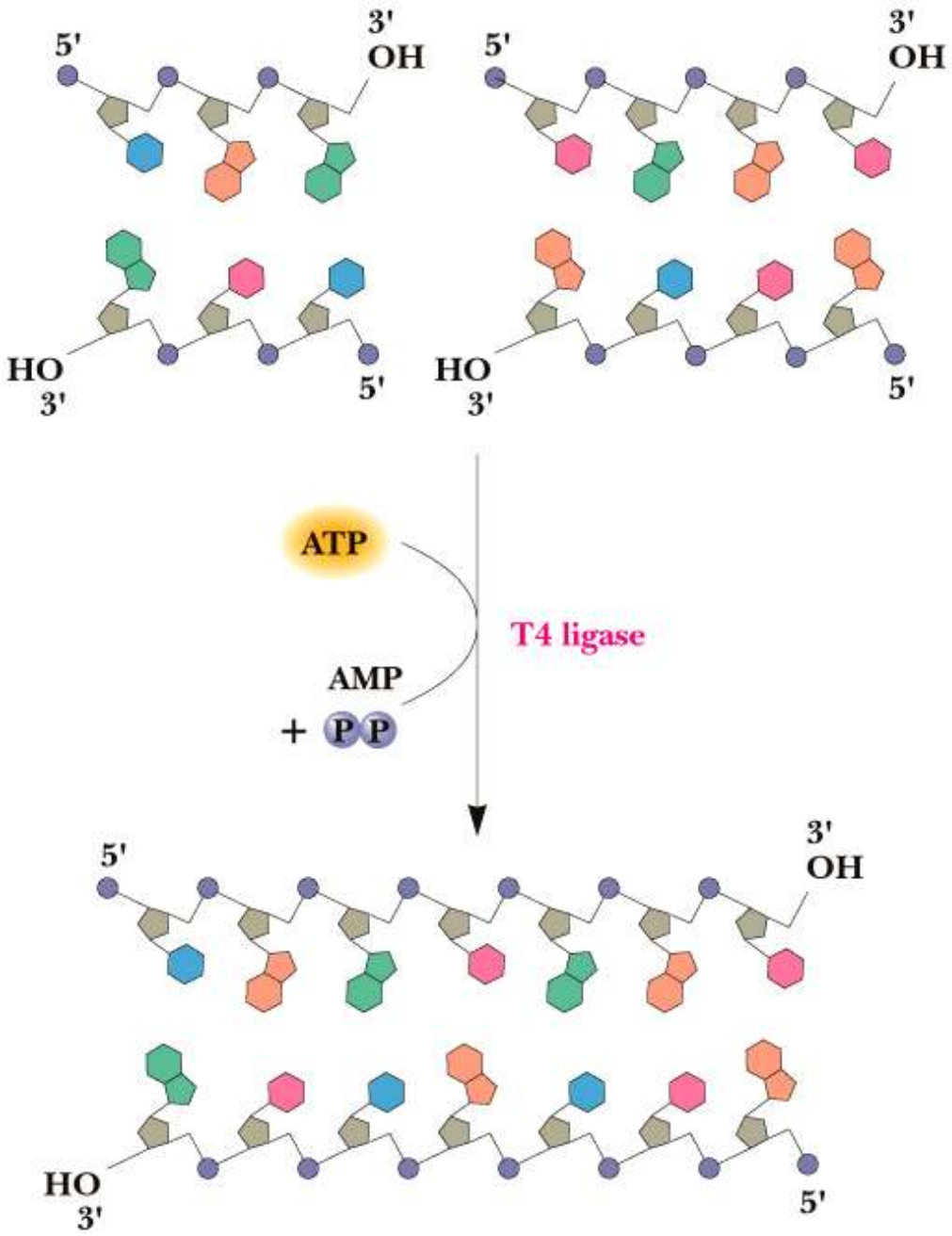
Enzymes that ligate nucleic acids

Action of DNA ligase



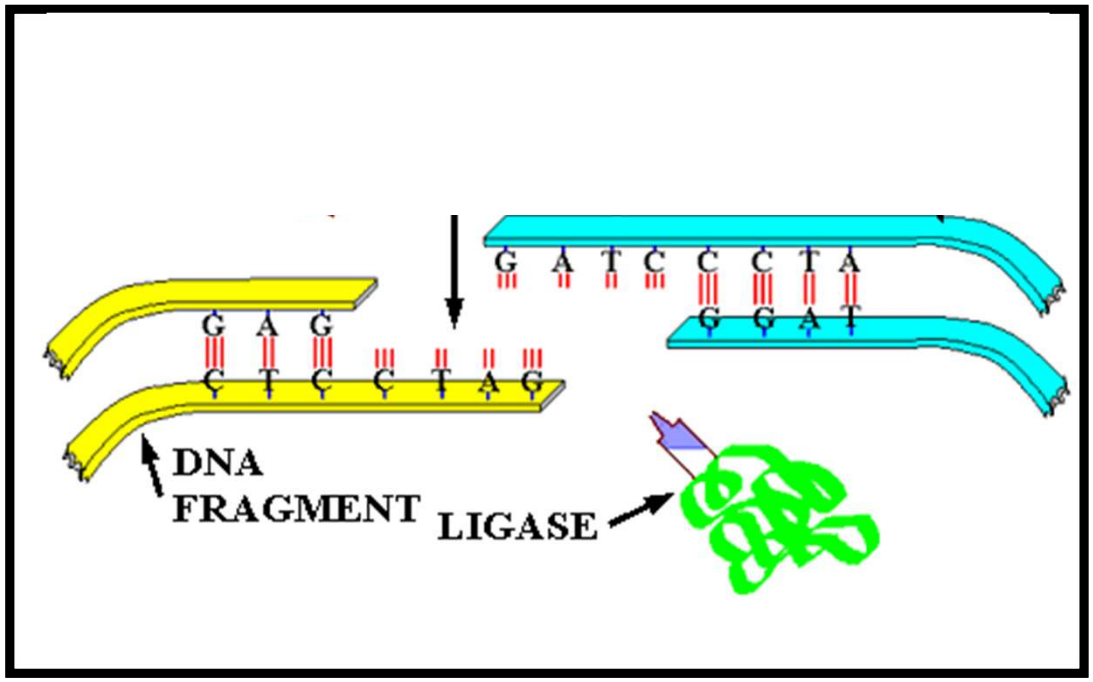
DNA ligases are enzymes that are capable of reforming the phosphoester bond between the 3'-OH carbon and the 5' phosphate of two neighboring nucleotides on a DNA strand

T4 DNA Ligase



Enzymes that ligate nucleic acids

- T4 DNA Ligase
  - Fast and efficient ligation of DNA and RNA.



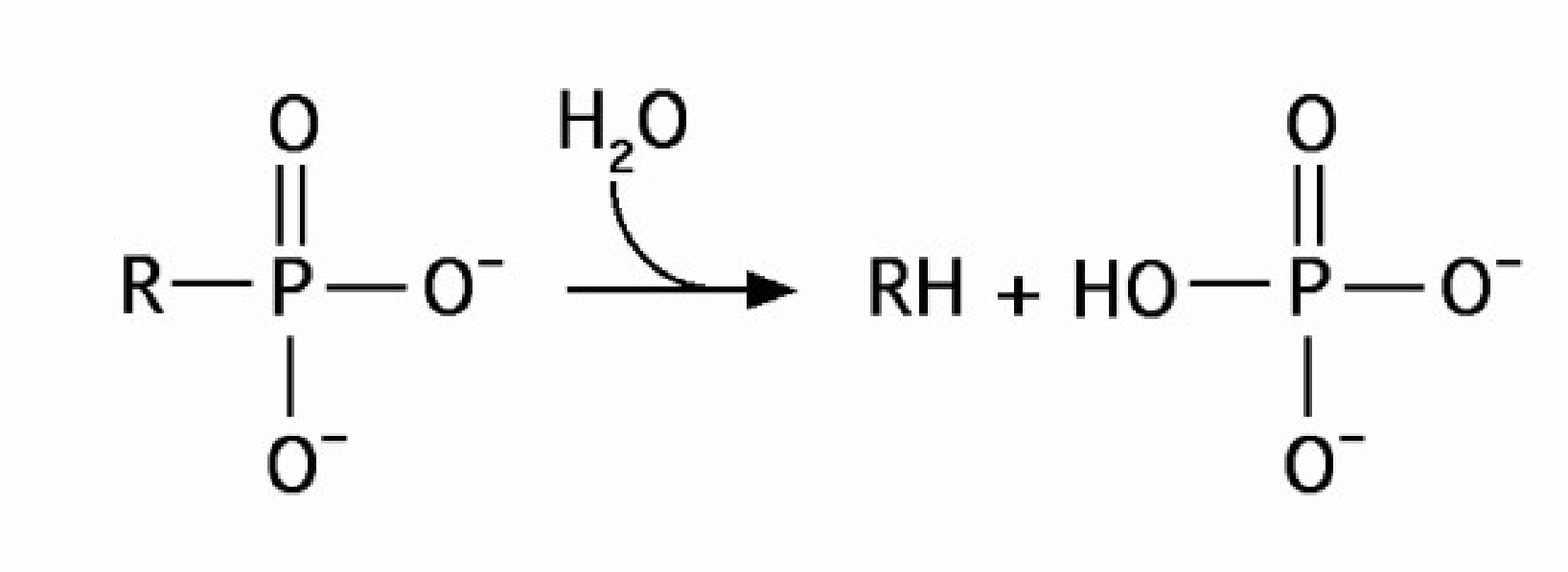
  - It also joins DNA fragments with either cohesive or blunt ends, but has no activity on single-stranded nucleic acids. T4 DNA ligase from phage T4 requires ATP as a cofactor.

Enzymatic tools for genetic engineering

- Enzymes that cut nucleic acids
- Enzymes that stick pieces of DNA together
- Enzymes that work on phosphates
- Enzymes that copy nucleic acids
- Enzymes that modify DNA

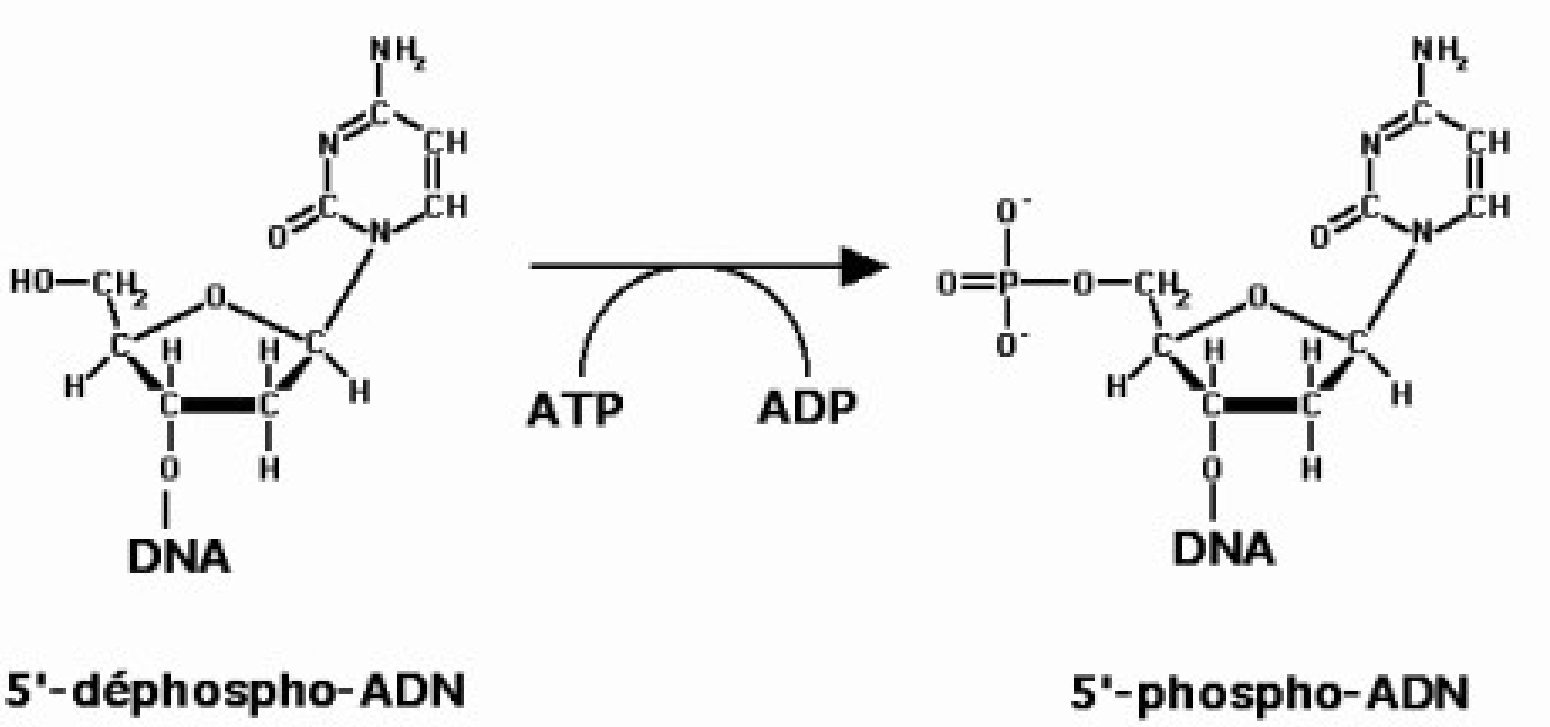
Enzymes that work on phosphates

- **Alkaline phosphatase** removes the 5' phosphate from DNA, RNA, and free nucleotides. The removal of 5' phosphates prevents any action of ligases. An open vector treated in this way will not be able to close itself, and this closure will become possible again through the integration of foreign DNA.



Enzymes that work on phosphates

- T4 polynucleotide kinase: it transfers the phosphate from an ATP molecule to the 5' phosphate group of a polynucleotide. This 5' labeling is performed to determine the DNA sequence.



Enzymatic tools for genetic engineering

- Enzymes that cut nucleic acids
- Enzymes that stick pieces of DNA together
- Enzymes that work on phosphates
- Enzymes that copy nucleic acids
- Enzymes that modify DNA

Enzymes that copy nucleic acids

-**Polymerases** synthesize copies of nucleic acid molecules and are used in many genetic engineering procedures

-When describing a polymerase, the terms "DNA-dependent" or "RNA-dependent" can be used to indicate the type of nucleic acid template (or template) that the enzyme uses.

-So,

-a 'DNA-dependent' DNA polymerase, copies DNA into DNA,

-an RNA-dependent DNA polymerase, which copies RNA into DNA,

-A 'DNA-dependent' RNA polymerase transcribes DNA into RNA.

Enzymes that copy nucleic acids

-These enzymes are template-dependent and can be used to copy long fragments of DNA or RNA.

-These enzymes synthesize nucleic acids by joining together nucleotides whose bases are complementary to the bases of the template strand.

-The synthesis product in the 5'→3' direction, and that each addition of nucleotide requires a free 3'-OH group for the formation of the phosphodiester bond.

-These enzymes have polymerase activity (5'→3'), and exonuclease activity (whether 5'→3' and/or 3'→5').

-The 3' → 5' exonuclease activity is often referred to as proofreading activity.

Enzymes that copy nucleic acids

-DNA-dependent DNA polymerases

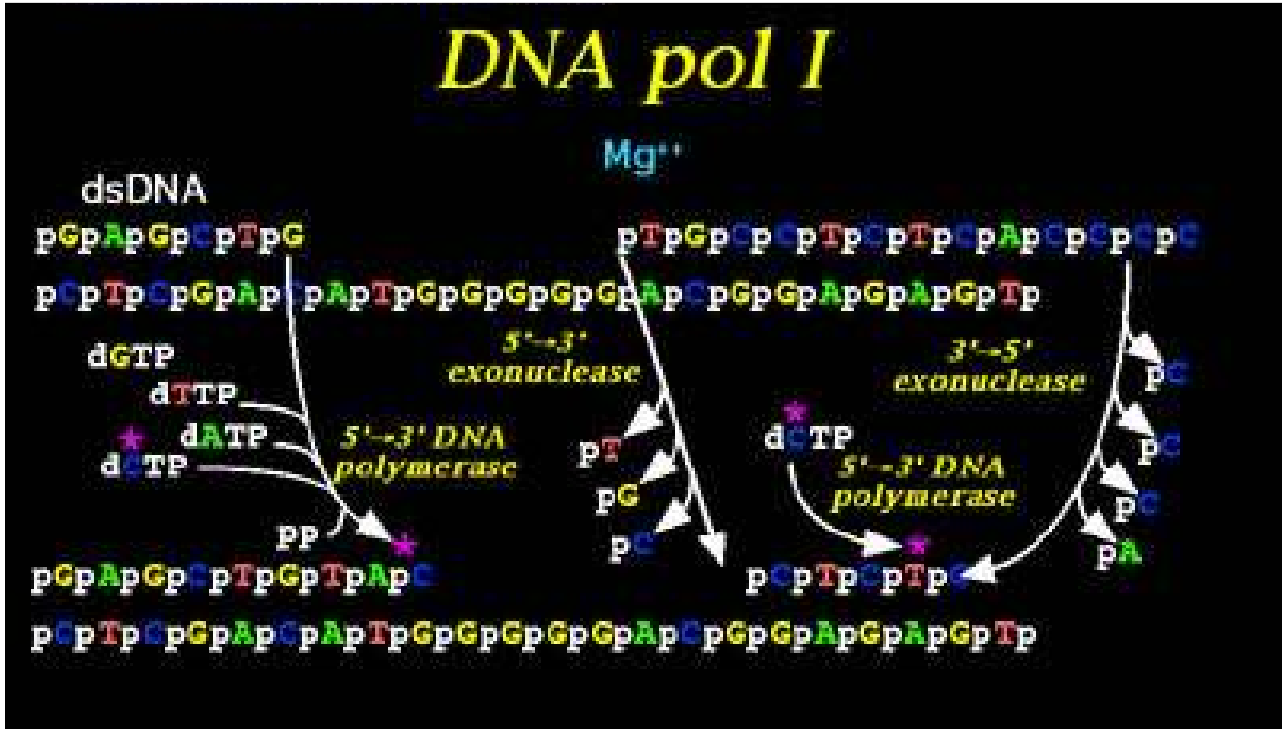
DNA polymerases

-DNA polymerase I

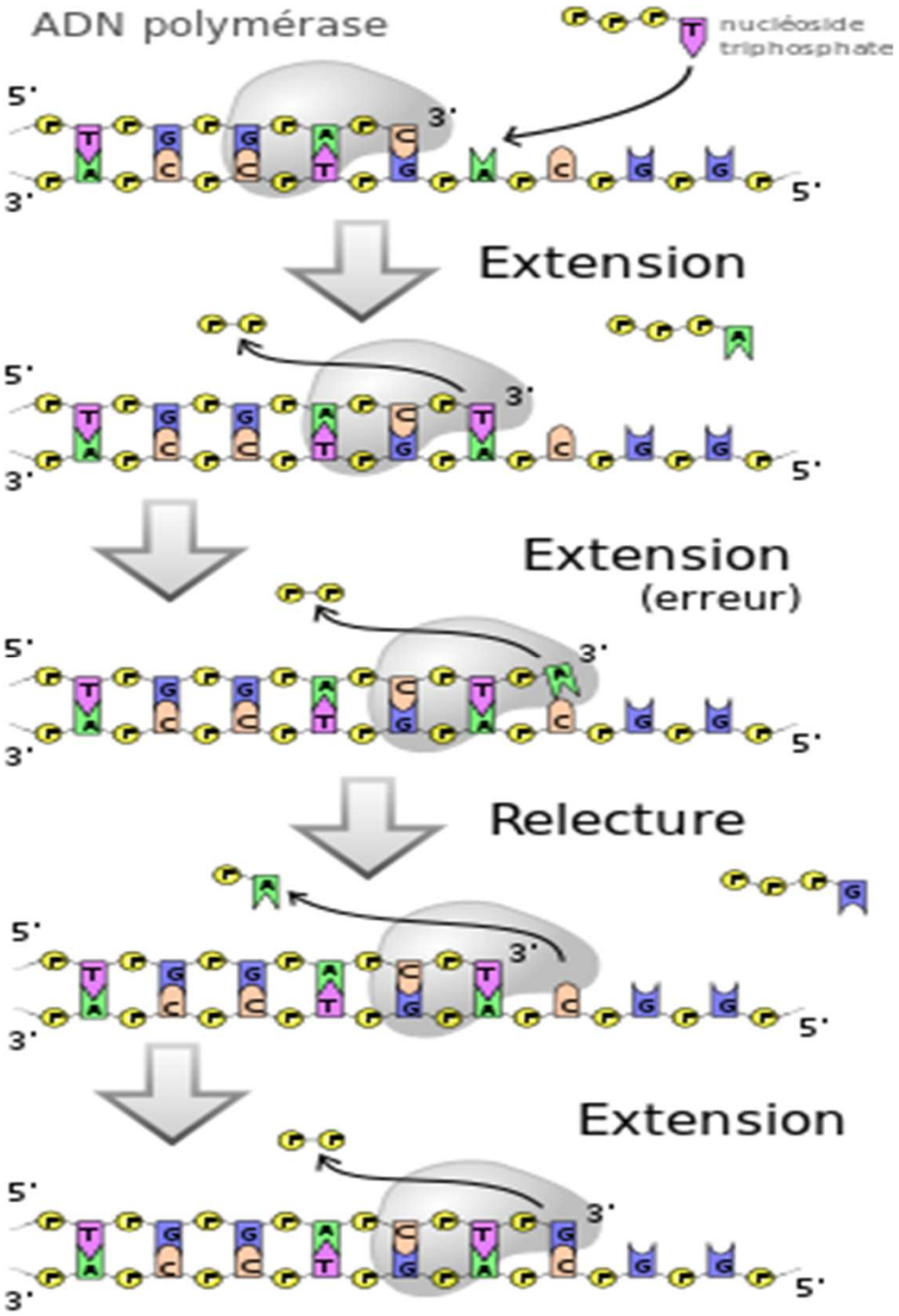
-A DNA polymerase that catalyzes DNA synthesis in the 5'→3' direction.

-The enzyme also exhibits 3'→5' exonuclease (proofreading) and 5'→3' exonuclease activity.

-Incorporates modified nucleotides (e.g., biotin, digoxigenin, aminoallyl, and fluorescently labeled nucleotides)



DNA polymerases

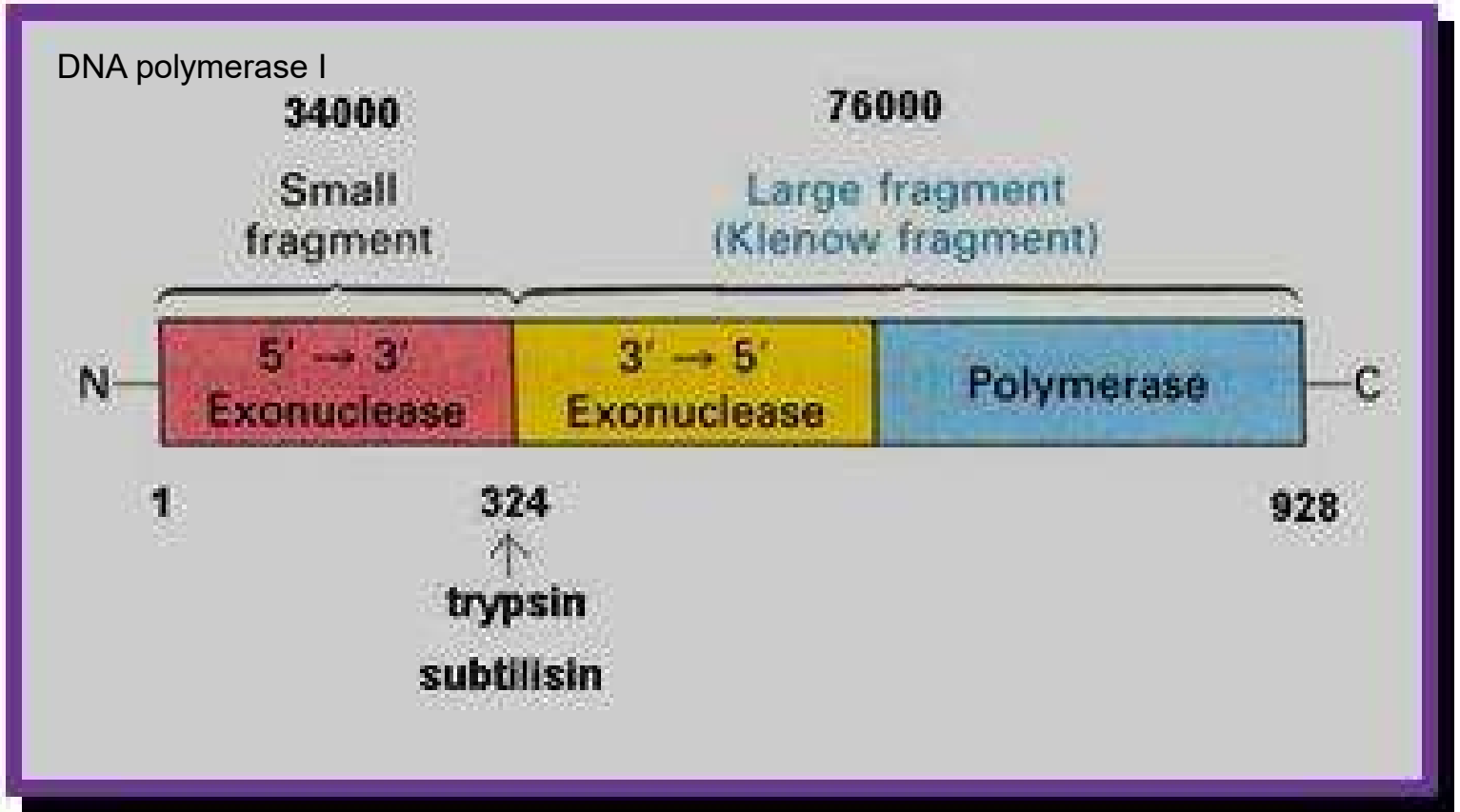


DNA polymerases

-Fragment of Klenow

-Klenow fragment is the large fragment of DNA polymerase I.

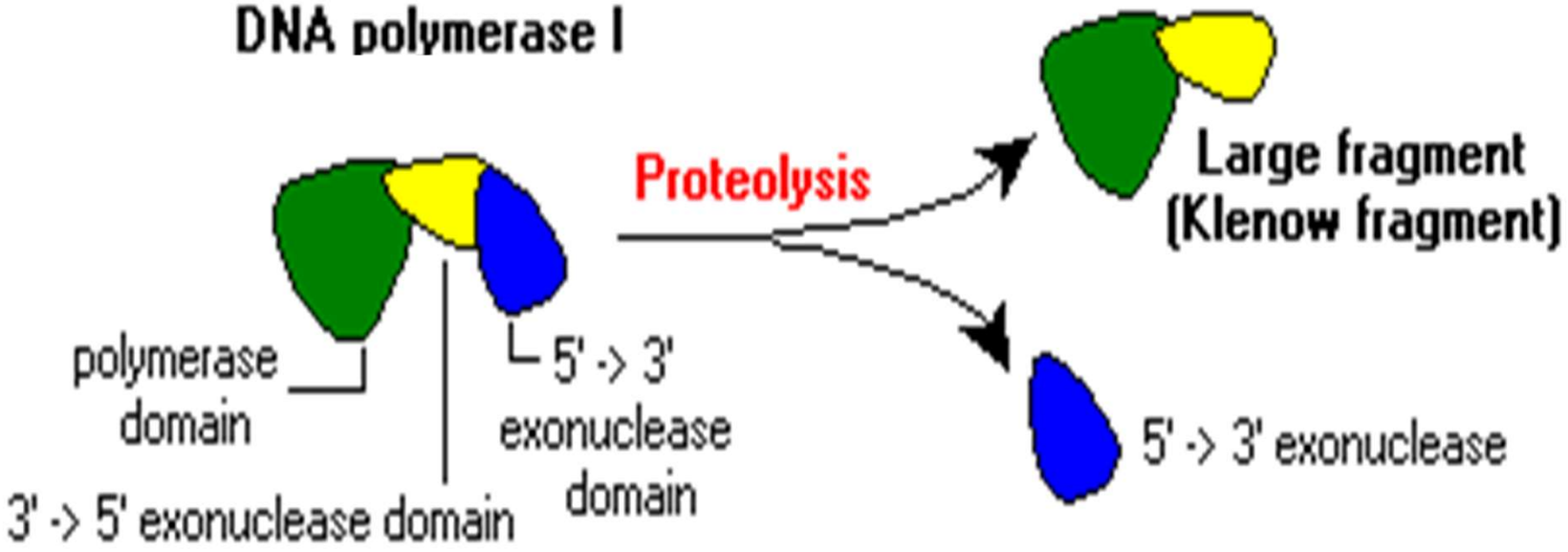
-It exhibits 5'→3' polymerase activity and 3'→5' exonuclease (proofreading) activity, but it lacks the 5'→3' exonuclease of DNA polymerase I.



DNA polymerases

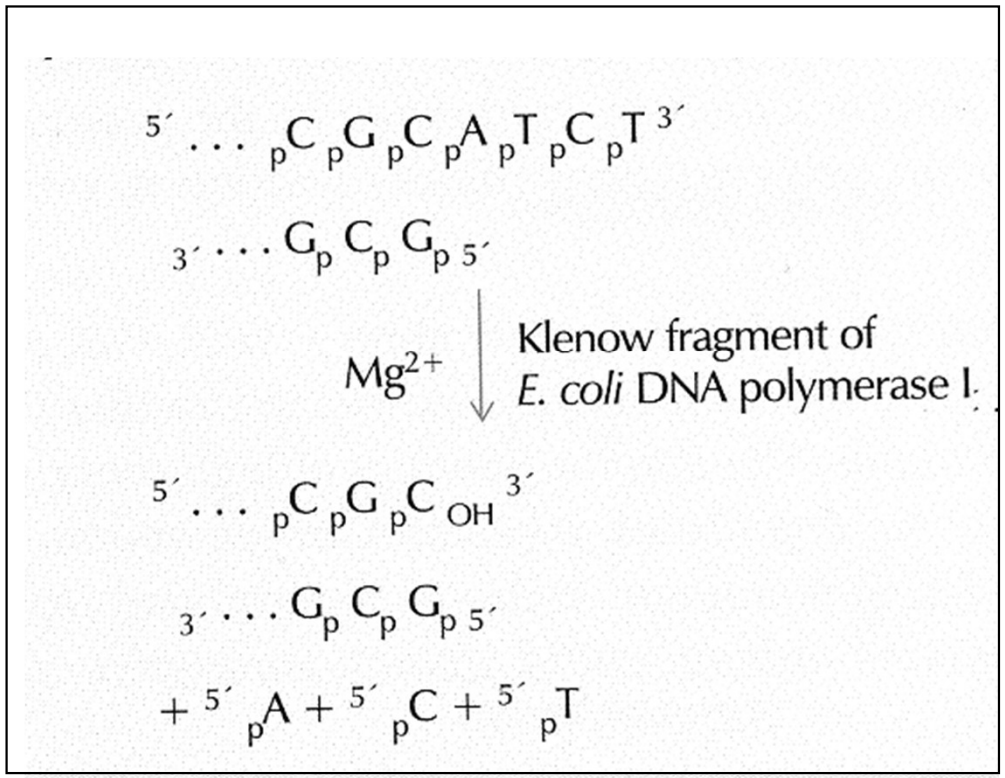
-Fragment of Klenow

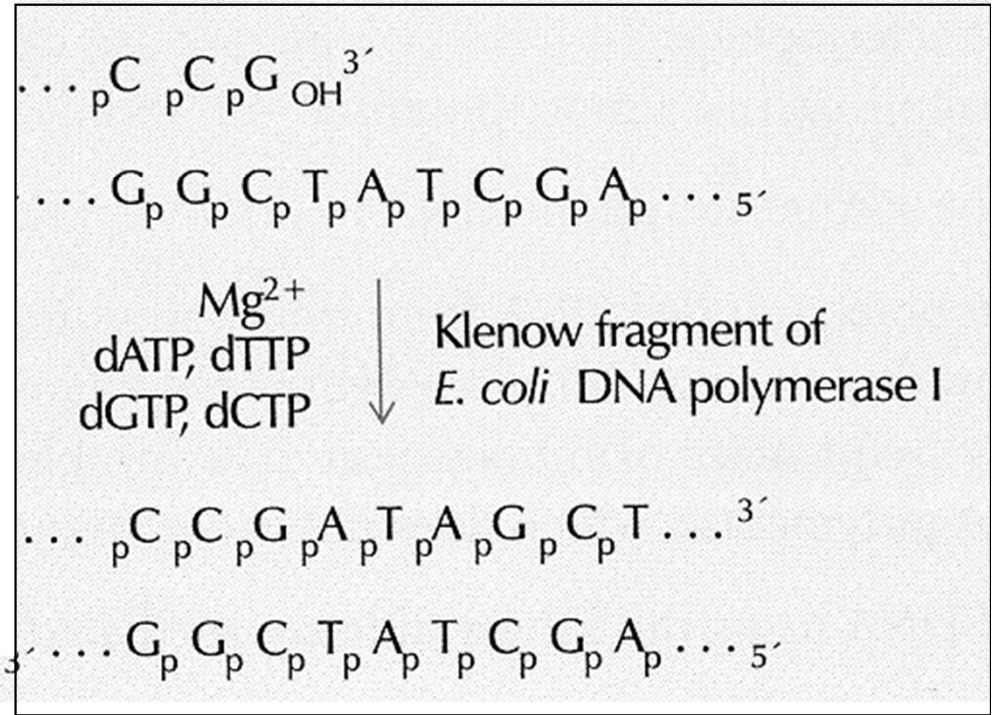
-The Klenow fragment is the larger of the two protein fragments formed by the hydrolysis of *Escherichia coli DNA polymerase I* by a protease (subtilisin).



Fragment of Klenow

- It is used, for example, to make outbound ends clear, whether they are 3' outbound using exonuclease activity or 5' outbound using T4 DNA polymerase activity



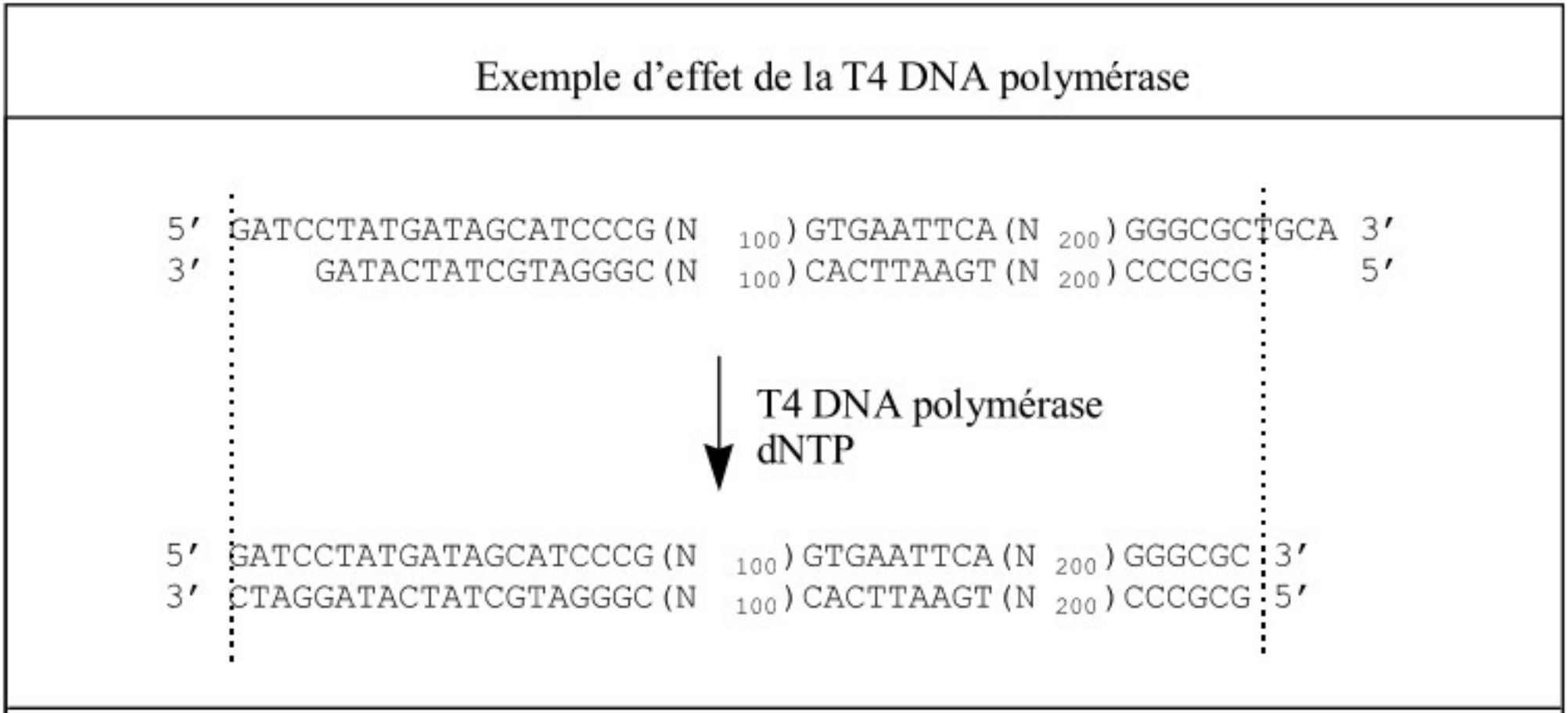


DNA polymerases

- T4 and T7 DNA polymerases:

-They have the same activities as Klenow (5'-3' polymerase and 3'-5' exonuclease).

-The exonuclease activity of T4 DNA polymerase is greater than the exonuclease activity of Klenow, therefore it will be preferred to Klenow when this activity is required.



DNA polymerases

-T7 modified DNA polymerase (Sequenase):

-They are deprived of any editing activity (5'→3' exonuclease and 3'→5' exonuclease) by a modification of the gene.

-Sequenases are the fastest of all DNA polymerases.

-Sequenases are used in dideoxyribonucleotide (Sanger) sequencing techniques.

-They are responsible for a few errors on the order of 1 mismatch per 1000 base pairs.

DNA polymerases

- **Taq DNA polymerase :**

  - It has 5'-3' polymerase and 5'->3' exonuclease activities.

  -Its advantage is that it is thermostable (resistant to very high temperatures).

  -Since it possess 3'-5' exonuclease activity, the error rate is approximately 10^5 per duplicated base.

  -It is widely used in PCR and RT-PCR.

Enzyme Activity

Pol I 5′→3′ polymerase
3′→5′ exonuclease (proofreading)
5′→3′ exonuclease

Pol II 5′→3′ polymerase
3′→5′ exonuclease

Pol III 5′→3′ polymerase
3′→5′ exonuclease

Klenow 5′→3′ polymerase
(large 3′→5′ exonuclease fragment
of Pol I)

T4 DNA Pol 5′→3′ polymerase
3′→5′ exonuclease

T7 DNA Pol 5′→3′ polymerase
3′→5′ exonuclease

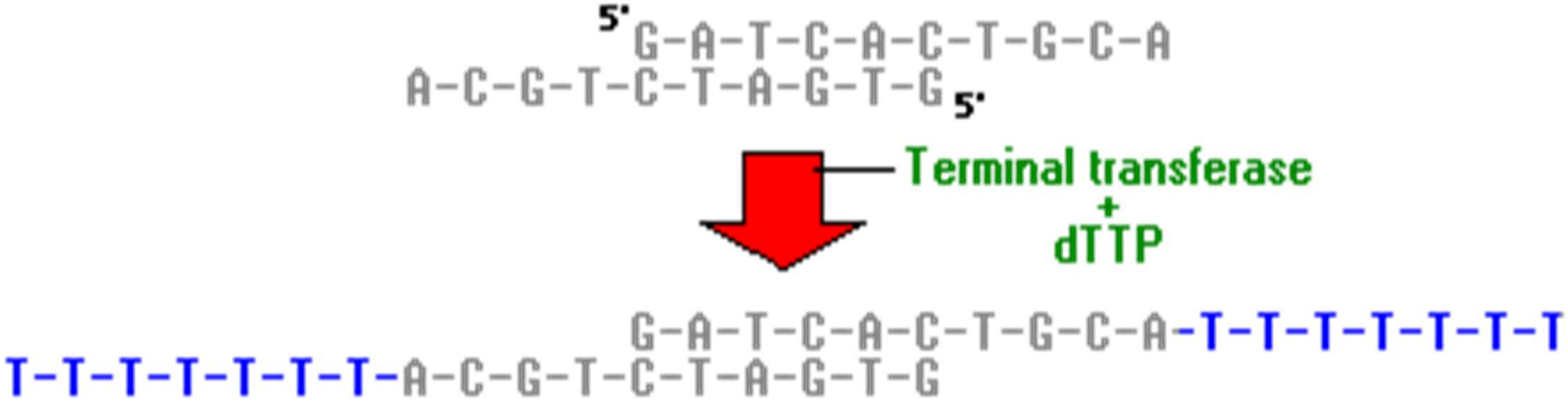
Taq polymerase 5′→3′ polymerase
5′→3′ exonuclease

Thermostable 5′→3′ polymerase
DNA polymerase 5′→3′ exonuclease
3′→5′ exonuclease (proofreading)

DNA polymerases

- Terminal transferase

- This polymerase does not need a template like other polymerases; it adds nucleotides at the 3' end to the end of the DNA strand.
- It is a rare enzyme (it is not discussed in general cell mechanisms), it adds nucleotides in 3' in the presence of dNTPs.
- If we only want to add one, we add a ddNTP.



Enzymes that copy nucleic acids

-RNA-dependent DNA polymerases

RNA-dependent

- **Reverse transcriptase:**

-This enzyme has the same activity as DNA-dependent DNA polymerase, but it synthesizes DNA from RNA. Therefore, it exhibits 5'3' DNA polymerase activity on a substrate composed of a primer hybridized to an RNA.

-The main use of this enzyme is the synthesis of complementary DNA (cDNA) from messenger RNA.

-Three types of primer can be used:

-either an oligo dT in which case we obtain a population of cDNAs.

-If a primer is used at random, we then obtain a population of cDNAs whose 5' end is variable

-Using a primer specific to a sequence present on an RNA, we then obtain cDNAs with a unique sequence corresponding to a single gene from RNA

RNA-dependent

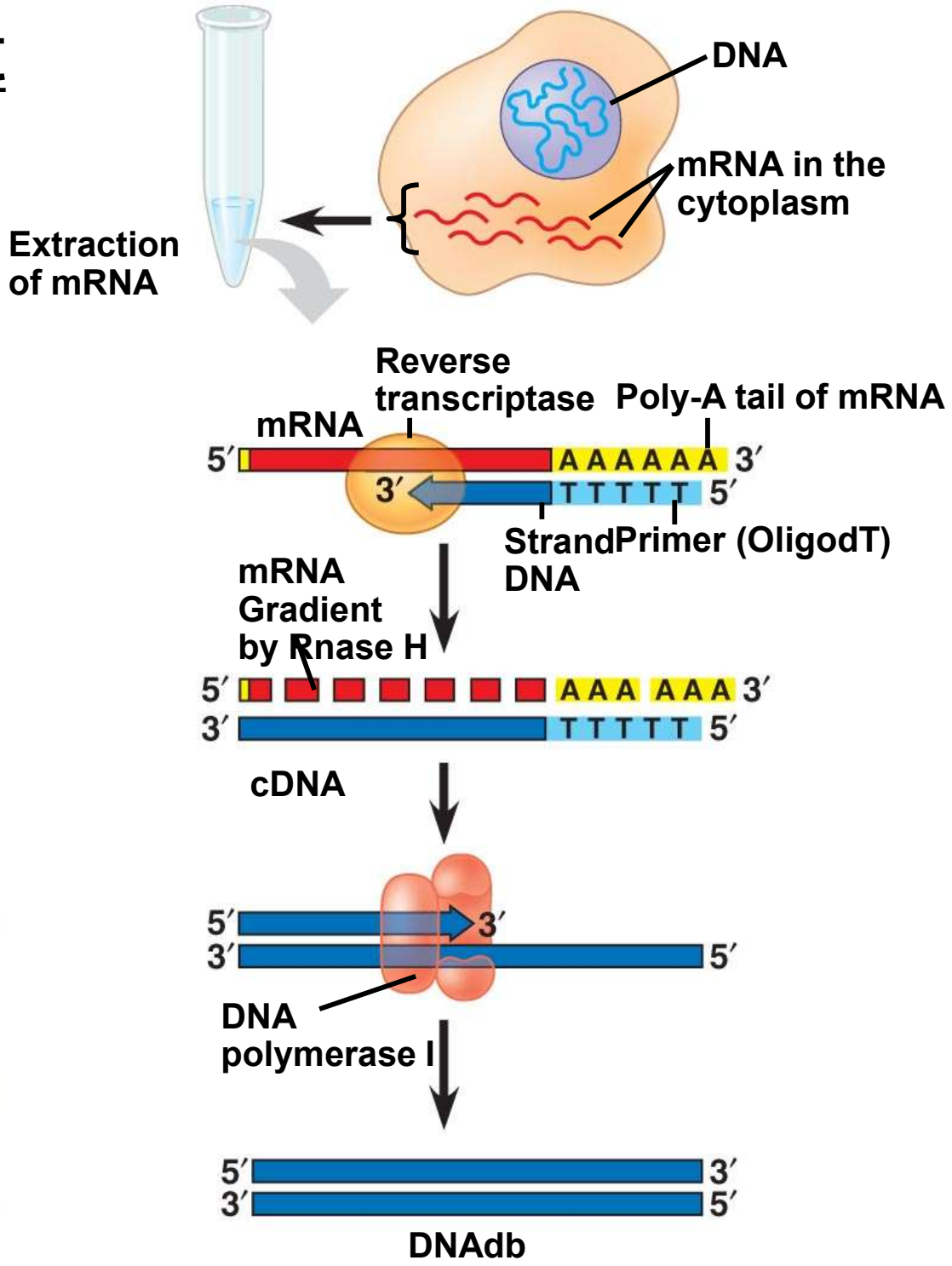
- **Reverse transcriptase**

(Retro transcriptase):

Réplication à partir de l'ARNm

Brèches dans l'ARNm

Réplication de l'ADN en utilisant les ARN comme amorces puis resynthèse au niveau des amorces



Enzymes that copy nucleic acids

-DNA-dependent RNA polymerases

DNA-dependent RNA polymerase

-RNA polymerase:

-RNA polymerase recognizes a promoter and synthesizes an RNA complementary to the strand downstream of that promoter.

-The synthesis takes place in the 5'-3' direction in the presence of ribonucleotide triphosphates.

-The gene of interest must therefore be cloned behind a promoter specific to the RNA polymerase we want to use.

-Three main RNA polymerases are used: T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase.

-These three polymerases originate from T7, T3 or SP6 phages and each recognizes a specific promoter; thus, the T7 RNA polymerase recognizes the T7 promoter but not the T3 or SP6 promoters.

Enzymatic tools for genetic engineering

- Enzymes that cut nucleic acids
- Enzymes that stick pieces of DNA together
- Enzymes that work on phosphates
- Enzymes that copy nucleic acids
- Enzymes that modify DNA

Enzymes that modify DNA

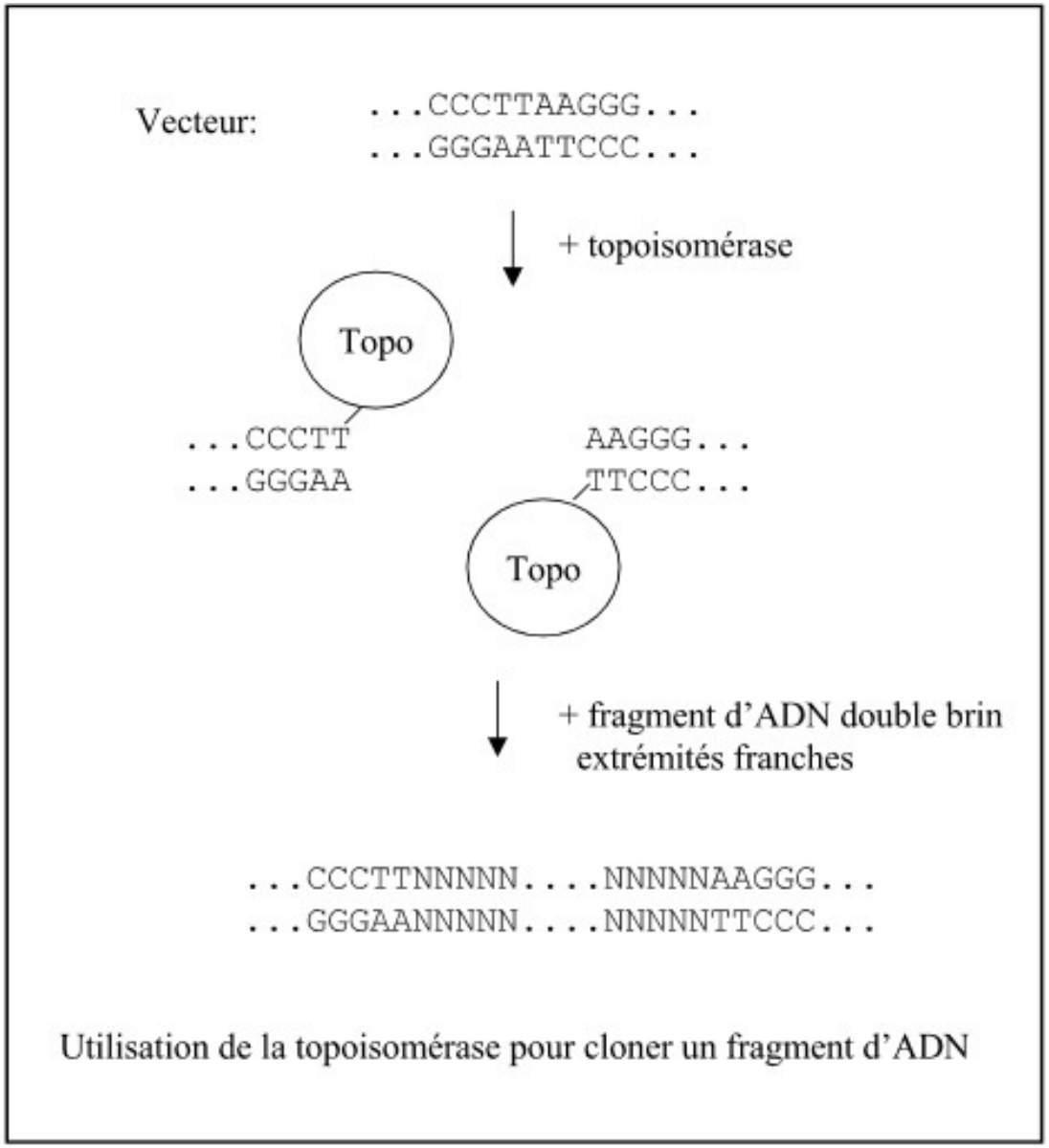
- **Methylases**
  - In order to protect bacterial DNA from hydrolysis by the enzyme, a methylase, encoded by the methylation gene, will modify the nucleotides of bacterial DNA by methylating them so that they are no longer recognized by the restriction enzyme.

Enzymatic tools for genetic engineering

- Other enzymes:
  - **Topoisomerases:**
    - Removes the supercoiling of DNA during replication.
    - A restriction enzyme and a ligase in one.
    - It forms a 3' phosphate-enzyme and a 5' OH. It can then reform the phosphodiester bond.
    - In the presence of a DNA fragment, it can reform the bond provided that the DNA sequence is 5'OH.

Enzymatic tools for genetic engineering

- Other enzymes:
  - **Topoisomerases**



Utilisation de la topoisomérase pour cloner un fragment d'ADN