Department of BSBE
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Introduction to Recombinant DNA Technology

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BT 207 – Genetic Engineering

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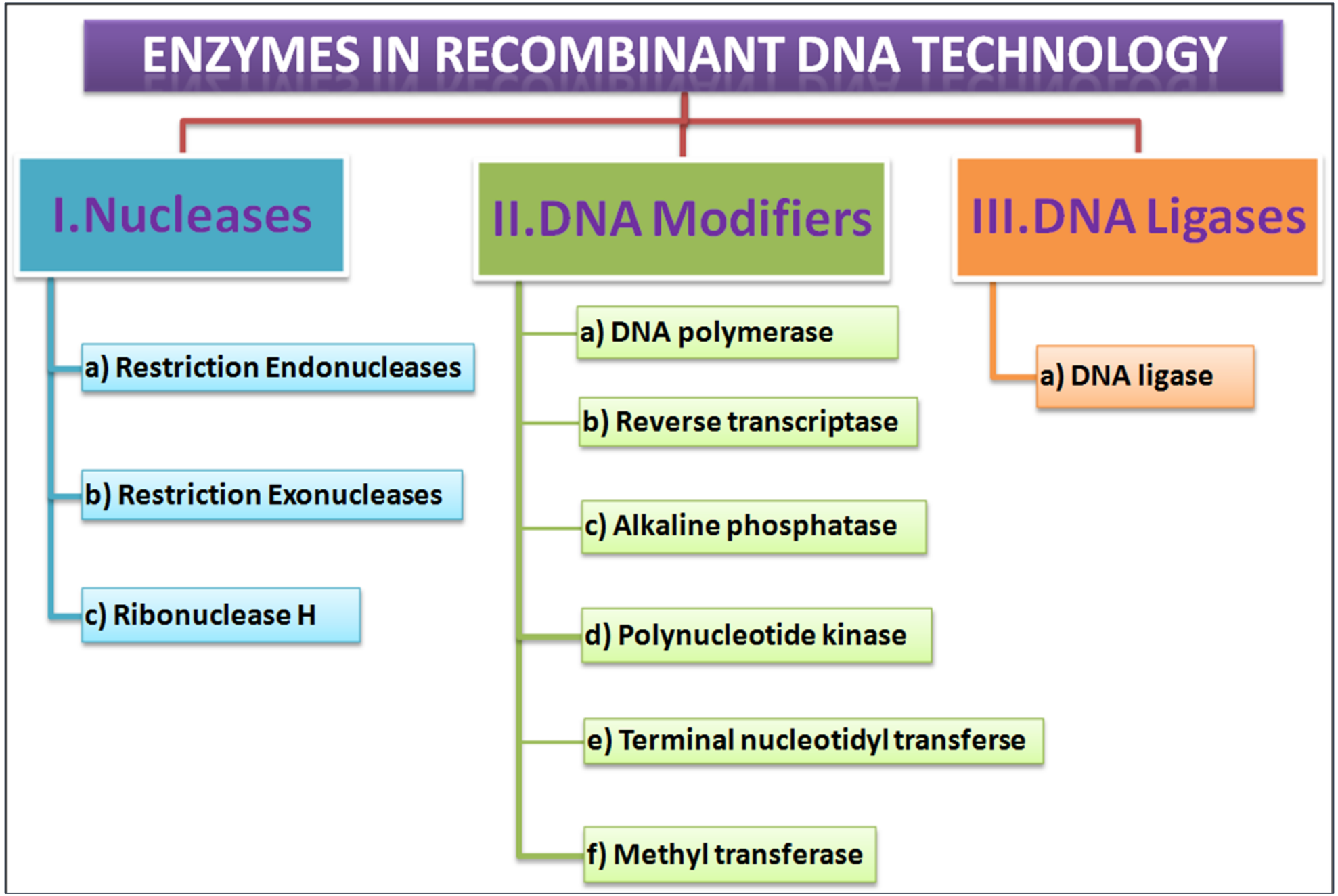
Tools that allow DNA to be manipulated.

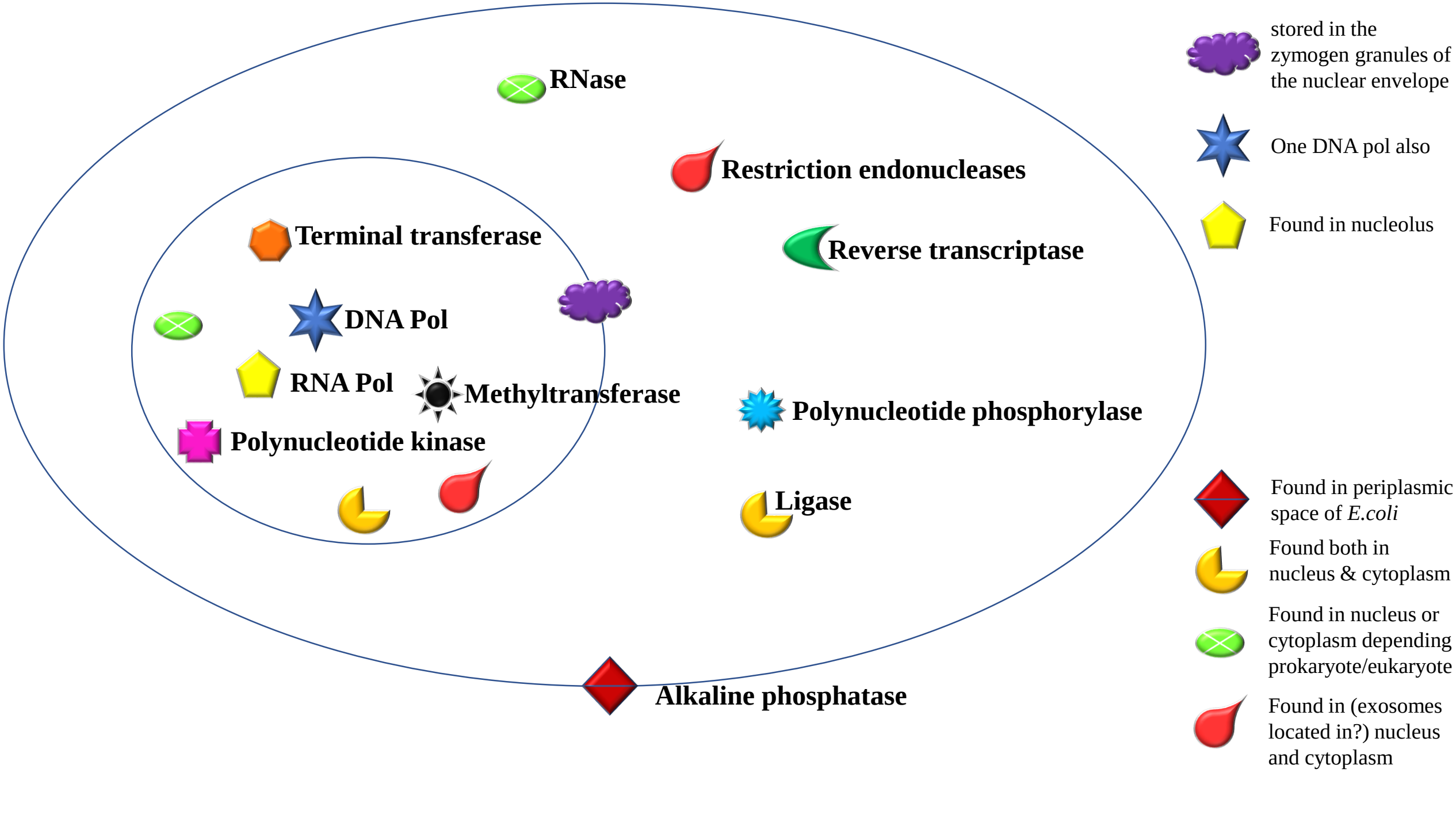
- Restriction enzyme - In 1970 Hamilton Smith's lab discovered restriction enzymes that allowed DNA to be cut at specific places and separated out on an electrophoresis gel. This enabled scientists to isolate genes from an organism's genome.
- Ligase - DNA ligases, that join broken DNA together, discovered earlier in 1967 and by combining the two enzymes it was possible to "cut and paste" DNA sequences to create recombinant DNA.
- Other enzymes
- Polymerase chain reaction (PCR), developed by Kary Mullis in 1983, allowed small sections of DNA to be amplified and aided identification and isolation of genetic material.
- Vector - Plasmids, discovered in 1952, became important tools for transferring information between cells and replicating DNA sequences.
- Host – Expression host
- Sequencing - Frederick Sanger developed a method for sequencing DNA in 1977, greatly increasing the genetic information available to researchers.

Examples of enzymes used in Gene cloning

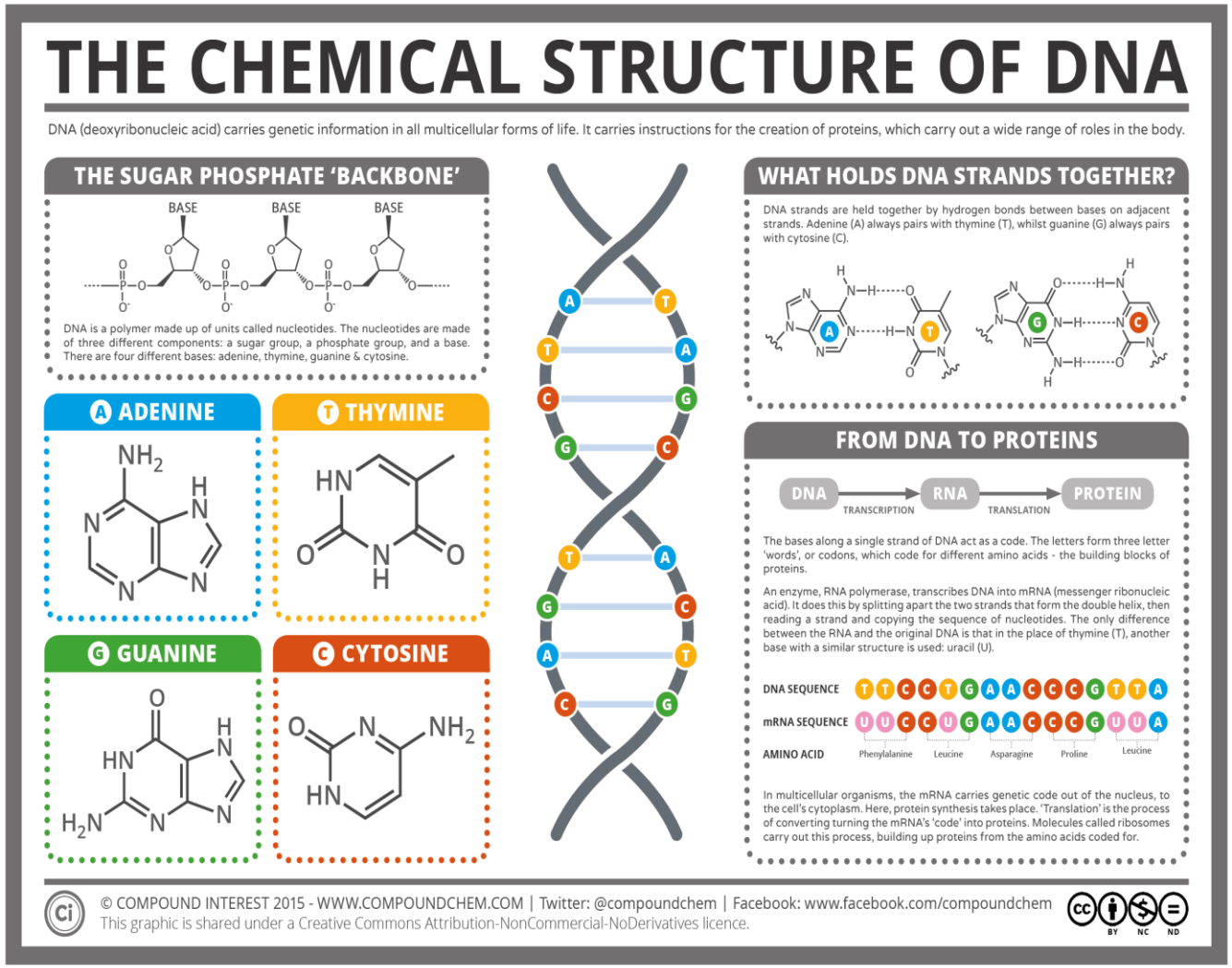
Enzyme(s)	Activity	Comments
Restrictionendonucleases	recognize specific nucleotide sequences and cleaves the DNA within or near to the recognition sequences	Used to specifically recognise and cleave the double stranded DNA
Reverse transcriptase(RT)	retrovirally encoded RNA-dependent DNA polymerase	used to convert mRNA into a complimentary DNA (cDNA) copy for the purpose of cloning cDNAs
RNase H	recognizes RNA-DNA duplexes and randomly cleaves the phosphodiester backbone of the RNA	used primarily to cleave the mRNA strand that is annealed to the first strand of cDNA generated by reverse transcription
DNA polymerase	synthesis of DNA	used during most procedures where DNA synthesis is required, also used in in vitro mutagenesis
Klenow DNApolymerase	proteolytic fragment of DNA polymerase that lacks the 5' ----> 3' exonuclease activity	used to incorporate radioactive nucleotides into restriction enzyme generated ends of DNA, also can be used in place of DNA polymerase
DNA ligase	covalently attaches a free 5' phosphate to a 3' hydroxyl	used in all procedures where to molecules of DNA need to be covalently attached
Alkaline phosphatase	removes phosphates from 5' ends of DNA molecules	used to allow 5' ends to be subsequently radiolabeled with the phosphate of ATP in the presence of polynucleotide kinase, also used to prevent self-ligation of restriction enzyme digested plasmids and lambda vectors

Enzyme(s)	Activity	Comments
Polynucleotide kinase	introduces phosphate of ATP to 5' ends of DNA	used to allow 5' ends to be subsequently radiolabeled with the phosphate of ATP in the presence of polynucleotide kinase, also used to prevent self-ligation of restriction enzyme digested plasmids and lambda vectors.
DNase I	randomly hydrolyzes the phosphodiester bonds of double-stranded DNA	is used in the identification of regions of DNA that are bound by protein and thereby protected from DNase I digestion, also used to identify transcriptionally active regions of chromatin since they are more susceptible to DNase I digestion
S_1 Nuclease	exonuclease that recognizes singlestranded regions of DNA	used to remove regions of single strandedness in DNA or RNA-DNA duplexes
Exonuclease III	exonuclease that removes nucleotides from the 3' end of DNAs	used to generate deletions in DNA for sequencing, or to map functional domains of DNA duplexes
Terminal transferase	DNA polymerase that requires only a 3'-OH, lengthens 3' ends with any dNTP	used to introduce homopolymeric (same dNTP) *tails* onto the 3' ends of DNA duplexes, also used to introduce radiolabeled nucleotides on the 3' ends of DNA
T3, T7, and SP6 RNA polymerases	bacterial virus encoded RNA polymerase, recognize specific nucleotide sequences for initiation of transcription	used to synthesize RNA *in vitro*
Taq and Vent DNA polymerase	thermostable DNA polymerases	used in PCR


ENZYMES IN RECOMBINANT DNA TECHNOLOGY
I.Nucleases
a) Restriction Endonucleases
b) Restriction Exonucleases
c) Ribonuclease H
II.DNA Modifiers
a) DNA polymerase
b) Reverse transcriptase
c) Alkaline phosphatase
d) Polynucleotide kinase
e) Terminal nucleotidyl transferse
f) Methyl transferase
III.DNA Ligases
a) DNA ligase


RNase
Restriction endonucleases
Terminal transferase
Reverse transcriptase
DNA Pol
RNA Pol
Methyltransferase
Polynucleotide phosphorylase
Polynucleotide kinase
Ligase
Alkaline phosphatase
stored in the zymogen granules of the nuclear envelope
One DNA pol also
Found in nucleolus
Found in periplasmic space of E.coli
Found both in nucleus & cytoplasm
Found in nucleus or cytoplasm depending prokaryote/eukaryote
Found in (exosomes located in?) nucleus and cytoplasm

Nucleases


THE CHEMICAL STRUCTURE OF DNA
DNA (deoxyribonucleic acid) carries genetic information in all multicellular forms of life. It carries instructions for the creation of proteins, which carry out a wide range of roles in the body.
THE SUGAR PHOSPHATE 'BACKBONE'
BASE
BASE
BASE
DNA is a polymer made up of units called nucleotides. The nucleotides are made of three different components: a sugar group, a phosphate group, and a base. There are four different bases: adenine, thymine, guanine & cytosine.
A ADENINE
T THYMINE
G GUANINE
C CYTOSINE
WHAT HOLDS DNA STRANDS TOGETHER?
DNA strands are held together by hydrogen bonds between bases on adjacent strands. Adenine (A) always pairs with thymine (T), whilst guanine (G) always pairs with cytosine (C).
FROM DNA TO PROTEINS
DNA
TRANSCRIPTION
RNA
TRANSLATION
PROTEIN
The bases along a single strand of DNA act as a code. The letters form three letter 'words', or codons, which code for different amino acids - the building blocks of proteins.
An enzyme, RNA polymerase, transcribes DNA into mRNA (messenger ribonucleic acid). It does this by splitting apart the two strands that form the double helix, then reading a strand and copying the sequence of nucleotides. The only difference between the RNA and the original DNA is that in the place of thymine (T), another base with a similar structure is used: uracil (U).
DNA SEQUENCE T T C C T G A A C C C G T T A
mRNA SEQUENCE U U C C U G A A C C C G U U A
AMINO ACID
Phenylalanine
Leucine
Asparagine
Proline
Leucine
In multicellular organisms, the mRNA carries genetic code out of the nucleus, to the cell's cytoplasm. Here, protein synthesis takes place. 'Translation' is the process of converting turning the mRNA's 'code' into proteins. Molecules called ribosomes carry out this process, building up proteins from the amino acids coded for.
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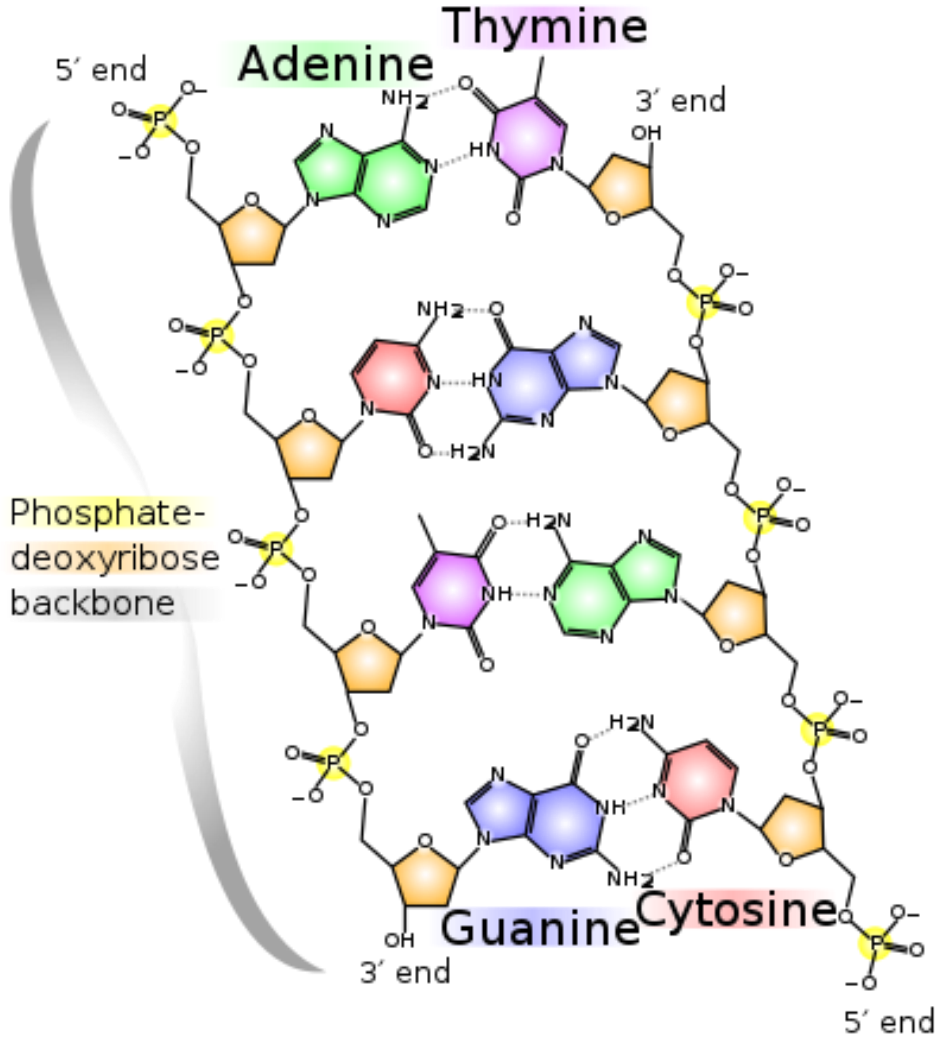

5′ end
Adenine
Thymine
3′ end
Phosphate-
deoxyribose
backbone
Guanine
Cytosine
3′ end
5′ end

Restriction Enzymes

In the early 1950s, experiments by two teams of researchers, Salvador Luria working with Mary Human and Joe Bertani working with Jean Weigle, showed that some strains of bacteria were more resistant to viral infections than others.

Those strains of bacteria appeared to be less vulnerable to bacteriophage infections than others and resisted the hijacking of their cell machinery by bacteriophages.

A self-defense mechanism - of these bacteriophage-resistant bacteria revealed their secret weapon: a group of enzymes called restriction endonucleases, or restriction enzymes.

The hypothesis:
In the 1960s, Werner Arber observed a dramatic change in the bacteriophage DNA after it invaded these resistant strains of bacteria: It was degraded and cut into pieces.
Arber then posited that bacteriophage-resistant bacterial cells might express a specific enzyme that degrades only invading bacteriophage DNA, but not their own DNA.

The Nobel Prize in Physiology or Medicine 1978, Biozentrum der Universität, Basel, Switzerland

Prize motivation: "for the discovery of restriction enzymes and their application to problems of molecular genetics"

What are restriction enzymes?

- Molecular scissors that cut double stranded DNA molecules at specific points.
- Found naturally in a wide variety of prokaryotes
- An important tool for manipulating DNA.

Discovery

- 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.

Biological Need of bacteria

- Most bacteria use Restriction Enzymes as a defense against bacteriophages.
- Restriction enzymes prevent the replication of the phage by cleaving its DNA at specific sites.
- The host DNA is protected by Methylases which add methyl groups to adenine or cytosine bases within the recognition site thereby modifying the site and protecting the DNA.

How, though, would a DNA-degrading enzyme distinguish between the two?

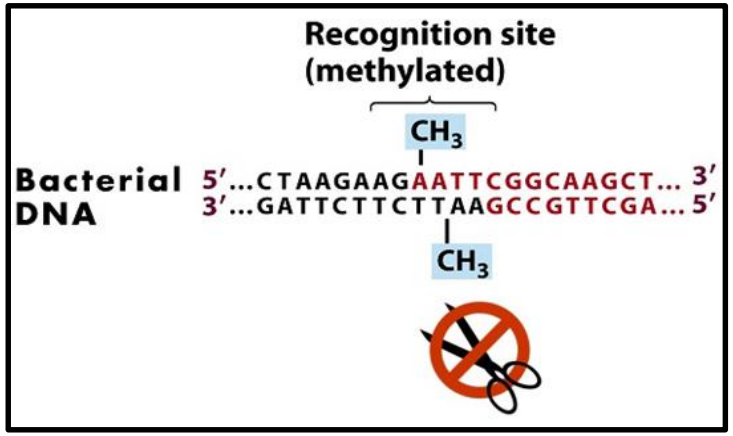
Arber hypothesized that bacterial cells might express two types of enzymes: a restriction enzyme that recognizes and cuts up the foreign bacteriophage DNA and a modification enzyme that recognizes and modifies the bacterial DNA to protect it from the DNA-degrading activity of its very own restriction enzyme.

He predicted that the restriction enzyme and the modification enzyme act on the same DNA sequence, called a recognition sequence.

This prediction was confirmed in the late 1960s by Stuart Linn and Arber when they isolated a modification enzyme called methylase and a restriction enzyme responsible for bacteriophage resistance in the bacterium *Escherichia coli*. The methylase enzyme added protective methyl groups to DNA, and the restriction enzyme cut unmethylated (unprotected) DNA at multiple locations along its length.

Methylation specific PCR – An application

How does bacteria protect their own DNA against restriction enzymes?

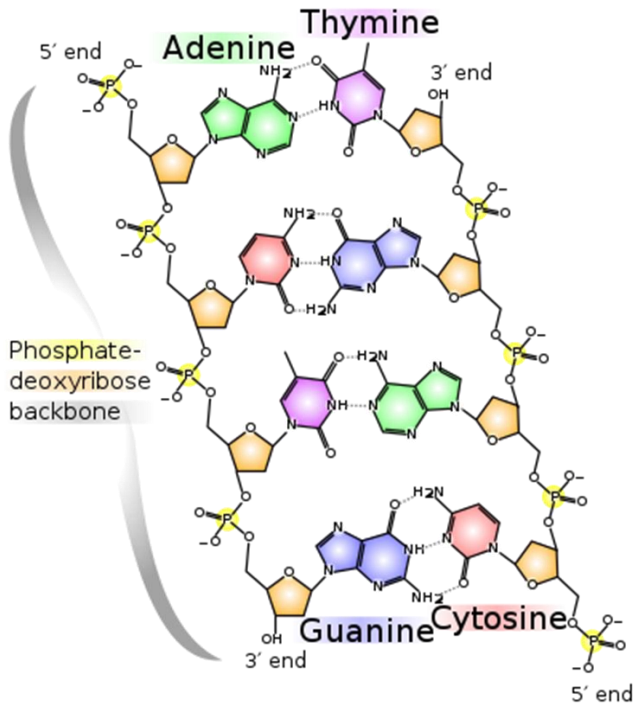


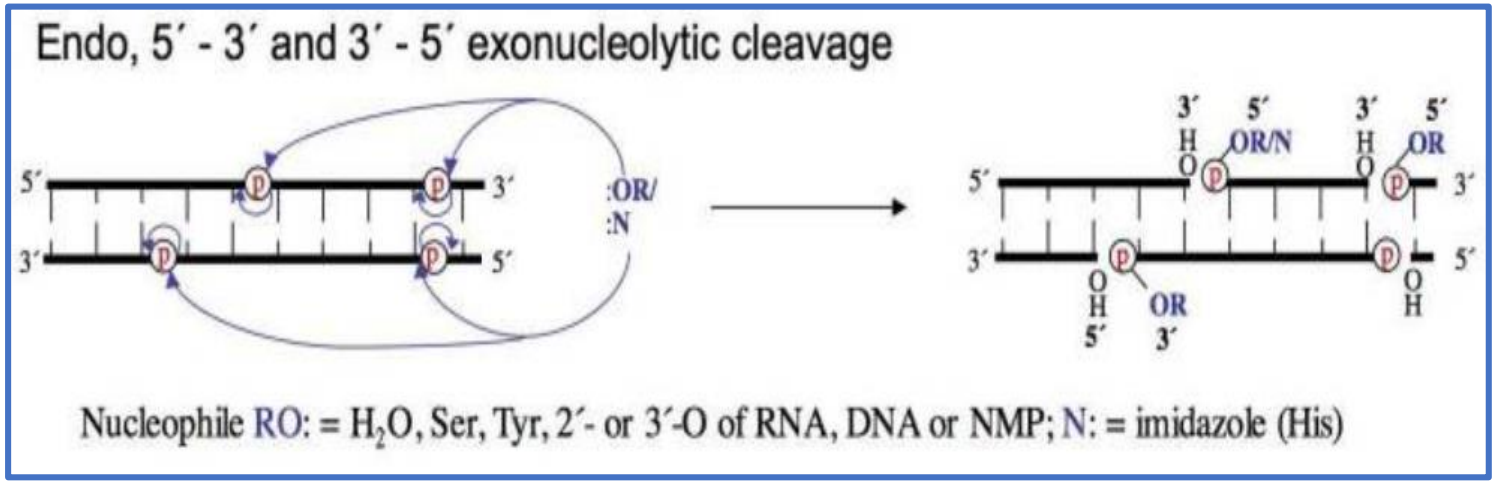
Bacteria prevent eating away their own DNA by masking the restriction sites with **methyl groups** (CH3). Methylation of DNA is a common way to modify DNA function and bacterial DNA is highly methylated. In this case it functions to make the restriction sites unrecognizable for the restriction enzymes.

Nuclease - a phosphodiesterase that cleaves one of the two bridging P-O bonds, 3′ or 5′, in a nucleic acid polymer.

Nucleases utilize a variety of nucleophiles to cleave a scissile phosphate bond.

The most common nucleophiles are water molecules deprotonated by a general base for direct hydrolysis.





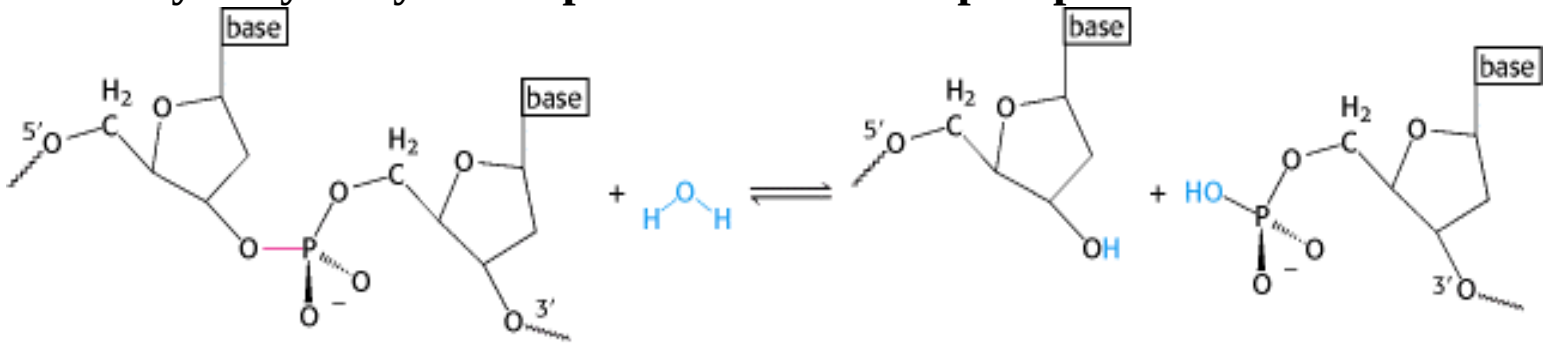
Restriction Endonuclease scans the length of the DNA

Binds to the DNA molecule when it recognizes a specific sequence.

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.

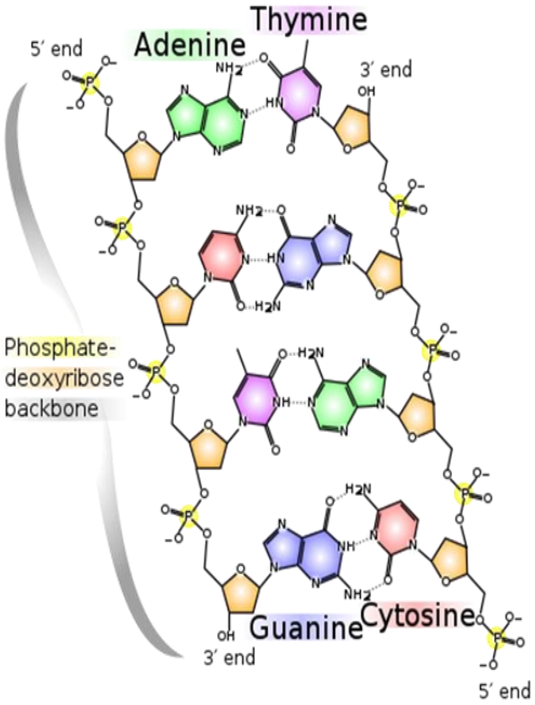
Direct hydrolysis by nucleophilic attack at the phosphorous atom



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.



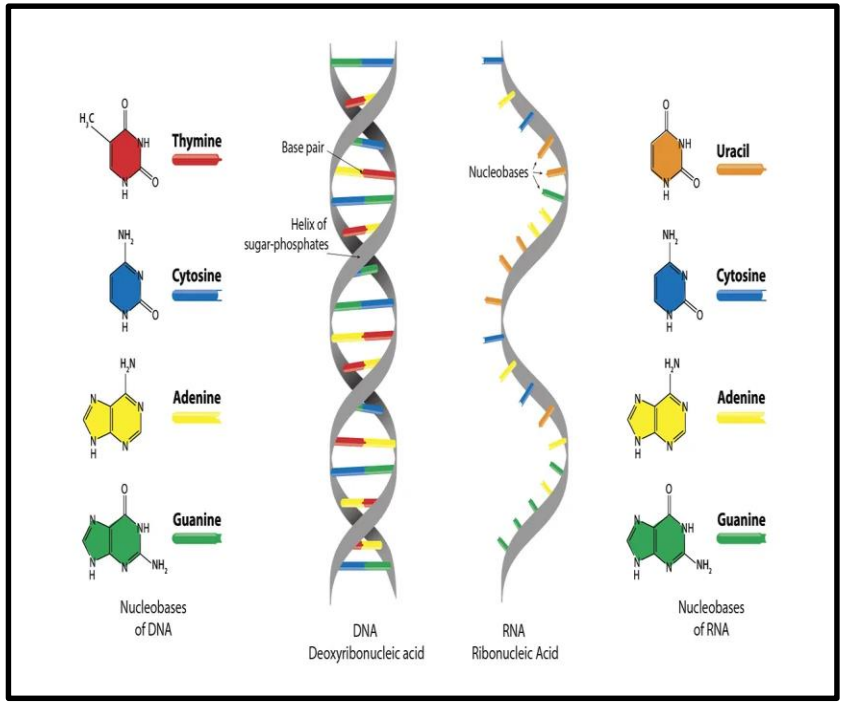
Nucleases variously affect single and double stranded breaks in their target molecules.

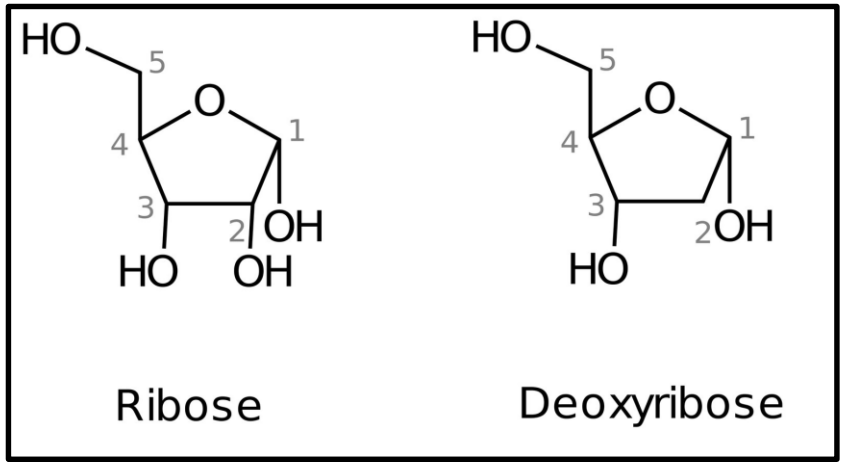
In vivo -
(1) They are essential machinery for many aspects of DNA repair.
Defects in certain nucleases can cause genetic instability or immunodeficiency.
(2)Nucleases in Bacteria – for defense

In vitro - Nucleases are used in molecular cloning.

Two primary classifications based on the position of activity.
Exonucleases digest nucleic acids from the ends.
Endonucleases act on regions in the *middle* of target molecules.

Further subcategorized as:
Deoxyribonucleases - acts on DNA
Ribonucleases - on RNA





Site recognition

- A nuclease must associate with a nucleic acid before it can cleave the molecule.
- That entails a degree of recognition.
- Nucleases variously employ both nonspecific and specific associations in their modes of recognition and binding.

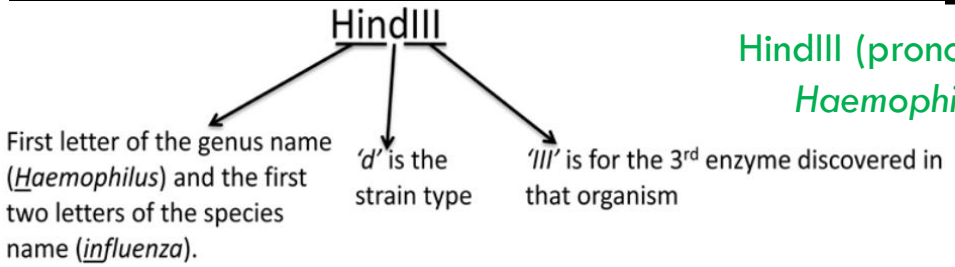
- Both modes play important roles in living organisms, especially in DNA repair.

- Nonspecific endonucleases involved in DNA repair can scan DNA for target sequences or damage. Such a nuclease diffuses along DNA until it encounters a target, upon which the residues of its active site interact with the chemical groups of the DNA.

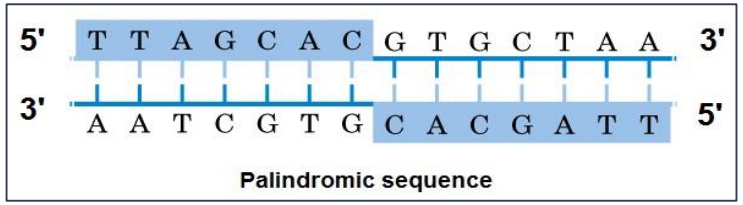
- However most are nonspecific, recognizing structural abnormalities produced in the DNA backbone by base pair mismatches.

The First Restriction endoucleases

In 1970 the first restriction endonuclease enzyme Hind III was isolated. For the subsequent discovery and characterization of numerous restriction endonucleases, in 1978 Daniel Nathans, Werner Arber, and Hamilton O. Smith awarded for Nobel Prize for Physiology or Medicine. Since then, restriction enzymes have been used as an essential tool in recombinant DNA technology

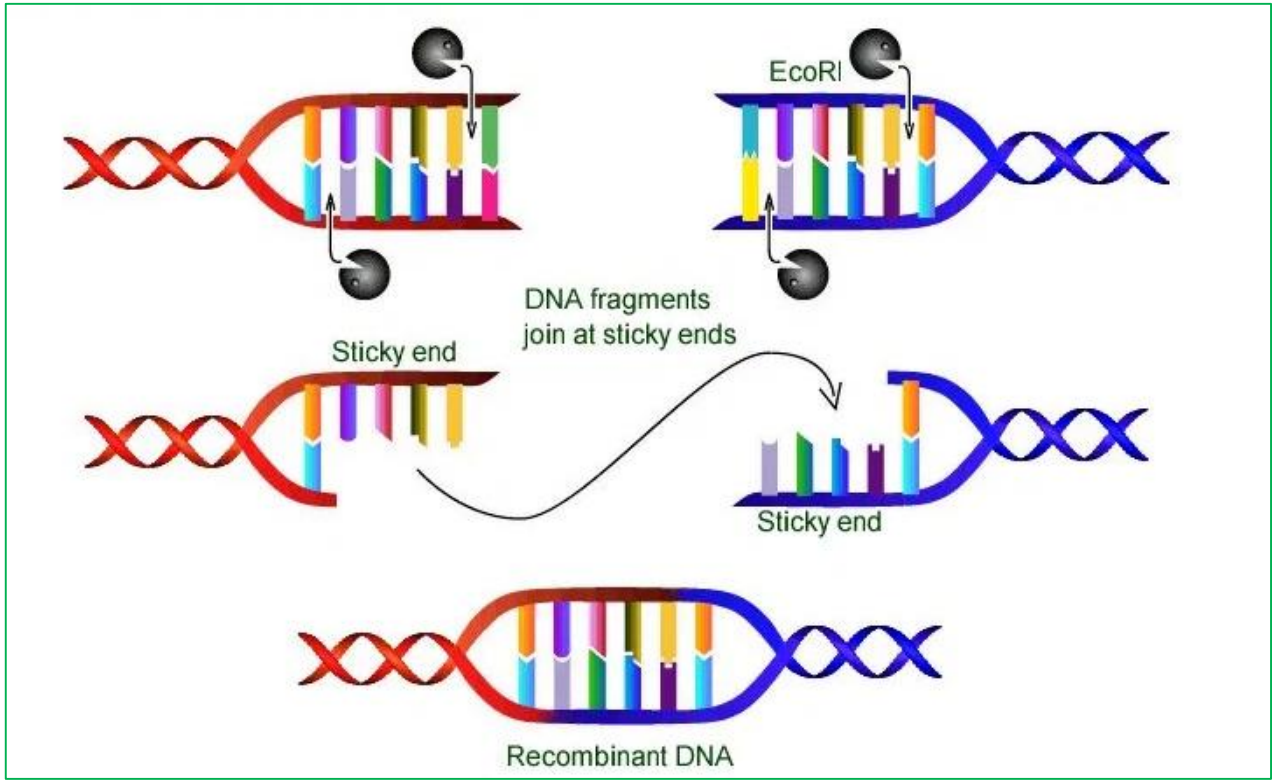


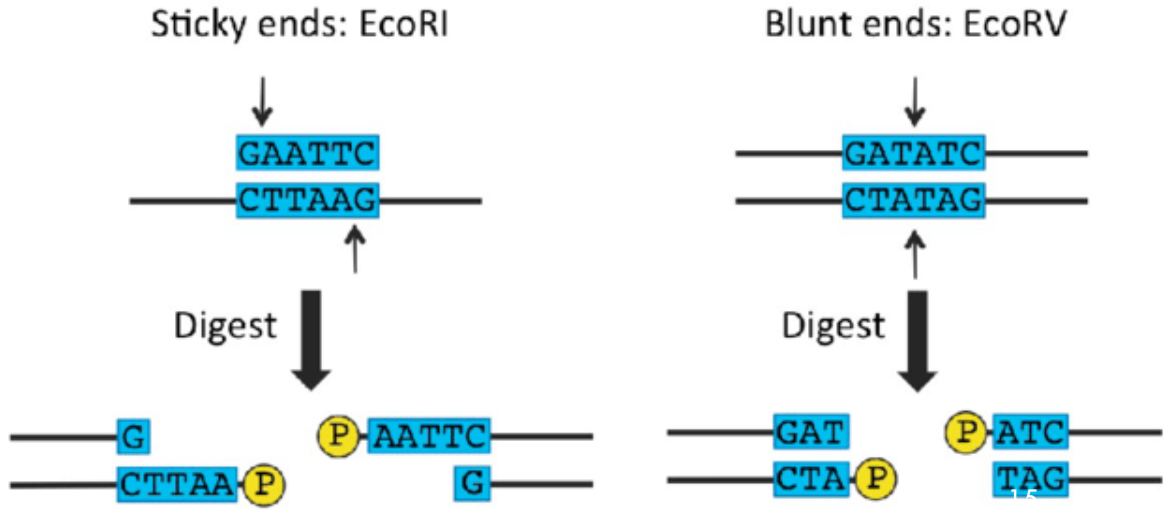
HindIII (pronounced) was discovered in *Haemophilus influenza* (strain d).



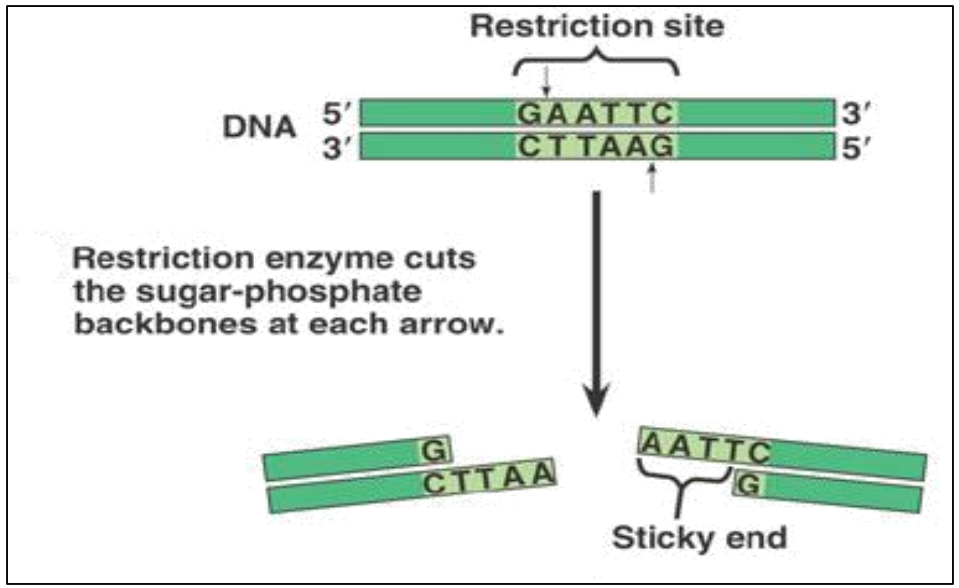
We will learn more about:

- Type I restriction enzymes
- Type II restriction enzymes
- Type III restriction enzymes





DNA Cutters- Restriction endonucleases



enzymes)

- sticky ends
- blunt ends

- Nomenclature
  - *Eco*RI
  - *E* = genus (*Escherichia*)
  - *co* = species (*coli*)
  - R = strain
  - I = # of enzyme

Blunt & Sticky ends

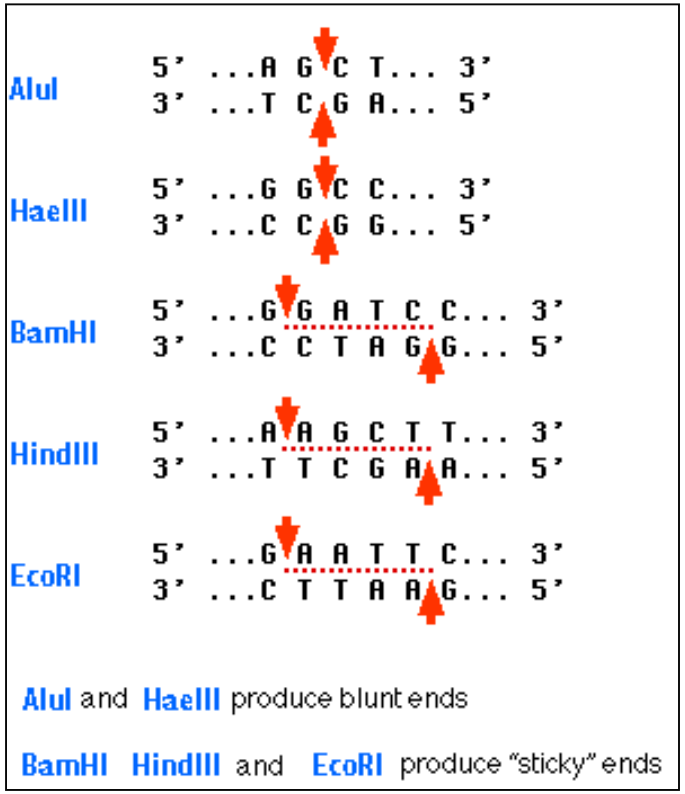


Table 16.1 Common restriction endonucleases (enzymes) and their DNA recognition sequences

Microorganism	Restriction Endonuclease	Target Sequences, Showing Axis of Symmetry (|) and DNA Cleavage Sites (▼)
Generates cohesive ends:		
Escherichia coli RY13	*Eco*RI	G ▼ A A | T T C C T T A A ▲ G
Bacillus amyloliquefaciens H	*Bam*HI	G ▼ G A | T C C C C T A G ▲ G
Bacillus globigii	*Bgl*II	A ▼ G A | T C T T C A A G ▲ A
Haemophilus aegyptius	*Hae*II	Pu ▼ G C | G C Py Py C G C G ▲ Pu
Haemophilus influenza R_d	*Hind*III	A ▼ A G | C T T T T C G A ▲ A
Providencia stuartii	*Pst*I	C T G | C A ▼ G G ▲ A C G T C
Streptomyces albus G	*Sal*I	G ▼ T C | G A C C A G C T ▲ G
Xanthomonas badrii	*Xba*I	T ▼ C T | A G A A G A T C ▲ T
Thermus aquaticus	*Taq*I	T▼ | C G A A G C▲T
Generates flush ends:		
Brevibacterium albidum	*Bal*I	T G G ▼ | C C A A C C ▲ G G T
Haemophilus aegyptius	*Hae*III	G G ▼ | C C C C ▲ G G
Serratia marcescens	*Sma*I	C C C ▼ | G G G G G G ▲ C C C

MICROBIAL LIFE , **Table 16.1** © 2002 Sinauer Associates, Inc.

Recognition site

Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA.

Frequency of occurrence - The recognition sequences can also be classified by the number of bases in its recognition site, usually between 4 and 8 bases, and the number of bases in the sequence will determine how often the site will appear by chance in any given genome **4^n,** e.g., a 4-base pair sequence would theoretically occur once every 4^4 or 256bp, 6 bases, 4^6 or 4,096bp, and 8 bases would be 4^8 or 65,536bp.

Many of them are palindromic, meaning the base sequence reads the same backwards and forwards.

(1)The *mirror-like* palindrome is similar to those found in ordinary text, in which a sequence reads the same forward and backward on a single strand of DNA, as in GTAATG.

(2)The *inverted repeat* palindrome is also a sequence that reads the same forward and backward, but the forward and backward sequences are found in complementary DNA strands (i.e., of double-stranded DNA), as in GTATAC (GTATAC being complementary to CATATG).

Inverted repeat palindromes are more common and have greater biological importance than mirror-like palindromes.

The frequency of cutting in a random DNA sequence for a given restriction enzyme is once per every 4^n, where n is the number of bases in the restriction enzymes recognition sequence.

The “4” derives from the fact that there are four different possible nucleotides that may be inserted at any one position (G, A, T, or C).

Eco RI and *Hin* dIII have a six-base recognition site, so they will cut once per every 4^6, or 4096 bases.

Hin dII has a five-base recognition site, so it will cut once per every 4^5, or 1024 bases.

Q1. To determine the number of sites, divide the number of bases in the DNA by the average length of the restriction fragments resulting from the given enzyme. The size of genome of a λ is 48,502 base pairs, so the number of expected sites with Eco R I and Hind III are?

Q2. Cutting with *Hin* dIII will yield fragments of 1, 2, and 2.5 kilobases. Cutting with *Eco* RI will yield fragments of 1.5, 2, and 3.5 kilobases. Cutting with both *Hin* dIII and *Pvu* II will yield fragments of 1, 1.5, and 2 kilobases. Construct the plasmid map indicating restriction sites.

Types

Naturally occurring restriction endonucleases are categorized into four groups (Types I, II III, and IV) based on:

- their composition and enzyme cofactor requirements
- the nature of their target sequence
- the position of their DNA cleavage site relative to the target sequence

Type I enzymes (EC 3.1.21.3) cleave at sites remote from a recognition site; around 1000bp away from recognition site; require both ATP and S-adenosyl-L-methionine to function; multifunctional protein with both restriction digestion and methylase (EC 2.1.1.72) activities. EcoK I, EcoA I, CfrA I

Type II enzymes (EC 3.1.21.4) cleave within or at short specific distances from a recognition site; most require magnesium; they do not use ATP, Inverted palindromic and 4–8 nucleotides in length, single function (restriction digestion) enzymes independent of methylase. Blunt and cohesive end cuts possible, EcoR I, BamH I, Hind III

Type III enzymes (EC 3.1.21.5) cleave at sites a short distance from a recognition site; Random 24-26 bp away from recognition site; require ATP; S-adenosyl-L-methionine stimulates the reaction but is not required; recognize two separate non-palindromic sequences that are inversely oriented, exist as part of a complex with a modification methylase (EC 2.1.1.72). EcoP I, Hinf III, EcoP15 I

Type IV enzymes target modified DNA, e.g. methylated, hydroxymethylated and glucosyl-hydroxymethylated DNA. McrBC and Mrr systems of *E. coli.*

Restriction endonucleases classification

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non- specific location > 1000 bp away from recognition site	Cut both strands at a specific, usually palindromic recognition site (4-8 bp)	Cleavage of one strand, only 24-26 bp downstream of the 3′ recognition site
Restriction and modification	Single multifunctional enzyme	Separate nuclease and methylase	Separate enzymes sharing a common subunit
Nuclease subunit structure	Heterotrimer	Homodimer	Heterodimer
Cofactors	ATP, Mg2+, SAM	Mg2+	Mg2+ (SAM)
DNA cleavage requirements	Two recognition sites in any orientation	Single recognition site	Two recognition sites in a head-to-head orientation
Enzymatic turnover	No	Yes	Yes
DNA translocation	Yes	No	No
Site of methylation	At recognition site	At recognition site	At recognition site

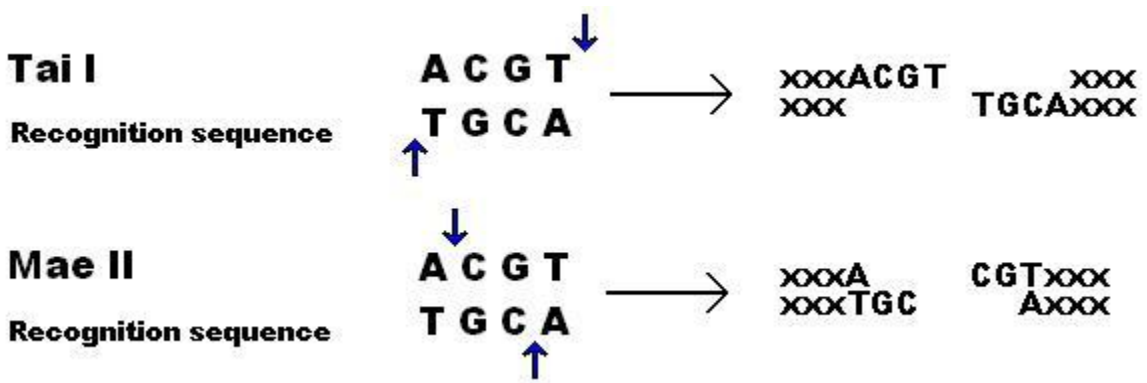
Isoschizomers and Neochischizomers

- **Isoschizomers** –are pairs of restriction enzymes specific to the same recognition sequence. For example, 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 identified enzymes that recognize that sequence are isoschizomers.

Isoschizomers are isolated from different strains of bacteria and therefore may require different reaction conditions.

- **Neochischizomers -**Restriction enzymes that recognize the same nucleotide sequence as their prototype but cleave at a different site.



Types of Restriction enzymes

Restriction Enzymes

- **Isoschizomer**
  - Enzymes that **recognize the same target** DNA sequence and **cleave it in the same way**
  - e.g. SphI and BbuI (CGTAC/G)
- **Neoschizomer**
  - Enzymes that **recognizes the same target** DNA sequence but **cleave at different points**
  - e.g. SmaI (CCC/GGG) and XmaI (C/CCGGG)
- **Isocaudomers**
  - Enzymes that **produce the same nucleotide extensions** but **have different recognition sites**
  - e.g. BamHI (G/GATCC) and Sau3AI (/GATC)

EXAMPLE

Isoschizomers		Neoschizomers	
Sac I	GAGCT↓C	Sma I	CCC↓GGG
Sst I	GAGCT↓C	Xma I	C↓CCGGG

Which of the following are iso/neoschizomers?

AvaI 5′...C↓YCGRG...3′ 3′...GRGCY↑C...5′	XmaI 5′...C↓CCGGG...3′ 3′...GGGCC↑C...5′	SalI 5′...G↓TCGAC...3′ 3′...CAGCT↑G...5′
PvuI 5′...CGAT↓CG...3′ 3′...GC↑TAGC...5′	BsrFI 5′...R↓CCGGY...3′ 3′...YGGCC↑R...5′	PacI 5′...TTAAT↓TAA...3′ 3′...AAT↑TAATT...5′

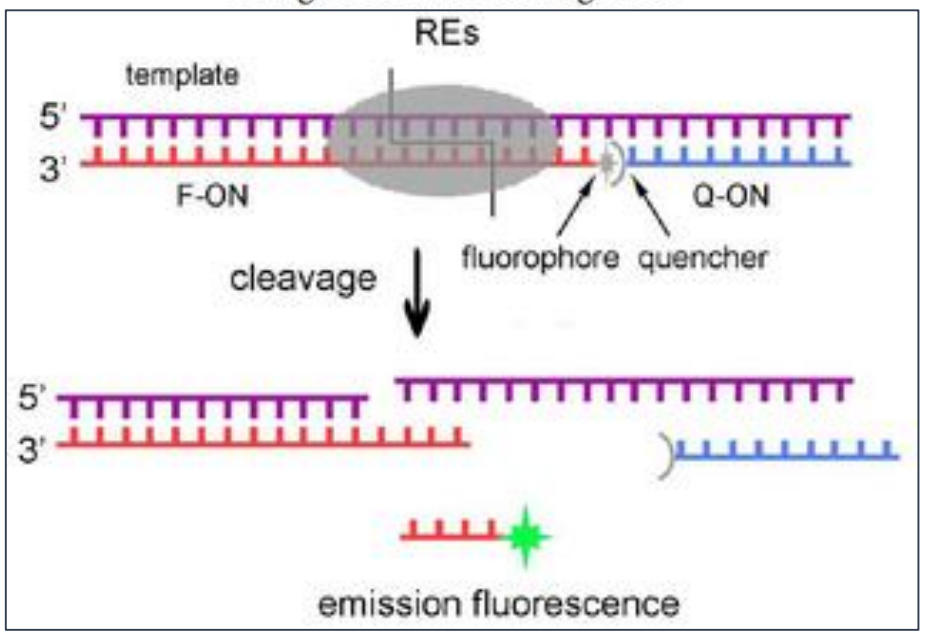
- **Type I restriction enzymes**

Type II enzymes (EC 3.1.21.4) cleave within or at short specific distances from a recognition site; most require magnesium; single function (restriction digestion) enzymes independent of methylase. EcoR I, BamH I, Hind III

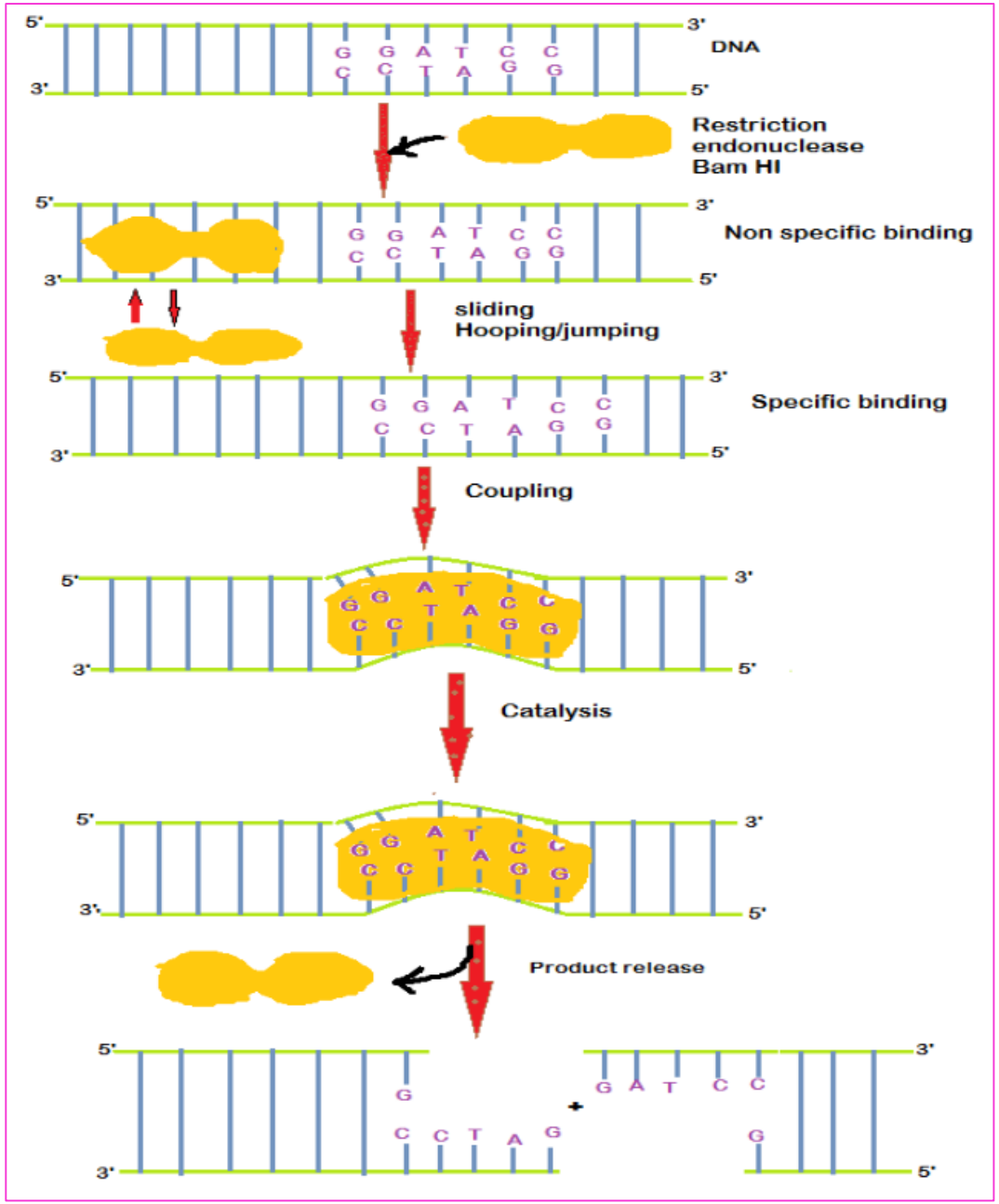
• Type II restriction enzymes

Restriction enzyme	Microbial source	Recognition sequence	Fragments
Alu I	Arthrobacter luteus	5'AG/CT3' 3'TC/GA5'	A-G C-T / T-C G-A — Blunt ends
BamHI	Bacillus amyloliquefaciens	5'G/GATCC3' 3'CCTAG/G5'	G / C-C-T-A-G — G-A-T-C-C / G — Sticky ends
EcoRI	Escherichia coli	5'G/AATTC3' 3'CCTAG/G5'	G / C-T-T-A-A — A-A-T-T-C / G — Sticky ends
HaeIII	Haemophilus aegyptus	5'GG/CC3' 3'CC/GG5'	G-G C-C / C-C G-G — Blunt ends
HindIII	Haemophilus influenza	5'A/AGCTT3' 3'TTCGA/A5'	A / T-T-C-G-A — A-G-C-T-T / A — Sticky ends

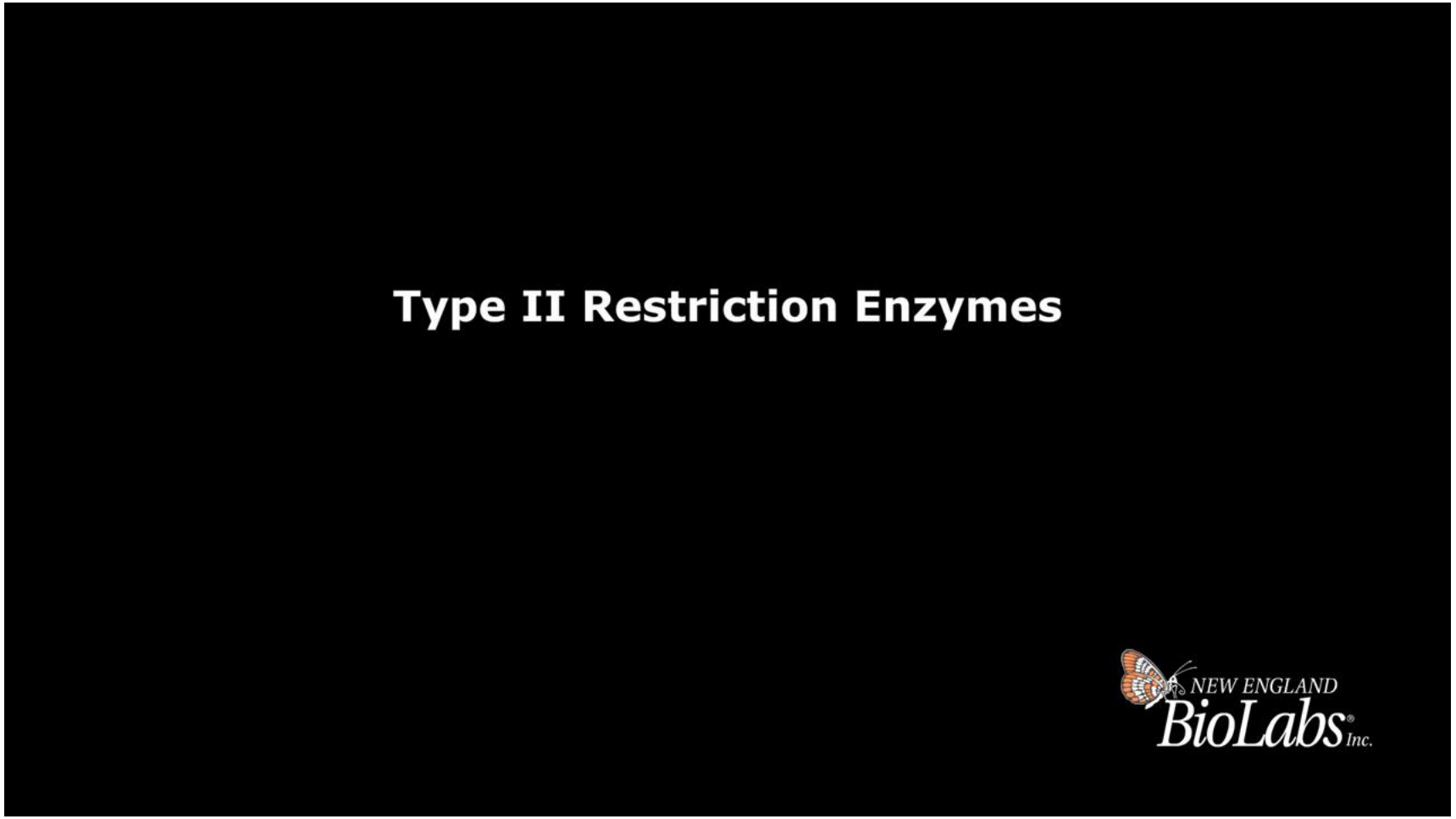
Table 4.1: Type II restriction enzyme with source, recognition and cleavage site.



Application of TypeII RE: FRET assay

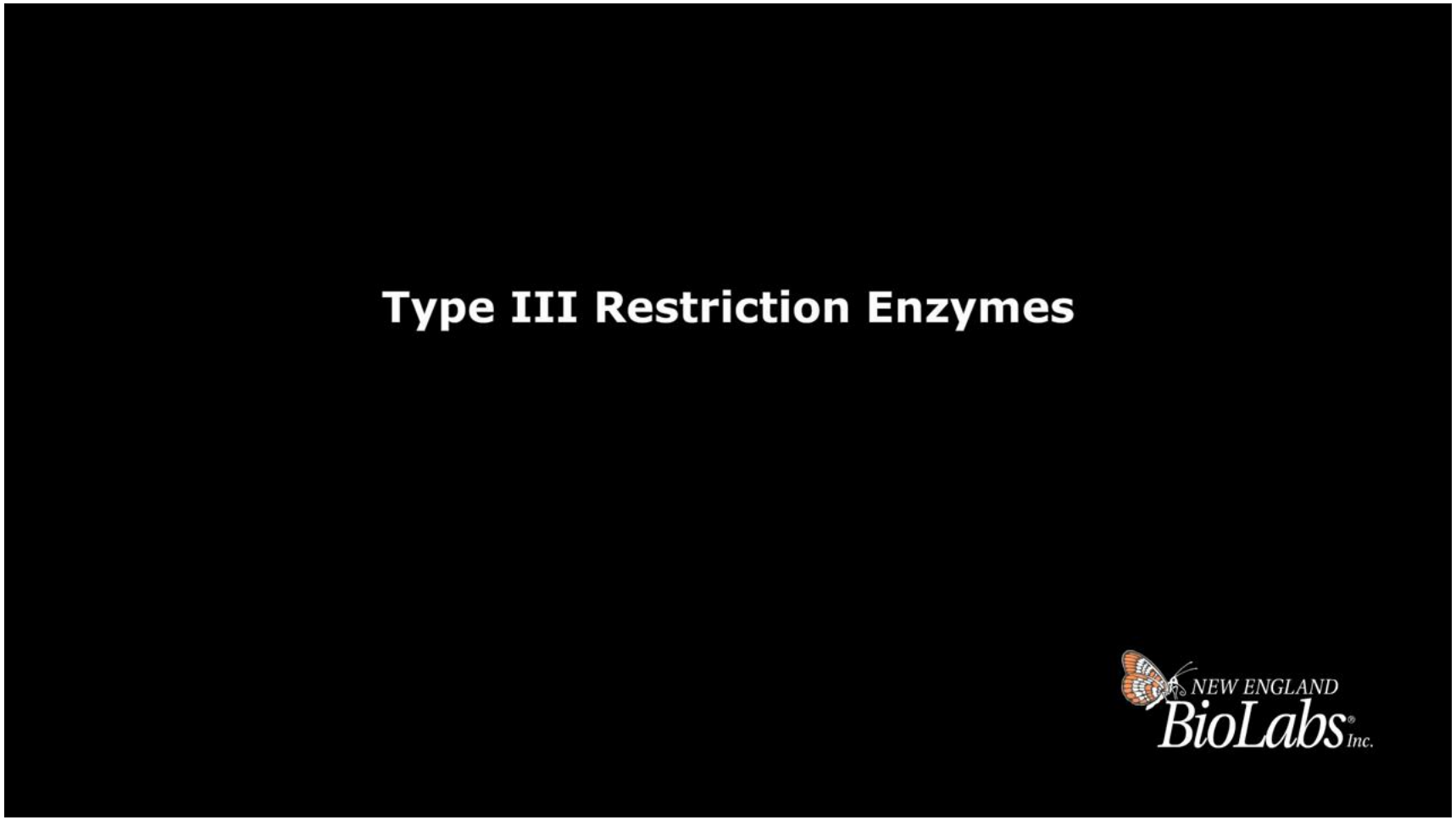


- **Type II restriction enzymes**



Type III enzymes (EC 3.1.21.5) cleave at sites a short distance from a recognition site;
Random 24-26 bp away from recognition site; require ATP (but do not hydrolyse it); S-adenosyl-L-methionine stimulates the reaction but is not required; exist as part of a complex with a modification methylase (EC 2.1.1.72).
EcoP I, Hind III, EcoP15 I

- **Type III restriction enzymes**



Applications

In **various applications** related to genetic engineering DNA is cleaved by using these restriction enzymes.

1. They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.

2. 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.

3.Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species

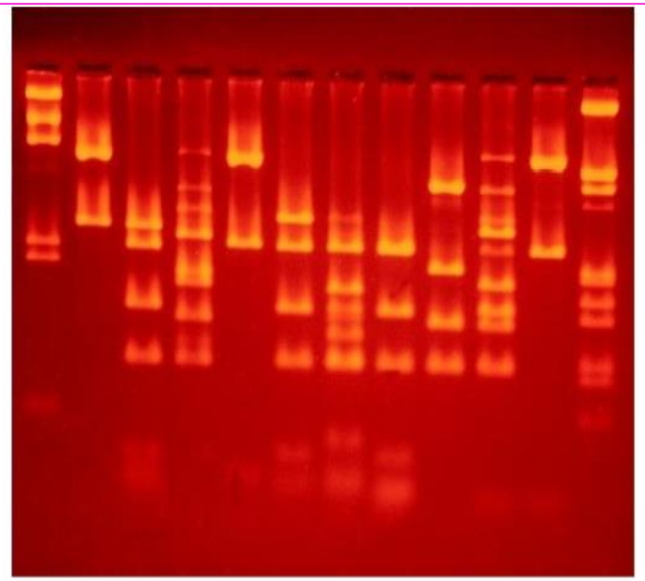
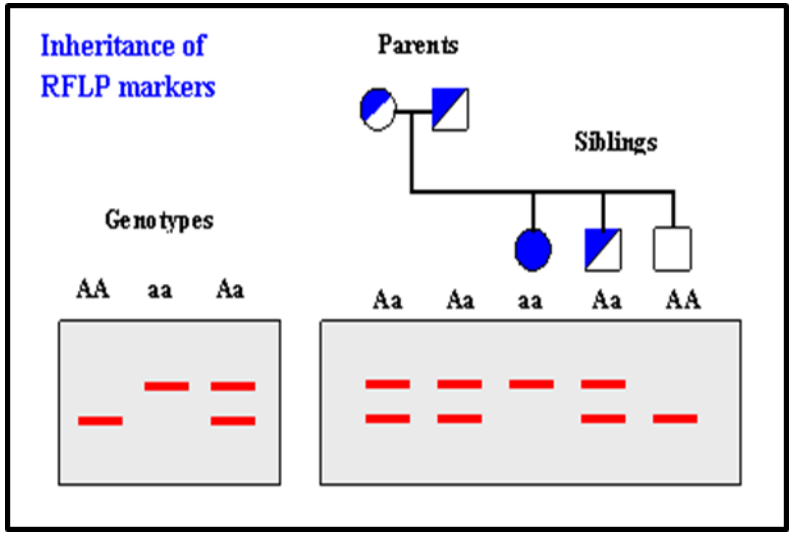


Fig 2-1.4.3: Cleaving a DNA sequence by a restriction enzyme creates a specific pattern .

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.



Star Activity

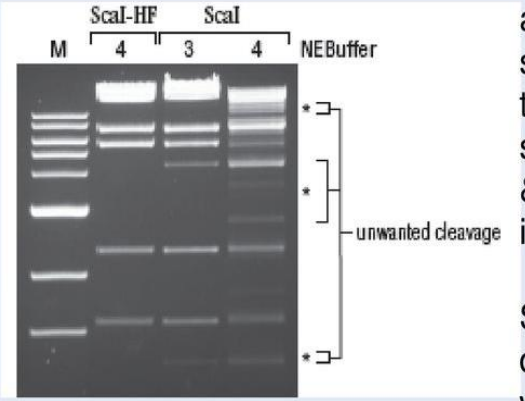
„Star activity“ of restriction enzymes

= relaxed specificity that is exhibited by some restriction enzymes under suboptimal reaction conditions.

	Correct cleavage	Star activity
*Eco*RI	G/AATTC	Pu/PuATPyPy /AATT

Factors provoking star activity:

- >5% glycerol
- low ionic strength (NaCl)
- high pH
- high enzyme:DNA ratio
- Mn^{2+} instead of Mg^{2+}



Star activity is due to the restriction enzyme cutting at DNA sequences in addition to the normal recognition site. This often occurs at sequences that contain subsets of the full sequence (e.g., ScaI site is AGTACT & star sites might include only the internal 4 bases (XGTACX).

ScaI-**HF** (high fidelity recombinant derivative of the ScaI enzyme without the star activity.

Deoxyribonuclease I (DNaseI):

An endonuclease which cleaves double - stranded DNA or single stranded DNA.

The cleavage preferentially occurs adjacent to pyrimidine (C or T) residues.

The major products are 5'-phosphorylated bi -, tri- and tetranucleotides.

Unlike restriction enzymes, DNase does not have any specific recognition/restriction site and cleave DNA sequence at random locations.

DNA Footprinting ..

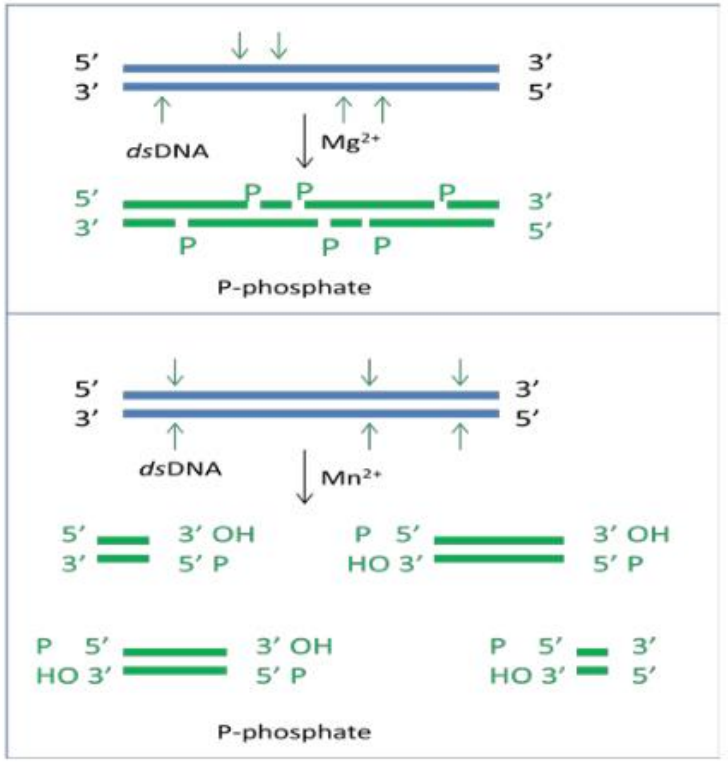


Fig 2-2.2 Action of DNase I in the presence of Mg^{+2} and Mn^{+2} ions. (Arrowhead denoting random site of cleavage in double stranded DNA by DNase I)

Some of the common applications of DNase I in rDNA technology have been mentioned below:

- Eliminating DNA contamination (e.g. plasmid) from preparations of RNA.
- Analyzing the DNA-protein interactions via DNA footprinting.
- Nicking DNA prior to radio-labeling by nick translation.

DNA footprinting is a method of investigating the sequence specificity of DNA-binding proteins in vitro. This technique can be used to study protein-DNA interactions both outside and within cells.

The simplest application of this technique is to assess whether a given protein binds to a region of interest within a DNA molecule.

Polymerase chain reaction (PCR) amplify and label region of interest that contains a potential protein-binding site, ideally amplicon is between 50 and 200 base pairs in length.

(i)Add protein of interest to a portion of the labeled template DNA; (ii)a portion should remain separate without protein, for later comparison.

Add a cleavage agent - a chemical or enzyme that will cut at random locations in a sequence independent manner.

The reaction should occur just long enough to cut each DNA molecule in only one location. A protein that specifically binds a region within the DNA template will protect the DNA it is bound to from the cleavage agent.

Run both samples side by side on a polyacrylamide gel electrophoresis. The portion of DNA template without protein will be cut at random locations, and thus when it is run on a gel, will produce a pattern.

The DNA template with the protein will result in a changed distribution pattern, the "footprint", where the DNA has been protected from the cleavage agent.

DNA electrophoretic mobility shift assay (EMSA)

The DNA electrophoretic mobility shift assay (EMSA) is used to study proteins binding to known DNA oligonucleotide probes and can be used to assess the degree of affinity or specificity of the interaction.

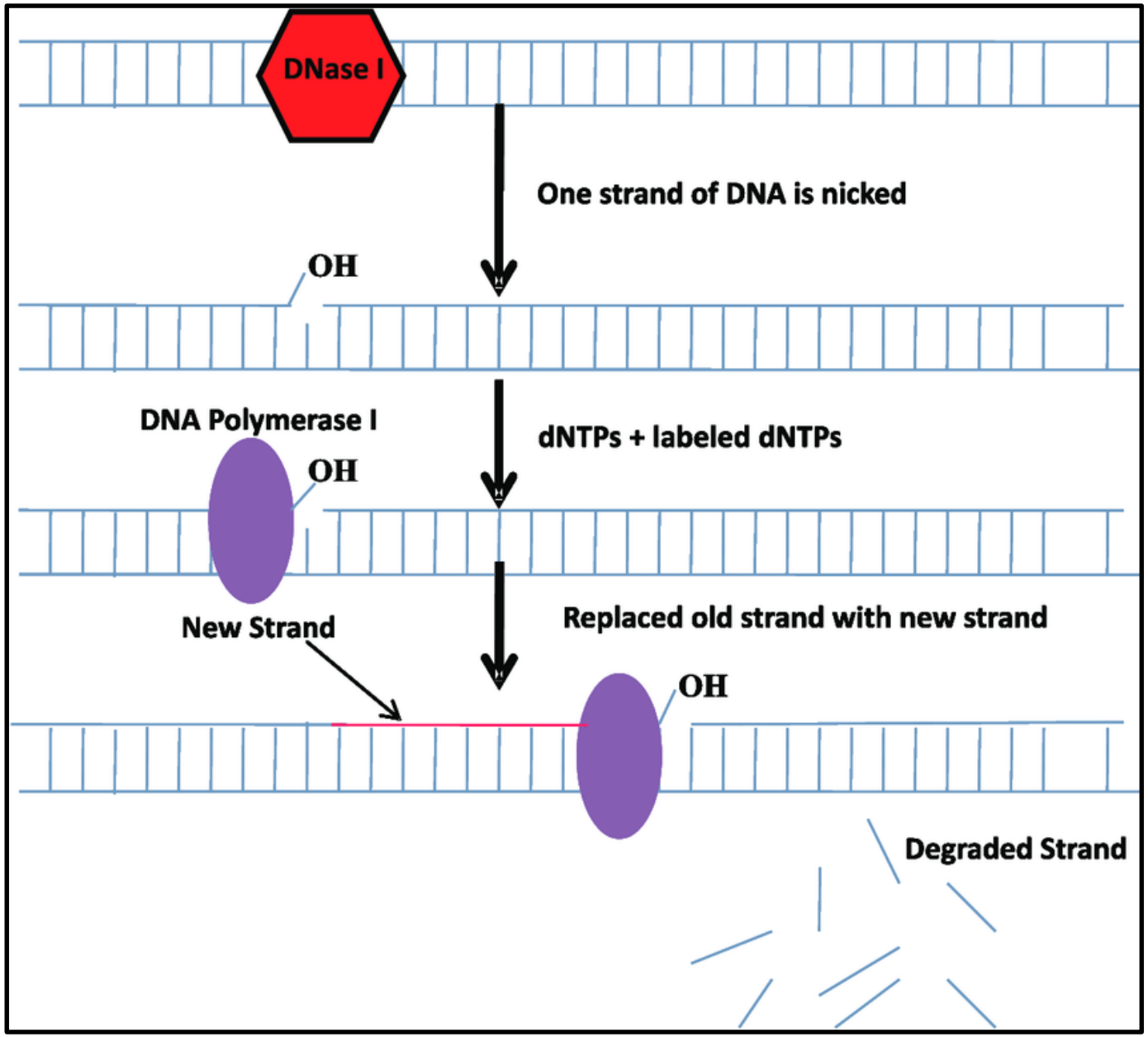
The technique is based on the observation that protein–DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis.

Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay.

Deoxyribonuclease II (DNaseII):

- It has a non-specific with optimal activity at acidic pH (4.5 -5.5) and conserved from human to *C.elegans*.

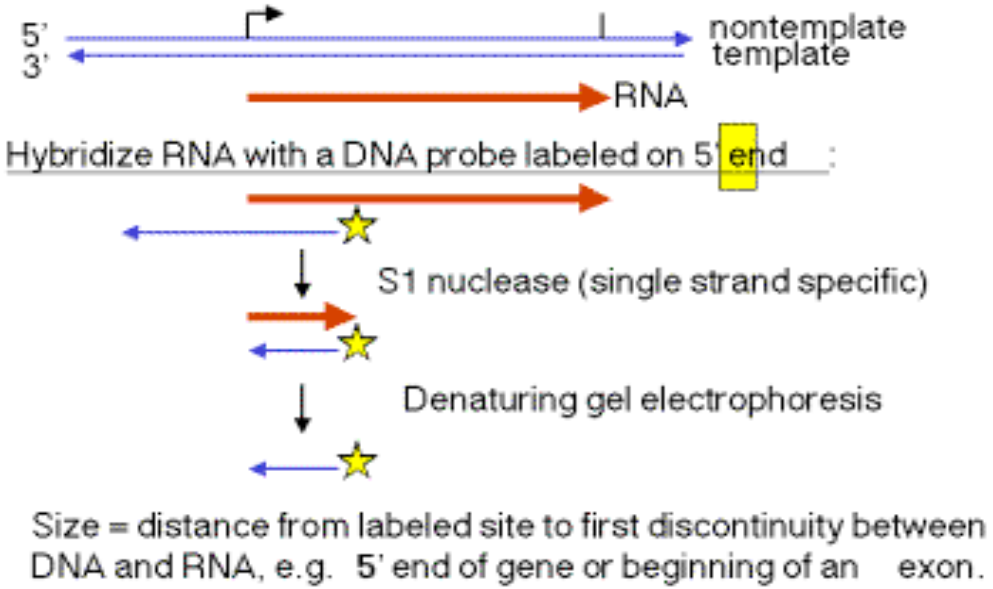
- Dnase II initially introduces multiple single stranded nicks in DNA backbone and finally generates 3’ phosphate groups by hydrolyzing phosphodiester linkages

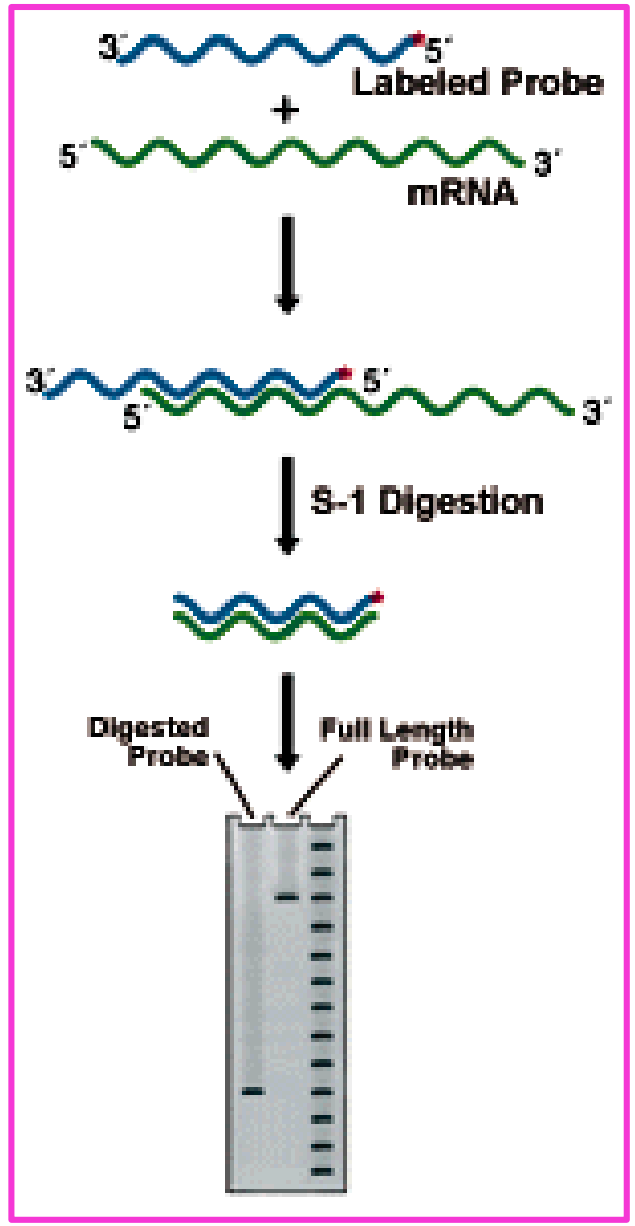


Nick translation (or head translation), developed in 1977 by Peter Rigby and Paul Berg, is **a tagging technique in molecular biology in** which DNA Polymerase I is used to replace some of the nucleotides of a DNA sequence with their labeled analogues, creating a tagged DNA sequence which can be used as a probe/ radiolabeling techniques.

S1 nuclease is an endonuclease specific for single-stranded DNA or RNA and can be used to **study nucleic acid hybridization, mapping RNA start sites and RNA splice sites**. This enzyme is five times more active on DNA than RNA, and it will digest all nucleic acids if the enzyme is added to the reaction in excess.

Mung bean nuclease enzymes can degrade single stranded DNA as well RNA.
Under high enzyme concentration, they can degrade double stranded DNA, RNA or even DNA/RNA hybrids. Mung bean nuclease can cleave single stranded DNA or RNA to produce 5' –phosphoryl mono and oligonucleotides. It has been used to create blunt end DNA by cleaving protruding ends from 5' ends.





Application: S1 mapping in RDT

Exonucleases

Exonucleases are enzymes that cleave DNA sequences in a polynucleotide chain from either the 5' or 3' end one at a time.

•Exonuclease, like endonuclease, is a hydrolyzing enzyme that cleaves the phosphodiester bond between the nucleotides.

APPLICATION

•(i)Exonulceases are important **during replication** as one of these enzymes works together with RNA polymerase II degrade the newly formed RNA primer present on the new transcript which is then replaced by DNA nucleotides.

•(ii)Exonuclease activity is also exploited during editing and proofreading DNA for errors.

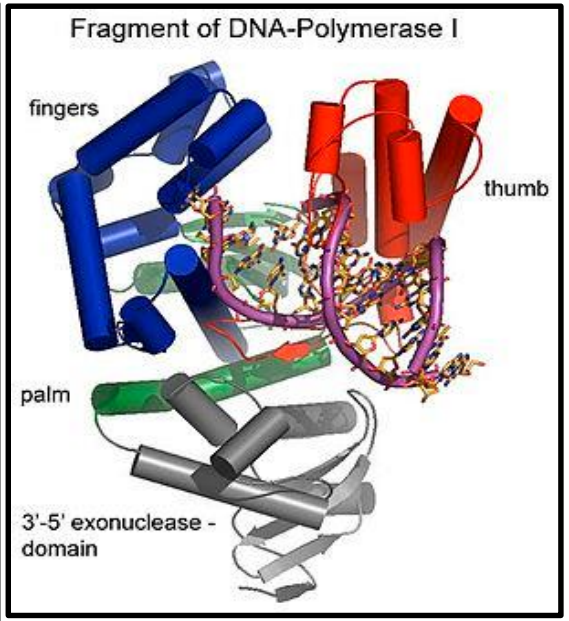
•The exonuclease in prokaryotes and eukaryotes are of three types; a decapping 5' to 3' exonuclease (Xrn1), an independent 5'to 3' exonuclease and a polyA-specific 3' to 5' exonuclease.

•All of these exonucleases are involved in the formation, replication, and transcription of RNAs.

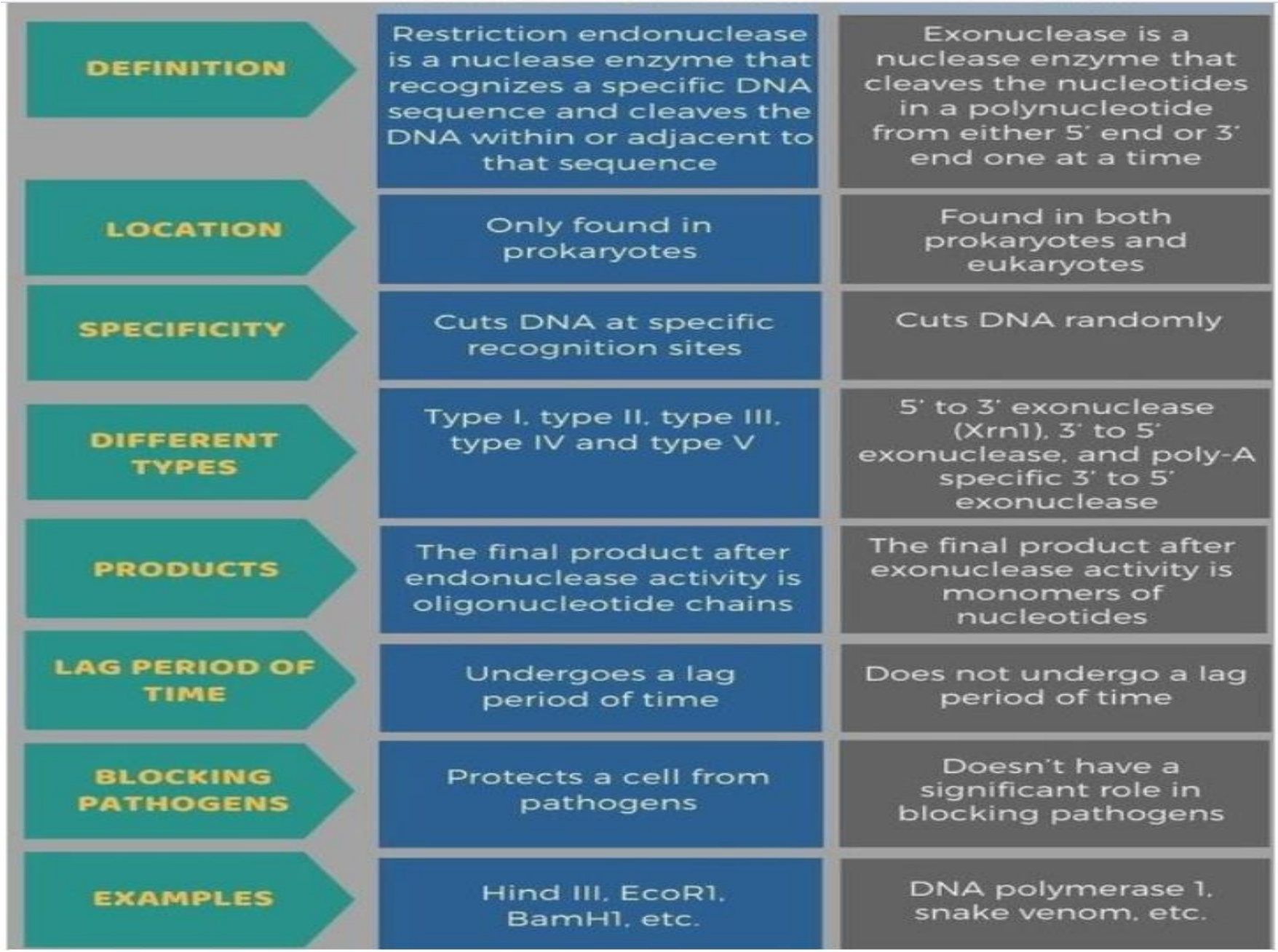
•Exonucleases, unlike endonucleases, do not have a lag period as they cleave the sequences from the ends, resulting in sticky ends.

•Similarly, exonucleases also cleave individual nucleosides from either of the ends instead of resulting in oligonucleotides.

•Exonuclease does not have defensive properties against the entry of pathogenic microbes

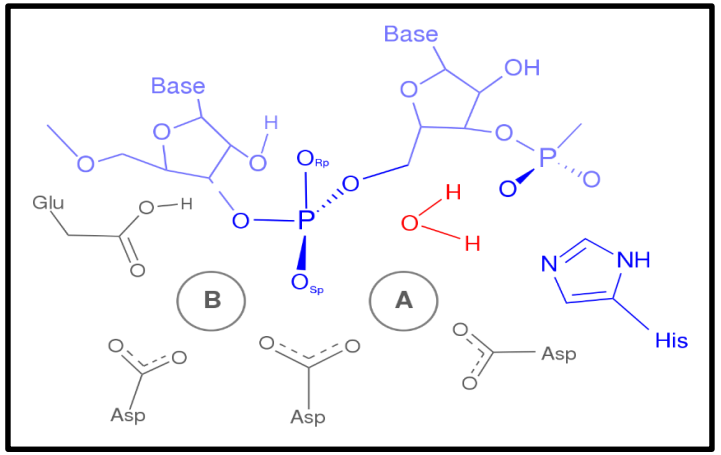


- One of the most important routes of RNA degradation in both archaea and eukaryotes is by the multi-protein exosome, which consists of multiple exoribonucleases.
- Besides, exonucleases are also found in the venoms of snakes and lizards. These toxins work by the cleavage of DNA coding for essential proteins within the body


DEFINITION
Restriction endonuclease is a nuclease enzyme that recognizes a specific DNA sequence and cleaves the DNA within or adjacent to that sequence
Exonuclease is a nuclease enzyme that cleaves the nucleotides in a polynucleotide from either 5' end or 3' end one at a time
LOCATION
Only found in prokaryotes
Found in both prokaryotes and eukaryotes
SPECIFICITY
Cuts DNA at specific recognition sites
Cuts DNA randomly
DIFFERENT TYPES
Type I, type II, type III, type IV and type V
5' to 3' exonuclease (Xrn1), 3' to 5' exonuclease, and poly-A specific 3' to 5' exonuclease
PRODUCTS
The final product after endonuclease activity is oligonucleotide chains
The final product after exonuclease activity is monomers of nucleotides
LAG PERIOD OF TIME
Undergoes a lag period of time
Does not undergo a lag period of time
BLOCKING PATHOGENS
Protects a cell from pathogens
Doesn't have a significant role in blocking pathogens
EXAMPLES
Hind III, EcoR1, BamH1, etc.
DNA polymerase 1, snake venom, etc.

Ribonuclease H

- Ribonuclease H (abbreviated RNase H or RNH) is a family of non-sequence-specific endonuclease enzymes that cleave the phosphodiester bonds of RNA in an RNA:DNA hybrid substrate via a hydrolytic mechanism.
- Members of the RNase H family can be found in nearly all organisms, from bacteria to archaea to eukaryotes.
- All RNases H have an active site centered on a conserved sequence motif composed of aspartate and glutamate residues, often referred to as the **DEDD motif.** These residues interact with catalytically required magnesium ions.
- Depending on the differences in their amino acid sequences, these RNases H are classified into type 1 and type 2 RNases H. Type 1 RNases H have prokaryotic and eukaryotic RNases H1 and retroviral RNase H. Type 2 RNases H have prokaryotic and eukaryotic RNases H2 and bacterial RNase H3.



Reaction mechanism for RNase H catalysis using two metal ions in the HIV-1 RNase H domain

- RNase HI is often used to destroy the RNA template after first-strand complementary DNA (cDNA) synthesis by reverse transcription.
- RNase HII can be used to degrade the RNA primer component of an Okazaki fragment or to introduce single-stranded nicks at positions containing a ribonucleotide.
- A variant of hot start PCR, known as RNase H-dependent PCR or rhPCR, has been described using a thermostable RNase HII from the hyperthermophilic archaeon *Pyrococcus abyssi*.

Because RNase H specifically degrades only the RNA in RNA:DNA duplexes, it is commonly used as a laboratory reagent in molecular biology


WHO? WHERE? WHEN? WHY? WHAT? HOW?