MODULE 2: LECTURE 1

ENZYMES IN GENETIC ENGINEERING: RESTRICTION NUCLEASES: EXO & ENDO NUCLEASES

2-1.1 Introduction:

A **restriction enzyme** is a nuclease enzyme that cleaves DNA sequence at a random or specific recognition sites known as restriction sites. In bacteria, restriction enzymes form a combined system (restriction + modification system) with modification enzymes that methylate the bacterial DNA. Methylation of bacterial DNA at the recognition sequence typically protects the own DNA of the bacteria from being cleaved by restriction enzyme.

There are two different kinds of restriction enzymes:

(1) Exonucleases catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5'to 3' direction or 3' to 5' direction. Example: exonuclease I, exonuclease II etc.

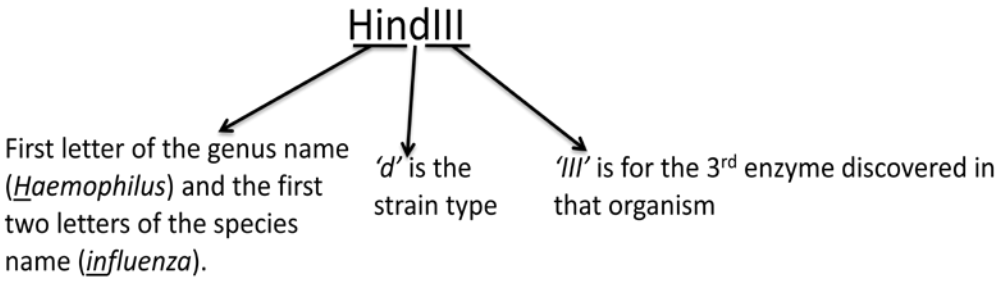
(2) Endonucleases can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. Example: EcoRI, Hind III, BamHI etc.

2-1.2History:

In 1970 the first restriction endonuclease enzyme *Hind*II 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.

2-1.3 Restriction Endonuclease Nomenclature:

Restriction endonucleases are named according to the organism in which they were discovered, using a system of letters and numbers. For example, *HindIII* (pronounced "*hindee-three*") was discovered in *Haemophilus influenza* (strain d). The Roman numerals are used to identify specific enzymes from bacteria that contain multiple restriction enzymes indicating the order in which restriction enzymes were discovered in a particular strain.



2-1.4 Classification of Restriction Endonucleases:

There are three major classes of restriction endonucleases based on the types of sequences recognized, the nature of the cut made in the DNA, and the enzyme structure:

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

2-1.4.1 Type I restriction enzymes:

- These enzymes have both restriction and modification activities. Restriction depends upon the methylation status of the target DNA.
- Cleavage occurs approximately 1000 bp away from the recognition site.
- The recognition site is asymmetrical and is composed of two specific portions in which one portion contain 3–4 nucleotides while another portion contain 4–5

nucleotides and both the parts are separated by a non-specific spacer of about 6–8 nucleotides.

- They require S-adenosylmethionine (SAM), ATP, and magnesium ions (Mg2+) for activity.
- These enzymes are composed of mainly three subunits, a specificity subunit that determines the DNA recognition site, a restriction subunit, and a modification subunit

2-1.4.2 Type II restriction enzymes:

- Restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification. Although the two enzymes recognize the same target sequence, they can be purified separately from each other.
- Cleavage of nucleotide sequence occurs at the restriction site.
- These enzymes are used to recognize rotationally symmetrical sequence which is often referred as palindromic sequence.
- These palindromic binding site may either be interrupted (e.g. BstEII recognizes the sequence 5´-GGTNACC-3´, where N can be any nucleotide) or continuous (e.g. KpnI recognizes the sequence 5´-GGTACC-3´).
- They require only Mg^{2+} as a cofactor and ATP is not needed for their activity.
- Type II endonucleases are widely used for mapping and reconstructing DNA *in vitro* because they recognize specific sites and cleave just at these sites.

The steps involved in DNA binding and cleavage by a type II restriction endonuclease:

- These enzymes have nonspecific contact with DNA and initially bind to DNA as dimmers.
- 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.

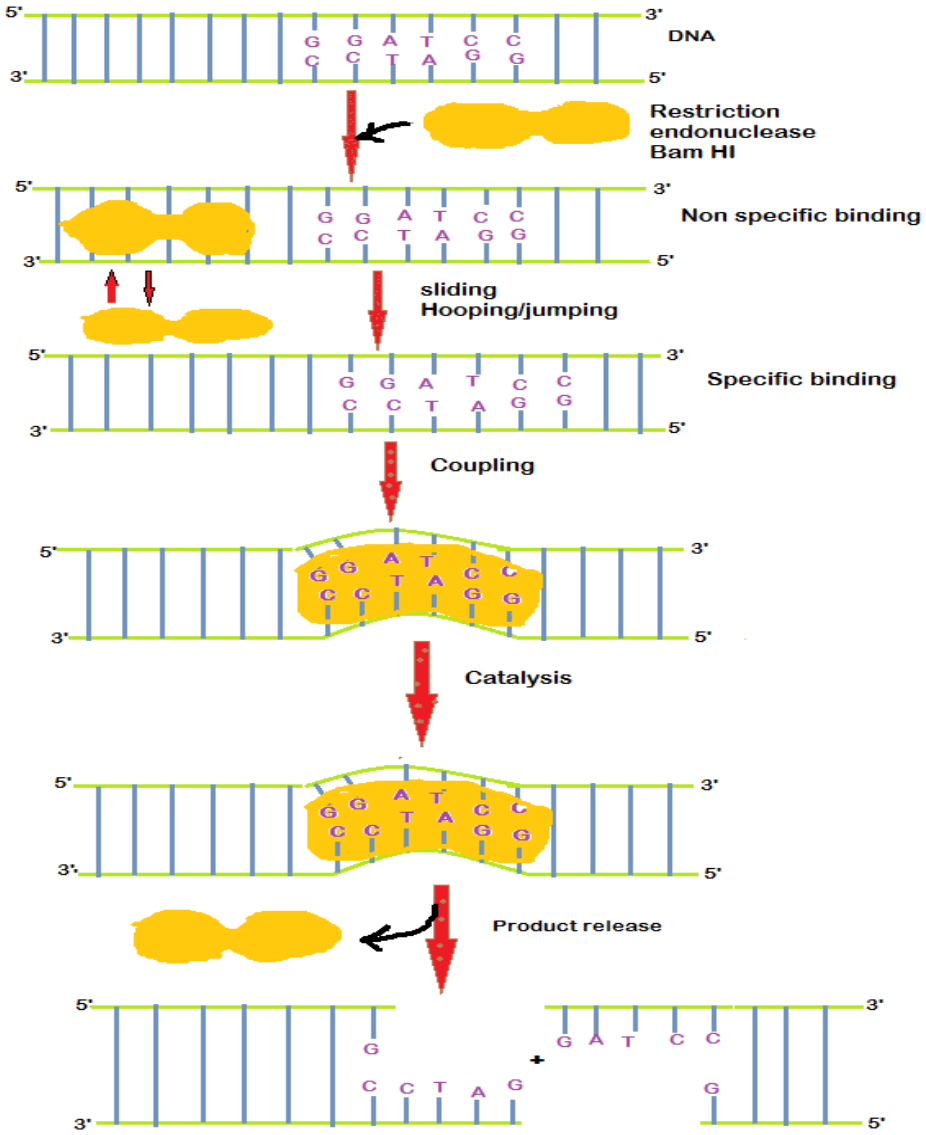


Fig 2-1.4.2: Structures of free, nonspecific, and specific DNA-bound forms of BamHI.

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.

2-1.4.3 Type III restriction enzymes:

- These enzymes recognize and methylate the same DNA sequence but cleave 24–26 bp away.
- They have two different subunits, in which one subunit (M) is responsible for recognition and modification of DNA sequence and other subunit (R) has nuclease action.
- Mg^{+2} ions, ATP are needed for DNA cleavage and process of cleavage is stimulated by SAM.
- Cleave only one strand. Two recognition sites in opposite orientation are necessary to break the DNA duplex.

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

Table 2-1.4: Comparative properties of restriction enzymes

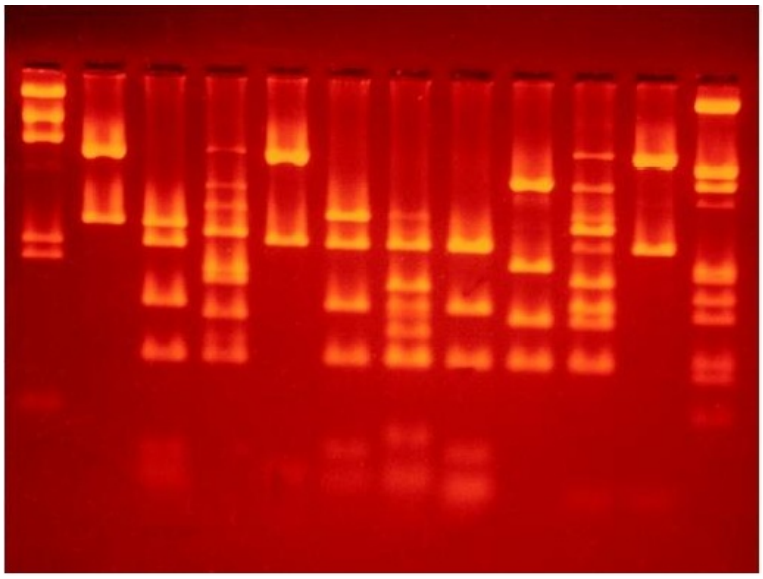


Fig 2-1.4.3: Cleaving a DNA sequence by a restriction enzyme creates a specific pattern .

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.

2-1.5 Cleavage Patterns of Some Common Restriction Endonucleases:

The recognition and cleavage sites and cleavage patterns of *HindIII*, *SmaI*, *EcoRI*, and *BamHI* are shown. Cleavage by an endonuclease creates DNA sequence with either a sticky end or blunt end. The blunt ended fragments can be joined to any other DNA fragment with blunt ends using linkers/adapters, making these enzymes useful for certain types of DNA cloning experiments.

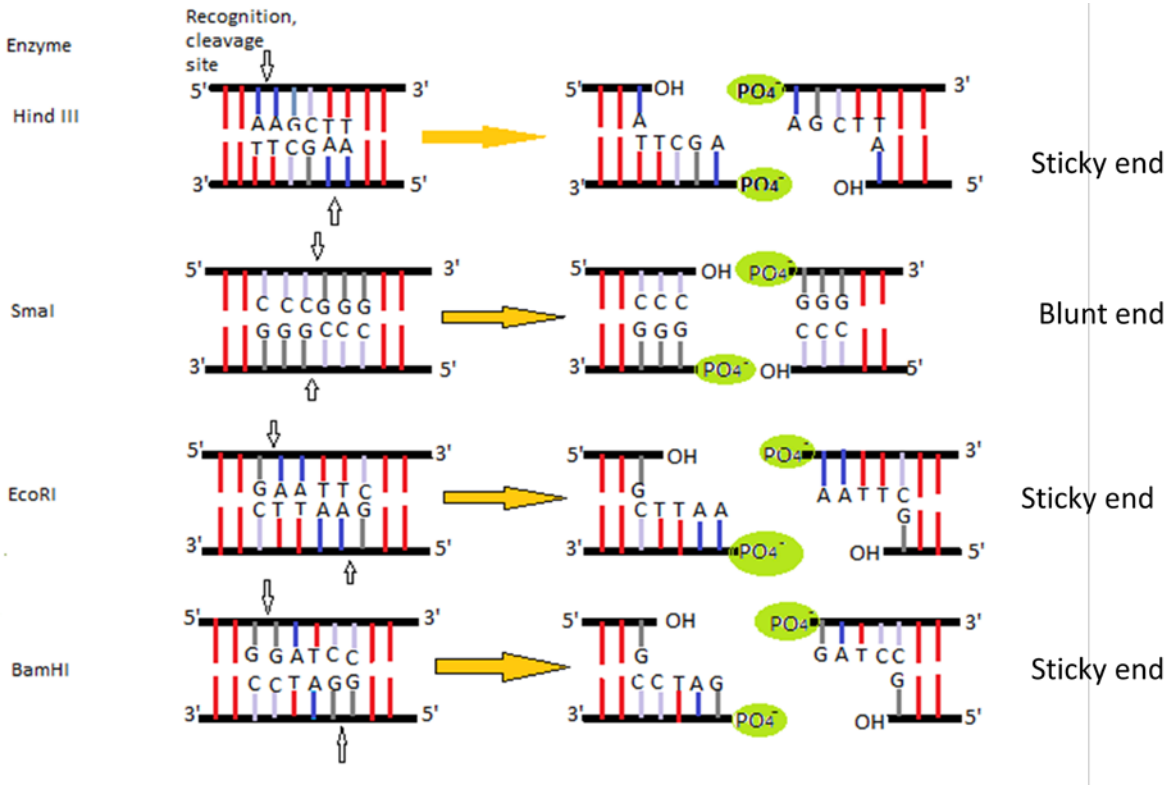


Fig 2-1.5: Cleavage patterns of *HindIII, SmaI, EcoRI* and *BamHI*

2-1.6 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.

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MODULE 2- LECTURE 2

ENZYMES IN MODIFICATION- POLYNUCLEOTIDE PHOSPHORYLASE, DNASE AND THEIR MECHANISM OF ACTION

2-2.1 Polynucleotide phosphorylase:

- Polynucleotide phosphorylase was first discovered from extracts of *Azotobacter agile* by Grunberg-Manago and Ochoa.
- Polynucleotide phosphorylase (PNPase) catalyzes the synthesis of long chain polyribonucleotides (RNA) in 5’ to 3’ direction from nucleotide diphosphates as precursors and reversibly catalyzes phosphorolytic cleavage of polyribonucleotides in 3’ to 5’ direction with a release of orthophosphate in presence of inorganic phosphate.
- PNPase is a bifunctional enzyme and functions in mRNA processing and degradation inside the cell.
- Structural and physiochemical studies in enzymes showed that it is formed of subunits. The arrangements of the subunits may vary from species to species which would alter their properties.
- These enzyme can catalyze not only the synthesis of RNA from the mixtures of naturally occurring ribonucleoside diphosphates, but also that of non-naturally occurring polyribonucleotides

2-2.1.1 Mechanism of action:

As mentioned earlier, polynucleotide phosphorylase is a bifunctional enzyme. The mechanism of action of this enzyme can be represented by following reactions:

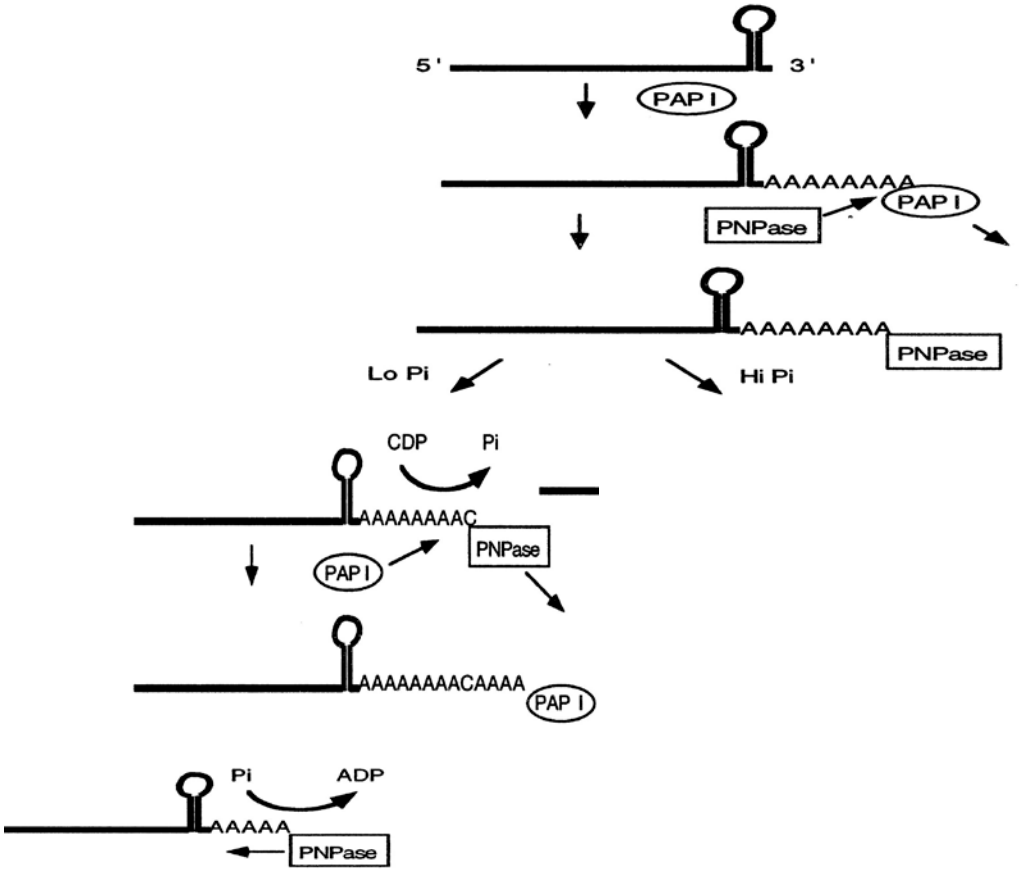

Fig 2-2.1: Schematic representation of the role of PNPase in poly(A) tail metabolism in *E. coli*.

(Source: Mohanty B K, and Kushner S R PNAS 2000; 97:11966-11971, copyright 2000, National Academy of Science*)*

In *E.coli*, polynucleotide phosphorylase regulates mRNA processing either by adding ribonucleotides to the 3' end or by cleaving bases in 3' to 5' direction. The function of PNPase depends upon inorganic phosphate (Pi) concentration inside the cell. The transcripts are polyadenylated using enzyme polyadenylate polymerase I (PAPI). After primary polyadenylylation of the transcript by PAP I, PNPase may bind to the 3′ end of the poly(A) tail. PNPase works either degradatively or biosynthetically inside the cell depending on the Pi concentration. Under high Pi concentration, it degrades the poly(A) tail releasing adenine diphosphates. If the Pi concentration is low, PAP I

initiates addition of one or more nucleotides to the existing poly (A) tail and in the process generates inorganic phosphate. On dissociation of PNPase, the 3′ end again is available to PAP I for further polymerization.

2-2.1.2 Function:

Different functions of Polynucleotide phosphorylase are:

- It is involved in mRNA processing and degradation in bacteria, plants, and in humans.
- It synthesizes long, highly heteropolymeric tails *in vivo* as well as accounts for all of the observed residual polyadenylation in poly(A) polymerase I deficient strains.
- PNPase function as a part of **RNA degradosome** in *E.coli* cell. RNA degradosome is a multicomponent enzyme complex that includes RNaseE (endoribinuclease), polynucleotide phosphorylase (3' to 5' exonuclease), RhlB helicase (a DEAD box helicase) and a glycolytic enzyme enolase. This complex catalyzes 3' to 5' exonuclease activity in presence of ATP. In eukaryotes, the exosomes are located in nucleus and cytoplasm. Degradsomes in bacteria and exosomes in eukaryotes are associated with processing, control and turnover of RNA transcripts.
- In rDNA cloning technology, it has been used to synthesize radiolabelled polyribonucleotides from nucleoside diphosphate monomers.

2-2.2 Deoxyribonuclease (DNase):

- A nuclease enzyme that can catalyze the hydrolytic cleavage of phosphodiester bonds in the DNA backbone are known as deoxyribonuclease (DNase).
- Based on the position of action, these enzymes are broadly classified as endodeoxyribonuclease (cleave DNA sequence internally) and exodeoxyribonuclease (cleave the terminal nucleotides).

- Unlike restriction enzymes, DNase does not have any specific recognition/restriction site and cleave DNA sequence at random locations.
- There is a wide variety of deoxyribonucleases known which have different substrate specificities, chemical mechanisms, and biological functions. They are:

1) **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. It requires divalent ions (Ca^{2+} and Mn^{2+}/Mg^{2+}) for its activity and creates blunt ends or 1-2 overhang sequences.

DNaseI is the most widely used enzyme in cloning experiments to remove DNA contamination from mRNA preparation (to be used for cDNA library preparation, northern hybridization, RT-PCR etc). The mode of action of DNaseI varies according to the divalent cation used.

In the presence of magnesium ions (Mg^{+2}), DNaseI hydrolyzes each strand of duplex DNA producing single stranded nicks in the DNA backbone, generating various random cleavages.

On the other hand, in the presence of manganese ions (Mn^{+2}), DNaseI cleaves both strands of a double stranded DNA at approximately the same site, producing blunt ended DNA fragments or with 1-2 base overhangs. The two major DNases found in metazoans are: deoxyribonuclease I and deoxyribonuclease II.

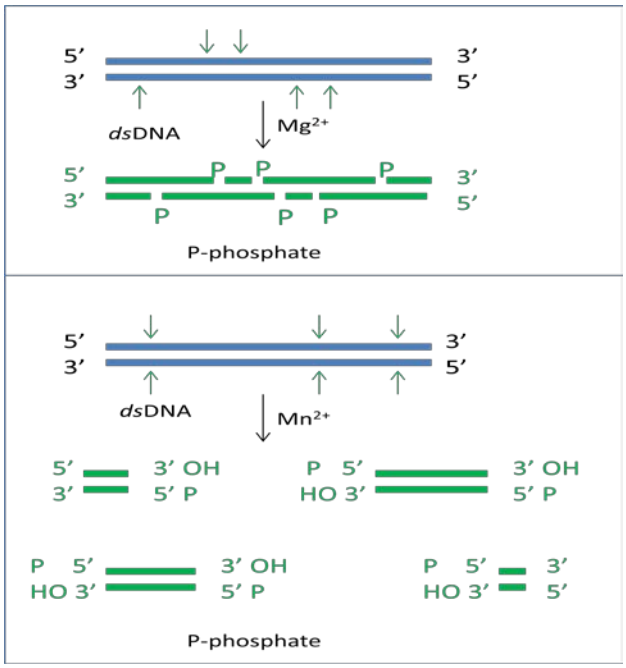

Fig 2-2.2 Action of DNase I in the presence of Mg^{+2} and Mn^{+2} ions. (Arrowhead denoting random site of cleavage in double stranded DNA by DNase I)

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.

2) **DeoxyribonucleaseII (DNaseII)**:

It is a non-specific endonuclease with optimal activity at acidic pH (4.5-5.5) and conserved from human to *C.elegans*.. It does not require any divalent cation for its activity. DNaseII initially introduces multiple single stranded nicks in DNA backbone and finally generates 3' phosphate groups by hydrolyzing phosphodiester linkages.

This enzyme releases 3'phosphate groups by hydrolyzing phosphodiester linkage and creating nicks in the DNA backbone. DNaseII acts by generating multiple single stranded nicks followed by production of acid soluble nucleotides and oligonucleotides.

The catalytic site of the enzyme contains three histidine residues which are essential for enzyme activity.

Some of the common applications of DNase II are as follows:

- DNA fragmentation
- Molecular weight marker
- Cell apoptosis assays etc.

3)**Exonuclease III:**

Exonuclease III is a globular enzyme which has 3'→5' exonuclease activity in a double stranded DNA. The template DNA should be double stranded and the enzyme does not cleave single stranded DNA. The enzyme shows optimal activity with blunt ended sequences or sequences with 5' overhang.

Exonuclease III enzyme has a bound divalent cation which is essential for enzyme activity. The mechanism of the enzyme can be affected by variation in temperature, monovalent ion concentration in the reaction buffer, and structure and concentration of 3'termini. The enzyme shows optimal activity at 37°C at pH 8.0.

Various application of exonuclease III in molecular cloning experiments are:

- To generate template for DNA sequencing
- To generate substrate for DNA labeling experiments
- Directed mutagenesis
- DNA-protein interaction assays (to find blockage of exonuclease III activity by protein-DNA binding) etc.

4) **Mung bean nuclease:**

As the name suggest, this nuclease enzyme is isolated from mung bean sprouts (*Vigna radiata*). 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 requires Zn^{2+} ion for its activity and shows optimal activity at 37°C. The enzyme works in low salt concentration (25mM ammonium acetate) and acidic pH (pH 5.0). Treatment with EDTA or SDS results in irreversible inactivation of the enzyme.

Mung bean nuclease is less robust than S1 nuclease and easier to handle. It has been used to create blunt end DNA by cleaving protruding ends from 5' ends. This enzyme cannot produce nicks in a double stranded DNA but at higher concentration, it can generate nicks and cleave double stranded DNA.

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MODULE 2-LECTURE-3

ENZYMES IN MODIFICATION- PHOSPHATASES AND METHYLASES AND THEIR MECHANISM OF ACTION

2-3.1 Phosphatase:

- Phosphatase catalyses the cleavage of a phosphate (PO_4^{-2}) group from substrate by using a water molecule (hydrolytic cleavage).
- This reaction is not reversible.
- This shows totally opposite activity from enzyme like kinase and phosphorylase that add a phosphate group to their substrate. On the basis of their activity there are two types of phosphatase i.e acid phosphatase and alkaline phosphatase. In both forms the alkaline phosphatase are most common.
- Special class of phosphatase that remove a phosphate group from protein, called "Phosphoprotein phosphatase".

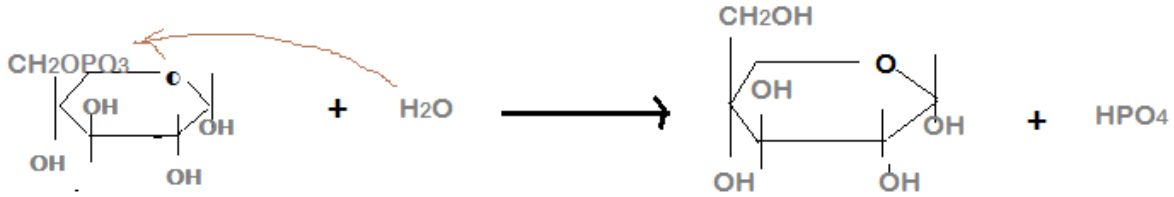

Fig 2-3.1: Schematic representation of hydrolytic cleavage of phosphate group ($-PO_4^{-2}$).

2-3.1.1 Acid phosphatase:

- It shows its optimal activity at pH between 3 and 6, e.g. a lysosomal enzyme that hydrolyze organic phosphates liberating one or more phosphate groups. They are found in prostatic epithelial cells, erythrocyte, prostatic tissue, spleen, kidney etc.

2-3.1.2 Alkaline phosphatase:

- Homodimeric enzyme which catalyzes reactions like hydrolysis and transphosphophorylation of phosphate monoester.
- They show their optimal activity at pH of about 10.

- Alkaline phosphatase was the first zinc enzyme discovered having three closed spaced metal ion. Two Zn^{+2} ions and one Mg^{+2} ion, in which Zn^{+2} ions are bridges by *Asp* 51. The mechanism of action is based on reaction where a covalent serine – phosphate intermediate is formed to produce inorganic phosphate and an alcohol.
- In human body it is present in four isoforms, in which three are tissue specific isoform i.e. placental, germ cell, intestinal and one is non tissue specific isoform. The genes that encode for tissue specific isoforms are present on chromosome -2 p37-q37, while the genes for one non tissue specific are present on chromosome -1 p34- p36.1.
- During post-translational modification, alkaline phosphatase is modified by N-glycosylation. It undergoes a modification through which uptake of two Zn^{+2} ion and one Mg^{+2} ion occurs which is important in forming active site of that enzyme. Alkaline phosphatases are isolated from various sources like microorganisms, tissue of different organs, connective tissue of invertebrate and vertebrate, and human body.

Fig 2-3.1.2: Action of alkaline phosphatase

There are several AP that are used in gene manipulation-

- **Bacterial alkaline phosphatase (BAP) -** Bacterial alkaline phosphate is a phosphomonoester that hydrolyzes 3' and 5' phosphate from nucleic acid (DNA/ RNA). It more suitably removes phosphate group before end labeling and remove phosphate from vector prior to insert ligation. BAP generally shows optimum activity at temperature 65°C. BAP is sensitive to inorganic phosphate so in presence of inorganic phosphates activity may reduce.

- **Calf intestinal alkaline phosphatase (CIP) –** It is isolated from calf intestine, which catalyzes the removal of phosphate group from 5' end of DNA as well as RNA. This enzyme is highly used in gene cloning experiments, as to make a construct that could not undergo self-ligation. Hence after the treatment with CIP, without having a phosphate group at 5' ends a vector cannot self ligate and recircularise. This step improves the efficiency of vector containing desired insert.
- **Shrimp alkaline phosphatase (SAP) -** Shrimp alkaline phosphatase is highly specific, heat labile phosphatase enzyme isolated from arctic shrimp (*Pandalus borealis*). It removes 5' phosphate group from DNA, RNA, dNTPs and proteins. SAP has similar specificity as CIP but unlike CIP, it can be irreversibly inactivated by heat treatment at 65°C for 15mins. SAP is used for 5' dephosphorylation during cloning experiments for various application as follows:
  - Dephosphorylate 5'-phosphate group of DNA/RNA for subsequent labeling of the ends.
  - To prevent self-ligation of the linearized plasmid.
  - To prepare PCR product for sequencing.
  - To inactivate remaining dNTPs from PCR product (for downstream sequencing appication).

Two primary uses for alkaline phosphatase in DNA modification:

- Removing 5' phosphate from different vector like plasmid, bacteriophage after treating with restriction enzyme. This treatment prevents self ligation because unavailability of phosphate group at end. So, this treatment greatly enhances the ligation of desired insert. During ligation of desired insert, the complementary ends of the insert and vector will come to proximity of each other (only for sticky ends but not for blunt ends). One strand of the insert having 5'-phosphate will ligate with the 3'OH of the vector and the remaining strand will have a nick. This nick will be sealed in the next step by ligase enzyme in the presence of ATP. It is used to remove 5' phosphate from fragment of DNA prior to labeling with radioactive phosphate.

2-3.2 Methylase:

- Methyltransferase or methylase catalyzes the transfer of methyl group (-CH_3) to its substrate. The process of transfer of methyl group to its substrate is called methylation.
- Methylation is a common phenomenon in DNA and protein structure.
- Methyltransferase uses a reactive methyl group that is bound to sulfur in S-adenosyl methionine (SAM) which acts as the methyl donor.
- Methylation normally occurs on cytosine (C) residue in DNA sequence. In protein, methylation occurs on nitrogen atom either on N-terminus or on the side chain of protein.
- DNA methylation regulates gene or silence gene without changing DNA sequences, as a part of epigenetic regulation.
- In bacterial system, methylation plays a major role in preventing their genome from degradation by restriction enzymes. It is a part of restriction – modification system in bacteria.

Methyltransferase can be classified in three groups:

a) m6A-generates N6 methyladenosine,

b) m4C-generates N4 methylcytosine,

c) m5C-generatesN5 methylcytosine.

m6A and m4C methyltransferase are primarily found in prokaryotes. These enzymes are responsible for methylation of DNA sequences in order to prevent the host from digesting its own genome via its restriction enzyme.

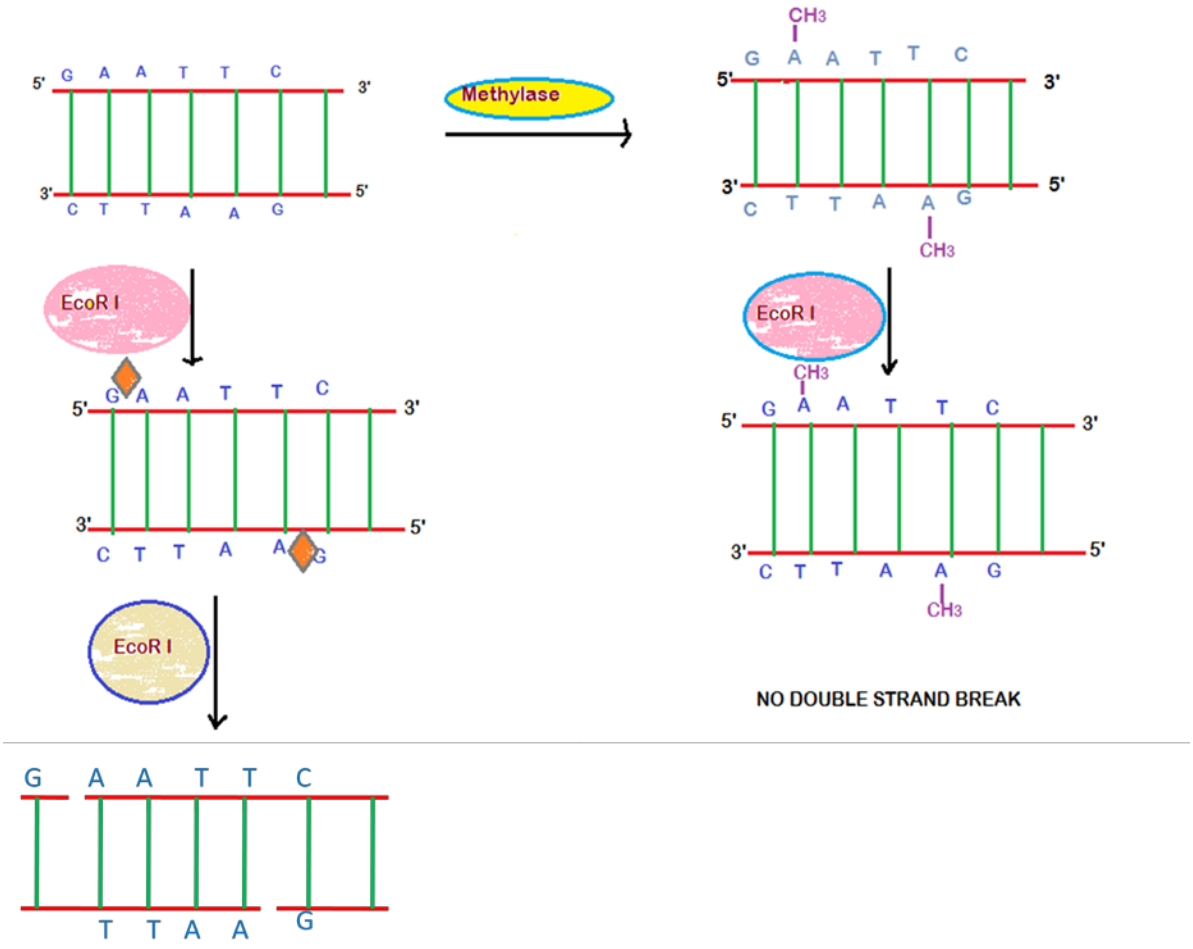

Fig 2-3.2: Activity of restriction and methylase enzymes

Restriction enzyme *EcoR*I cleaves within the recognition sequence if the DNA is unmethylated. On methylation by methylases, the restriction enzyme *EcoR*I is inhibited from cleaving within the restriction site.

Some common examples of methytransferases are: DNA adenyl methytransferase (DAM), histone methyltransferase, O-methyltransferase etc. DAM methylase is generally used in recombinant DNA technology which can methylate adenine (A) in the sequence 5'GATC3'. This enzyme can methylate a newly synthesized DNA strand on specific sites.

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MODULE 2-LECTURE 4

ENZYMES IN MODIFICATION- LIGASES, POLYNUCLEOTIDE KINASE, RNASE AND THEIR MECHANISM OF ACTION

2-4.1 Ligases:

- DNA ligase catalyses the formation of phosphodiester bond between two deoxynucleotide residues of two DNA strands.
- DNA ligase enzyme requires a free hydroxyl group at the 3´ -end of one DNA chain and a phosphate group at the 5´-end of the other and requires energy in the process.
- *E.coli* and other bacterial DNA ligase utilizes NAD^+ as energy donor, whereas in T4 bacteriophage, T4 DNA ligase uses ATP as cofactor.
- The role of DNA ligase is to seal nicks in the backbone of double-stranded DNA after lagging strand formation to join the okazaki fragments.
- This joining process is essential for the normal synthesis of DNA and for repairing damaged DNA. It has been exploited by genetic engineers to join DNA chains to form recombinant DNA molecules. Usually single stranded break are repaired using the complimentary strand as the template but sometimes double stranded breaks can also be repaired with the help of DNA ligase IV.
- The most widely used DNA ligase is isolated from T4 bacteriophage. T4 DNA ligase needs ATP as a cofactor. The enzyme from *E. coli* uses cofactor NAD. Except this, the catalysis mechanism is somewhat similar for both the ligases. The role of cofactor is splitting and forming an enzyme-AMP complex which further aids in formation of phosphodiester bonds between hydroxyl and phosphate groups by exposing them.

2-4.1.1 Mechanism of Action of DNA Ligases:

- ATP, or NAD+, reacts with the ligase enzyme to form a covalent enzyme–AMP complex in which the AMP is linked to ε-amino group of a lysine residue in the active site of the enzyme through a phosphoamide bond.
- The AMP moiety activates the phosphate group at the 5´-end of the DNA molecule to be joined. It is called as the donor.
- The final step is a nucleophilic attack by the 3´-hydroxyl group on this activated phosphorus atom which acts as the acceptor. A phosphodiester bond is formed and AMP is released.
- The reaction is driven by the hydrolysis of the pyrophosphate released during the formation of the enzyme–adenylate complex. Two high-energy phosphate bonds are spent in forming a phosphodiester bond in the DNA backbone with ATP serving as energy source.
- The temperature optimum for T4 DNA ligase mediated ligation *in vitro* is 16˚C. However ligation is also achieved by incubation at 4˚C by incubating over night or at room temperature condition by incubating for 30 minutes.
- Adenylate and DNA-adenylate are the important intermediates of the phosphodiester bond forming pathway.

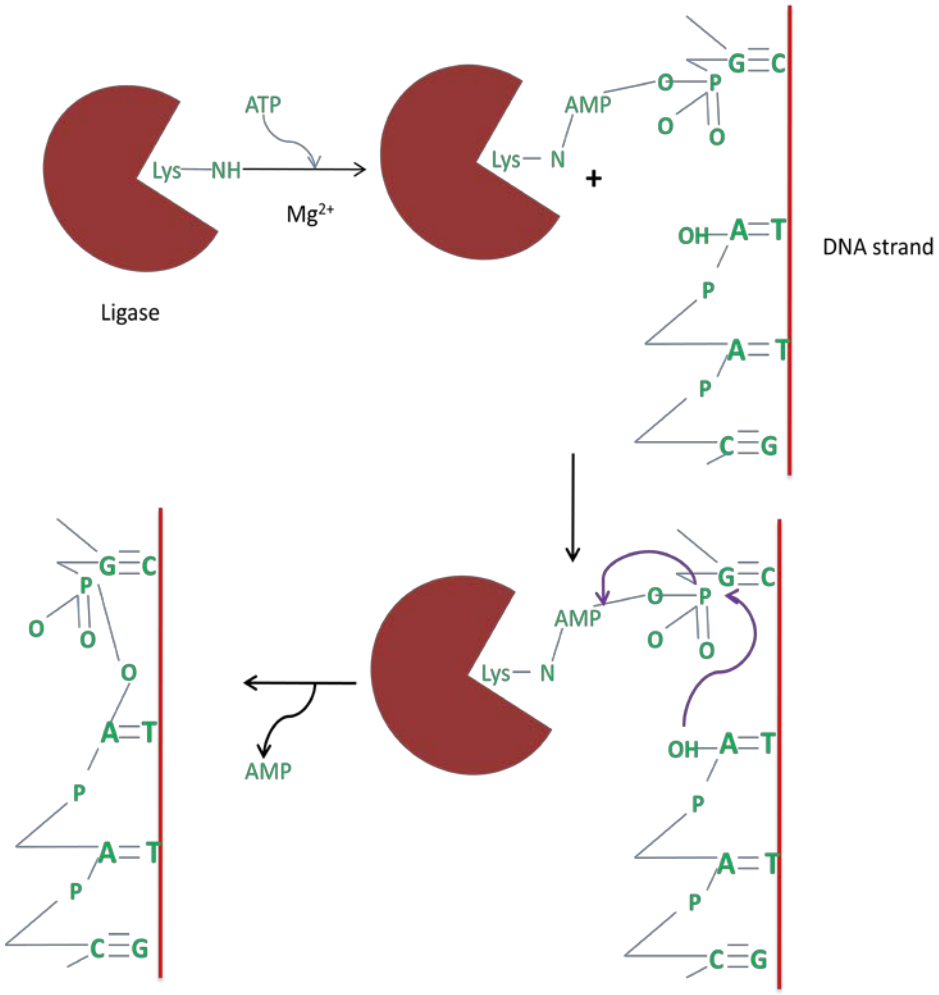

Fig 2-4.1.1: The mechanism of DNA joining by DNA ligase.

2-4.1.2 Application:

- DNA ligase enzyme is used by cells to join the “okazaki fragments” during DNA replication process. In molecular cloning, ligase enzyme has been routinely used to construct a recombinant DNA. Followings are some of the examples of application of ligase enzyme in molecular cloning.Joining of adapters and linkers to blunt end DNA molecule.

- Cloning of restricted DNA to vector to construct recombinant vector.

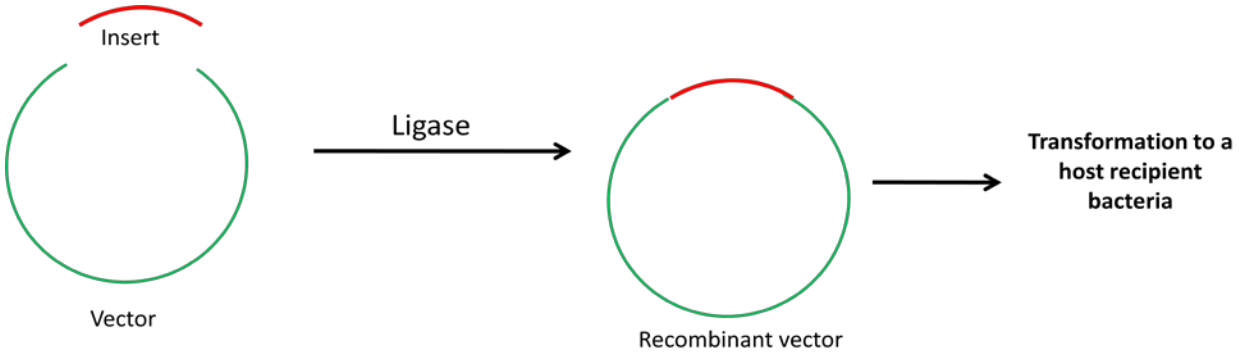

Fig 2-4.1.2: Ligation of a gene fragment into the vector and transformation of the cell.

2-4.2 Polynucleotide Kinase:

- PNK is a homotetramer with phosphatase activity at 3’ end and kinase activity at 5’ end with a tunnel like active site. The active site has side chains which interact with NTP donor’s beta-phosphate and 3’ phosphate of acceptor with an acid which activated 5’ –OH. Lys-15 and Ser-16 are important for the kinase activity of the enzyme.

- The basic residues of active site of PNK interact with the negatively charged phosphates of the DNA.

- Polynucleotide kinase (PNK) catalyzes the transfer of a phosphate group (PO_4^{-2}) from γ position of ATP to the 5' end of either DNA or RNA and nucleoside monophosphate.

- PNK can convert 3' PO_4/5' OH ends into 3' PO_4/5' PO_4 ends which blocks further ligation by ligase enzyme.
- PNK is used to label the ends of DNA or RNA with radioactive phosphate group.
- T4 polynucleotide kinase is the most widely used PNK in molecular cloning experiments, which was isolated from T4 bacteriophage infected *E.coli.*

PNK carries out two types of enzymatic activity:

- ***Forward reaction****: γ-phosphate is transferred from ATP to the 5' end of a polynucleotide (DNA or RNA). 5' phosphate is not present either due to chemical synthesis or dephosphorylation. The 5' OH nucleophile is activated by abstraction of the proton. Asp35 of PNK forms the co-ordinate bond with 5' OH and attacks γ phosphorus forming an intermediate.*
- **Exchange reaction:** target DNA or RNA having a 5' phosphate is incubated with an excess of ADP - where PNK transfers the phosphate from the nucleic acid to an ADP, forming ATP. PNK then performs a forward reaction and transfer a phosphate from ATP to the target nucleic acid. Exchange reaction is used to label with radioactive phosphate group.

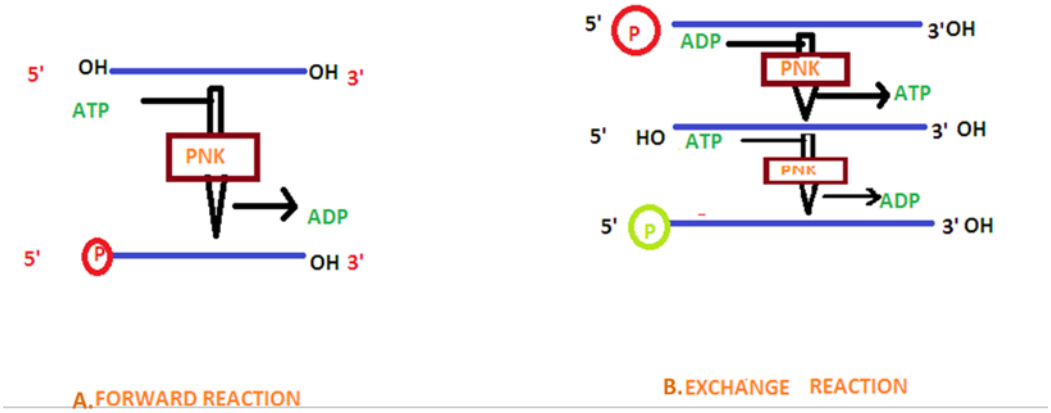

Fig 2-4.2: Polynucleotide kinase reaction (A) forward (B) exchange.
(Adapted from http://www.vivo.colostate.edu/hbooks/genetics/biotech/)

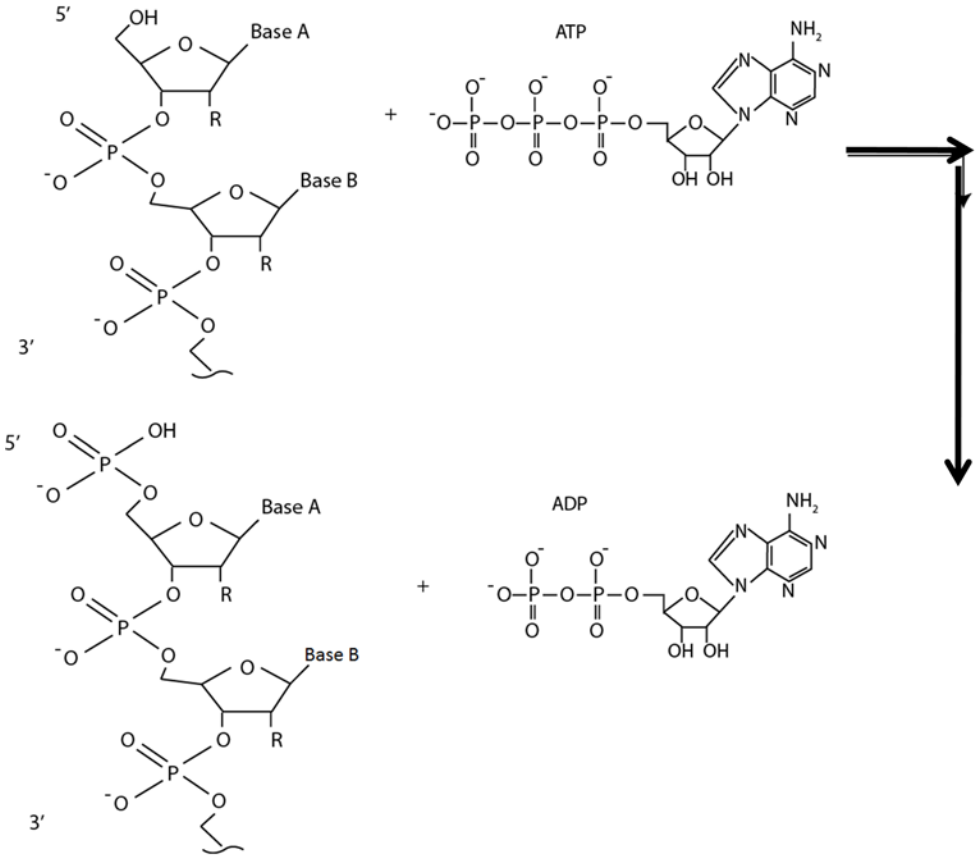

Fig. 2-4.2.1: Conversion of 5’ dephospho-(deoxy) ribonucleic acid to 5’ phospho-(deoxy) ribonucleic acid by the action of PNK.

The efficiency of phosphorylation is less in exchange reaction compared to forward reaction. Along with the phosphorylating activity, PNK also has 3' phosphatase activity.

There are two major uses of PNK:

- The linkers and adopters are phosphorylated along with the fragments of DNA before ligation, which requires a 5' phosphate. This includes products of polymerase chain reaction, which are generated by using non-phosphorylated primers.
- PNK is also used for radio labelling oligonucleotides, generally with ^{32}P for preparing hybridization probes.

PNK is inhibited by ammonium ions, so ammonium acetate cannot be used to precipitate nucleic acids before phosphorylation. Sometimes phosphate ions or NaCl of greater than 50 mM concentration can also inhibit this enzyme.

2-4.3 Ribonuclease (RNase):

- Nuclease that can catalyze hydrolysis of ribonucleotides from either single stranded or double stranded RNA sequence are called ribonucleotides (RNase).
- RNase are classified into two types depending on position of cleavage, i.e. endoribonuclease (cleave internal bond) and exoribonuclease (cleave terminal bond).
- RNase is important for RNA maturation and processing.
- RNaseA and RNaseH play important role in initial defence mechanism against RNA viral infection.

Two common types of ribonucleases are discussed below:

2-4.3.1 RibonucleaseA (RNaseA):

- An endo-ribonuclease that cleaves specifically single-stranded RNA at the 3' end of pyrimidine residues.
- The RNA is degraded into 3'-phosphorylated mononucleotides C and U residues and oligonucleotides in the form of 2', 3'-cyclic monophosphate intermediates.
- Optimal temperature for RNaseA is 60˚C (activity range 15-70˚C) and optimal pH is 7.6.
- RNaseA has two histidine residues in its active site (His12 and His119). In the first step, His12 acts as a base; accepting proton forming a nucleophile which then attacks positively charged phosphorus atom. His119 acts as an acid in this case, donating a proton to oxygenated P-O-R’ bond. The imidazole side chain acts as base in His 12 here.

- The side chain of Lys41 and Phe120 further stabilize the transition state. Nitrogen of the main chain of Phe120 donates hydrogen, thus bonding with the unbound oxygen atom.
- In the second step the acid base activities get reversed and His119 accepts proton from water causing hydroxyl attack on cyclic intermediate.
- Activity of RNaseA can be inhibited by alkylation of His12 and His119 residue essential for activity of the enzyme.

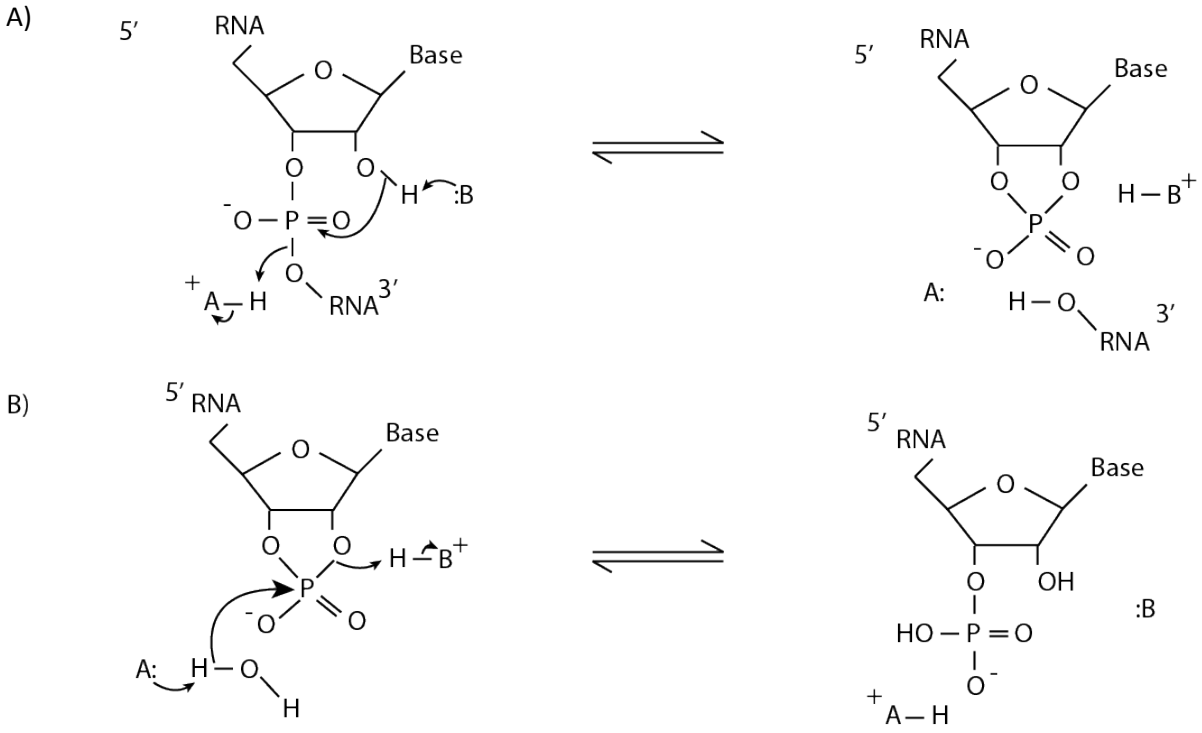

Fig : (A) Transphosphorylation reaction by RNase A
(B) Hydrolysis reaction catalyzed by RNase A

"B" → His 12
"A" → His 119

Fig. 2-4.3.1: Mechanism of action of RNase A

Application:

- It is used to remove RNA contamination from DNA sample.

2-4.3.2 RibonucleaseH:

- Non-specific endoribonuclease that degrades RNA by hydrolytic mechanism from DNA/RNA duplex resulting in single stranded DNA.
- Enzyme bound divalent metal ion is a cofactor here. The product formed is 5' phosphorylated ssDNA.
- During cDNA library preparation from RNA sample, RNaseH enzyme is used to cleave RNA strand of DNA-RNA duplex.

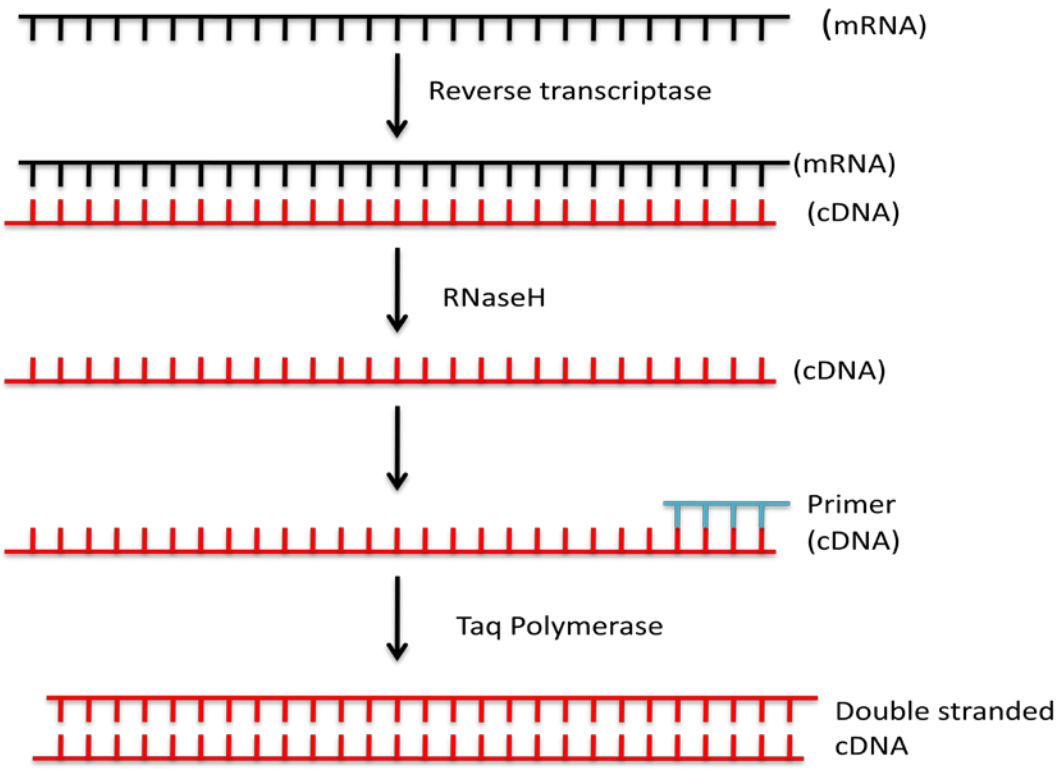

Fig 2-4.3.2: Schematic representation of cDNA preparation from mRNA

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