TELANGANA TRIBAL WELFARE RESIDENTIAL DEGREE COLLEGE (Girls), MAHABUBABAD.

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SUBJECT:ZOOLOGY- PAPER IV

UNIT II: MOLECULAR BIOLOGY

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Designation: DL in ZOOLOGY

2.1 DNA Structure

2.2 RNA structure and types

2.3 DNA Replication

2.4 Transcription and Translation.

2.5 Gene expression

2.6 Molecular Biology Techniques

Introduction to Molecular Biology

Molecular biology is the study of the Structure, organization, functions & regulation of the genome. It is a branch of biology concerning biological activity at the molecular level. Molecular biology looks at the molecular mechanisms behind processes such as Replication, Transcription, Translation and cell function I,e how genes regulate life activities.

Genome refers to the genetic material associated with applied cell.the quantity of DNA present in the genome is given as a diploid cell before application content to cDNA. was first isolated nucleic acids isolated phosphate Bridge from pus cells called the nucleus.

Erwin chargaff proposed chemical structure for DNA based on analysis of double stranded DNA. chargaff hydrolyzed double-stranded DNA and separated the free nucleotides by paper chromatography method.

DNA identification of genetic Material by **Griffith transformation** effect this experiment identify the phenomenon of transformation in diplococcus pneumonia. the causative agent of pneumonia in higher vertebrates. Griffith studies to different states of diplococcus smooth strains and rough strains.

The smooth strains were capsulated having a heteropolysaccharide capsule of glucose and glucuronic acid. The presence of capsule made the virulent factor and was responsible for pneumonia.

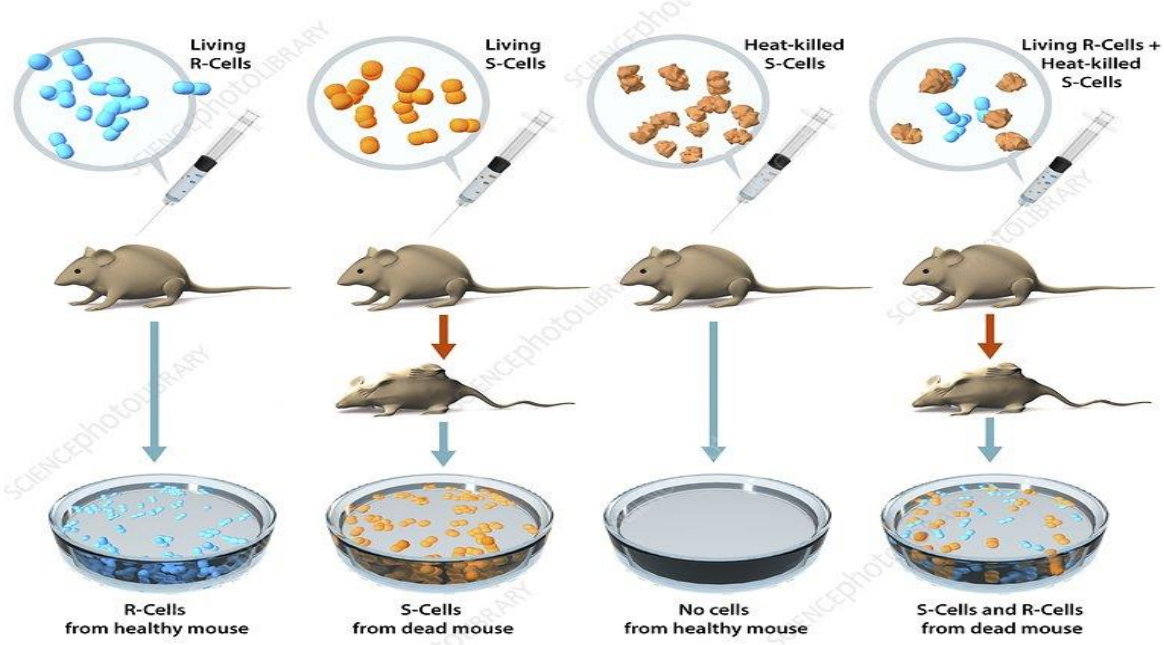
The presence of capsule made the smooth strains to appear agranulated under the microscope and therefore these strains were main smooth strains. griffith used type-IIIS smooth strains for transformation experiments.

The rough strainlack a heteropolysaccharide capsule and where avirulent. due to the lack of capsule this strains appears granulated under the microscope has the peptidoglycan is uneven griffith used type-IIR strains for transformation experiment.

When Griffith used live-III Stain per administer to mice, the mice develop pneumonia and such pneumonia mice were subjected to death. the autopsy of Dead mice revealed the presence of large number of types IIIS cells.

When griffith injected live-IIR strains into the mice the mice remain healthy similarly when heat killed III- S strains were injected into the mice remain healthy. However on a mixture of live II R cells and heat Killed III S strains was injected into the mice. Some mice develop pneumonia andwere subjected to death.

The autopsy of Dead mice revealed the presence of live III S cells. Griffith concluded that a component in the strain converted some of the live IIR cells into live IIIS cells.This conversion of one bacterial strain into another was named transformation by griffith and compound responsible for the conversion was named transforming density.



2.1 DNA STRUCTURE

- DNA is a double helix structure
- The most well accepted DNA Double helix model was proposed by Watson&Crick in 1953 & published to the journal nature.
- The double helix model is based on the following evidence
  i.X-Ray Diffraction study of franklin &wilkins : this indicates the double stranded nature of the molecule the helical parameter
  ii. Chargaff chemical analysis: provided the basis for complementary base pairing
- Watson,Crick&wilkins jointly received the noble prize in physiology or medicine in 1962 for proposing model. Franklin was died in 1958 his name didn't mentioned in noble prize because he was die before announcement of noble prize
- Watson &crick found that the hydrogen bonded base pairs G with C & A withT.

Components of DNA:-

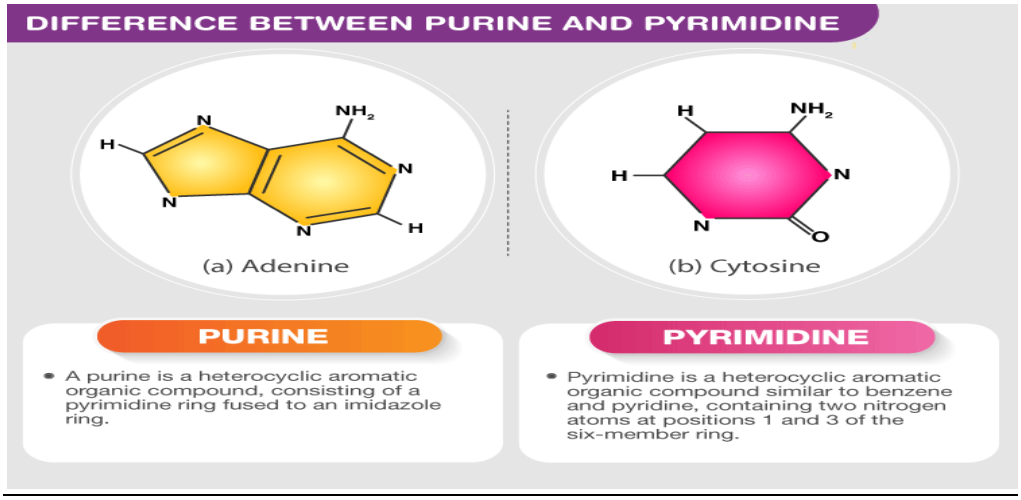
- Nucleic acidsare polymers of Nucleotides. DNA contains 2- DeoxyRibo nucleotide units and the RNA contain Ribonucleotide units
- Nucleotide= Nitrogenous base +Pentose sugar +Phosphate or (Nucleoside + phosphate group)
- Nucleoside = Nitrogenous base + Pentose sugar

1.Nitrogenous base :-

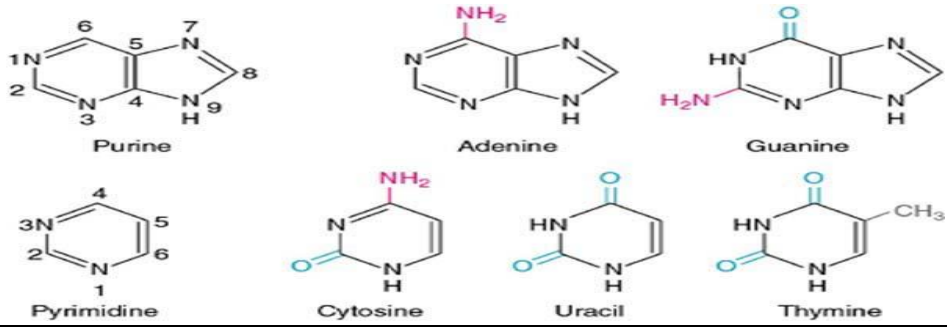
Nitrogenous bases are two types

i) Pyrimidine:- pyrimidine bases have six membered ring
ii) Purine :- purins are bases which are having one extra imidazole ring in their structure

Purine= pyrimidine ring+ one imidazole ring

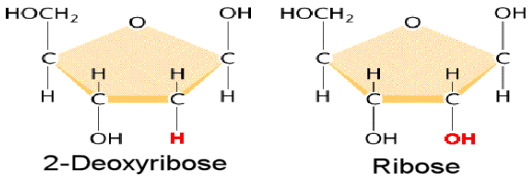


Both DNA & RNA contains two major purine bases Adenine (A) & Guanine (G) ,two major pyrimidines in both DNA & RNA one of the pyrimidine is Cytosine (C) ,but the second major pyrimidine is not the same in both it is Thymine (T) in DNA & Uracil(U) in RNA



2.Pentose sugar :-

DNA contains De-oxy Ribse sugar where as RNA contains Ribose sugar

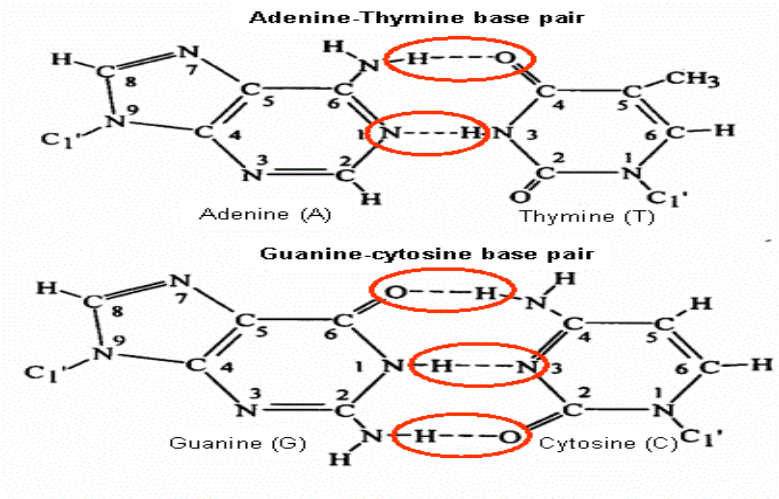


3. Phosphate group :-

- Phosphate groups connects Nucleosides
- The nitrogenous base of a nucleotide is joined covalently in an β- glycosidic bond to the 1' carbon of pentose & the phosphate is esterified to the 5' carbon
  - Adenine+ pentose sugar+phosphate = Adenosine 5' mono phosphate
  - Guanine+pentose sugar+phosphate =Guaninosine 5' mono phosphate
  - Thymine+pentose sugar+phosphate = Thymidine 5' mono phosphate
  - Cytosine+pentose sugar+phosphate =cytidine 5' mono phosphate

Structure:-

- DNA consists of two helical chains wound around the same axis to form right handed double helix.
- The model corresponds to B- DNA.
- Base pairing occurs in DNA .
- Base pairing involves complementary bases.
- A pyrimidine pairs with a purine (T=A ,C≡G).
- A purine pairs with a pyrimidine (A=T, G≡C).
- An amino base pairs with a keto base (A=T, C≡G)
- A keto base pairs with an amino base (T=A, G≡C)
- Amino bases having amino group as a functional group (A&C)
- Keto bases are havingketol group as a functional group (G,T&U)
- NH&NH2 are hydrogen bonds donors where as O&N are hydrogen bond acceptors that is why three hydrogen bonds in between G≡C& two hydrogen bonds in A=T

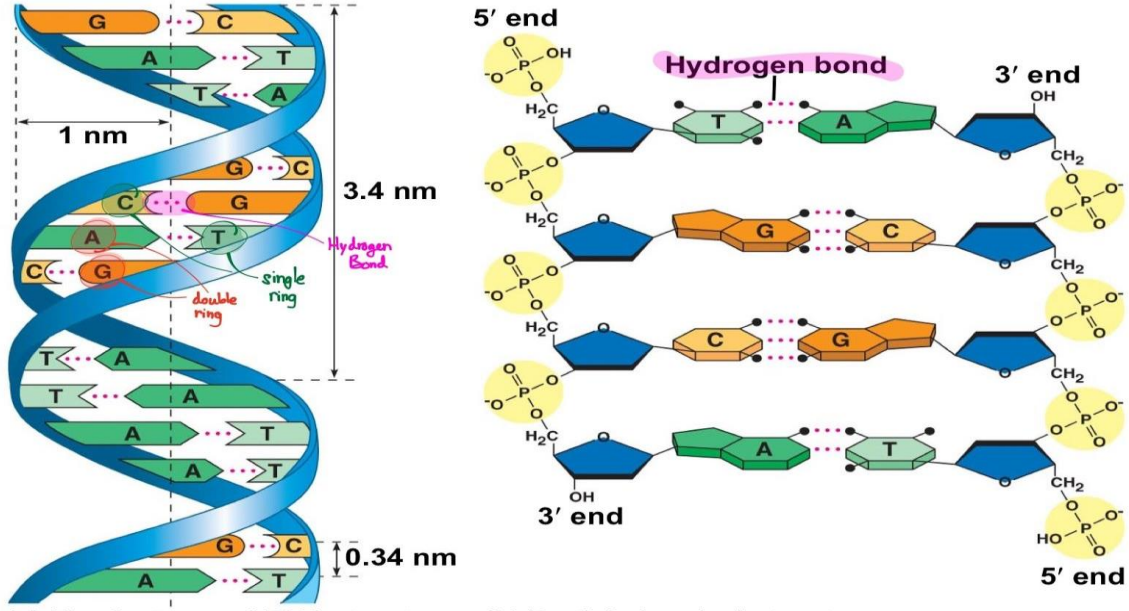


- The two strands are anti-parallel and exist in opposite directions; one strand is 5'-3' in one direction whereas the second strand is 5'-3' in opposite directions
- The two strands are complementary that is the base sequence in one strand is complementary but not identical to the base sequence of another strand
- The pitch of helix corresponds to one turn is 3.4nm or 34 A^0 (1nm =10A^0)
- There are 10 base pairs per turn in the crystal structure but 10.5 base pairs in solution.
- The helical rise or the distance between adjacent base pairs is 0.34nm or 3.4 A^0
- The helix makes a twist of 360^0 for every base pair
- The diameter of helix is 2nm or 20 A^0
- The bases are almost perpendicular to helical axis
- The sugar phosphate back bone is hydrophilic & occurs in the exterior of molecule whereas the hydrophobic bases are interior.
- There are two grooves in DNA, major groove and minor groove. The regions are where proteins interact with the DNA.
- Histone proteins interact with minor groove region whereas Non-Histones such as transcription factors interact with major groove region
- Transcription binding proteins is the only non histone protein interact with minor groove region

Chargaff's proposed the following rules

- the sum totla of Purins=The sum total of pyrimidines
  [A+G]=[C+T]
- The sum total of amino bases =the sum total of keto bases
  [A+C]=[G+T]
- The molar proportion of Adenine = that of Thymine and molar proportion of Guanin = Cytosine
  [A]=[T] , [G]=[C].

Fig. 16-7a



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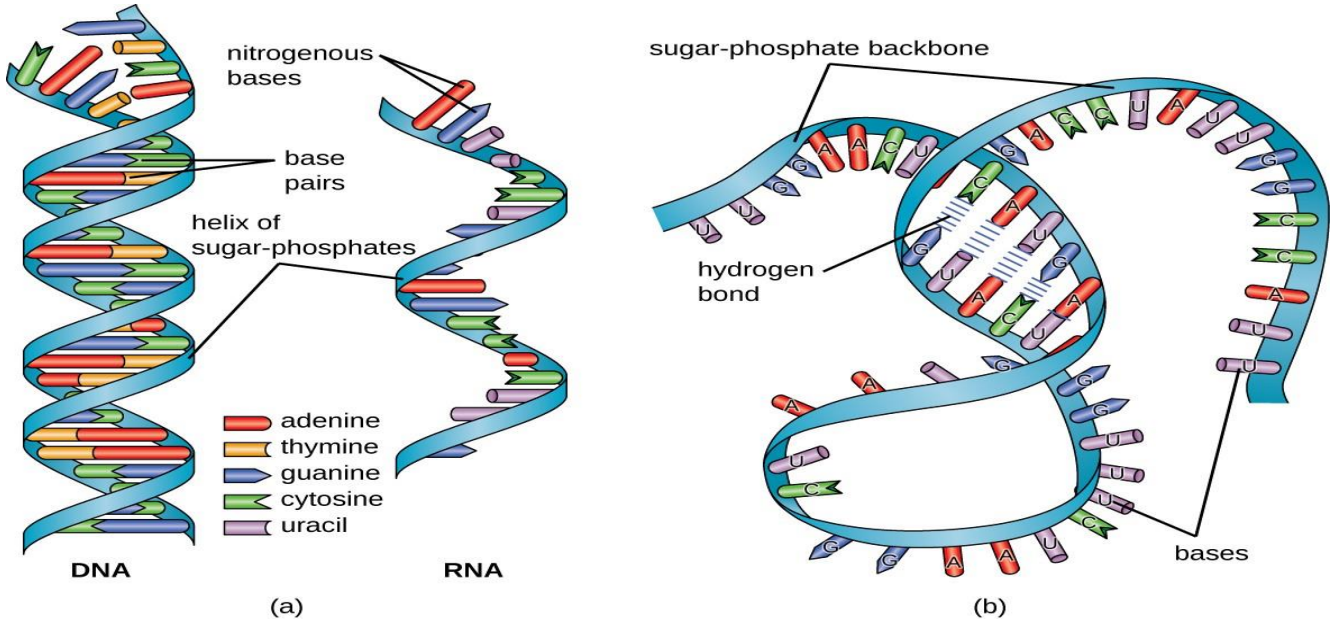
2.2 RNA

- Ribo nucleic acid is a poly nucleotide that consists of a long chain of nucleotide units.
- Each nucleotide consists of nitrogenous base ,a Ribose sugar of Deoxy ribose sugar and among pyrimidine Uracil instead of Thymine
- The structure of nucleotide, the bonds that link Pentose sugar with Nitrogenous base and phosphate group remain same
- Nucleotide that contain Adenine Nitrogenous base that nucleotide is called as Adenylate or Adenosine 5'mono phosphate
- Adenine+ pentose sugar+phosphate = Adenosine 5' mono phosphate
- Guanine+pentose sugar+phosphate =Guaninosine 5' mono phosphate
- Uracil+pentose sugar+phosphate = uridine 5' mono phosphate
- Cytosine+pentose sugar+phosphate =cytidine 5' mono phosphate
- RNA is the genetic material of some viruses.
- Most cellular RNA is single stranded, Right handed helix. It may fold on itself to form Double (Retro viruses have double stranded RNA).
- Common nitrogenous bases are Adenine,Guanine,Cytosine,and Uracil.
- Base pairing involves Purins pairs with Pyrimidines. pyrimidines pairs with Purines A=U , G≡C. U=A, C≡G
- Amino bases pairs with ketobases and vice versa
- A=U, C≡G . U=A, G≡C
- RNA is synthesized in Nucleus but function in cytoplasm
- Function as the machinery of protein synthesis
- RNA transcribed from DNA by RNA polymerases

- The sequence of Nucleotides in the strand is complementary to the Template or Non coding strand of DNA and is called its primary structure

RNA is a single stranded, although some viruses have double stranded RNA .the RNA strand folds upon itself either entirely or in a certain regions, any self complementary sequences in the molecule produce more complex secondary structure.

An important structural feature of RNA that distinguishes it from DNA in the presence of Ribose sugar in the Nucleotide which has a Hydroxyl group at the 2' position. Presence of this functional group causes the helix to adopt the A-form right handed double helix rather than B- form (mostly found in DNA).



TYPES OF RNA

RNA'S are mainly three types

1. Messenger RNA (m-RNA)

2. Transfer RNA(t-RNA)

3. Ribosomal RNA(r-RNA)

Messenger RNA (m-RNA)

Jacob and monad proposed the name messenger RNA for the RNA that interact information (code) for protein synthesis from the DNA to the site of protein synthesizing machinery (Ribosomes).

- It consists only 3-5% of total cellular RNA
- m-RNA is created during transcription where the enzyme RNA polymerase converts genes into primary transcript of m-RNA. The pre-m RNA usually still contains introns regions that are noncodan regions , these are removed in the process of RNA splicing leaving only exans these regions that will encode protein like in DNA .m-RNA genetic information is in the sequence of nucleotides which are arranged in to codans consisting three base pairs of each
- start codan on m-RNA is AUG – methionine
- Terminal codans are UAA,UAG,UGA.these codans are terminates transcription.
- m-RNA is having 5'capping at the 5'end

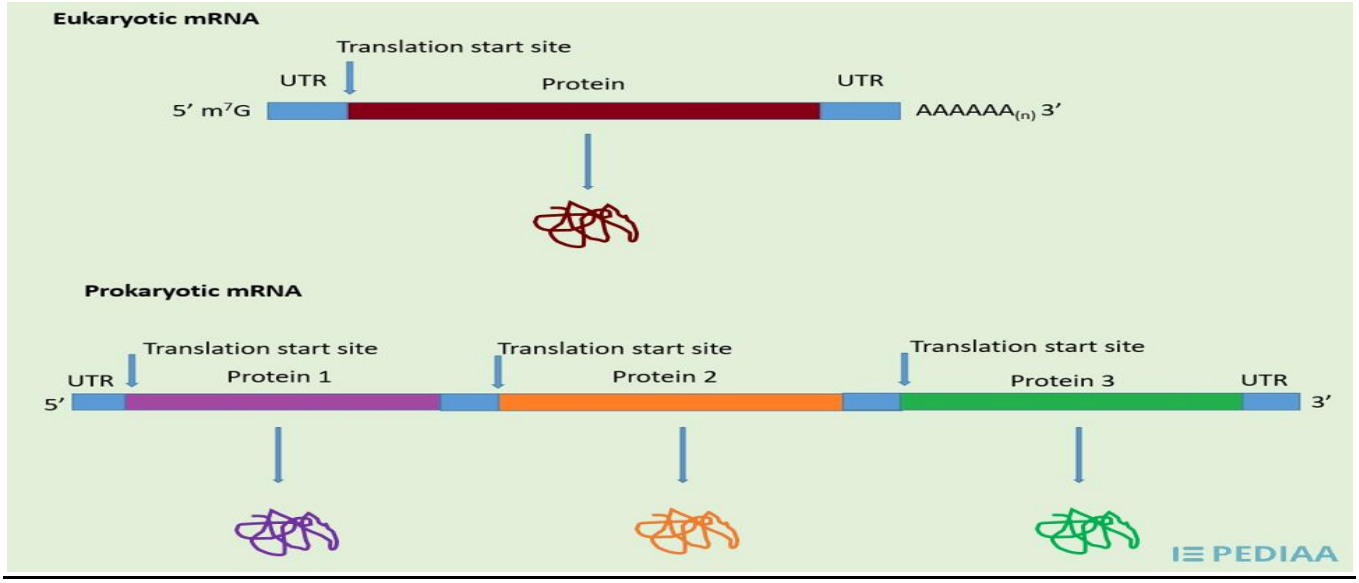
Prokaryotic m-RNA

m-RNA of prokaryotes is usually poly cistronic each coding region at the 5'end contains untranslated regions (UTR) followed by shine dalgarno sequences which helps m-RNA to bind to small ribosomal unit.

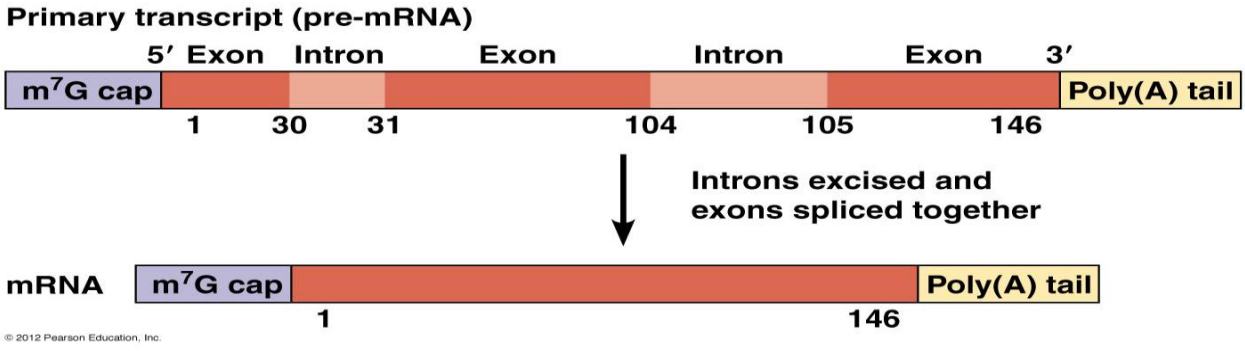
- An m-RNA molecule is said to be poly cistronic when it contains the genetic information to translate many protein chains.
- Poly cis tronic chains m-RNA carries several openreading frames. Each of which translate into a poly peptide.

Eukaryotic m-RNA

An m-RNA molecule is said to be monocistronic when it contains the genetic information to translate only one polypeptide chain



- Eukaryotic m-RNA's at 5' end the m-RNA has a cap of 7-methyl guanosine residue known as 5'cap.
- The 5' cap is essential for binding of m-RNA to the ribosome and protection from RNases.
- Next to 5'cap there is a UTR sequence &kozak sequence that are recognized by ribosome as the translation site and followed by termination codans and poly A- tail sequence.
- Poly A-tail protects the m-RNA from exonucleases.



Ribosomal RNA (r-RNA)

Ribosomal ribonucleic acid is a type of non-coding RNA which is primary components of ribosomes

- About 80% of total RNA
- Ribosomes are ribonucleo protein particles which are approximately 65% rRNA and 35% proteins
- The r-RNA genes in bacteria exist as a single cluster containing the 16s,23s&5s r-RNA genes

- The transcription of r-RNA genes cluster produces a pre-r RNA transcript that is processed and to release 16s,23s&5s r-RNA
- In case of eukaryotes the r-RNA gene cluster contains 18s,5.8s&28s r-RNA genes where as 5s gene is separately found
- The transcription of r-RNA gene cluster carried out by RNA polymerase in the nucleolus
- Prokaryotic ribosomes are 70s type :-

70s=50s large sub unit +30s small sub unit

50s large sub unit is made up of 23s+5s rRNA +34 proteins

30s small sub unit is comprises 16s r-RNA +21 proteins

- Eukaryotic ribosomes are 80s type :-

80s=60s large sub unit +40 s small sub unit

60s sub unit comprises 5.8s+28s+5s r-RNA +49 proteins

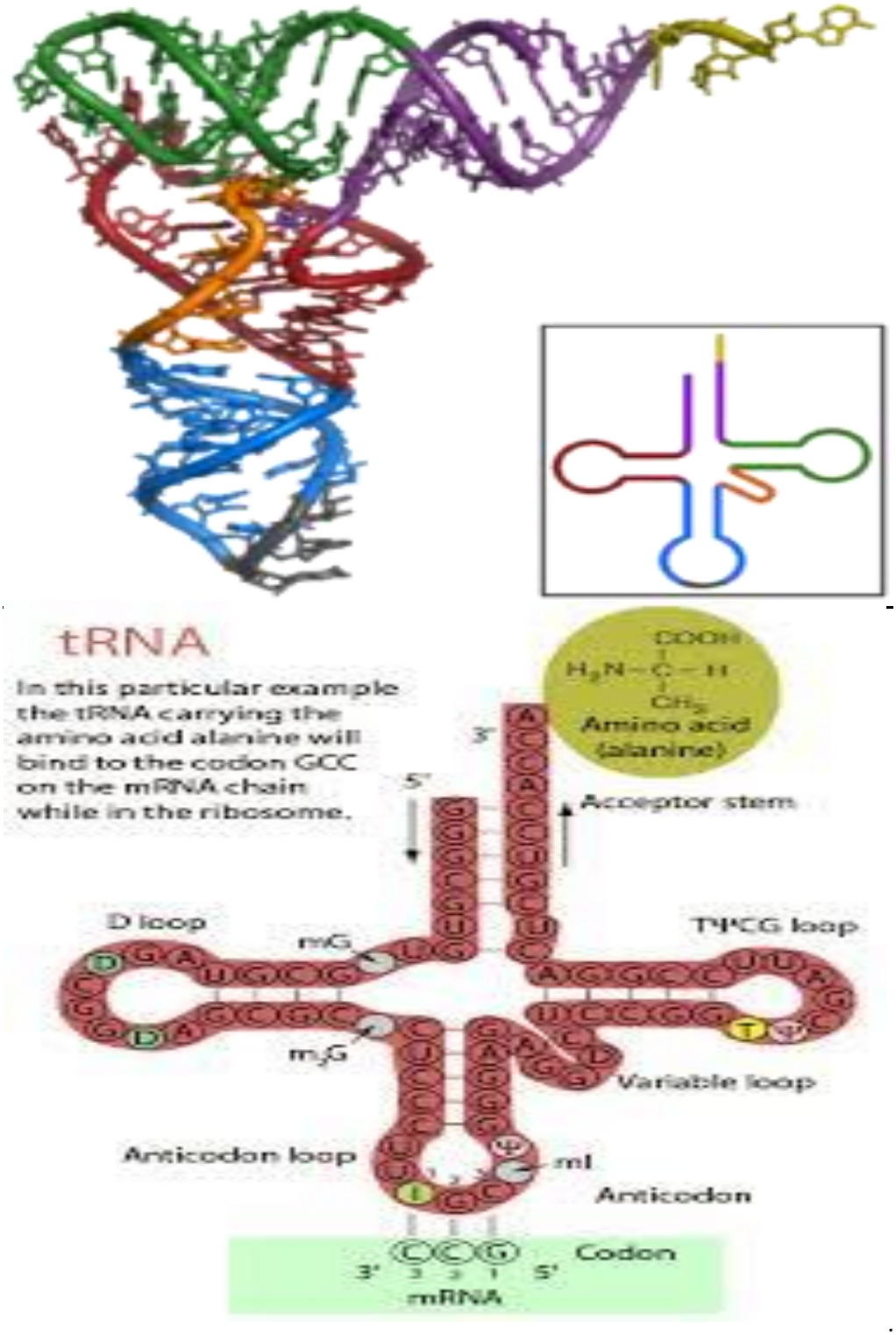
40s small sub unit comprises 18s r-RNA +33 proteins

- Ribosomal sub units are self assembling entities.
- The RNA of large sub unit is aRibozyme and catalyses peptide bond formation.

Transfer RNA (t-RNA)

- A t-RNA molecule is used in Translation and consists of a single RNA strand that is only about 80 nucleotides long, contains an anticodan on other end.
- The Anti codan base pairs with a complementary codan on m-RNA and transfer RNA
- The structure of t- RNA can be decomposed into its primary structure
- The secondary structure usually visualized as the clover leaf structure
- the tertiary structure is similar to twisted L-shaped structure
- It's about 10-20% of total RNA
- At least 32 t-RNA's are required to recognize all the amino acid codans
- The t-RNA structure consists of the following
- A 5'- terminal phosphate group
- Most t-RNA's have Guanylate residue at the 5'end and all have the trinucleotide sequence CCA at the 3' end.
- Clover leaf structure of t RNA has four arms .the longer t-RNA have a short fifth arm or extra arm.
- The amino acid arm can carry a specific amino acid is esterified by its carboxyl group to the 3' hydroxyl group at the 3'end
- The anti codan arm contains the anti codan which base pairs with codan of m-RNA
- The other major arms are the D-arm which contains the unusual nucleotide Di hydro uridine (D)
- TφC arm contains a Ribo thymidine (T), not usually present in RNA's,pseudo uridine (φ) a modified uridine and cytidine (C) in that order.
- The variable arm is variable and in some t-RNA's it is barely noticeable

- The D& TφC arms contribute important interactions for the overall folding of t-RNA molecule
- TφC arm interacts with a large sub unit of Ribosome.
- Some of the unusual bases of t-RNA are methyl guanine, di methyl guanine, methylcytosine,Ribo thymie, pseudo uridine, di hydro uridine, inosine and methyl inosine.

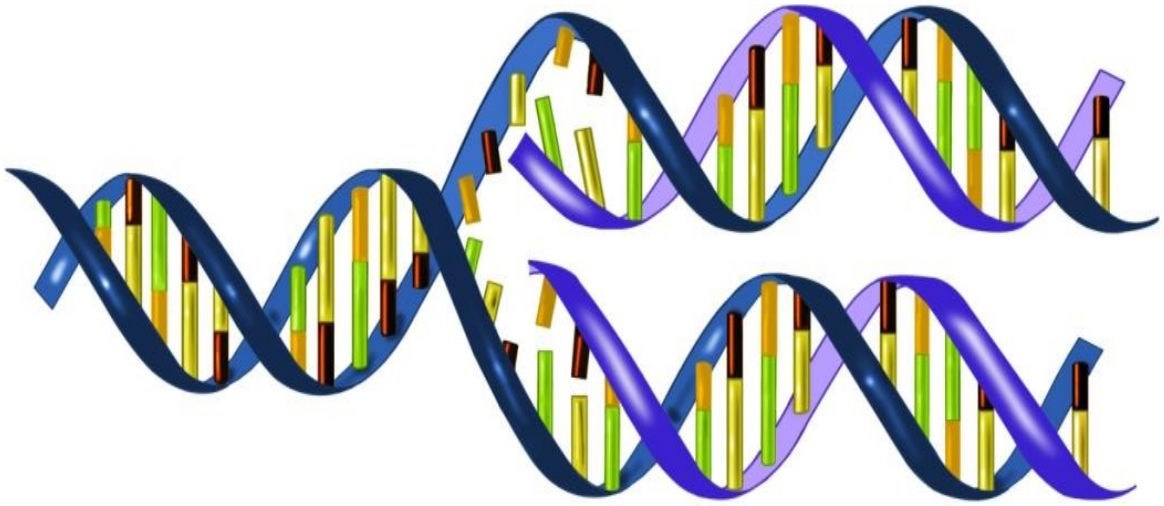


Digram: Clover leaf structureTwisted L- shaped structure

2.3 DNA REPLICATION

Replication is a self duplication process in which DNA acts as a template for the synthesis of its own copies.

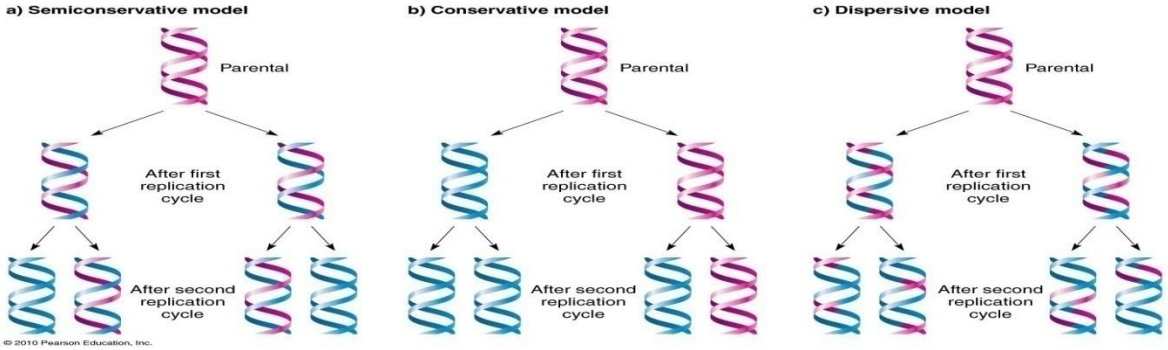
- DNA replication must occur before a cell can divide to produce two genetically identical daughter cells.
- Watson &crick proposed the hypothesis of semi conservative replication.
- According to them two strands of DNA unzip by the breakdown of hydrogen bonds between the complementary bases and then each strand serves as template for the synthesis of complementary strand.



- In semi-conservative mechanism each newly synthesized daughter DNA molecule contains one parental DNA strand &one newly synthesized strand.
- Experimentally proved by messelson &stahl
- Theoretically two other mode of replication are possible

a) Conservative: one of the daughter DNA molecules contains the two parental DNA strands whereas the second daughter DNA molecule contains two newly synthesized strands.

b) Dispersive: in this mechanism each daughter DNA strand is a hybrid of parental strand segment & newly synthesized segment.



Bidirectional replication: in this mechanism from the origin two oppositely moving replication forks are generated and replication occurs with both the replication forks; such a mechanism increases the replication rate & there by decreases the time taken for replication.

In case of circular chromosomes of bacteria bidirectional replication produces a theta (θ)shaped structure and it is called the theta mode of replication.

Components of DNA replication:

i)Deoxy ribo nucleotides:-four types of deoxy ribo nucleotides or deoxy ribo nucleotide tri phosphates (dNTPs) are required for the replication.

a) Deoxy adenosine tri phosphate (dATP)
b) Deoxy guanosine tri phosphate (dGTP)
c) Deoxy cytosine tri phosphate (dCTP)
d) Deoxy thymine tri phosphate (dTTP)

ii)Enzymes:-

a) DNA Helicase: an ATP ase that carries out the unwinding of double stranded DNA it uses one ATP molecule for un winding DNA.
- e.coli Dna-B protein is the helicase.
- Helicase attaches to Ori sequence of DNA and unwind.

b) DNA primase : DNA primase is an enzyme which synthesize a short chain RNA primer of 10-60 ribonucleotides complementary to parental DNA

- The RNA primer provides free 3'OH group to which dNTP's is added by DNA polymerase-III
- In eukaryotes one of the DNA polymerase-α synthesizes primers for both leading & lagging strands.

c)Topo isomearase(DNA gyrase):- Required for the removal of +ve supercoils generated ahead of the replication fork by the DNA unwinding

- Topo isomerase Type-II enzymes are widely used in both prokaryotes & eukaryotes.

d) DNA polymerase: DNA polymerase is responsible for synthesis of DNA molecule and also has exonuclease and proof reading activity.

e) DNA ligase: a ligase enzyme that fills the gap in leading and lagging strand during replication, gaps are created by primer removal.

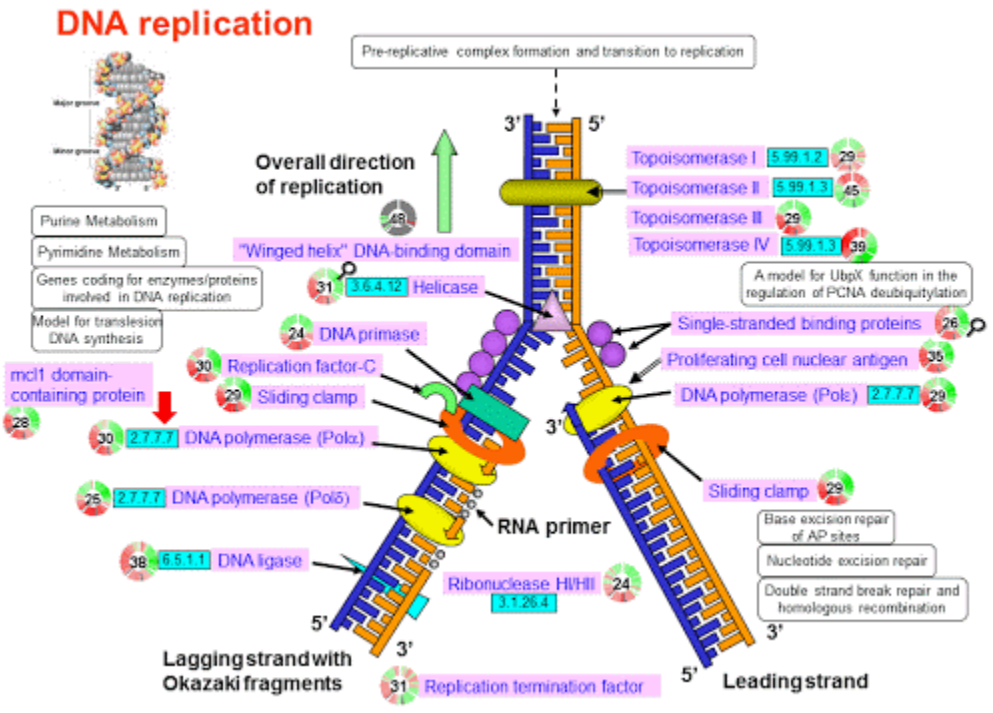
f) RNase H: is responsible for cutting out the RNA primer in leading and lagging strands.

iii) Required proteins for the replication:-

a) Replication initiation proteins: proteins that recognize a specific DNA sequence within the origin of replication.
b) Single stranded binding proteins (SSB Proteins):these proteins bind to Single stranded regions in double stranded molecule & prevent their re association into double stranded regions as well as protect them from single stranded specific nucleases.
c) Sliding clamp proteins:Clamp proteins bind DNA polymerase and prevent this enzyme for dissociating from the template DNA strand.

Origin of replication (Ori sequence):-

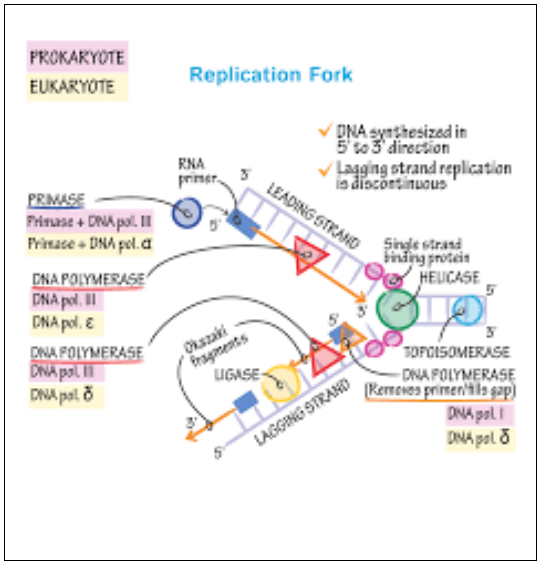
- Ori is a part of DNA where there replication begins.
- In bacterial cells there is a single Origin of replication
- **Eukaryotic** cells has much greater amount of DNA to replicate, there is hundreds to thousands of Origin of replication.



Initiation of Replication: -The process of DNA synthesis begins by the binding of initiation protein Dna A to 9-mer sequences of Ori, which is followed by binding of histone like proteins called Hu-proteins.

- The binding of DNa A & Hu proteins to 9-mer sequences it induces the localized melting of DNA at the AT rich sequence of 13-mer sequences of Ori sequence. Resulting in formation of single stranded regions in the Ori sequence
- In **eukaryotes** origin recognition complex is a analogue of DnaA
- SSB proteins are helix destabilization proteins and prevent re association into double stranded regions.
- The Topoisomerase (DNA gyrase) binds to DNA which is unwinds DNA by adding opposite super coils.
- Followed by helicase loaded on to the DNA it binds to one of the strand, it is loaded by DNa B proteins.
- In **eukaryotes** MCM complex is analogue of Dna B helicase
- Helicase will break hydrogen bonds between the bases.
- RNA primer loads on to the DNA strand it will give free 3' OH group for the attachment of deoxy ribonucleotides
- Because all known DNA polymerases will synthesize 3'-5' (The bond formation between 3'carbon of pre de oxy ribonucleotide and 5' carbon of post de oxy ribonucleotide) polymerase activity.
- DNA polymerase –III will add nucleotides to RNA primer.
- Helicase needs ATP for its activity

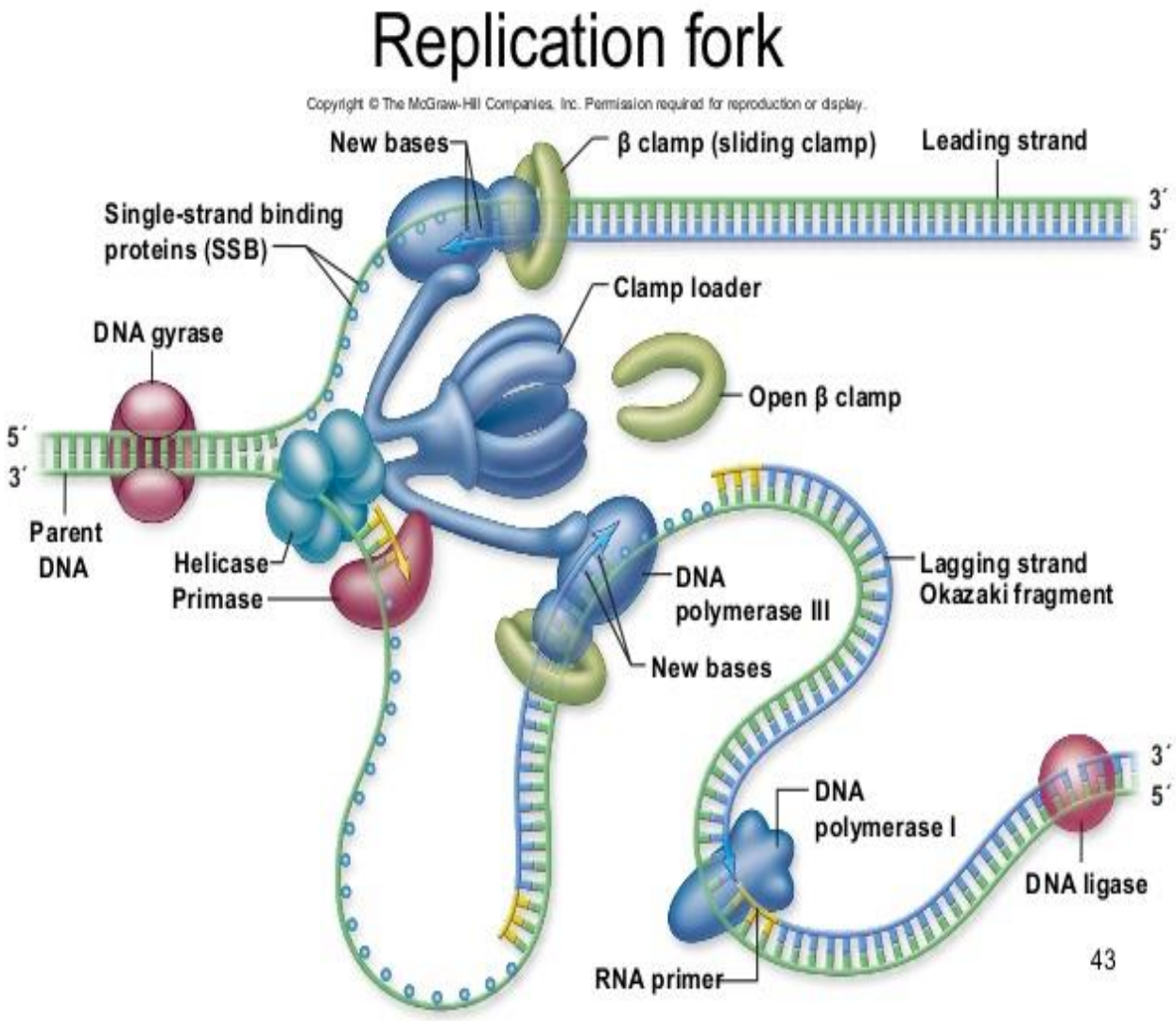
- The primosome complex makes the first primer for the leading strand; this will be the only primer require for the leading strand synthesis as leading strand continuously synthesized.
- The primer formation promotes the clamp loader to assemble the sliding clamp on the leading strand template; clamp loader requires ATP for its activity.
- The formation of sliding clamp is followed by loading of core polymerase on to the sliding clamp
- The core polymerase then begins the extension of leading strand.
- **Eukaryotic**pre replicative complex is an active initiation complex requiring DNA pol-α, DNA pol-ε Than initiation begins.



Elongation:-

- DNA polymerase –III continuously adds dNTPs to 3' OH group as long as DNA template is available.
- The nucleotides are always added to the 3'carbon of the last nucleotide so synthesize always proceeds from 5'→3'.

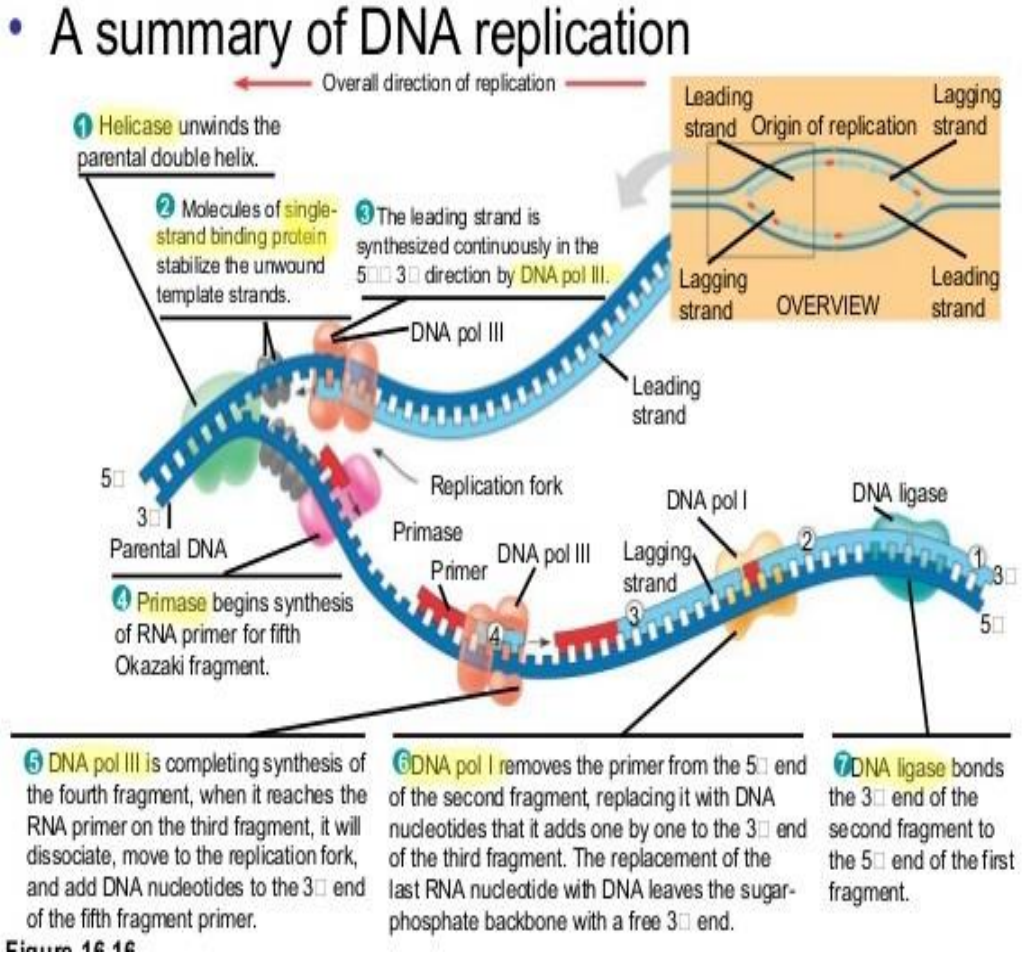
- No additional charges are needed for the phosphodiester bond formation since the hydrolysis of dNTP to dNMP releases energy for the phosphor diester bond formation.
- The rate of polymerization by DNA pol-III is 200-1000 nucleotides per second or approximately 50,000 nucleotides per minute in one direction.
- In **Eukaryotes** PCNA is analogue of $\beta -$ unit of DNA pol-III
  DNA pol-α is involved in primer synthesis & lagging strand replication.
  DNA pol-β $-$is involved in DNA repair; gap filling enzyme.
  DNA pol-$\varepsilon, \delta -$ are involved in leading strand synthesis.
- during the replication of DNA one strand is continuously synthesize by the extension of a single primer & this strand is called the leading strand whereas second strand is synthesized by extension of a large number of primers & is called the lagging strand
- Leading strand continuously synthesizes& lagging strand is discontinuously synthesized.
- The leading strand synthesize away from origin in one direction of replication, whereas lagging strand is synthesized away from replication fork in direction of origin.
- Lagging is synthesized in the form of fragments called Okazaki fragments which are joined by DNA ligase to complete the lagging strand synthesis.
- Okazaki fragments are 1000-2000 nucleotides in bacteria but 100-200 nucleotides in eukaryotes.
- When synthesis of an Okazaki fragment has been completed, replicationhalts, and the core sub units of DNA pol-III dissociate from their sliding clamp & associate with new clamp.
- The lagging strand template must loop around to permit the enzyme to extend the lagging strand.
- The enzyme releases lagging strand template when it encounters the previously synthesized okazaki fragment.
- And again loop will form and polymerization of new okazaki fragment will take place.
- The 5'→3' exonuclease activity of DNA pol-I removes the primers & gap filling activity of the polymerase incorporates de oxy nucleotides.
- DNA ligase finally completes the lagging strand synthesis by joining Okazaki fragments.
- DNA pol-II synthesizes DNA during repair, proof reading 3'→5' exonuclease activity.



Termination:-

- Termination of replication occurs when the core polymerase encounters the terminus regions having Tus proteins.
- The Ter sequence of DNA functions as binding sites for protein called Tus.
- The Tus –Ter complex can arrest a replication fork from only one direction.
- E.coli requires Topo isomerase –IV, the separated chromosomes then segregate into daughter cells at cell division.

- **Eukaryotic**chromosomes are linear, meaning that they have ends pose a problem for DNA replication .the DNA at the end of the chromosome cannot be fully copied in each round of replication, resulting in a slow gradual shortening of the chromosome.
  - Telomerase is a RNA directed DNA polymerase or reverse transcriptase that is involved in the replication of telomeres.
  - Telomerase is found only in germ cells and stem cells
  - Telomerase is absent in somatic cells



2.4 TRANSCRIPTION

The process of formation of an RNA transcript based on a DNA template by DNA directed RNA polymerase is called as Transcription.

- In the case of transcript at any given time only one of the two strands of the DNA will be used as a template which strand can be used as a template depends on the orientation of strand with respect to the promoter.
- Promoter is the regulatory region to which RNA polymerase binds at the time of transcription initiation.
- The DNA strand oriented 3'→5' with respect to the promoter acts as template strand where as the DNA strand which is oriented 5'→3' with respect to the promoter acts as the non template strand.
- The template strand has orientation i,e opposite to the orientation of transcript & therefore the template strand is called antisense DNA strand.
- The template strand has nucleotide sequence that is complementary but not identical to the nucleotide sequence in the transcript & template strand is called anti non coding DNA strand.
- The orientation of non template strand is similar to the orientation of transcript & the non template strand is called sense DNA strand
- The non template strand has nucleotide sequence i,e identical to the nucleotide sequence of the transcript except that DNA contains T in place of U in RNA and therefore non template strand is called the coding strand.

Transcriptional unit:

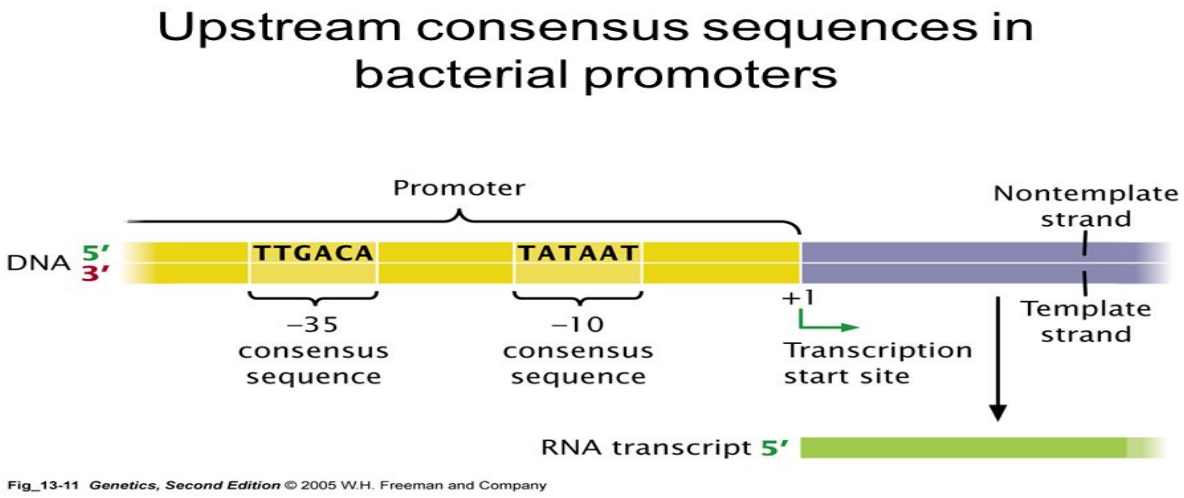
- The entire DNA segment which id transcribed as a single unit is called as a Transcriptional unit.
- The transcriptional unit consists of start point (initiator region), Termination region, Transcribed region and a promoter sequence.
- The start point indicates the first nucleotide that is transcribed;this position is indicated as +1; the region preceding the start point is called the upstream region whereas the region that comes after the start point is called downstreamregion.
- Upstream region is regulatory region in function and it is not transcribed.
- The downstream region contains the transcribed region which is expressed in the transcript.
- The transcribed region corresponds to the region between the start point and the termination region.
- The promoter is the upstream region that acts as the binding site for the RNA Pol during the initiation of transcription.

Promoter: promoter has three distinct regions.

i) Pribnow box (or) -10 upstream sequence: it is an AT rich region having the consensus sequences of 5'TATAAT3' it undergoes the initial melting (strand separation) when transcription initiation takes place.

ii) -35 upstream sequences: it has consensus sequence of 5'TAGACA3' and is bindng site for the β' subunit of RNA polymerase.

iii) Region between -10 sequence and -35 sequence: this sequence correctly orients the RNA polymerase so that only the downstream region is subjected to transcription.



Components involved in Transcription:

i) DNA template: strandof DNA on which mRNA is synthesized.
ii) Substrates: Ribonucleoside triphosphates (ATP,GTP,CTP,UTP)
iii) Helicase: DNA unwinding domain.
iv) RNA polymerase: initiates synthesis of mRNA from DNA template.

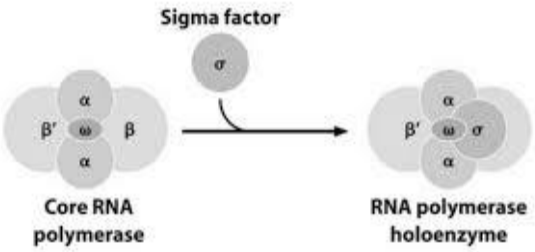
- RNA polymerase consists six polypeptide chains, in that five polypeptide ($2\alpha, \beta, \beta', \omega$)constitute Core enzyme. Sixth polypeptide is σ factor.
- Core enzyme + sigma factor (σ) = Holoenzyme.
- Functions of RNA polymerase sub units:

a)α-assembly of core enzyme, can bind to upstream of promoter.

b) β- Catalytic activity contains 5'-3' polymerase activity.

c)β'- binds to -35 sequence of promoter.

d)$\sigma\ factor$ - needed for successful initiation as it recognizes promotor region.



Initiation:

- The initiation process begins with Random binding of RNA polymerase core enzyme to the upstream of the promoter region.
- The core enzyme has low affinity for DNA
- A free $\sigma\ factor$ than binds to the core enzyme producing the holo enzyme having higher affinity for DNA
- The holoenzyme recognizes the promoter and tightly binds to the promoter region producing a closed binary complex consisting of DS DNA and the holoenzyme.
- The stabilization of holoenzyme at the promoter leads to the localized melting of DNA at the pribnow box region leading to the formation open binary complex which triggers the β subunit to synthesize an oligonucleotide segment of 8-12 nucleotides.
- After successful initiation $\sigma\ factor$ released from the holoenzyme.this leads to transition from initiation to elongation mode.

Elongation:

- The process of elongation involves the sequential incorporation of ribonucleotides into the growing chain of RNA based on the DNA template.
- RNA polymerase is a transferase enzyme that hydrolyses the nucleotide triphosphate and transfers the nucleotide mono phosphate into growing chain; hydrolysis of NTP (nucleotide tri phosphate) provides the energy for phosphor di ester bond formation.
- $(NMP)_n$+(NTP) $\quad - - - - - - - - \longrightarrow (NMP)_{n+1}$+PPi.
- $elongation\ can\ be\ carried\ out\ by\ the\ core$ Enzyme.
- The rate of elongation is 50-90 nucleotides per second.

Termination:

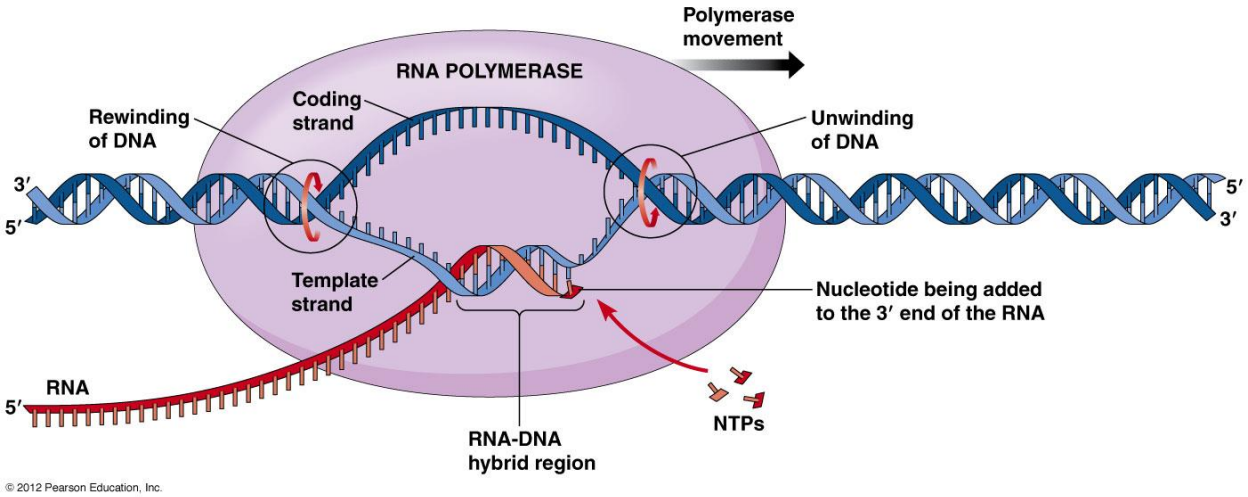
Termination in bacteria occurs by two different mechanisms.

i) ρ_ factor independent mechanism (or) intrinsic mechanism:

- The intrinsic termination is a passive process in which a termination hair pin look structure is form at the 3' end of the transcription. This termination hair loop characterized by a G-C rich stem and poly (U) sequence.
- The G-C rich stem prevents the migration of RNA polymerase and the poly (U) sequence unwinds from DNA template releasing the transcript.

ii) ρ_ factor dependent mechanism or Extrinsic mechanism :

- The extrinsic termination is an active process that require Rho factor .Nus A & Nus B
- The Rho factor as an ATP dependent helicase that binds to the transcript at the Rho binding site; when Nus A & Nus B interact with the Rho factor, the helicase activity of Rho factor is stimulated and the RNA transcript is released from the DNA template.
- Under normal conditions genes involving intrinsic & extrinsic termination are expressed in bacteria; however during starvation most of the genes express passive intrinsic termination.



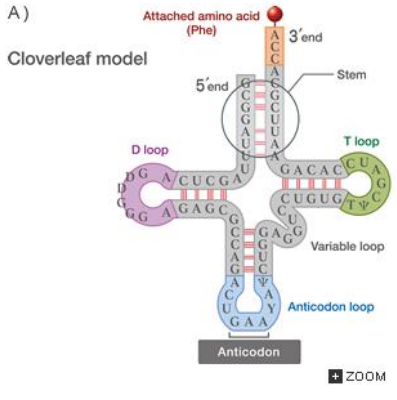
2.4.2 TRANSLATION

Translation is the second stage of gene expression in which polypeptide chain or protein is synthesized based on m-RNA template on ribosomes.

- It involves the translation of the language of nucleotide sequences of mRNA into the language of amino acid sequence of proteins.
- The m-RNA template is used in 5'→3' direction and the polypeptide synthesizes from 'N' terminus to C terminus.

Components required for Translation:

1. Amino acid pool: all the 20 amino acids are essential for translation and the deficiency of a single amino acid can inhibit translocation.
2. Energy: Translation requires GTP is required for initiation, elongation& termination. Whereas ATP is required for the activation of amino acid during the charging of t-RNA.
3. m RNA : transcribed from DNA
4. Ribosomes: work benchers of protein synthesis.
5. tRNAs: Adaptors in translation; have anticodans on one arm to recognize codans of mRNA and have specific amino acids attached to another arm.
6. Enzymes:
   Amino acyl t-RNA synthetases: for activation of amino acids.
   Peptidyl transferase: for peptide bond formation between amino acids.
7. Protein factors:
   i) Initiation factors: IF1,IF2,IF3 IN Bacteria
   eIF1,eIF2,eIF3,eIF5,eIF6 In Eukaryotes.
   ii) Elongation factors : EF-TU,EF-TS&EF-G IN Bacteria
   eEFor α & eEF-2 or β in eukaryotes.
   iii)Releasing factors: RF-1,RF-2,&RF-3 In Bacteria.eRF in eukaryotes



t-RNA

Ribosomes:ribosomes have binding sites for t-RNAs known as peptidyl site (P site), Aminoacyl site (A site), and exit site (E site)

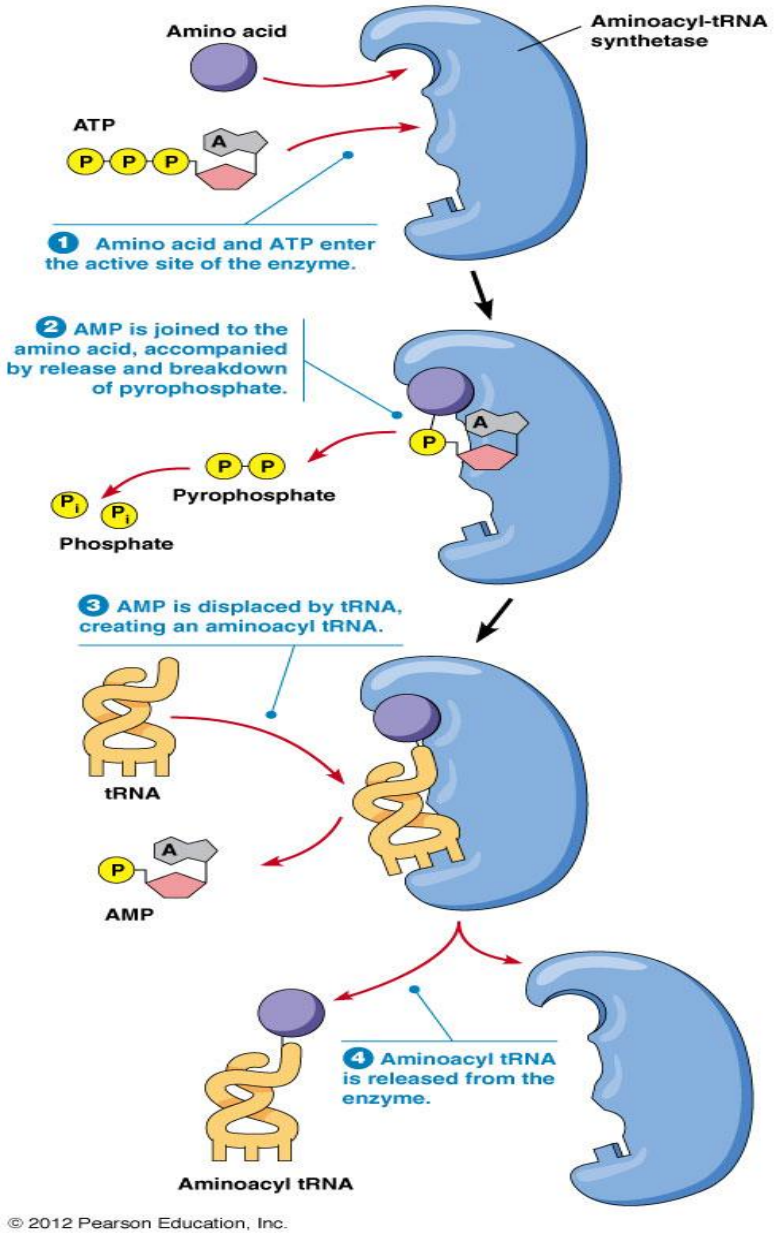
t-RNA: t-RNA secondary structure is clover leaf , an amino acid attached to the 3’

Charging of t-RNA molecule:

- first step: each amino acid reacts with ATP in presence of specific aminoacyl t-RNA synthetase to give aminoacyladenylic acid , the molecule remain associated with enzyme
- Second step: the amino acid transferred to the appropriate tRNA and bonded covalently to the Adenine residue at the 3’ end. This process is called Charging.

Amino acid +ATP → aminoacyladenylic acid + ppi

aminoacyladenylic acid+ t-RNA →amino acyl t-RNA +AMP

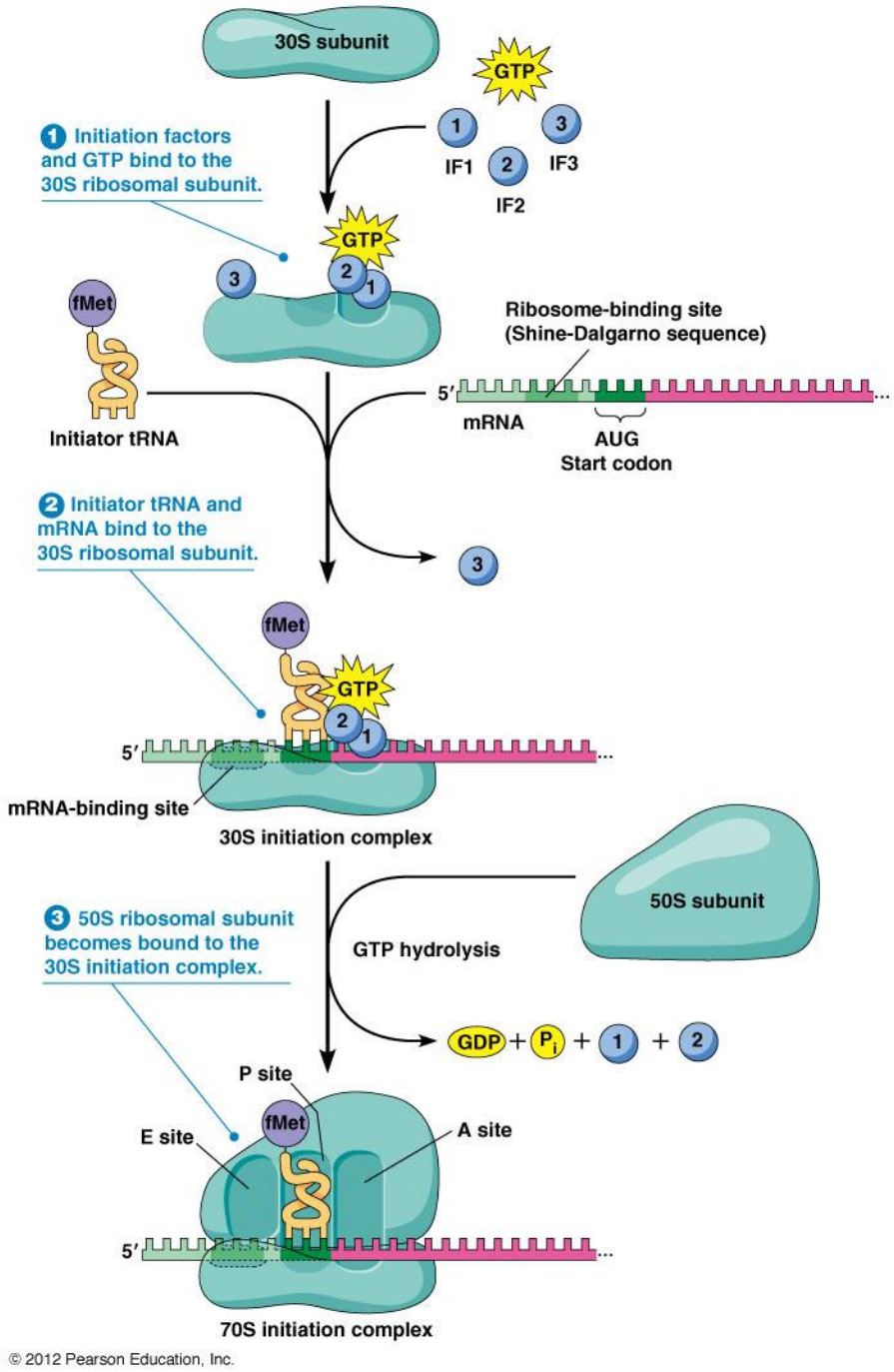


Stages in Translation:

Initiation:

- Prokaryotic initiation: Begins with the binding of the initiation factor IF1, to the small subunit of 70s complex of ribosome.
- The binding of IF1 causes the dissociation of Ribosomal subunits; however due to the high mg^{+2} concentration that two sub units reassociated to form the 70s complex. The binding of IF3 to the small subunit prevents the formation of 70s complex.
- The initiation codan (5') AUG is guided to its correct positions by the Shine dalgarno sequence in the m-RNA.

- AUG sequence of the m-RNA in the correct position of the P site so that the initiating tRNA farmyl methionine can bind to initiating codan.
- IF2 which uses GTP promotes the binding of the initiator tRNA to the start codan in the P site. The anticodans of this t-RNA now pairs correctly with the mRNAs initiating codan.
- Now all three initiation factors dissociate from the ribosome, it leads to attachment of 50s sub unit to the 30s sub unit and formation of 70s complex takes place.
- Now the complex is ready for elongation.
- Eukaryotic initiation:The m-RNA is recognized by eIF4, which is a multi protein complex that recognizes the 7-Methyl guanosine cap and facilitates the binding of the m RNA to the 40s subunit.
- The initiator sequence is AUG which codes for methionine eIF2 binds directly to tRNAMet to recruit the 40s subunit. Shine dalgarno sequences are absent instead of that kozak sequences are present.
- The initiating 5' (AUG) codan detected within the mRNA by eIF4F complex which scans the mRNA from the 5' end until first AUG is encountered.



Elongation:

- The elongation process begins with the transfer of an aminoacyl t-RNA by the elongation factors
- Cells use three steps to add each amino acid residue, and the steps are repeated as many times as there are residues added.
-

i) Binding of an incoming Aminoacyl t-RNAs: if the aminoacyl t-RNA forms stable base pairing at the A-site ,the GTPase activity of EF-TU releases the aminoacyl t-RNA at the

A-site thus one high energy bond is needed for the transfer of aminocyl t-RNA to the A-site

- The inactivated EF-TU is activated by EF-TS
- The presence of aminoacyl t-RNA at the A- site promotes the peptidyl transferase activity

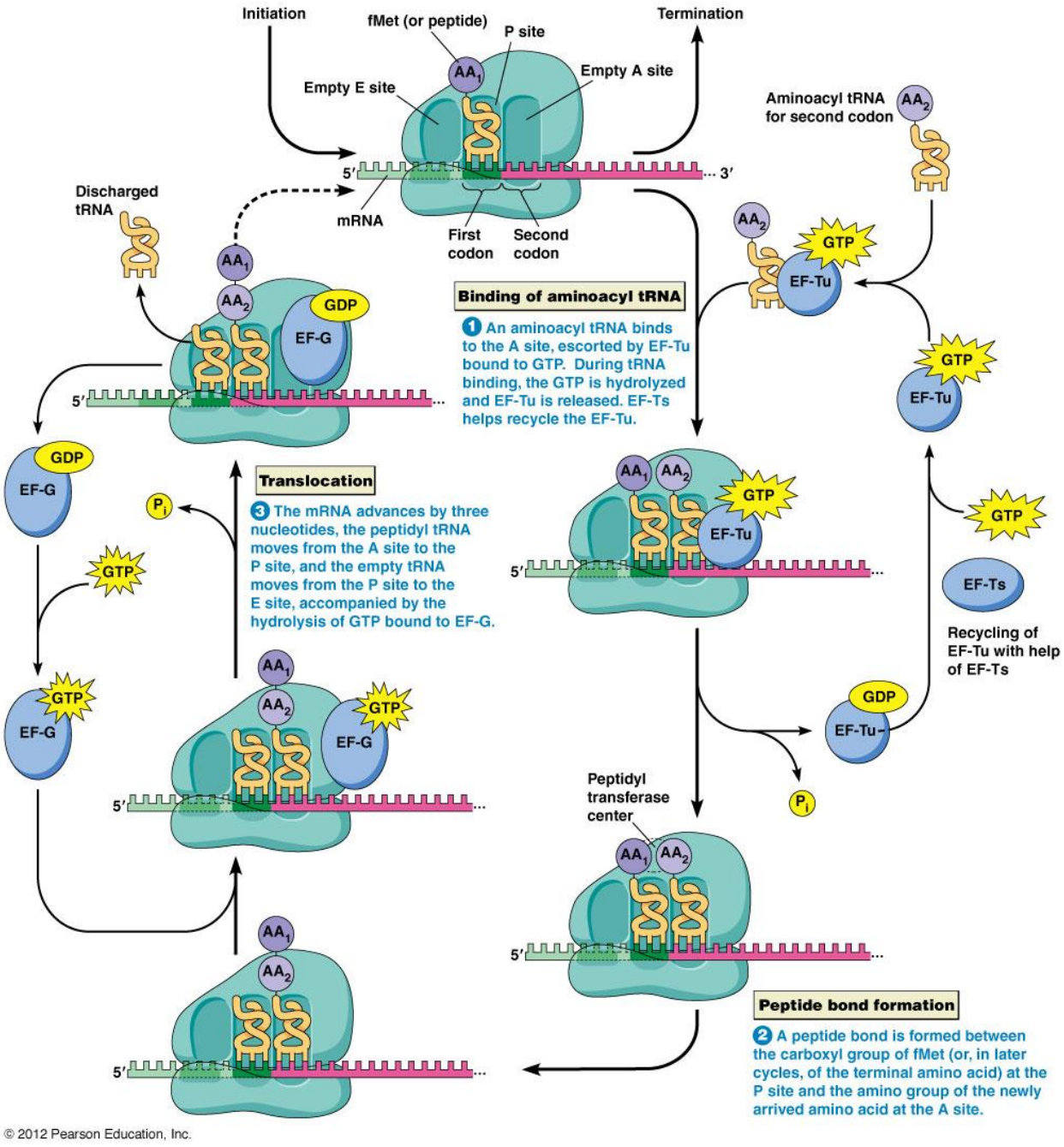
ii) Peptide bond formation: the peptide bond formation between the amino acid at P-site & A- site; the amino acyl t-RNA at the A- site will have the peptide whereas the P-site t-RNA becomes empty I,e called uncharged t-RNAfMet is remains bound to the P site.

iii) Translocation: peptide bond formation is followed by translocation which requires translocase (EF-G), a GTPase protein.

- The movement of m-RNA by one codan.
- The transferase of empty t-RNA from the p-site to the E-site.
- The transfer of aminoacyl t-RNA from the A-site to the P-site.
- Formation of an empty A-site.

Erythromycin is an antibiotic that selectively inhibits the translocase & there by inhibit protein synthesis.

Eukaryotic Elongationprocess requires eEF-1 which contains bacterial EF-TU & EF-TS activity.

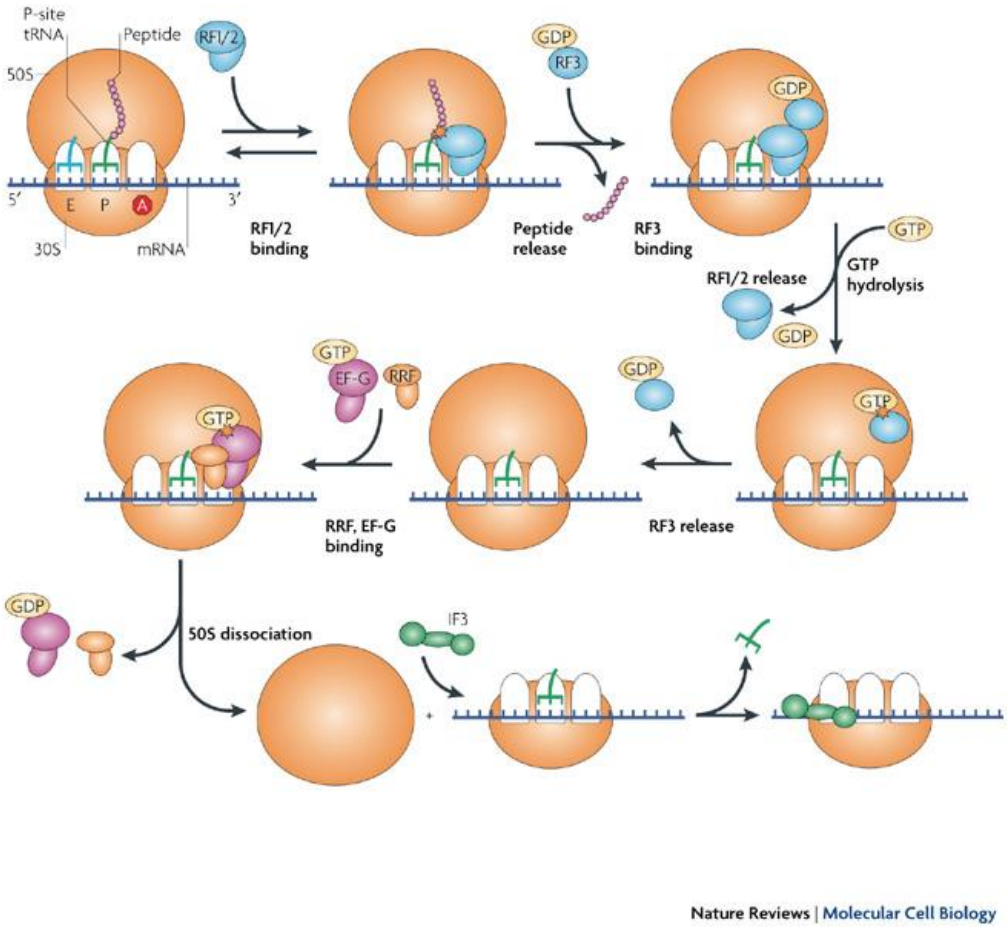


Termination:

- Elongation continues until the ribosome adds the last amino acid coded by the m-RNA. Termination.
- When the A- site on the ribosome reaches the termination codan, the ribosomes temporarily halts the transcription has it fails to get t-RNA having anti codans for termination codans; this allows the releasing factor RF-1 or RF-2 to bind to the A- site
- RF-1 recognizes UAA, & UAG whereas RF-2 recognizes UAA & UGA.

- RF-3 is a regulatory factor having the GTPase activity that releases the protein from the p- site t-RNA
- Termination process requires one highenergy bond

Eukaryotic termination requires a single releasing factor that can recognize all the 3 termination codans and also contains the GTPase activity to release the protein.



2.5 GENE EXPRESSION

2.5.1 Genetic code

A Dictionary or compilation that contains codon and their means in the form of amino acids or termination for translation.

- The concept of genetic code was given by George gamow.
- George gamow proposed a Triplet overlapping code. This was later modified by Fransis & crick to triplate non overlapping code.
- Codon is a sequence of nucleotides on DNA or m-RNA that recognizes a specific aminoacyl t RNA with complementary anticodan. DNA has 4 bases A,T,G&C and when transcribed into mRNA, the will be U,A,C&G and proteins contain 20 natural amino acids.
- Francis crick and his colleagues in their land mark paper in Nature entitled ''General nature of the genetic code for proteins'' concluded that the genetic code is a triplet code,the code is degenerate, triplets are not overlapping, there are no commas, and each nucleotide sequence is read from a specific starting point.

Triplet code:

- Each codan consist of 3 nucleotides successively present either in the coding strand of DNA or mRNA.
- Since there are four different bases, triplet code it contains $4^3 = 64$ codans.
- 61 codans specify the 20 standard amino acids and are called the sense codans.
- Addition or deletion of one or two nucleotide bases in DNA it causes frame shift mutations and that sequence alters the genetic message resulting in defective protein.
- 3 codans specify the termination of translation and are called Stop codans or termination codans or non-sense codan
- The termination codans are
  UAA=Ochre, UGA=Opal, UAG=Amber

Degenerate code (or) Redundancy) :

- The phenomenon in which an amino acid is specified by more than one codan is called degeneracy.(or) Two or more codans specify one amino acid
- Degeneracy occurs by there are 61 sense codans specifying 20 codans.
- There is no degeneracy for methionine (AUG)& tryptophan(UGG).
- The highest degree of degeneracy occurs for leucine,Arginine& serine which have 6 codans.
- Particular codon will always code for the same amino acid ths is called non-ambiguous.

1st letter	Second Letter: U	Second Letter: C	Second Letter: A	Second Letter: G	3rd letter
U	UUU Phe UUC UUA Leu UUG	UCU UCC UCA UCG Ser	UAU Tyr UAC UAA Stop UAG Stop	UGU Cys UGC UGA Stop UGG Trp	U C A G
C	CUU CUC CUA CUG Leu	CCU CCC CCA CCG Pro	CAU His CAC CAA CAG Gln	CGU CGC CGA CGG Arg	U C A G
A	AUU AUC AUA Ile AUG Met	ACU ACC ACA ACG Thr	AAU Asn AAC AAA AAG Lys	AGU Ser AGC AGA AGG Arg	U C A G
G	GUU GUC GUA GUG Val	GCU GCC GCA GCG Ala	GAU Asp GAC GAA GAG Glu	GGU GGC GGA GGG Gly	U C A G

Non-overlapping code:

- In such a code a nucleotide at a given position will be part of a single codan;in non overlapping code the number of codans will be 1/3 of nucleotides present in the molecule. $\overline{AGC}\overline{AGC}\overline{ACC}\overline{AGA}$

Comma less or Punctuation less:

- In the genetic code the adjacent codans are not separated by means of any nucleotide; each nucleotide in the reading frame is part of a specific codan.

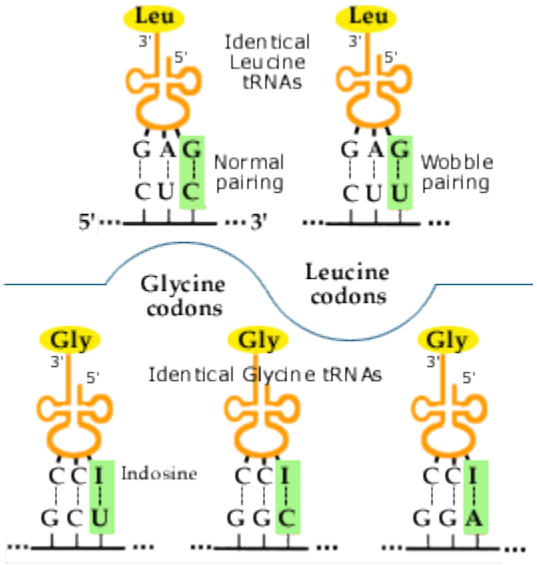
Universal code:

- The genetic code has the same meaning in all the organisms and this feature is called universality of the code.
- The universality of code allows the expression of genes from one group of organisms to another group for example: Bacterial proteins can be synthesized in eukaryotic cells and eukaryotic proteins in prokaryotic cells.
- However there are some exceptions to the universality of genetic code for example: in the universal code AGA &AGG specify Arginin whereas AGA &AGG are termination codans for mitochondria.

Wobble hypothesis

- Francis crick proposed the Wooble hypothesis for complementary base pairing between the Codans or m-RNA & the Anti codan in t-RNA.
- The 3’ Nucleotide position (Third Base) of the m-RNA codan and the 5’ nucleotide of t-RNA Anticodan(first Base) are called Wobble positions.
- the 5’ nucleotide of t-RNA Anticodan(first Base) is called Wobble Base.
- The pairing between third base of codan and first base of anticodan is wobble.
- When the pairing occurs between the Codan & Anticodan ,the pairing strictly follows the Watson crick pairing rules. In which A pairs with U & C pairs with G; however at the 3’ position of the codan the base pairing is relaxed.
- For example: the 5’ Nucleotide of t-RNA Anticodan is that of Ionosine and then it can pair with A,C,U
- Because of wobble pairing the number of t-RNAs required for translation is less than that of number of codans is specifying the amino acid. This kind of wobbling allows Economy of the number of t-RNAs.
- A minimum of 32 t-RNAs are required to translate all 61 sense sodans.For example:Anticodan IGC can recognize codans GCU,GCC,and GCA (coding for Alanine).

First base of an Anticodan	Pair with third base of codan
A	U
C	G
G	C,U
U	A,G
I	A,C,U



WOBBLE BASES.

2.5.2 GENE REGULATION – OPERON CONCEPT

An operon is a cluster of structural gene that is transcribe into a poly cistronic m-RNA that codes for protein products involve in a common biochemical pathway and the regulation of the operon occurs by a common regulatory mechanism.

- The concept of operon was proposed by Jacob & Monad based on the Lac operon of E.coli which codes for protein products involves in the catabolism of lactose
- An operon contains the following components.
- Regulatory gene: it is constitutively expressed and its expression is independent of the expression of structural genes.
- Promoter: it is a cis regulatory region that acts as the binding site for RNA polymerase
- Operator sequence: A cis regulatory region that acts as the binding site for active repressor molecule.
- Structural genes: The gene codes for protein products are called a structural gene. The structural genes are transcribed from a common promoter and produce a poly cistronic m-RNA.

LAC OPERON

Gene action is regulated by two different molecular mechanisms they are negative regulation and positive regulation.

i) Negative regulation (or) Inducible operon (or) Off-On operon:

- Elucidated by Jacob and monad.
- The operon has following organization.

Regulatory gene - Lac I :-

- Regulator gene; constitutively express;mono cistronic having this own promoter.
- Forms the active repressor which is a DNA binding protein.
- The tetrameric active repressor has very high affinity for the operator sequence.
- The binding of lac repressor to the operator inactivates the structural gene expression.

Promoter- Lac P :-

- The promoter region of the structural gene cluster and is the binding site for RNA polymerases involved in the +ve regulation of the Lac operon.

Operator – Lac O:-

- The operator sequence which is the binding site for active tetrameric repressor
- When the operator is bounded by the repressor the operon remains transcriptionally inactive

- When the operator is free from the repressor, the operon remains transcriptionally active.

Structural Genes (Lac Z, Y, A):

Lac-Z: codes for the enzyme β −galactosidase that has two functions.

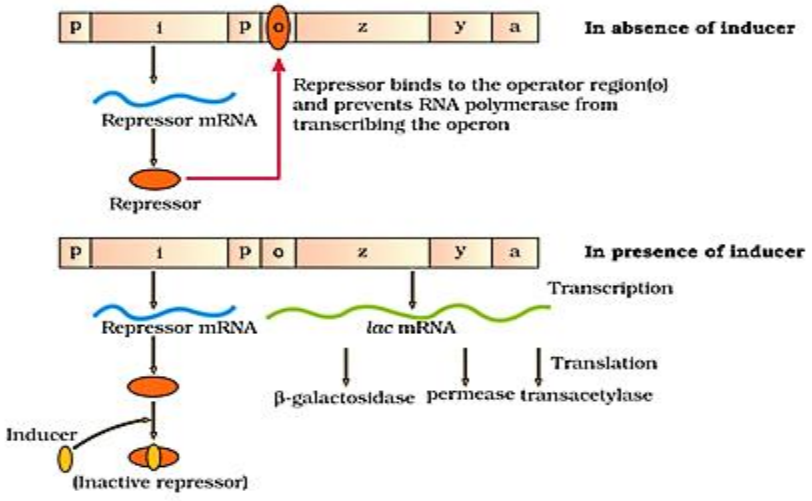
- It hydrolyzes lactose into galactose and glucose.
- It converts some of the lactose into allolactose; this is the true inducer of lac operon.

Lac- Y:codes for β −galactoside permiase, a Transmembranal carrier protein involved in the facilitated diffusion of lactose in to the bacterial cell.

Lac- A:encodes trans acetylase , an enzyme involved in the transfer of acetyl group from acetyl co-enzyme to galactoside.

Mechanism:

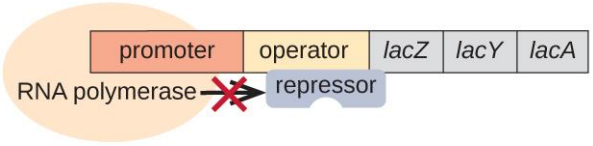
- The regulatory gene codes for the active repressor that binds to the operator sequence and inhibits the expression of the structural genes; the operon remain in the OFF state.
- When lactose is added to the growth medium and the basal activity of permease uptakes some of the lactose into the cell and basal activity of β −galactosidase converts lactose into allolactose and their by producing the inducer molecule.
- Inducer (Allo lactose) binds to free repressor as well as repressor bound to the operator causes the inactivation of the repressor. It leads to repressor is released from the operator.
- Now operator free from the repressor.
- Then RNA polymerase binds to the structural gene promoter and causes the expression of the structural genes (ON state of Operon).
- Once activated, within a short time the concentrations of β −galactosidase and permease increase by more than million fold.Then these enzymes synthesis will takes place. And these enzymes are used for the lactose catabolism process.



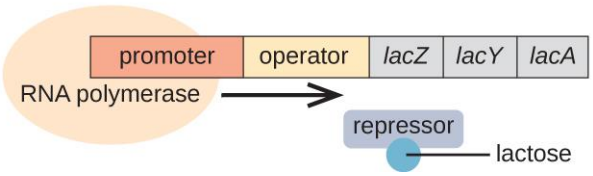
ii) Positive regulation (or) Repressor (or) ON-OFF Operon :-

- Some DNA binding proteins that stimulate transcription.
- Catabolite activator protein (CAP), which is also known as the cAMP response protein (CRP).
- It involves a CAP binding site upstream of the promoter.
- The CAP protein remains inactive when it is free from cAMP but gets activated when cAMP binds to it.
- The active state of CAP is determined by the availability of cAMP which intern is determined by the availability of ATP and the entry of glucose in to the cell.
- As glucose enters in to the cell, most of the cellular ATP is used for glucose Phaspharilation and therefore cyclic AMP levels will be low &CAP remains in the inactive state.
- When the glucose entry in to the cell is low due to low glucose in the growth medium, ATP will be available for the formation of cAMP.
- As cAMP is synthesized from ATP,cAMP binds to CAP and inactivates it.
- The activated CAP-cAMP complex binds to the CAP binding site and increases the affinity fo RNA polymerase;this increases the expression of Lac operon.
- By the expression of Lac operon enzyme synthesis will takes place and these enzymes are involved in the digestion of the lactose into galactose and glucose. Some amount of the lactose converts into allo lactose which is binds to the repressor and inactivates repressor.
- Then RNA polymerase action will begins.
- For optimal expression of Lac operon the concentration of glucose should be low and lactose should be high.

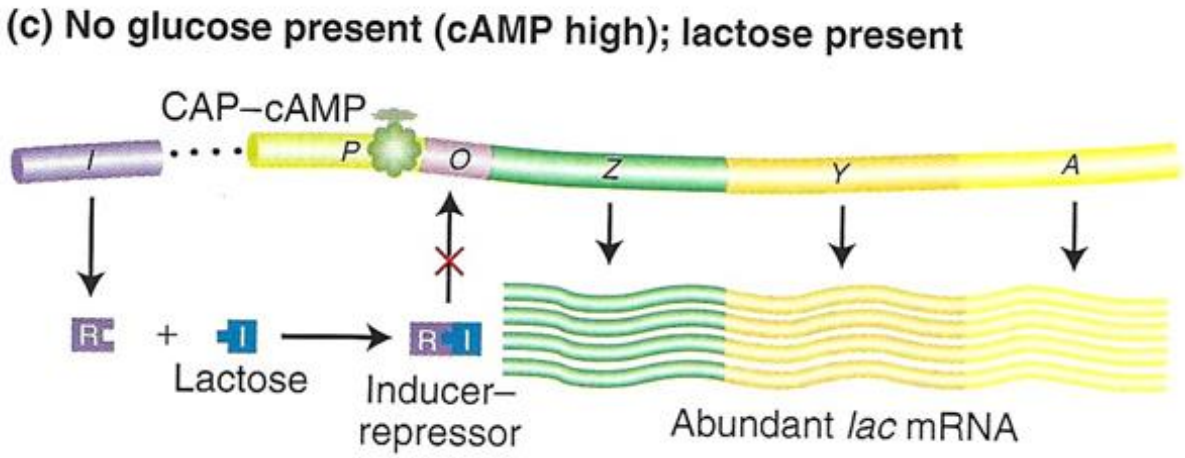
In the absence of lactose, the *lac* repressor binds the operator, and transcription is blocked.



In the presence of lactose, the *lac* repressor is released from the operator, and transcription proceeds at a slow rate.



Lactose, no glucose



2.6 Molecular Biology Techniques

2.6.1 Polymerase chain reaction (PCR)

PCR is a method widely used in molecular biology to rapidly make millions to billions of copies of a specific DNA sample allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail.

- PCR was invented in 1983 by Kary Mullis. It is fundamental to much of genetic testing including analysis of ancient samples of DNA and identification of infectious agents.
- Using PCR, copies of very small amounts of DNA sequences are exponentially amplified in a series or cycles of temperature changes.
- The majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions – specifically, DNA melting and enzyme-driven DNA replication.

The PCR reaction requires the following components.

***DNA* template**: that contains the DNA target region to amplify

DNA* polymerase*: enzyme that polymerizes new DNA strands; heat-resistant Taq polymerase is especially common, as it is more likely to remain intact during the high-temperature DNA denaturation process.

- Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the thermophilic bacterium Thermusaquaticus. If the polymerase used was heat-susceptible.

DNA primers: that are complementary to the 3' ends of each of the sense and anti-sense strands of the DNA target .

- Specific primers that are complementary to the DNA target region are selected. Two primers must be designed for PCR; the forward primer and reverse primer.

Primer length: the primer should be 18-24 nucleotides long so that they anneal to complementary sequences of templates with good specificity and strength.

No hairpin loops: each primer needs to be devoid of polindromic sequences which can give rise to stable intra strand structure that limit proper annealing.

Optimal distance between primers: In between the opposing primers is spaced 150-500 bp part.

Procedure: There are three major steps in PCR, which are repeated for 30 to 35 cycles. This is done on a automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98 °C for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

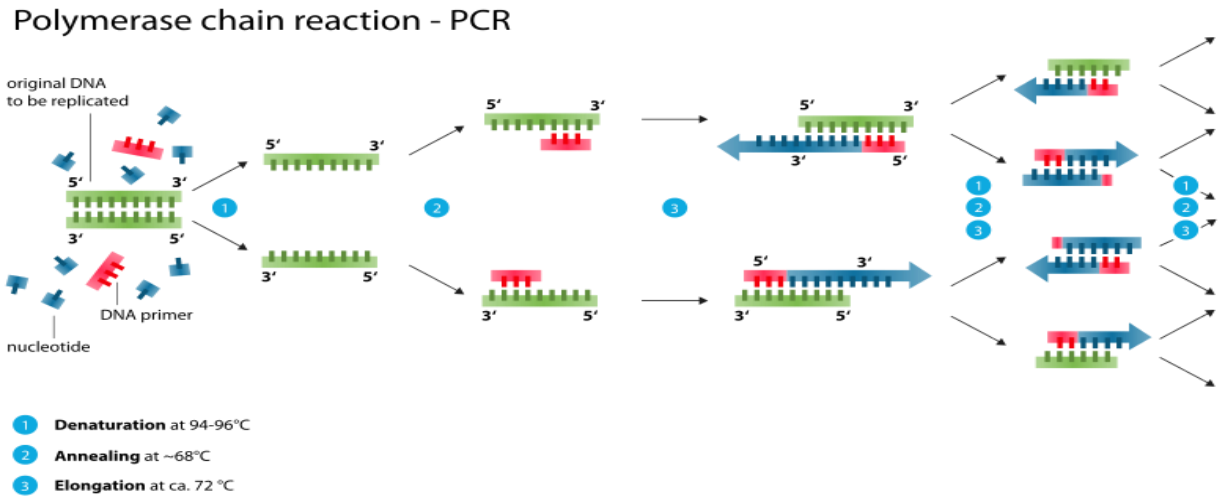
Annealing: In the next step, the reaction temperature is lowered to 50–65 °C for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates.

- Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region.
- The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.
- The primer should be consists of GC rich sequences because these sequence have three hydrogen bonds(between primer & template) these increase affinity with target DNA

Extension at 72 °C: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermo stable DNA polymerase of Taq (Thermus aquaticus) polymerase is approximately 75–80 °C though a temperature of 72 °C is commonly used with this enzyme.

- In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand.

Finalhold: first three steps are repeated 35-40 times to produce millions of exact copies of the target DNA. The final step cools the reaction chamber to 4–15 °C for an indefinite time, and may be employed for short-term storage of the PCR products.



PCR an exponential cycle: As both strands are copied during PCR there is an exponential increase in the number of copies of the genes

- Suppose there is only one copy of the desired gene before the PCR starts, after one cycle there will be a two copies after two cycles there will be a 4 copies ,so on at the end of 30th cycle, there will be billions copies of DNA. After 35 cycles the amplification reaches plateau.

Variation of PCR: Reverse transcription PCR it is a variant of PCR in which RNA is used as template to produce cDNA which is complimentary to RNA . this reaction is catalyzed by the enzyme Reverse transcriptase.

Real time PCR

- RT- PCR also known as quantitative PCR is used to amplify and simultaneously quantify a target DNA .
- Real time PCR is widely used in expression profiling to determine the expression of a gene or to identity the sequence of an RNA transcript
- Many probes are used for detection of amplified product in RT-PCR such as TaqMan probe, molecular beacons, dsDNA binding dyes of which TaqMan probes are most widely used.
- TaqMan probes are oligonucleotide probes which have a Flurophore at its 5' end and a quencher at its 3' end.
- The quencher molecules quenches the fluorescence emitted by flurophore when excited by the light source
- When DNA polymerase starts synthesizing the new strand, it degrades the probes which release the flurophore.
- This inhibits quenching effect of the quencher and allows fluorescence of the flurophore.
- Hence fluorescents detected in RT-PCR are directly proportional to the amount of DNA template.

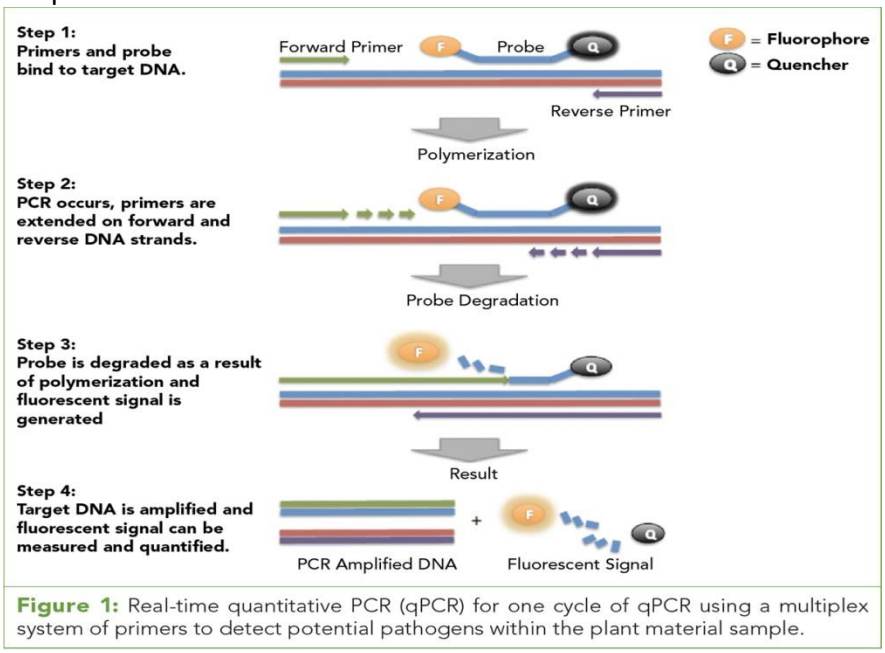


Figure 1: Real-time quantitative PCR (qPCR) for one cycle of qPCR using a multiplex system of primers to detect potential pathogens within the plant material sample.

PCR APPLICATIONS

- Applications of the technique include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis.
- construction of DNA-based phylogenies, or functional analysis of genes; diagnosis and monitoring of hereditary diseases; amplification of ancient DNA.

- analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.
- Viral targets such as HIV and HCV can also be identified and quantified by PCR.
- PCR permits early diagnosis of malignant disease such as leukemia and lymphomas. PCR assays can be performed directly on genomic DNA samples to detect translocation specific malignant cells infectious agents, like mycobacterium,bacteria,viruses.

2.6.2 ELECTROPHORESIS

Electrophoresis is an electro kinetic process which separates charged particles in a fluid using a field of electrical charge. It is most often used to separate protein molecules or DNA and can be achieved through several different procedures depending on the type and size of the molecules.

- This technique is used to separate large molecules such as DNA, RNA and proteins by size using an applied electric field and sieving matrix.
- Agarose and Acrylamide gels are common sieves.
- DNA and RNA are negatively charged molecule due to their phosphate backbone. and they naturally travel towards the positive charge at the far end of the gel
- Proteins are composed of amino acids that can be positively, negatively or uncharged. To give proteins a uniformity negative charge. They are coated with detergent, SDS prior to running them on Gel.

Principle:

- The law of electrostatics states that the electric force, Felectric, on an ion with charge q in an electric field of strength E is expressed by
- $F_{electric} = qE$
- The resulting electrophoretic migration of the ion through yhe solution is opposed by a frictional force.
- $F_{frictional} = vf$
- Where v is the rate of migration of the ion and f is its frictional coefficient.

Moving boundary electrophoresis:-

- Moving-boundary electrophoresis was developed by Arne Tiselius in 1930. Tiselius was awarded the 1948 Nobel Prize in chemistry for his work on the separation of colloids through electrophoresis, the motion of charged particles through a stationary liquid under the influence of an electric field.
- The moving-boundary electrophoresis apparatus includes a U-shaped cell filled with buffer solution and electrodes immersed at its ends.
- The sample applied could be any mixture of charged components such as a protein mixture. On applying voltage, the compounds will migrate to the anode or cathode depending on their charges.

- The change in the refractive index at the boundary of the separated compounds is detected using Schlieren optics at both ends of the solution in the cell.
- **Disadvantages** of **Moving Boundary electrophoresis**-The resolution of the technique is very low due to the mixing of the sample as well as over-lapping of the sample components.
- The electrophoresis technique is not good to separate and analyze the complex biological samples.

Zone Electrophoresis

- Zone electrophoresis in a chemically inert gel matrix, such as polyacrylamide or agarose. The sample is applied in a small volume as a narrow zone, e.g., in gel slots. As the electric field is applied, each sample component migrates according to its own mobility in a gel medium of constant pH and ionic strength.
- The separation into 'pure zones' is achieved by maximizing the differential rate of migration, while minimizing zone spreading (dispersion) due to heat convection and diffusion.
- We have two different geometrical forms in which gel electrophoresis can be carried out: horizontal gel electrophoresis, vertical gel electrophoresis.

Vertical Gel electrophoresis:

A **vertical** system utilizes a discontinuous buffer system, where the top chamber contains the cathode and the bottom chamber contains the anode.

1. **Sodium dodecyl sulphate Polyacrylamide Gel electrophoresis (SDS-PAGE):-**

   Instrument: It has two chambers, an upper and lower. Both chambers are fitted with the platinum electrodes connected to the external power supply from a power pack, which supplies a DC current. The upper and lower tanks filled with the running buffer are connected by the electrophoresis gel casted in between two glass plates

- There are additional accessories needed for casting the polyacrylamide gel such as comb,spacer, gel caster etc.

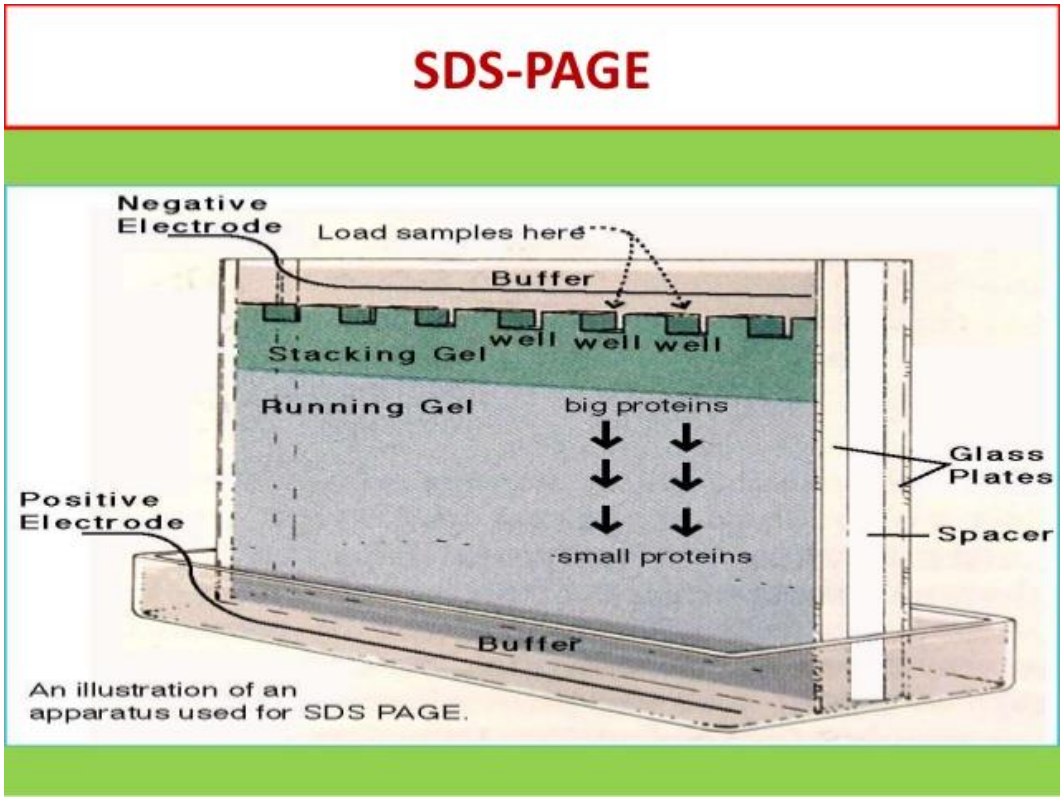
Buffers and reagents for electrophoresis:-

i. N,N,N,N-Tetra methyl ethylene diamine (TEMED):- it catalyses the acryl amide polymerization.
ii. Ammonium per sulfate (APS):- it is an initiator for the acylamide polymerization
iii. Tris-HCL: it is the component of running and gel casting buffer.
iv. Glycine :it is the component of running buffer.
v. Bromophenol blue: it is the tracking dye to monitor the progress of gel electrophoresis.
vi. Coomassie brilliant blue R250: it is used to stain the polyacrylamide gel.

vii. Sodium dodecyl sulphate : it is used to denature and provide negative charge to theprotein.
viii. Acrylamide: monomer used to prepare gel.
ix. Bis- acrylamide : cross linker for polymerization of acrylamide monomer to form gel.

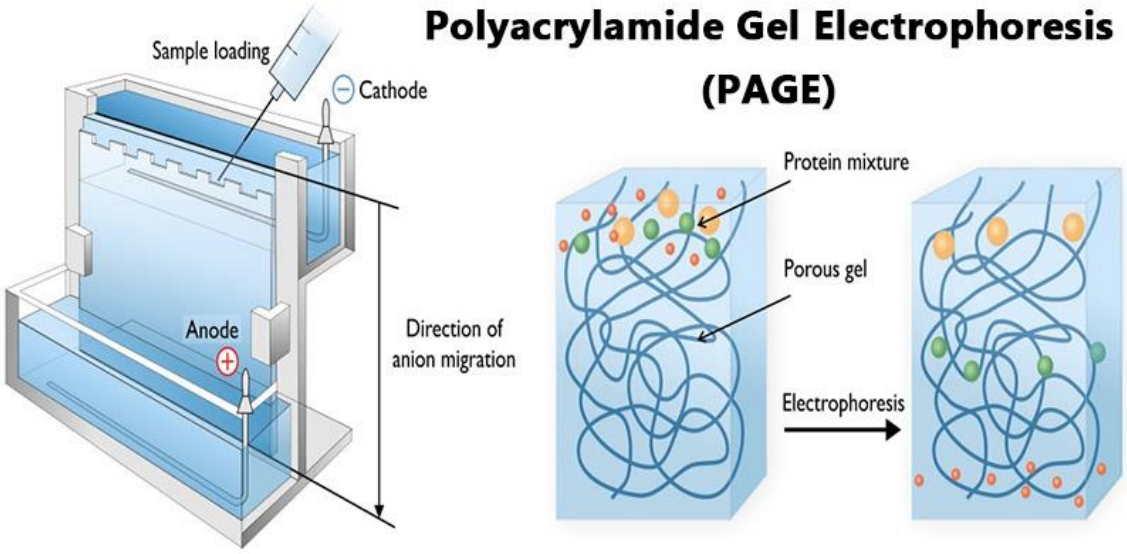
Casting of the Gel :

- The acrylamide solution is mixed with TEMED and APS and poured in between the glass plates fitted into the gel caster.
- Ammonium persulfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acrylamide monomer into linear polymer
- These linear polymers are interconnected by the cross linking bis-acrylamide monomer to form 3-D mesh with pores.
- The size of pore is controlled by the concentrations of acrylamide and amount of bis-acrylamide in the gel.
- In a vertical gel electrophoresis system two types of gels are cast,stacking gel and resolving gel.
- First the resolving gel solution is prepared and poured into the gel cassette for polymerization
- A thin layer of organic solvents is layered to stop the entry of oxygen and make the top layer smooth
- After polymerization of the resolving gel, the stacking gel is poured and comb is fitted into the gel for construction of different lanes for the samples.



Proteins are separated by SDS-PAGE:

- Proteins can be separated largely on the basis of mass by electrophoresis in a poliy acrylamide gel under denaturing conditions.
- The mixture of protein is first dissolved in a solution of sodium dodecyl sulfate , an anionic detergent that disrupts nearly all non covalent interactions in native proteins.
- Mercaptoethanol is added to reduce di sulfide bonds.
- Anions of SDS binds to main chains at a ratio of about one SDS anion for every two amino acid residues
- The complex of SDS with a denatured protein has a large net negative charge that is roughly proportional to the mass of the protein.
- The SDS –protein complexes are then subjected to electrophoresis. When the electrophoresis is complete, the proteins in the gel can be visualized by staining them with silver or a dye such as coomssie blue, which reveals a series of bands.



Horizontal Gel electrophoresis :

This technique is used to separate native proteins and nucleic acids. The electrophoresis in gel system is performed in a continuous fashion with both electrodes and gel cassette submersed within the buffer.

2. **<u>NATIVE-PAGE :-</u>**

- In the Native PAGE sample is prepared in the loading dyes does not contains detergents or denaturing agent and as a result sample runs on the basis of charge/mass
- In Native PAGE the D confirmation as well as activity of the protein remains unaffected.

Buffer and reagent for electrophoresis:

i) Agarose : polymeric sugar used to prepare horizontal gel for DNA analysis.
ii) Ethidium bromide : for staining of the agarose gel to visualize the DNA.
iii) Sucrose: for preparation of loading dye for horizontal gel.

iv) Tris-HCL- the component of the running buffer.

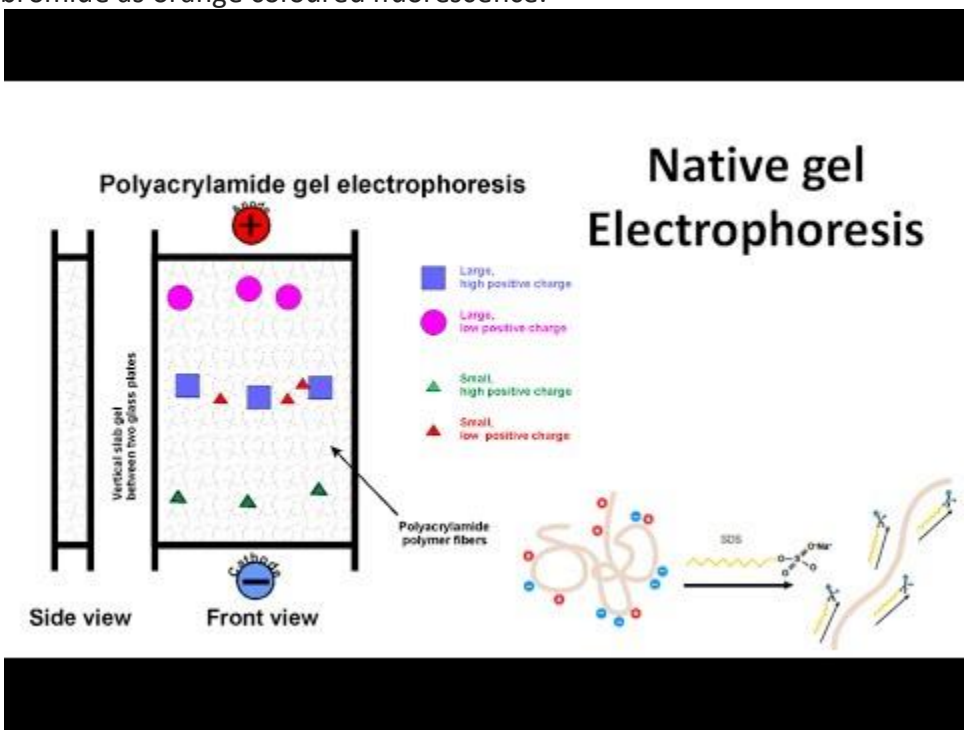
v) Bromophenol blue : Tracking dye to monitor the progress of the electrophoresis.

Casting of Agarose Gel:

- The agarose powder is dissolved in a buffer and heated to melt the agarose
- Hot agarose is poured into the gel cassette and allowed it to set
- A comb can be inserted into the hot agarose gel to cast the well for loading the sample.
- In few cases we can add ethidium bromide within the gel so that stains the DNA while electrophoresis.

Running and Staining:

- The gel cassette is placed in the electrophoresis tank submerged completely and DNA loaded into the well with the help of micropipette and run with constant voltage.
- DNA runs from negative to positive end and ethidium bromide present in the gel stain in the DNA .
- Observing the agarose gel in a UV- chamber shows the DNA stained with ethidium bromide as orange coloured fluorescence.



Applications of Horizontal agarose Gel electrophoresis :

- The size of a DNA can be determined by comparing the size of the known DNA molecules.
- The DNA s known sizes are resolved on 0.8% agarose along with the unknown sample.
- The value of the relative migration of each DNA band is calculated from the agarose gel.
- The value of relative migration and size of the DNA is used to draw the calibration curve to calculate the size of the unknown DNA samples.

Southern blotting:

Southern blotting is a method in molecular biology for detection of a specific DNA sequence in DNA samples.

- Southern blotting cannot be used in expression analysis as southern blotting only indicates the presence or absence of a specific DNA sequence in the genomic DNA.

Southern blotting involves following steps.

- Isolation of total genomic DNA
- Fragmentation of genomic DNA by restriction digestion by using suitable restriction endonucleases
- Electrophoretic separation on Agarose gels.
- Transfer of DNA fragments from the gel on to the nitrose cellulose membrane by capillary action by applying a uniform pressure either by suction pressure or by placing wet paper towels.
- Denaturation of transferred DNA fragments on the solid surface.
- Fixation of SSDNA by either vacuums banking of UV cross linking.
- Blocking the solid surface using casein,BSA,defeated milk or heparin
- Probing with labeled oligoribonucleotide probes
- Auto radiography or fluorescent analysis.
- Southern blot is used in sequencing and analyzing DNA for mutations, cancer,DNA finger printing, genetic engineering and in forensic science.