BIOTECHNOLOGY

INTRODUCTION

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Definition:

- **Biotechnology** is the use of living systems and organisms to develop or make useful products, or "any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use“
- European Federation of Biotechnology (EFB) has defined biotechnology as “The integration of natural science and organisms, cells, parts thereof, and molecular analogues for products and services”.

Oldest form of biotechnology

Making breads and curds with the help of micro organisms.

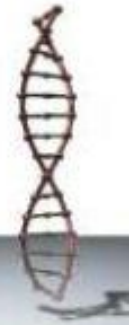
- Two important technique which enable development of modern biotechnology:

1. Alteration of chemistry of DNA & RNA to introduce into host organism to change phenotype of host- **Genetic engineering**
2. Maintenance of sterile ambience to enable growth of desired microbe/ eukaryotic cell in large quantities for manufacture of biotechnological products like *vaccine, enzymes, beverages, drugs etc.*- **Chemical engineering**

- Genetic engineering- isolate & introduce only one or set of desirable genes without introducing undesirable genes in target organism
- Techniques of genetic engineering- creation of **recombinant DNA**, use of **gene cloning** & **gene transfer** to *host*
- Recombinant DNA (rDNA)/ alien DNA- cannot multiply itself until integrated in host genome
- When inherited in host DNA- ability to replicate due to **origin of replication** (host DNA)- initiates replication
- Alien DNA- linked with host DNA replicates & multiply itself along with host DNA- **Cloning**

- Gene transfer to host require **Vector**
- Commonly used vector- **Plasmid** (small, circular, double stranded, self replicating extra chromosomal material of bacteria)
- First recombinant DNA was constructed- Stanley Cohen & Herbert Boyer (1972) by linking gene encoding for antibiotic resistance with plasmid of *Salmonella typhimurium*
- Isolation of desirable gene (antibiotic resistant)- cutting out piece of DNA from a plasmid responsible of antibiotic resistance which involve 'molecular scissors'- **restriction enzymes**
- Desirable gene/ alien DNA – linked with plasmid (vector) to transfer into host organism
- Linking of DNA involves **DNA ligase**- acts on cut DNA molecules & join their ends- new combination circular autonomously replicating DNA created *in vitro*- **Recombinant DNA**

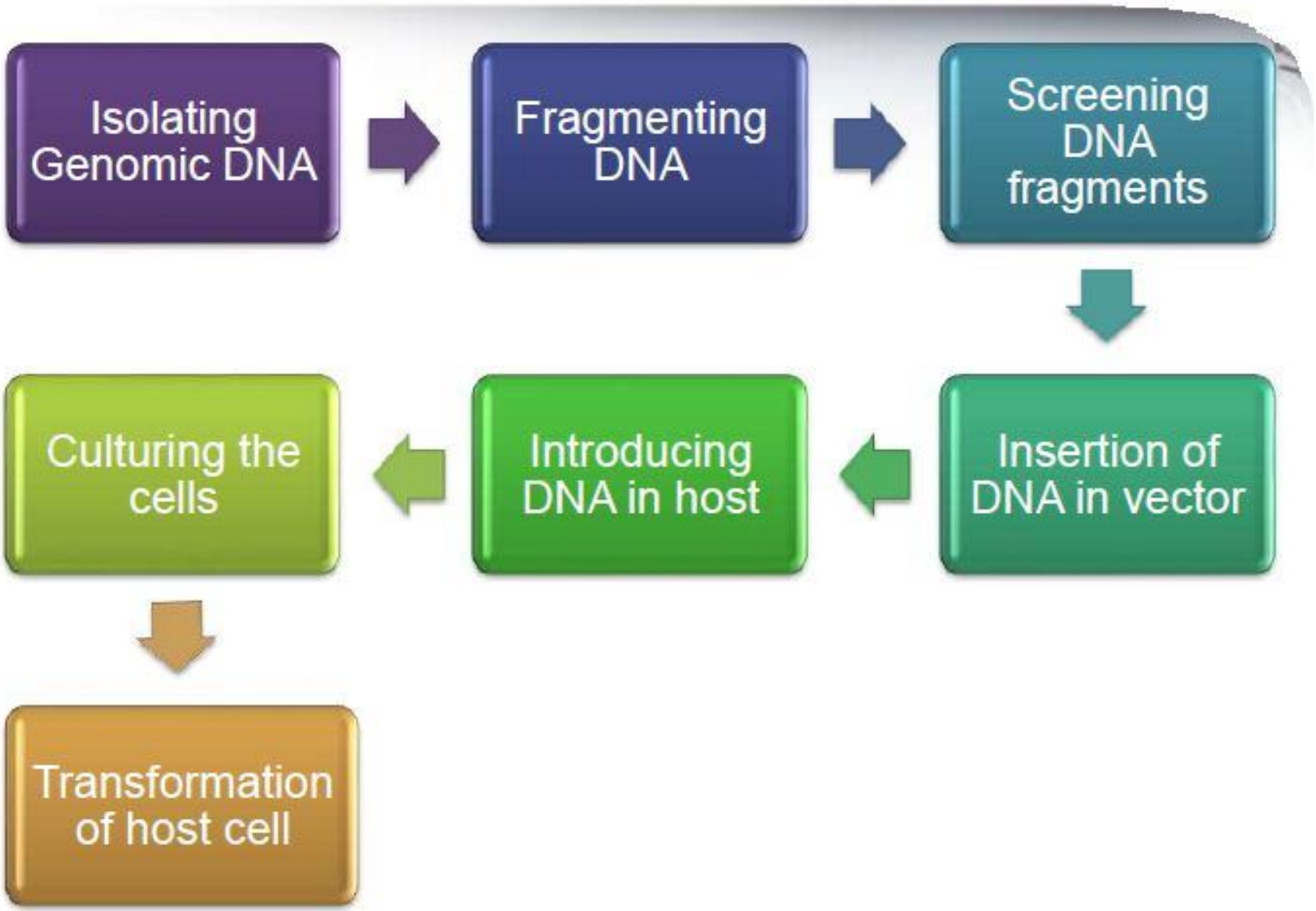
Process of recombinant DNA technology



- Recombinant DNA technology involves following steps:

1. Isolation of genetic material DNA
2. Fragmentation of DNA by restriction endonuclease
3. Isolation of desired DNA fragments
4. Ligation of the DNA fragments into a vector
5. Transferring rDNA into host
6. Culturing host cells in a medium at large scale
7. Extraction of desired product

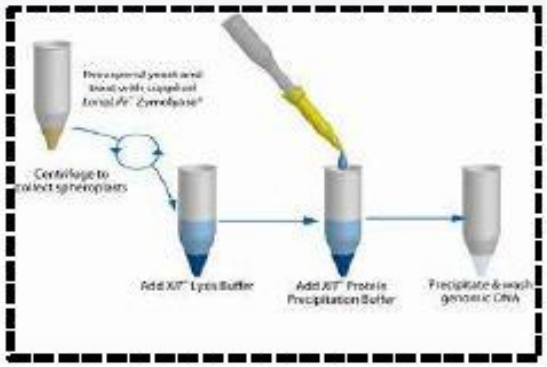
Basic steps involved in process



Basic steps involved in process

1. **Isolating genomic DNA**

Isolating genomic DNA from the donor.



2. **Fragmenting this DNA**

Fragmenting this DNA using molecular scissors.

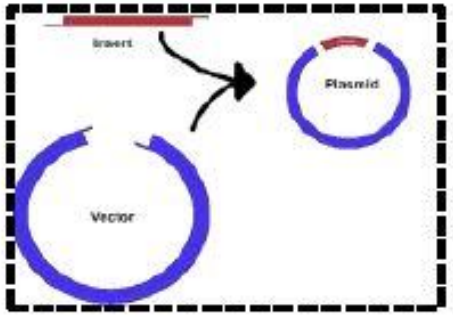
Basic steps involved in process

3. Screening the fragments

Screening the fragments for a "desired gene".

4. Insertion of DNA in a vector

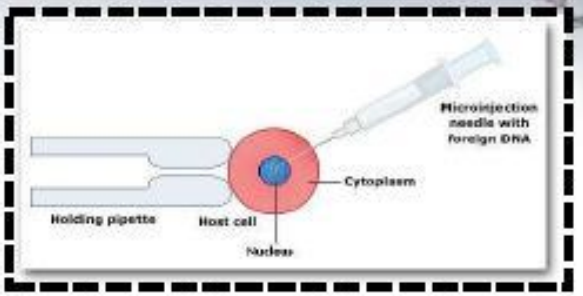
Inserting the fragments with the desired gene in a 'cloning vector'.



Basic steps involved in process

5. **Introducing in Host**

Introducing the recombinant vector into a competent host cell

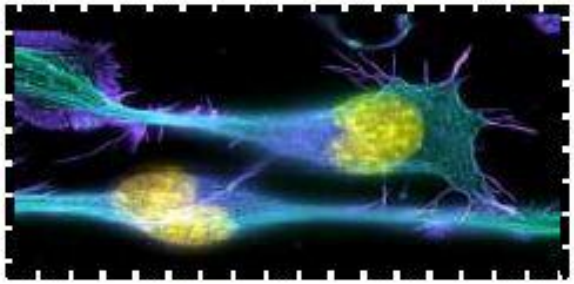


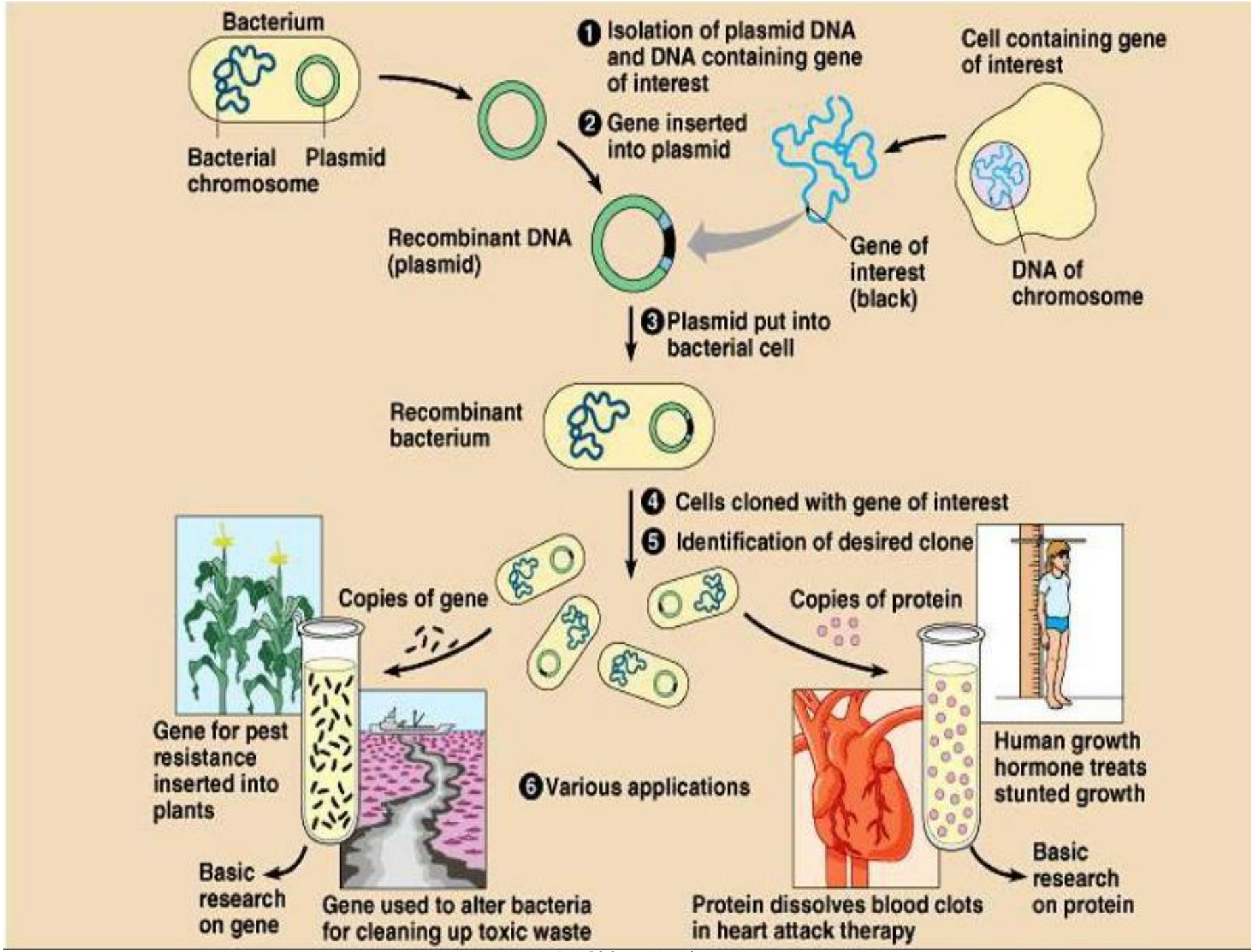
6. **Culturing the cells**

Culturing these cells to obtain multiple copies or clones of desired DNA fragments

7. **Transformation of host cell**

Using these copies to transform suitable host cells so as to express the desired gene.




Bacterium
Bacterial chromosome
Plasmid
1 Isolation of plasmid DNA and DNA containing gene of interest
2 Gene inserted into plasmid
Cell containing gene of interest
Recombinant DNA (plasmid)
Gene of interest (black)
DNA of chromosome
3 Plasmid put into bacterial cell
Recombinant bacterium
4 Cells cloned with gene of interest
5 Identification of desired clone
Copies of gene
Copies of protein
Gene for pest resistance inserted into plants
Human growth hormone treats stunted growth
6 Various applications
Basic research on gene
Gene used to alter bacteria for cleaning up toxic waste
Protein dissolves blood clots in heart attack therapy
Basic research on protein

Gene Cloning

- Definition: using bacteria to make multiple identical copies of a single stretch of DNA.
- Useful in understanding eukaryotic genome.
- Cloning Vector:
  - Any vehicle that inserts a fragment of foreign DNA into the genome of a host cell.
  - Example: virus or genetically engineered plasmid.
  - Used in gene therapy.

CLASSICAL DEFINITION OF GENE

Gene is the unit of-

- Function (one gene specifies one character),
- Recombination, and
- Mutation.

MORDERN DEFINITION OF GENE

- Unit of Genetic Information (Unit of DNA that specifies one polypeptide)
- Includes coding as well as non-coding regulatory sequences.

Exons and Introns

- **Exons** are segments of a gene that encode mature mRNA for a specific polypeptide chain.
- **Introns** are segments of a gene that do not encode mature mRNA. Introns are found in most genes in eukaryotes and in some gene of bacteriophage and archae .

ESSENTIAL FEATURES OF GENE

- Can duplicate themselves very accurately (Replication).
- Synthesizes a particular Protein.
- Determines the sequence of amino acid in the polypeptide chain

SOME TERMS RELATED TO GENE

- RECON - It is the smallest unit of DNA capable of undergoing Crossing Over & Recombination.
- MUTON - It is the smallest unit of DNA which can undergo Mutation.

SOME TERMS RELATED TO GENE

- COMPLON - It is the unit of complementation.
- CISTRON - The portion of DNA specifying a single polypeptide chain is termed as cistron.

ESSENTIAL FEATURES OF GENE

- Determines the physical as well as physiological characters.
- Situated in the chromosome.
- Occupies a specific position known as Locus.

ESSENTIAL FEATURES OF GENE

- Arranged in single linear order.
- Occur in functional states called Alleles.
- Some have more than 2 alleles known as Multiple Alleles.

ESSENTIAL FEATURES OF GENE

- Some may undergo sudden and permanent change in expression called as Mutant Gene (Mutation).
- May be transferred to its homologous (Cross-over) or non-homologous counterpart (Translocation).

Gene Cistron Relationship

- **Prokaryotes** : Genes and Cistrons are equivalent
- **Eukaryotes :** Cistron is equivalent to the exons

Cis and Trans position

***Cis position*:** *Genes* in the *cis position* are on the same chromosome of a pair of homologous chromosomes.

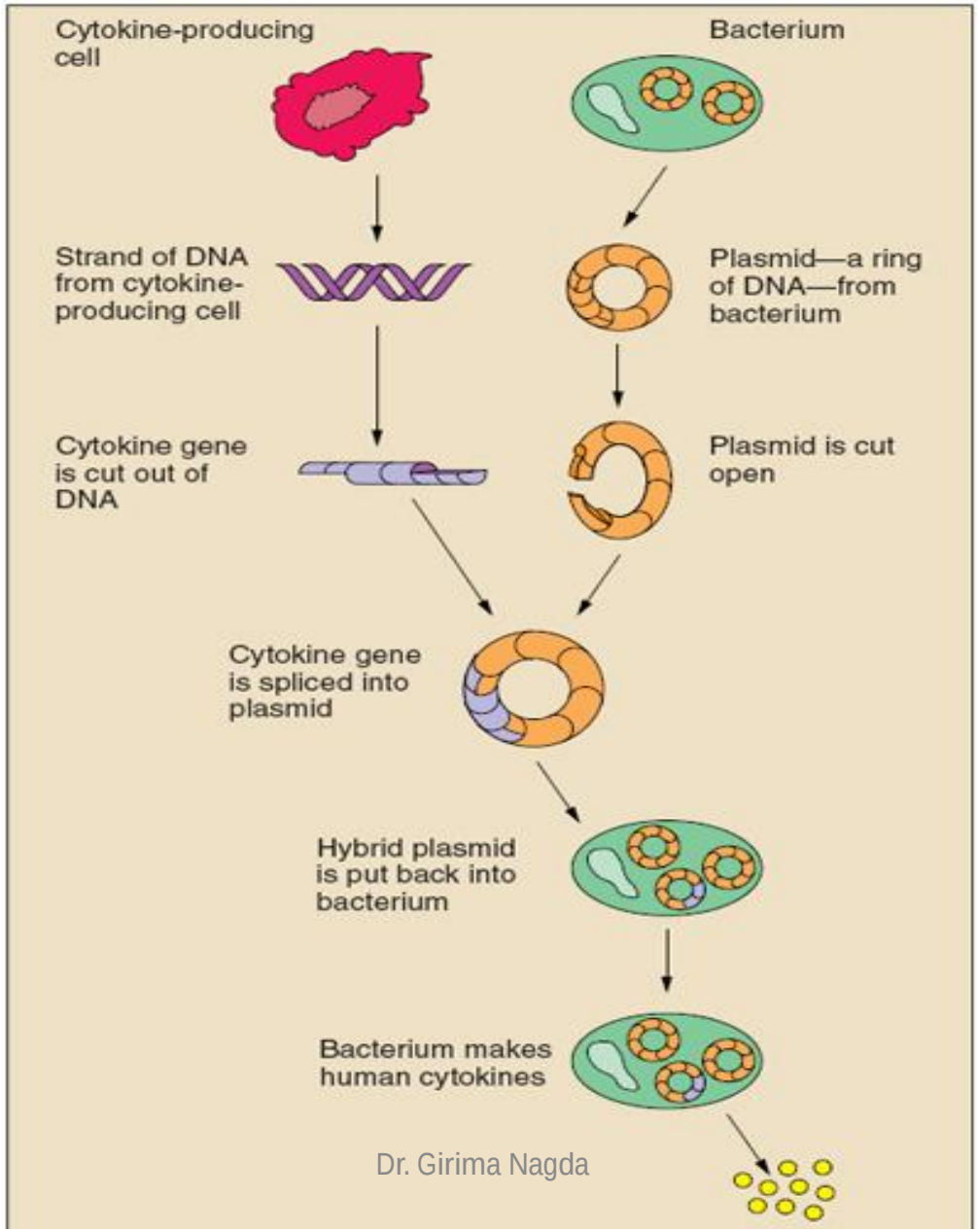
Trans position: *Genes* in the *trans position* are on the different chromosomes of a pair of homologous chromosomes.

Genetic Engineering

- Definition: Ability to precisely manipulate DNA sequences from widely different organisms.
- Process requires
  - Ability to cut DNA
  - To insert foreign DNA segment
  - “Glue” DNA sequences together

GENETIC ENGINEERING may be described as the introduction of manipulated genetic material in to a cell in such a way as to replicate and be passed on to the progeny cell.

Genetic Engineering Technique

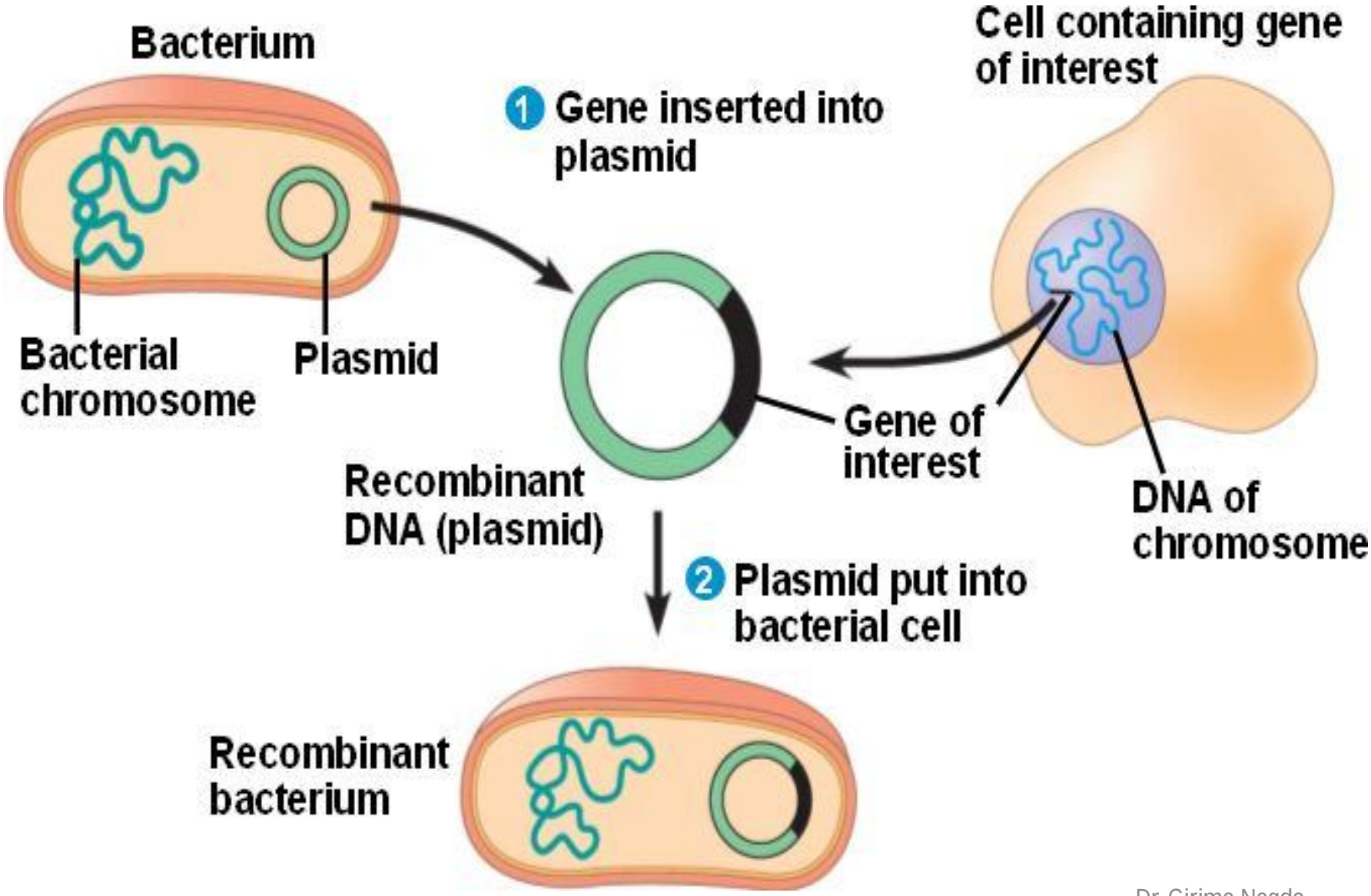


Gene Manipulation: Gene manipulation is defined as the formation of a new combination of a heritable material by the insertion of nucleic acid molecules (DNA molecules) into bacterial plasmid or any other vector so as to allow their incorporation in to the host organism, in which they are capable of containing propagation.

Microinjection Technique

Gene Cloning: The insertion of a fragment of DNA carrying a gene into a cloning vector and subsequent propagation of recombinant DNA molecules into many copies is known as gene cloning.

Gene Cloning Technique



BASIC STEPS OF GENE CLONING

- Construction of recombinant DNA molecule
- Transport of the recombinant DNA to the host cell
- Multiplication of recombinant DNA molecule
- Division of the host cell
- Numerous cell division resulting in a clone

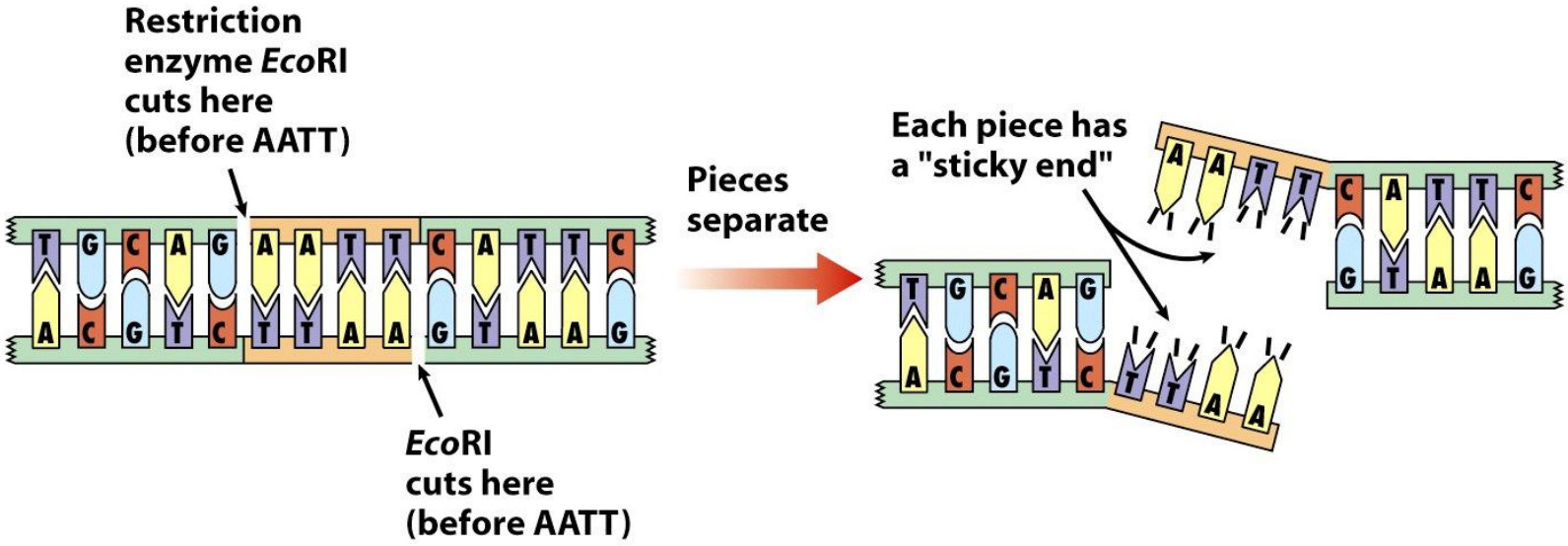
Gene cloning requires specialized tools and techniques:

Vehicles: The central component of a gene cloning experiment is the vehicle, which transport the gene into the host cell and is responsible for its replication. To act as a cloning vehicle a DNA molecule must be capable of entering a host cell and, once inside, replicating to produce multiple copies of itself.

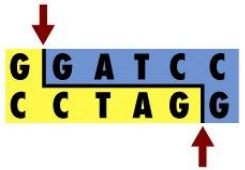
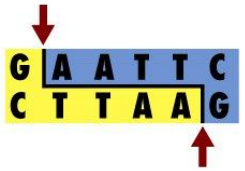
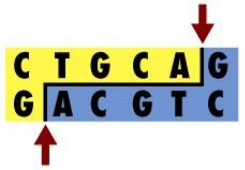
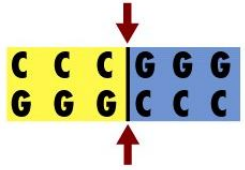
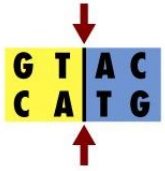
Vector: A DNA molecule, capable of replication in a host organism, into which a gene is inserted to construct a recombinant DNA molecule.

Molecular Scissors

- Restriction enzymes:
  - Cut DNA at specific places called recognition sites.
  - Form "sticky ends."



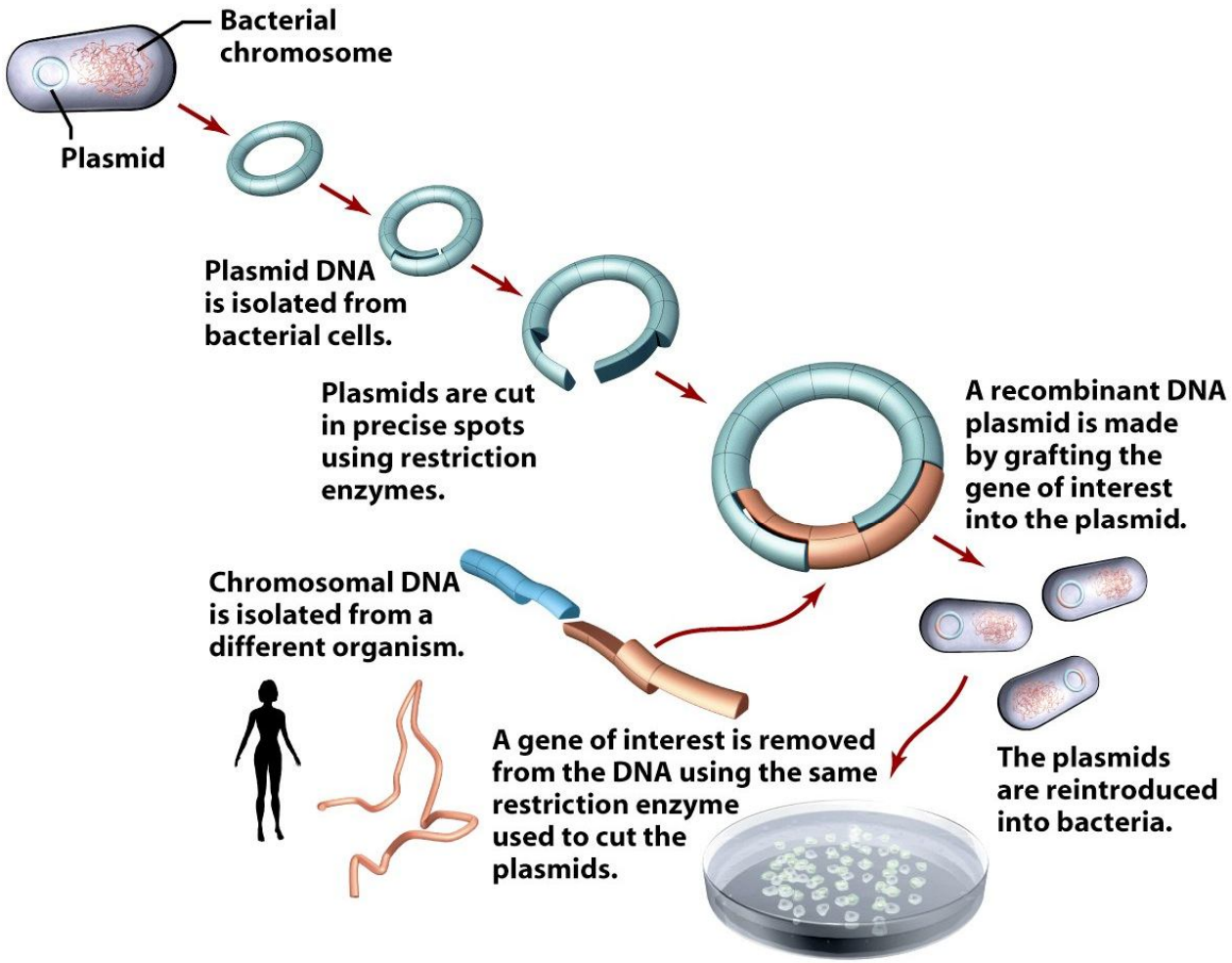
Restriction Sites

Bacterial strain	Enzyme name	Recognition sequences and cleavage sites
Bacillus amyloliquefaciens H	***Bam* H1**	
Escherichia coli Ry13	***Eco* R1**	
Providencia stuartii 164	***Pst* 1**	
Serratia marcescens SB	***Sma* H1**	
Rhodopseudomonas sphaeroides	***Rsa* 1**	

Molecular Paste

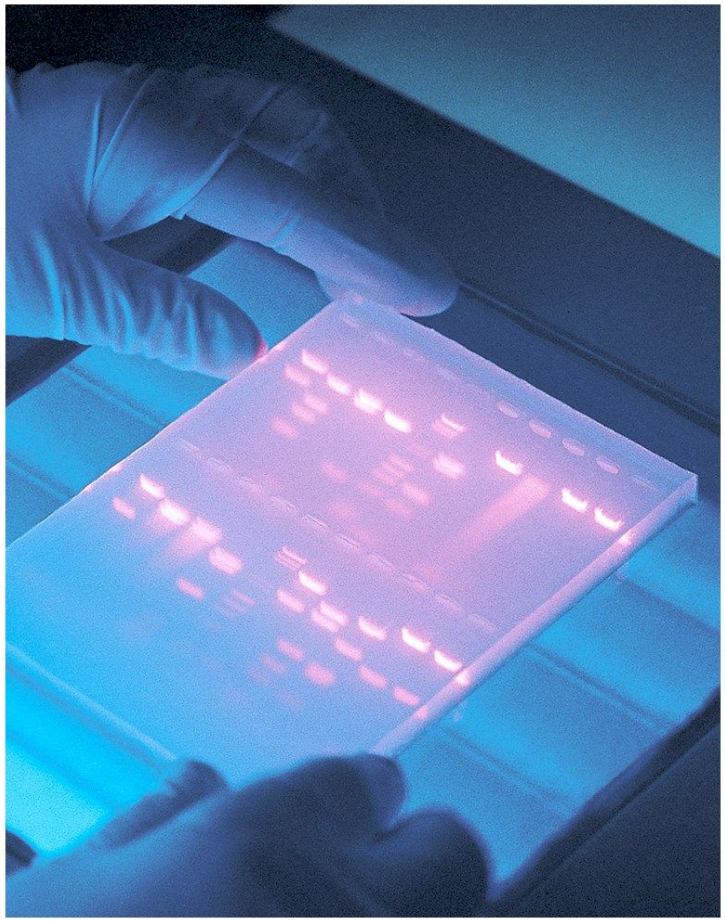
- DNA Ligase:
  - Form bonds between the sugar and phosphate backbone of the DNA molecule.
- Restriction enzymes and DNA ligase make possible the combination of DNA from different organisms into one DNA molecule
  - Called recombinant DNA

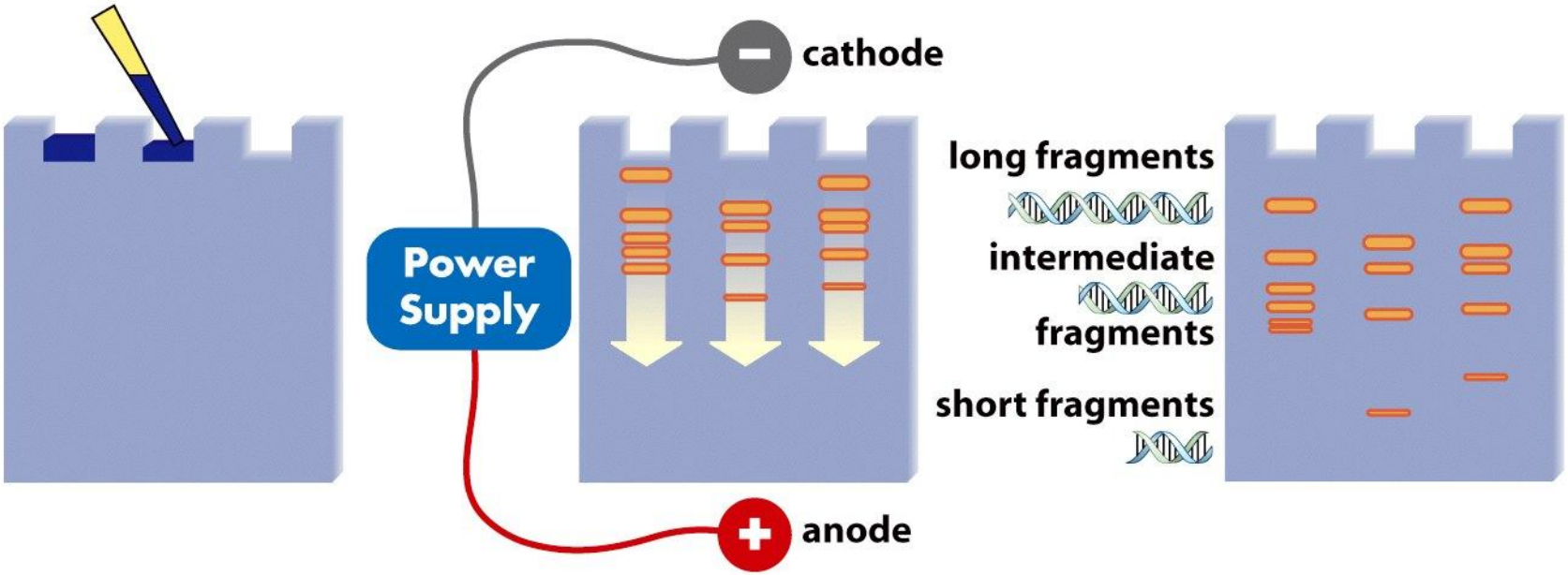
Making Recombinant DNA



How do we know what size DNA fragments we have?

- Agarose gel electrophoresis:
  - Allows separation of DNA on the basis of size.
  - Can visualize DNA to determine exactly how large it is.





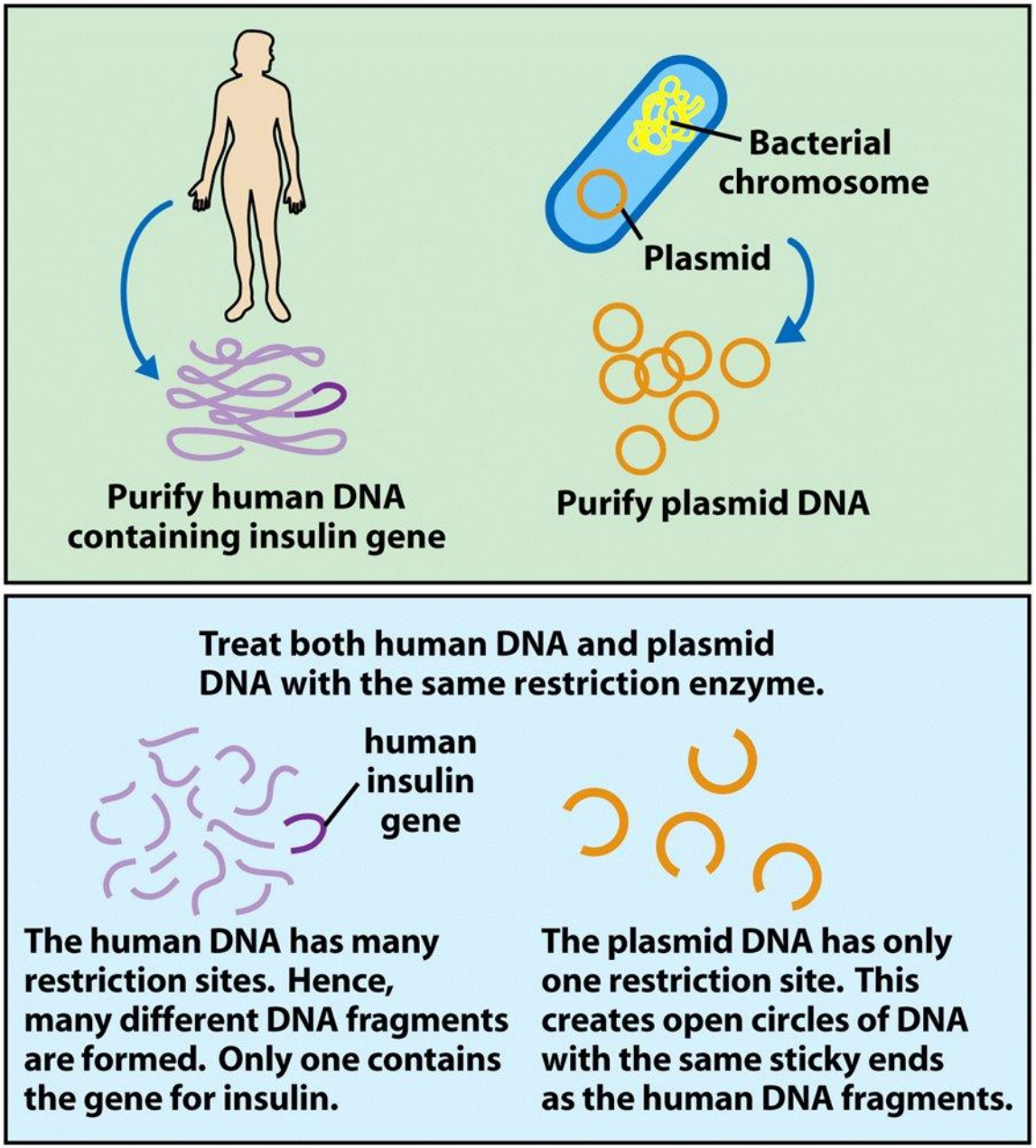
A mixture of DNA fragments is carefully added to the wells of an agarose gel. In this gel, three different mixtures are added to each of three different wells.

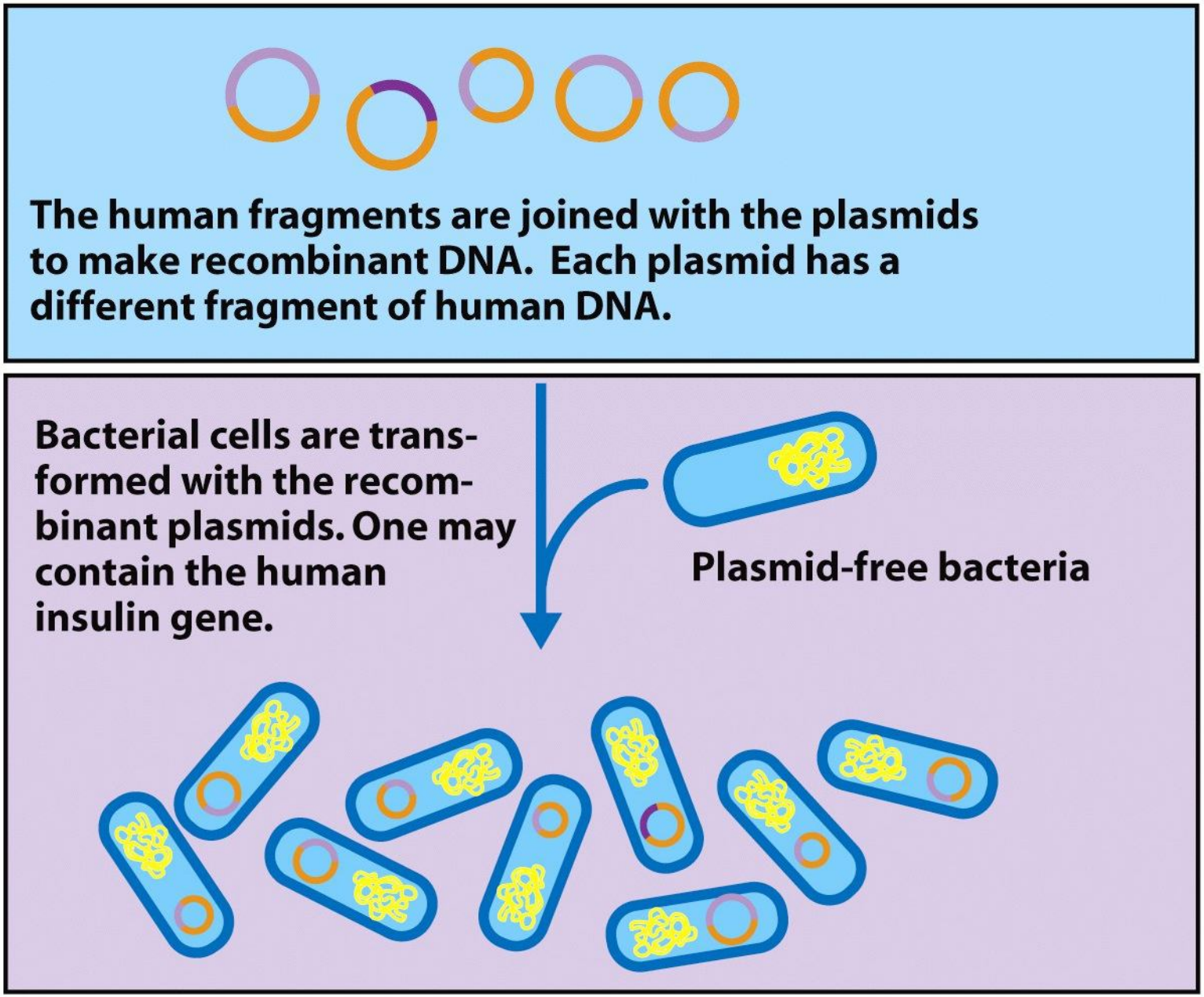
The entire agarose gel is placed in an electrical field, with the anode farthest from the wells. The DNA migrates through the gel toward the anode. Smaller fragments migrate farthest.

After the DNA fragments have been separated, the electrical field is removed. The number of bands indicates how many different-sized fragments of DNA were in the mixture. The position of the bands indicates how big each fragment is.

Making a DNA library

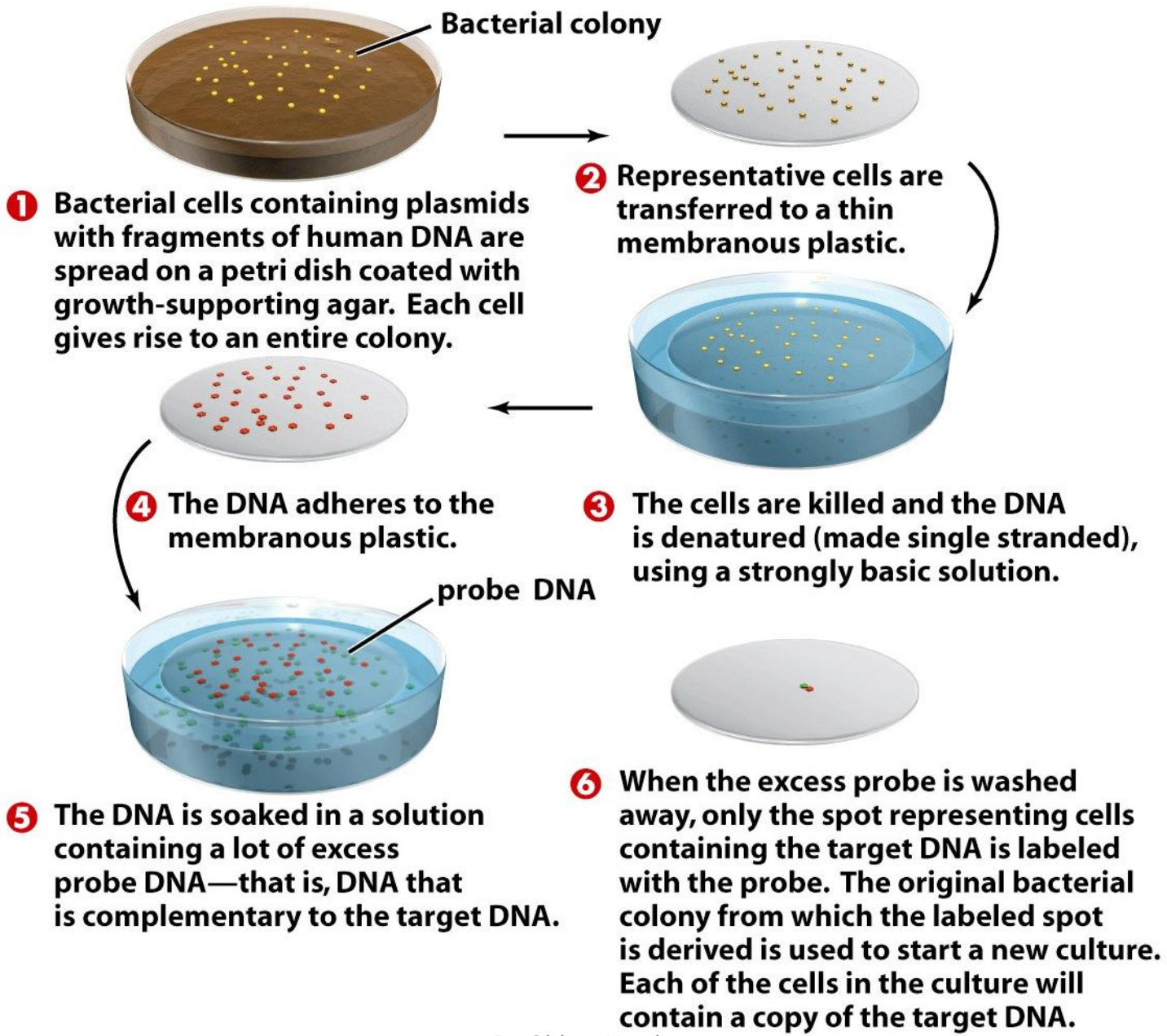
- Need the following:
  - A gene of interest
  - Restriction enzymes
  - Plasmids
  - DNA ligase
- Can create a cloning vector using these tools which can be inserted in a bacteria
- Allow bacteria to reproduce
- DNA library: entire collection of bacterial cells which contain cloned gene


Bacterial chromosome
Plasmid
Purify human DNA containing insulin gene
Purify plasmid DNA
Treat both human DNA and plasmid DNA with the same restriction enzyme.
human insulin gene
The human DNA has many restriction sites. Hence, many different DNA fragments are formed. Only one contains the gene for insulin.
The plasmid DNA has only one restriction site. This creates open circles of DNA with the same sticky ends as the human DNA fragments.


The human fragments are joined with the plasmids to make recombinant DNA. Each plasmid has a different fragment of human DNA.
Bacterial cells are trans-formed with the recom-binant plasmids. One may contain the human insulin gene.
Plasmid-free bacteria

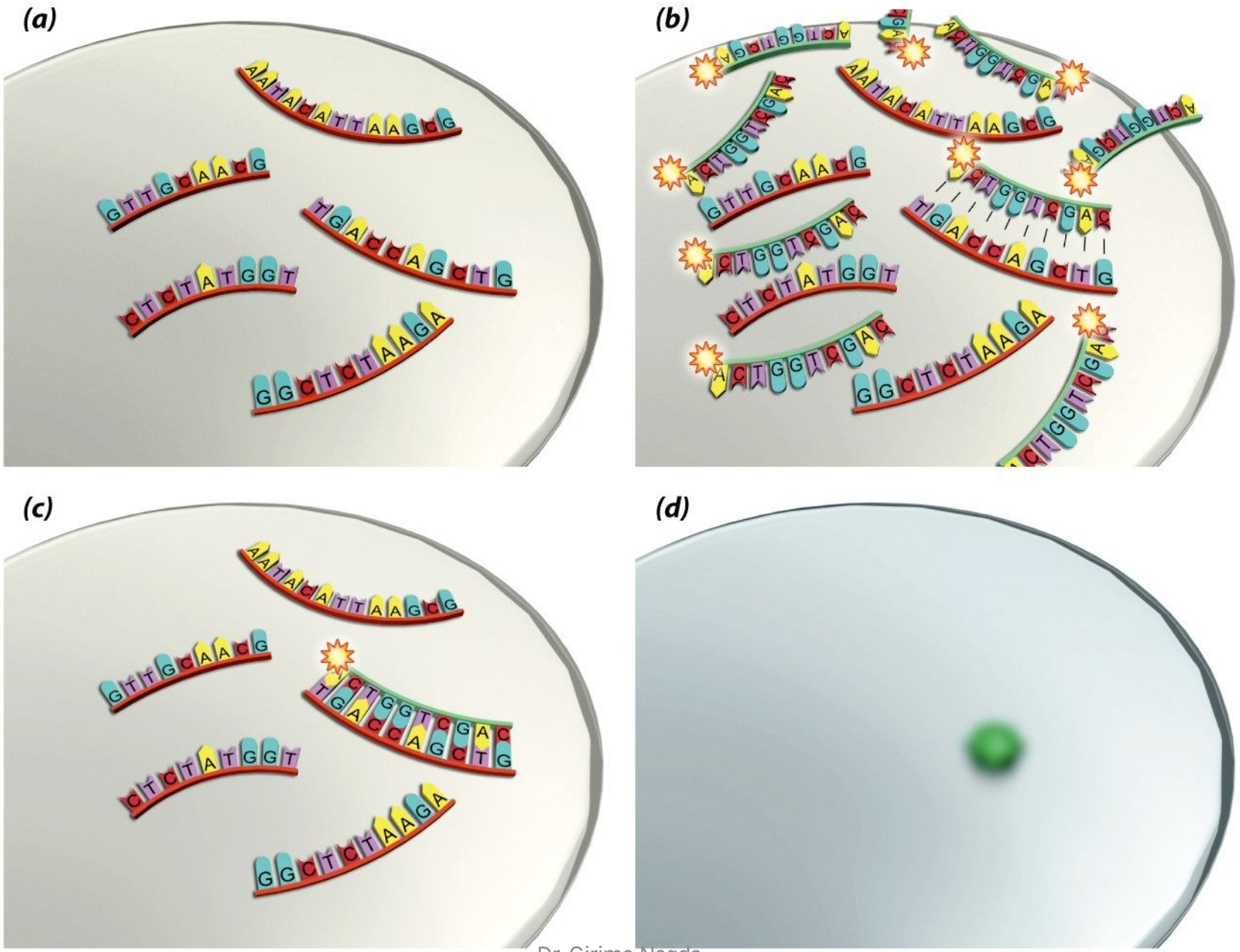
Screening a DNA Library

- Need to find the gene of interest in the bacteria or bacterial cells that possess the gene of interest.
- Use nucleic acid hybridization to find the gene of interest.


Bacterial colony
1 Bacterial cells containing plasmids with fragments of human DNA are spread on a petri dish coated with growth-supporting agar. Each cell gives rise to an entire colony.
2 Representative cells are transferred to a thin membranous plastic.
3 The cells are killed and the DNA is denatured (made single stranded), using a strongly basic solution.
4 The DNA adheres to the membranous plastic.
probe DNA
5 The DNA is soaked in a solution containing a lot of excess probe DNA—that is, DNA that is complementary to the target DNA.
6 When the excess probe is washed away, only the spot representing cells containing the target DNA is labeled with the probe. The original bacterial colony from which the labeled spot is derived is used to start a new culture. Each of the cells in the culture will contain a copy of the target DNA.

Nucleic Acid Hybridization

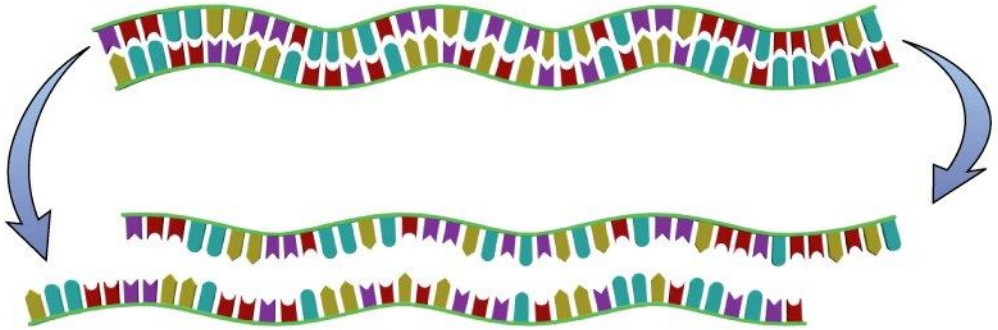
- Requires a molecular probe:
  - Probe is made of a synthetic single-stranded DNA whose sequence is complementary to the gene of interest.
  - Also has a built-in marker so scientists can find it.
- When probe binds to denatured gene of interest, a hybrid is formed.


(a)
(b)
(c)
(d)

Polymerase Chain Reaction

- Allows scientists to make copies of a small sample of DNA.
- Requires:
  - Primers: two synthetic short strands of DNA that are complementary to each of the two DNA sequences that flank the gene or DNA to be copied.
  - Heat-resistant DNA polymerase
  - Nucleotides

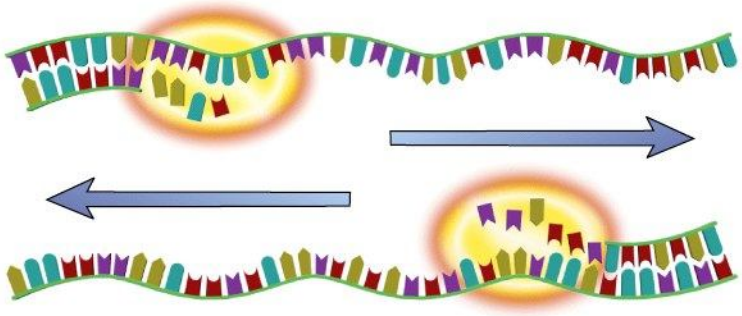
1. The tiny bit of starting DNA is "melted" so that the two strands separate.



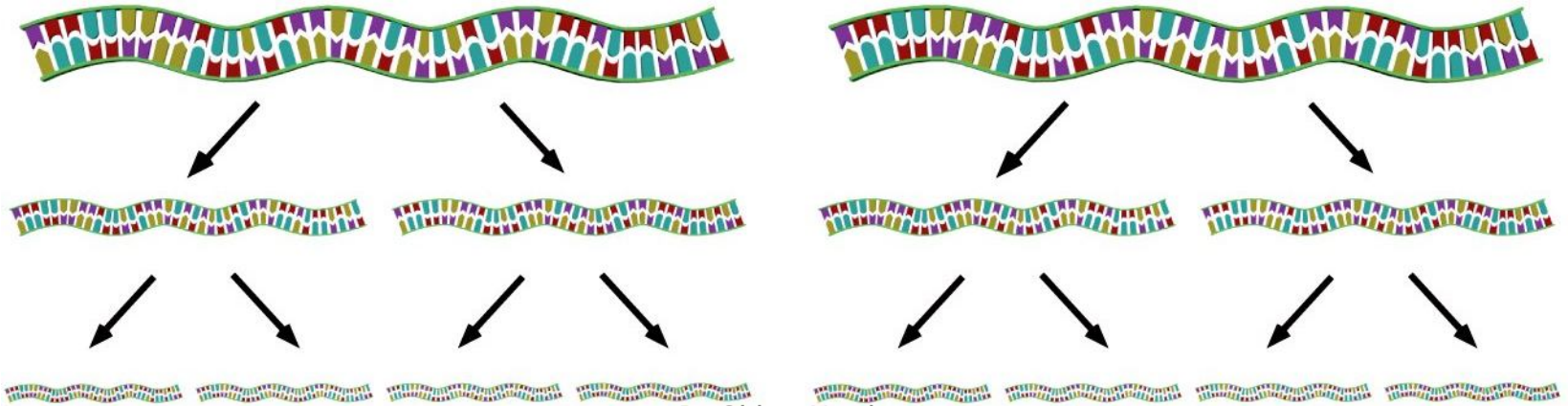
2. Primers are short DNA sequences that are complementary to the ends of the DNA. Primers are added to the mixture upon which new DNA strands can be made. The primers line up with their complementary bases on the separated strands.

Heat-resistant DNA Polymerase

❸ A heat-resistant DNA polymerase and nucleotides are part of the mixture. This enzyme adds nucleotides to the primers, using the original DNA as a template. This creates exact copies of the original DNA.



❹ This process (steps 1–3) is repeated many times. Each cycle doubles the amount of DNA, until the original DNA has been amplified into many identical copies.



DNA Sequencing

- Determining the base-by-base order of the nucleotides in a stretch of DNA.
- Can help us identify regions of DNA that contain genes.

Open Reading Frame	Random Sequence of RNA Nucleotides
AUG UUA CAG GUU CCA GCC GGA ACU CUA GAC ACU CUU AUA GAG CCC UCA ACC GGA GAA GCA UGG CUU CUA ACU CUC AGU UCG UUC UCG UCG GCG GAA CUG GCG ACG UCC CUG UCG CCC CAA GCA ACU CUA ACG GGG UCG CUG AAU UCA GCU CUA ACU CGC GUU AGG CUU ACA AAG ACC CCU GGC GUU CGU ACU ACC AUA AAC ACC CCA UUC CAA UUU CUA CGA AUU CCG ACU GCG UUA UUG UUA AGG CAA UGC GUC UCC ACC AAG GGC GAA CGA UUC CCU CAG CAA UCG CAA ACU CCG GCA UCU ACU AGA CGC CGG CCA UGA	ACC CUG GGA UAA GUC GCU CUA GUC AUC GCU AUC GCC GGU CGA UGC AAU GCU UAC CUG GAU GUU AGU AAG AUG GUA AAU CCU GUA CGA CGA CAG UUG CGA UGA AAG CGA UCG ACG GCA AAG CCG UUA UUG CCG AUC CGC UAA CGA UCG AUC GCU CGA CGA AGU CAU CGA GUA CGC AUA CUA CGG AUC UAU CGA AGC CGC UAG CCG UUA GCA CCC GUA CCG AGU UCU GGU AUA CGC AAG AUC GCU AUC CGA AAG UGU CUA UAU CGC AUC GCU CGA AUC GUA UUC AGC AUC GCA UAG CCC GGA CCA UAU CCG AAG CGA UGC UAU CCC

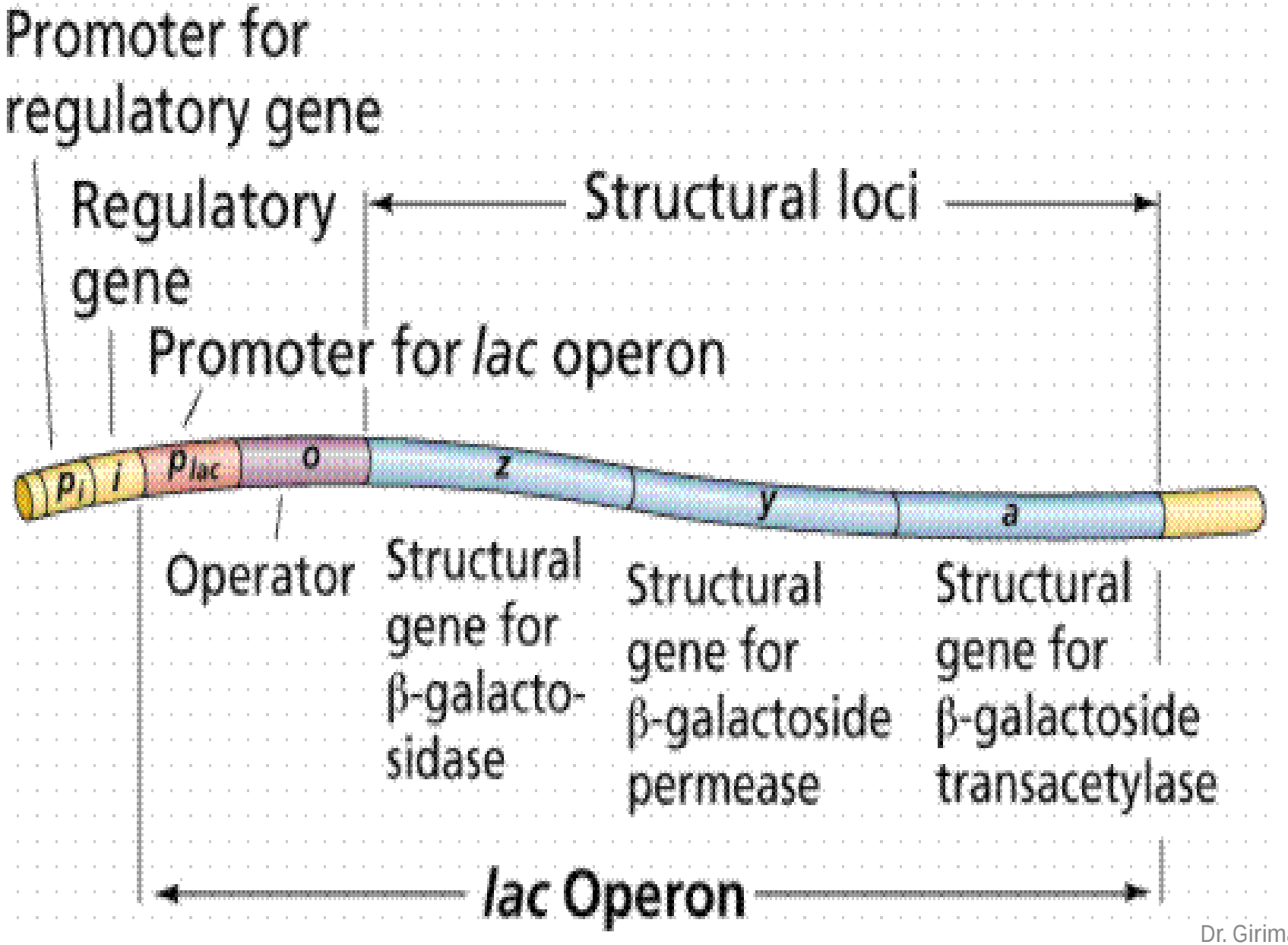
DNA Sequencing

- Makes possible comparisons of DNA sequences
  - between individuals to teach us about our susceptibility to disease.
  - between species to teach us about how we evolved.
- Also, DNA sequences teach us about the regulation of gene expression.

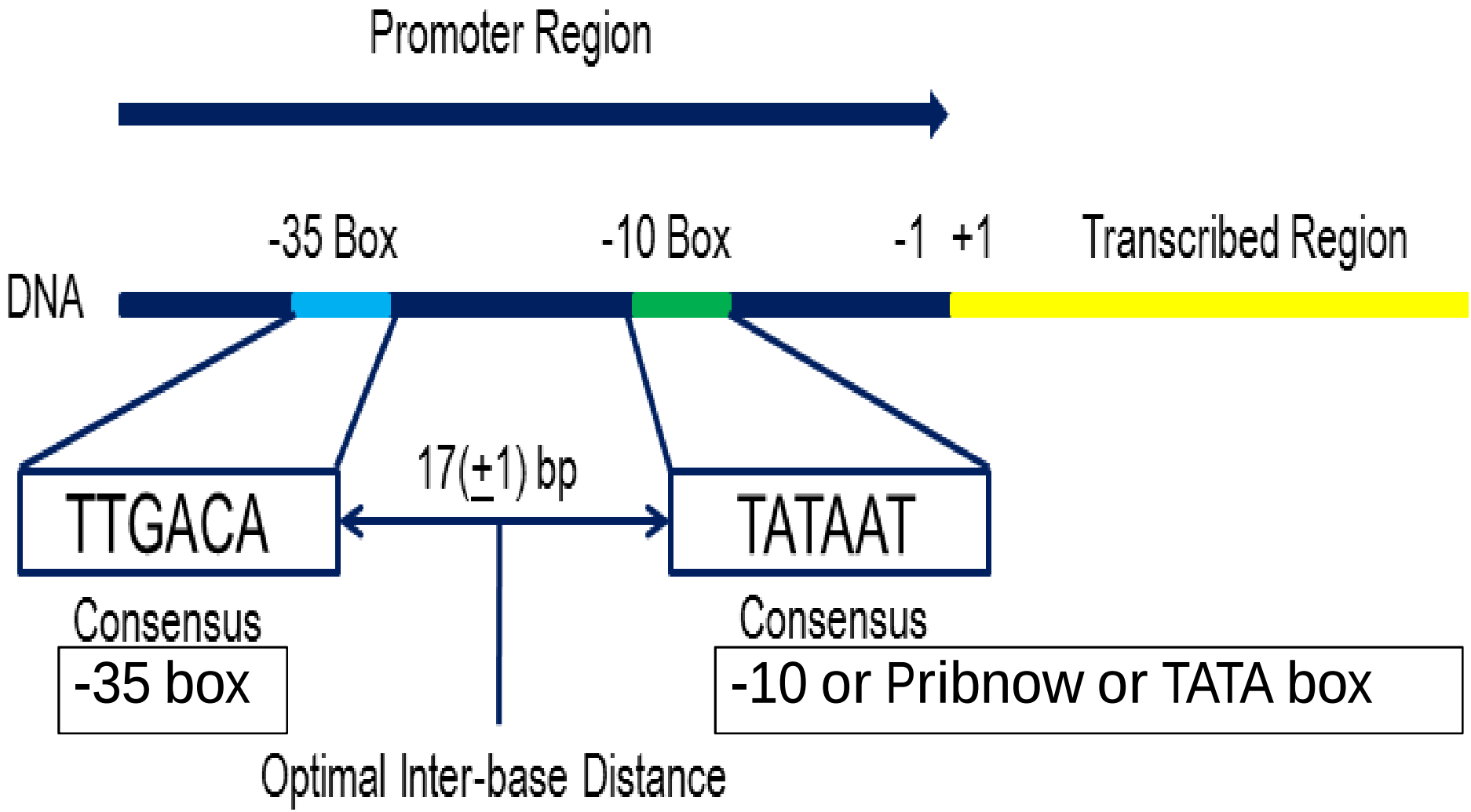
Operon(Gene cluster under control of single promoter)

- ***Structural gene*- gene that codes for a polypeptide**
- ***Promoter site*- region where RNA polymerase bind to initiate transcription of the structural genes (STG).**
- ***Operator Site* - region where the repressor attaches to control the access to STG**
- ***Regulatory gene*- codes for repressor proteins**

lac Operon


Promoter for regulatory gene
Regulatory gene
Structural loci
Promoter for lac operon
p_i
i
p_{lac}
o
z
y
a
Operator
Structural gene for β-galacto-sidase
Structural gene for β-galactoside permease
Structural gene for β-galactoside transacetylase
lac Operon

Bacterial Promoter



PROKARYOTIC Gene structure

Genes based on their activity:

1. House keeping genes
2. Specific genes.

STRUCTURAL FEATURES:

- Simple gene structure.
- Small genomes(0.5 to 10 million bp).
- Prokaryotic genes are collinear with their proteins.

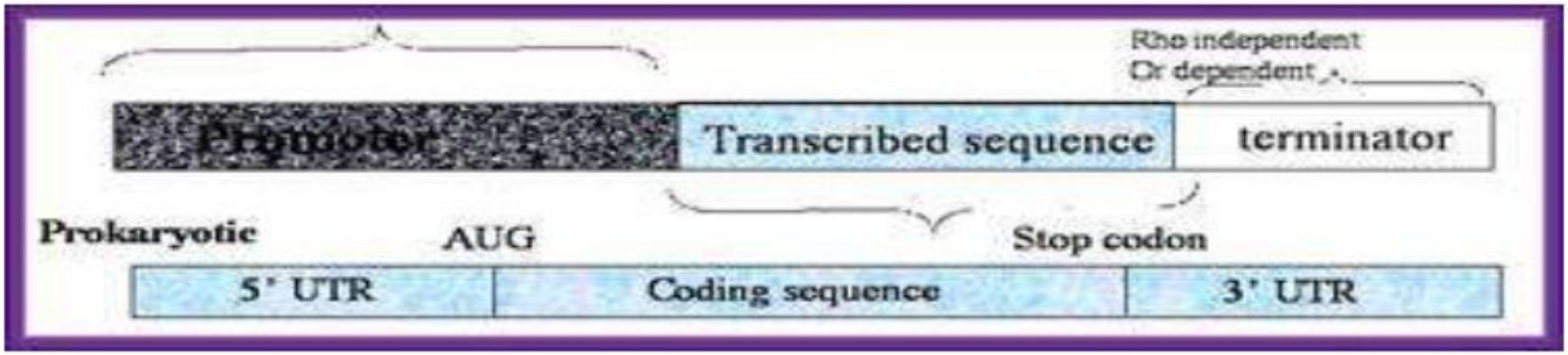
a. CODING REGION
b. PROMOTER ELEMENTS
c. TERMINAL REGION OR TERMINATOR.

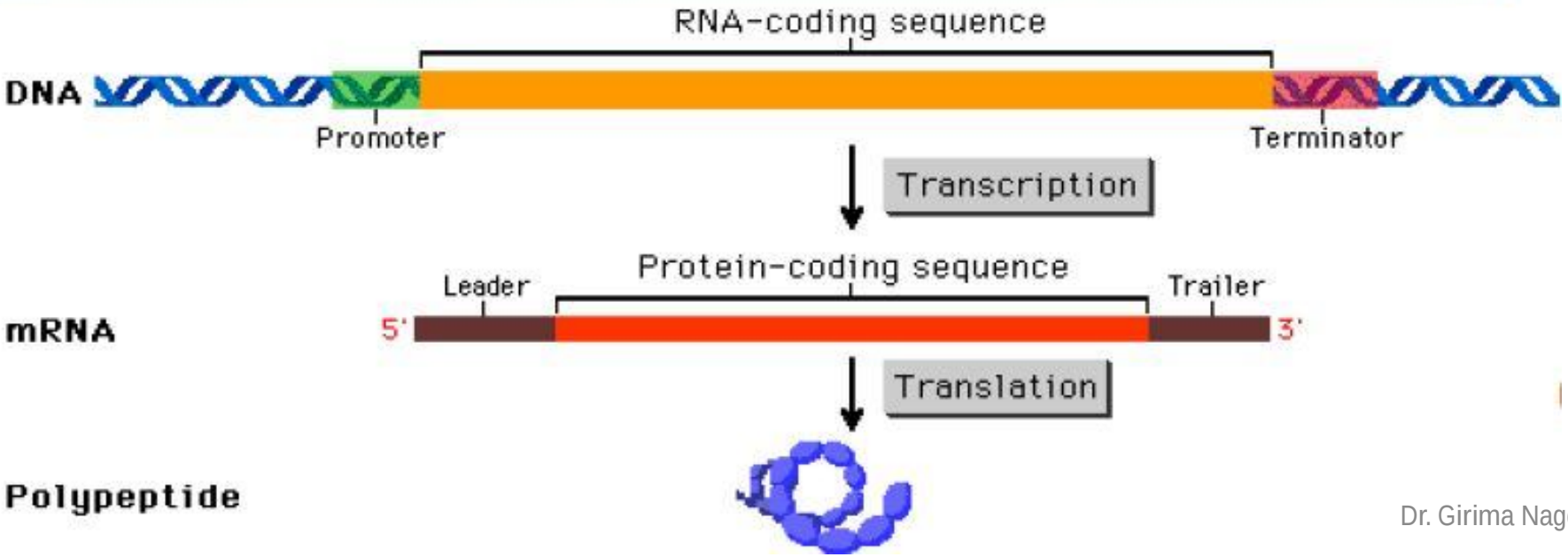
a. Coding region-

Starts with an initiator codon and ends with termination codon

No introns (uninterrupted).

Collinear to its mRNA.





b.Promoter elements-

The upstream elements from the start of the coding region include promoter sequences.

•50 to 100 ntds upstream of the start codon- transcriptional initiation site or START site.

•(any nucleotide present on the left is denoted by (-)symbol and the region is called upstream element. E.g. -10,-20,-35 etc.
Start site symbolized by +1.
Any sequence to the right of the start is downstream elements and numbered as +10,+35 etc.)

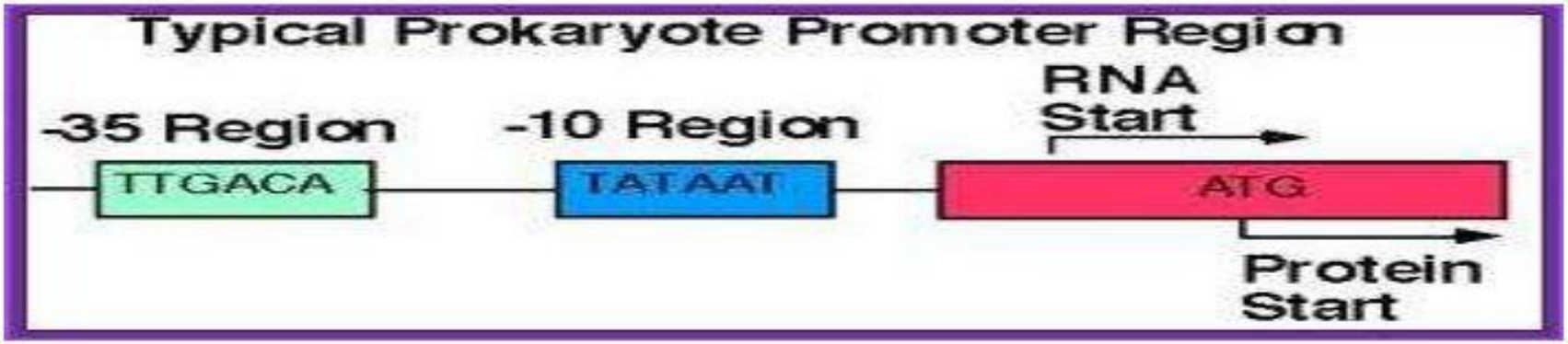
c. Terminal region of the gene-

Sequences for the termination of transcription.

It takes place by Rho dependent mode or Rho independent mode.

•At -10 there is a sequence TATAAT or PRIBNOW BOX.

•At -35 another consensus sequence TTGACA
These two are the most important promoter elements.
Recognized by transcription factors.



•At -65 to -60 activator elements. Activation of the polymerase.

•At -200 to -1000 enhancer sequence. Enhances transcription by 100 to 200 folds.

Eukaryotic gene structure

- Exons
- Introns
- Promoter sequences
- Terminator sequences
- Upstream sequences
- Downstream sequences
- Enhancers and silencers(upstream or downstream)
- Signals

(Upstream sequence signal for addition of cap. Downstream sequences signal for addition of poly A tail.)

- EXONS –coding sequence, transcribed and translated. Coding for amino acids in the polypeptide chain.

Vary in number ,sequence and length. A gene starts and ends with exons.(5' to 3').

Some exon includes untranslated(UTR)region.

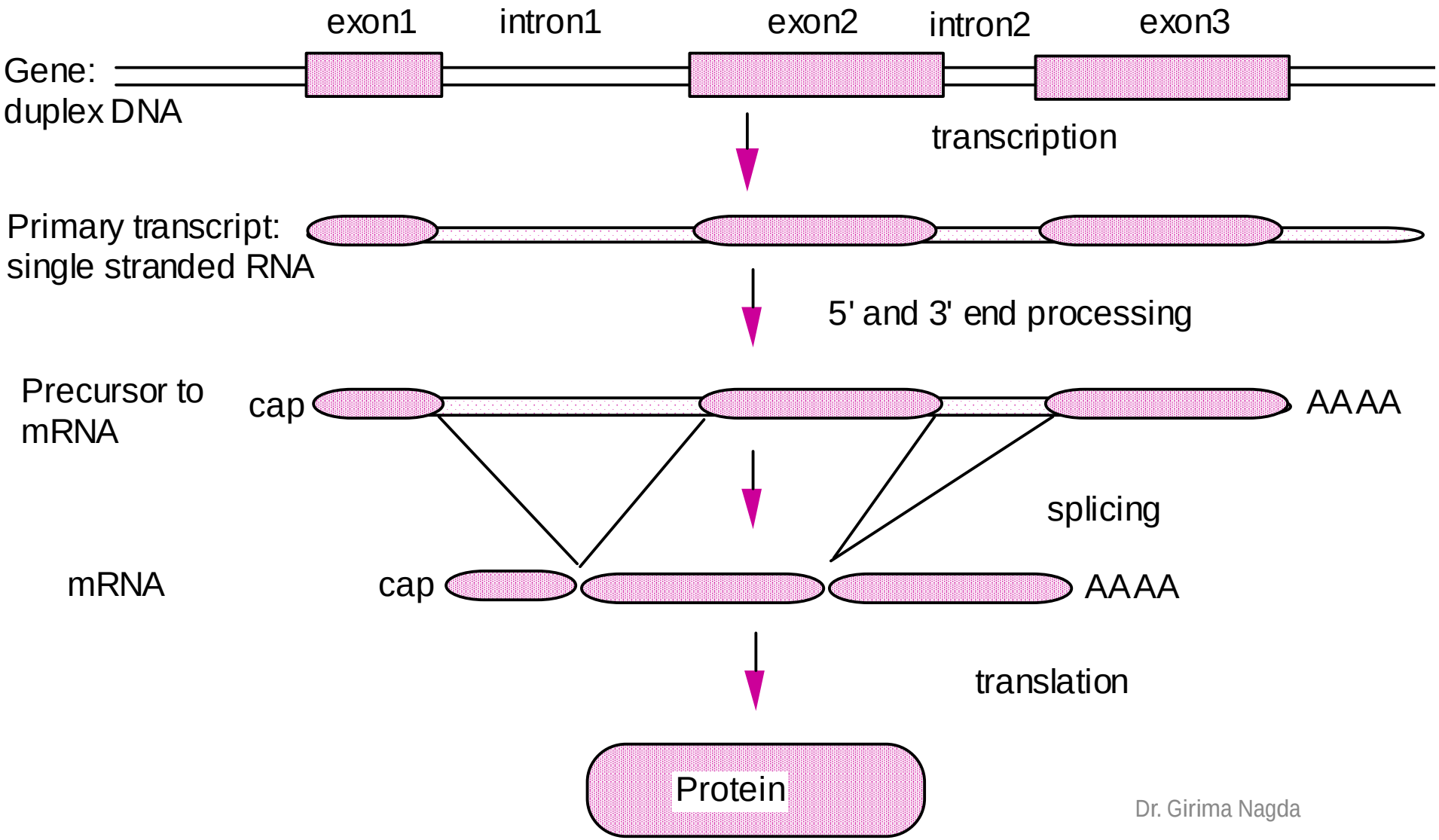
- INTRONS- coding sequences are separated by non-coding sequences called introns.

Any nucleotide sequence that are removed when the primary transcript is processed to give the mature RNA are called introns.

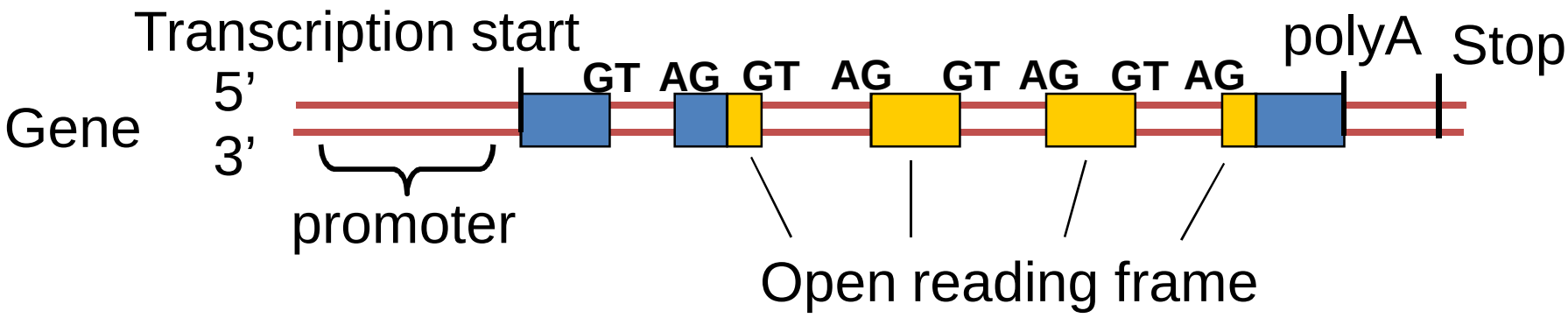
All introns share the base sequence GT in the 5'end and AG in the 3'end.

Introns were 1st discovered in 1977 independently by Phillip Sharp and Richard Roberts.

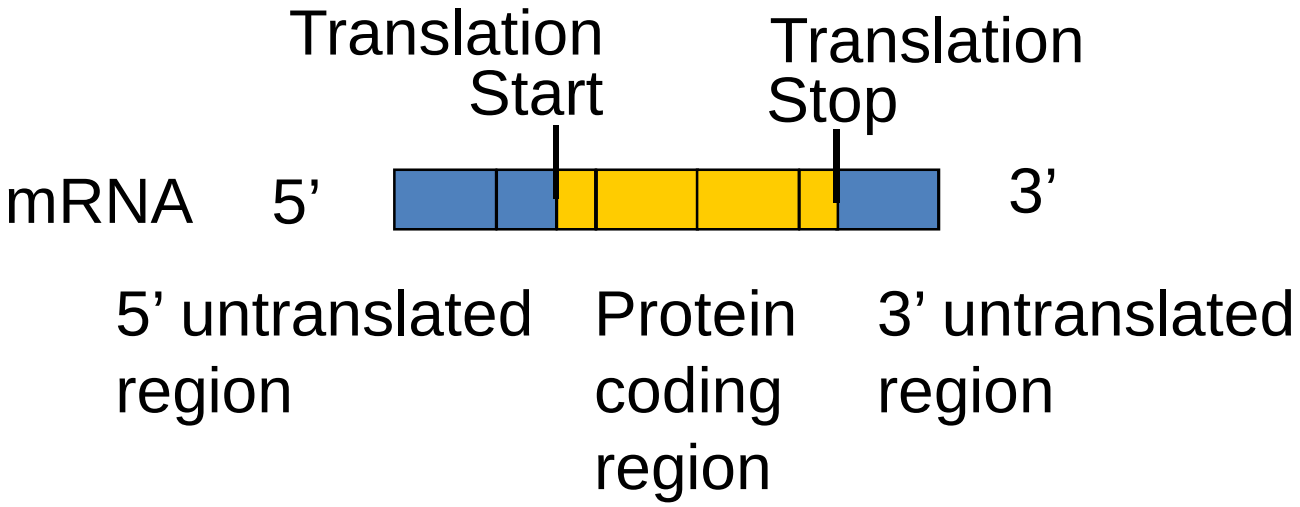
An eukaryotic Gene



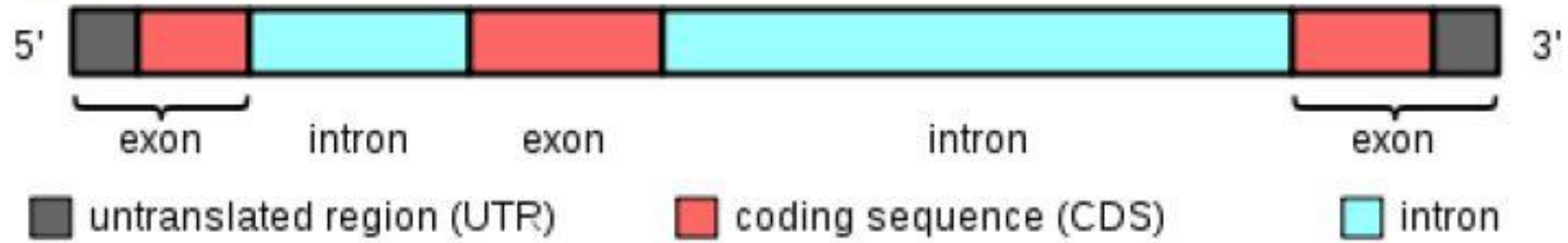
Types of exons

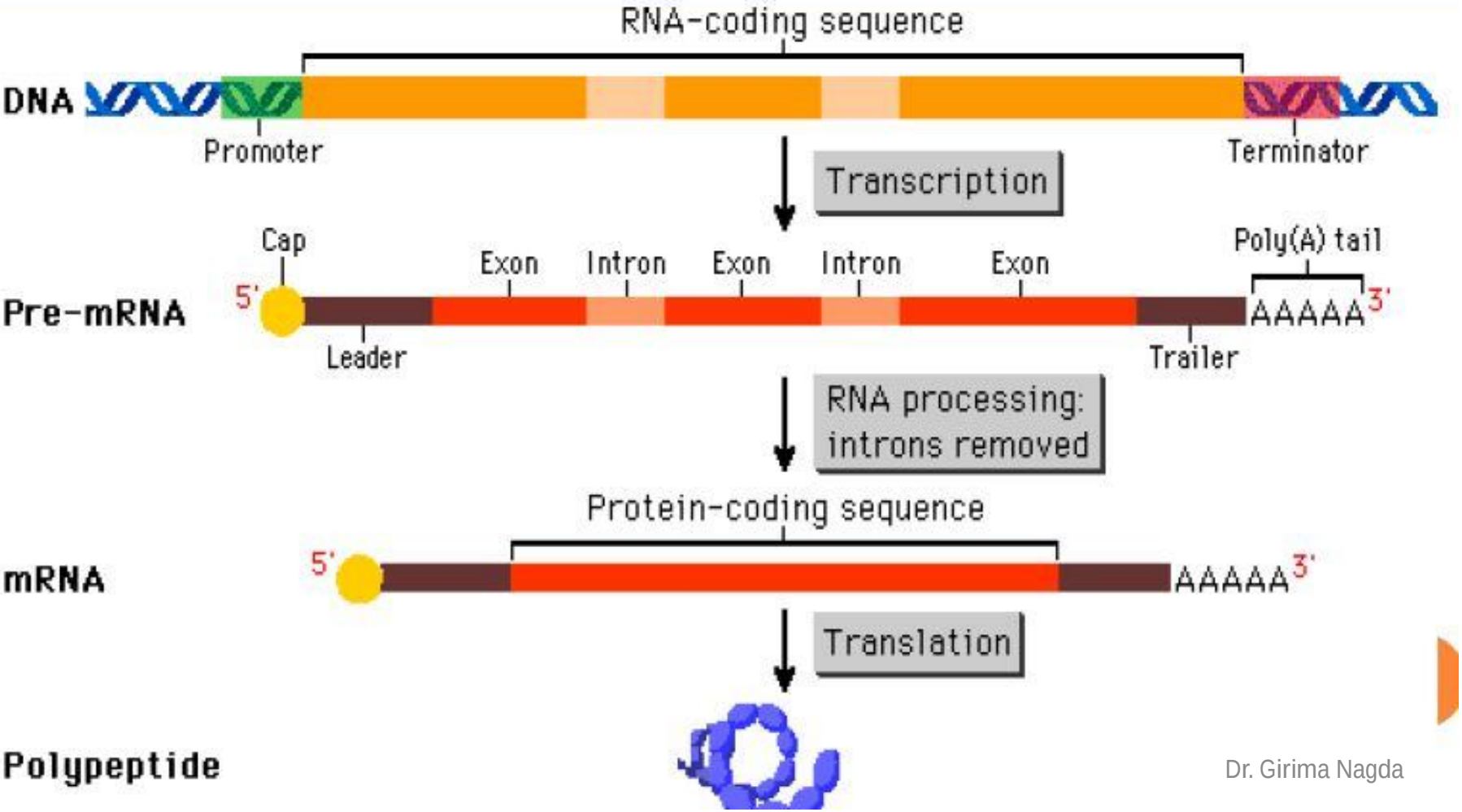


Initial exon
Internal exon
Terminal exon



Eukaryotic gene.





❑PROMOTERS- A promoter is a regulatory region of DNA located upstream controlling gene expression.

1.Core promoter – transcription start site(-34)

Binding site for RNA polymerase.

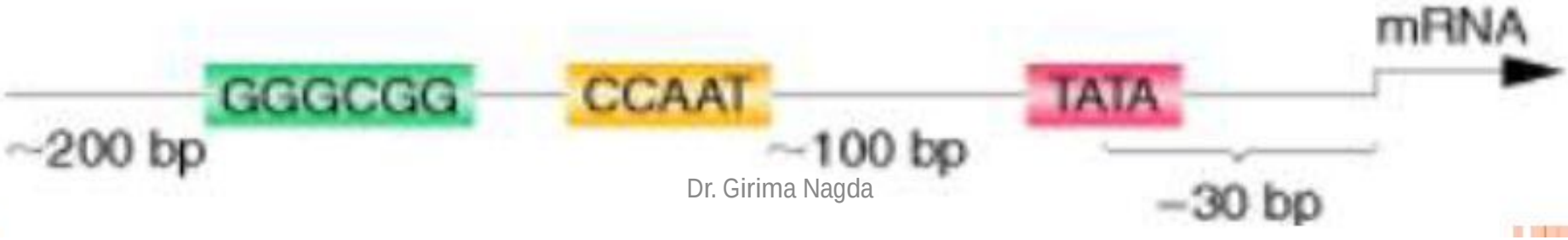
General transcription factor binding sites.

2. Proximal promoter-contain primary regulatory element

Apprx. -250,specific transcription factor binding sites.

❑TATA box or hogness box (-30 to -80)and CAAT(upstream TATA) are two distinct sequences.

These together are responsible for binding of RNA polymerase II which is responsible for transcription



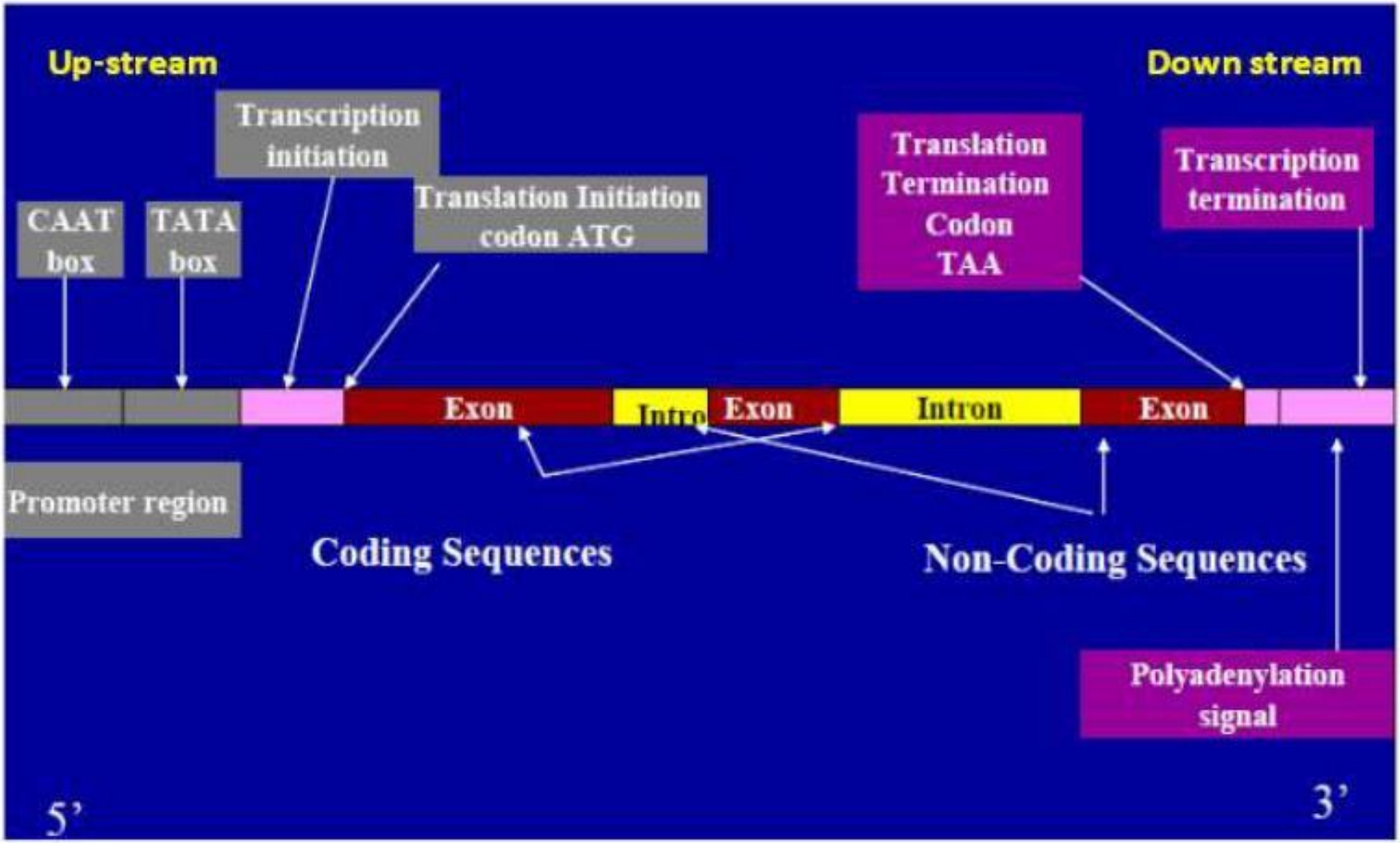
- UPSTREAM(5'END)- 5'UTR serve sevral functions including mRNA transport and initiation of translation.

Signal for addition of cap(7 methyl guanisine) to the 5'end of the mRNA.
The cap facilitates the initiation of translation.
Stabilization of mRNA.

- DOWNSTREAM(3'END)-3'UTR serves to add mRNA stability and attachment site for poly-A-tail.

The translation termination codon TAA.
AATAA sequence signal for addition of poly A tail.

Eukaryotic Gene Structure



- TERMINATOR- recognized by RNA polymerase as a signal to stop transcription

- ENHANCER-enhances the transcription of a gene. Upto few thousand bp upstream.

- SILENCERS-reduce or shut off the expression of a near by gene.

Significance of introns

- ✓Introns don't specify the synthesis of proteins but have other important cellular activities.
- ✓Many introns encodes RNA's that are major regulators of gene expression.
- ✓Contain regulatory sequences that control trancription and mRNA processing.
- ✓Introns allow exons to be joined in different combinations(alternative splicing), resulting in the synthesis of different proteins from the same gene.
- ✓Important role in evolution by facilitating recombination between exons of different genes(exon shuffling).

Performing the Restriction Digests

- You will need to set up a restriction digest of your plasmid vector and your DNA of interest
- Restriction enzymes all have specific conditions under which they work best. Some of the conditions that must be considered when performing restriction digest are: temperature, salt concentration, and the purity of the DNA

Purify your DNA Fragments

- The insert of interest that you want to clone into your plasmid needs to be separated from the other DNA
- You can separate your fragment using Gel Electrophoresis
- You can purify the DNA from the gel by cutting the band out of the gel and then using a variety of techniques to separate the DNA from the gel matrix

Ligation

- Ligation is the process of joining two pieces of DNA from different sources together through the formation of a covalent bond.
- DNA ligase is the enzyme used to catalyze this reaction.
- DNA ligation requires ATP.

Transforming Bacteria

- After you create your new plasmid construct that contains your insert of interest , you will need to insert it into a bacterial host cell so that it can be replicated.
- The process of introducing the foreign DNA into the bacterial cell is called transformation.

Competent Host Cells

- Not every bacterial cell is able to take up plasmid DNA.
- Bacterial cells that can take up DNA from the environment are said to be competent.
- Can treat cells (electrical current/divalent cations) to increase the likelihood that DNA will be taken up
- Two methods for transforming: heat shock and electroporation

Selecting for Transformants

- The transformed bacteria cells are grown on selective media (containing antibiotic) to select for cells that took up plasmid.
- For blue/white selection to determine if the plasmid contains an insert, the transformants are grown on plates containing X-Gal and IPTG.

What did the cells take up?

- Plasmid only
- Plasmid with insert cloned
- Foreign DNA from the environment
- Nothing

The markers on the plasmid will help you select bacterial colonies that contain the plasmid into which you have successfully cloned your DNA of interest.

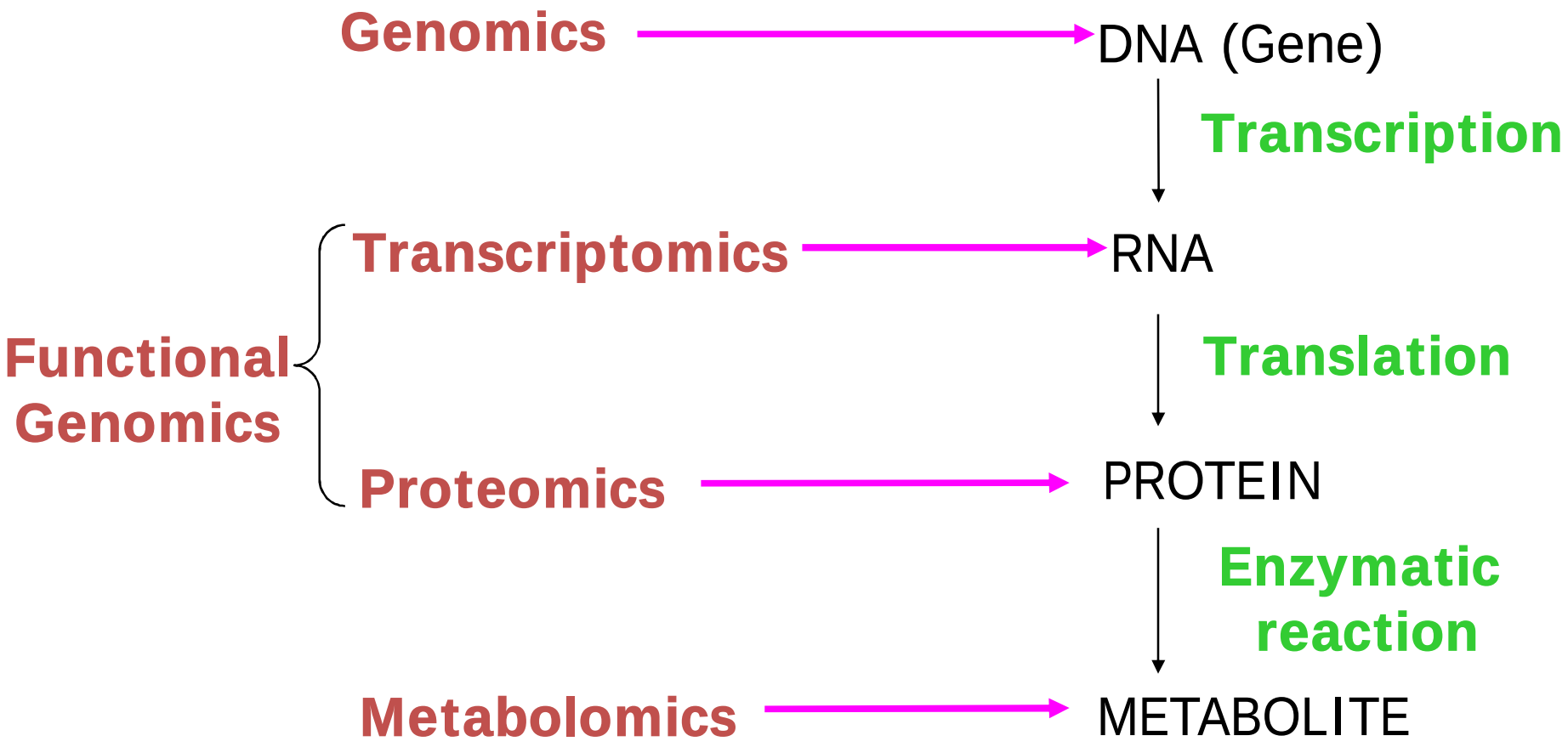
Expressing your cloned gene

- Even if your plasmid contains insert, it may not be able to generate functional protein from your cloned DNA.
  - The gene may not be intact, or mutations could have been introduced that disrupt it.
  - The protein encoded by the gene may require post-translational modifications (i.e., glycosylation or cleavage) to function.
  - Also, some enzymes are a complex of peptides expressed from separate genes.

Expressing your cloned gene

- Expression of a cloned gene can be accomplished by:
  - The *E. coli* host
  - Mammalian cells (if the plasmid used is designed for expression in mammalian cells)
  - Using an in vitro using a cell-free system

What about the other “omics”?



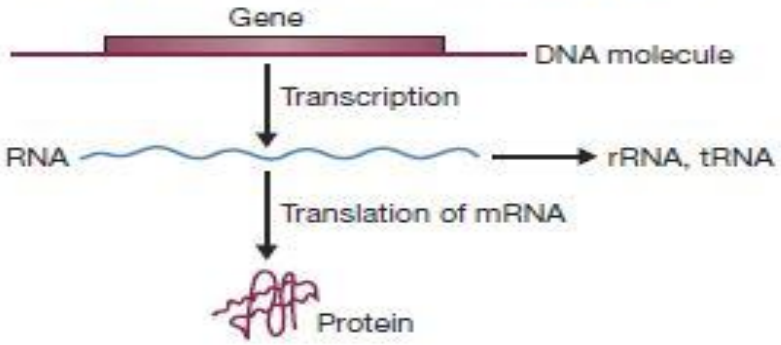
STUDYING GENE EXPRESSION

- All genes have to be expressed in order to function.
- The first step in expression is transcription of the gene into a complementary RNA strand
- For some genes—for example, those coding for transfer RNA (tRNA) and ribosomal RNA (rRNA) molecules—the transcript itself is the functionally important molecule. For other genes, the transcript is translated into a protein molecule.

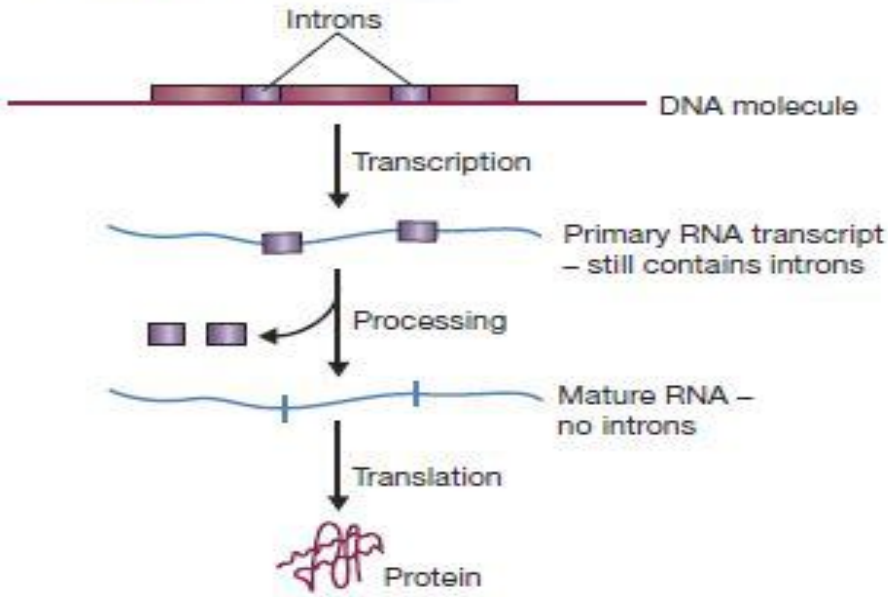
STUDYING GENE TRANSCRIPT

- To understand how a gene is expressed, the RNA transcript must be studied.
- We want to know whether the transcript is a faithful copy of the gene, or whether segments of the gene are missing from the transcript
- These missing pieces are called introns and considerable interest centers on their structure and possible function.
- In addition to introns, the exact locations of the start and end points of transcription are important. Most transcripts are copies not only of the gene itself, but also of the nucleotide regions either side of it .
- The signals that determine the start and finish of the transcription process are only partly understood, and their positions must be located if the expression of a gene is to be studied.

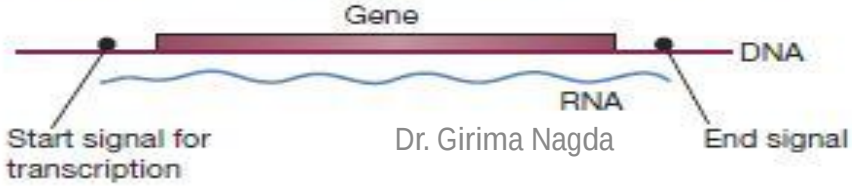
(a) Genes are expressed by transcription and translation



(b) Some genes contain introns



(c) RNA transcripts include regions either side of the gene



METHODS USED FOR **TRANSCRIPT ANALYSIS**.

- These methods can be used to map the positions of the start and end points for transcription and also to determine if a gene contains introns.