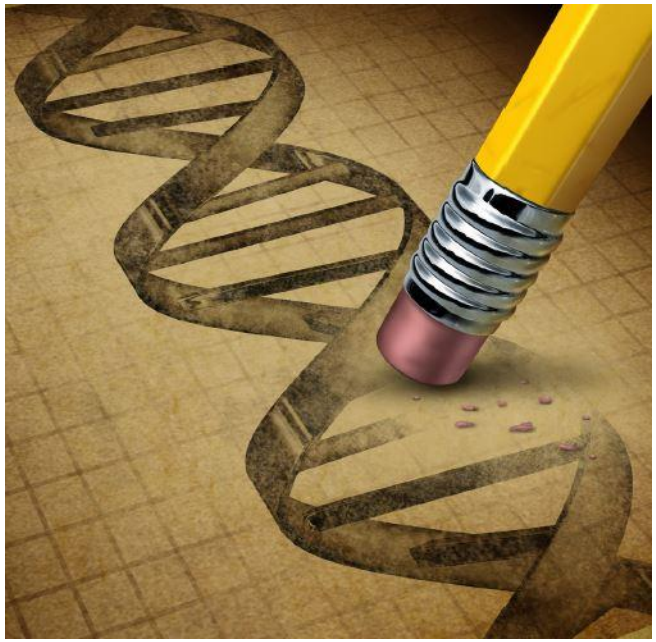
Advanced Biotechnological Methods

I. Fundamentals of Molecular Biotechonology

Principles and Applications of Recombinant DNA



Mahmut Çerkez Ergören, PhD

April 2015

Molecular Biotechnology

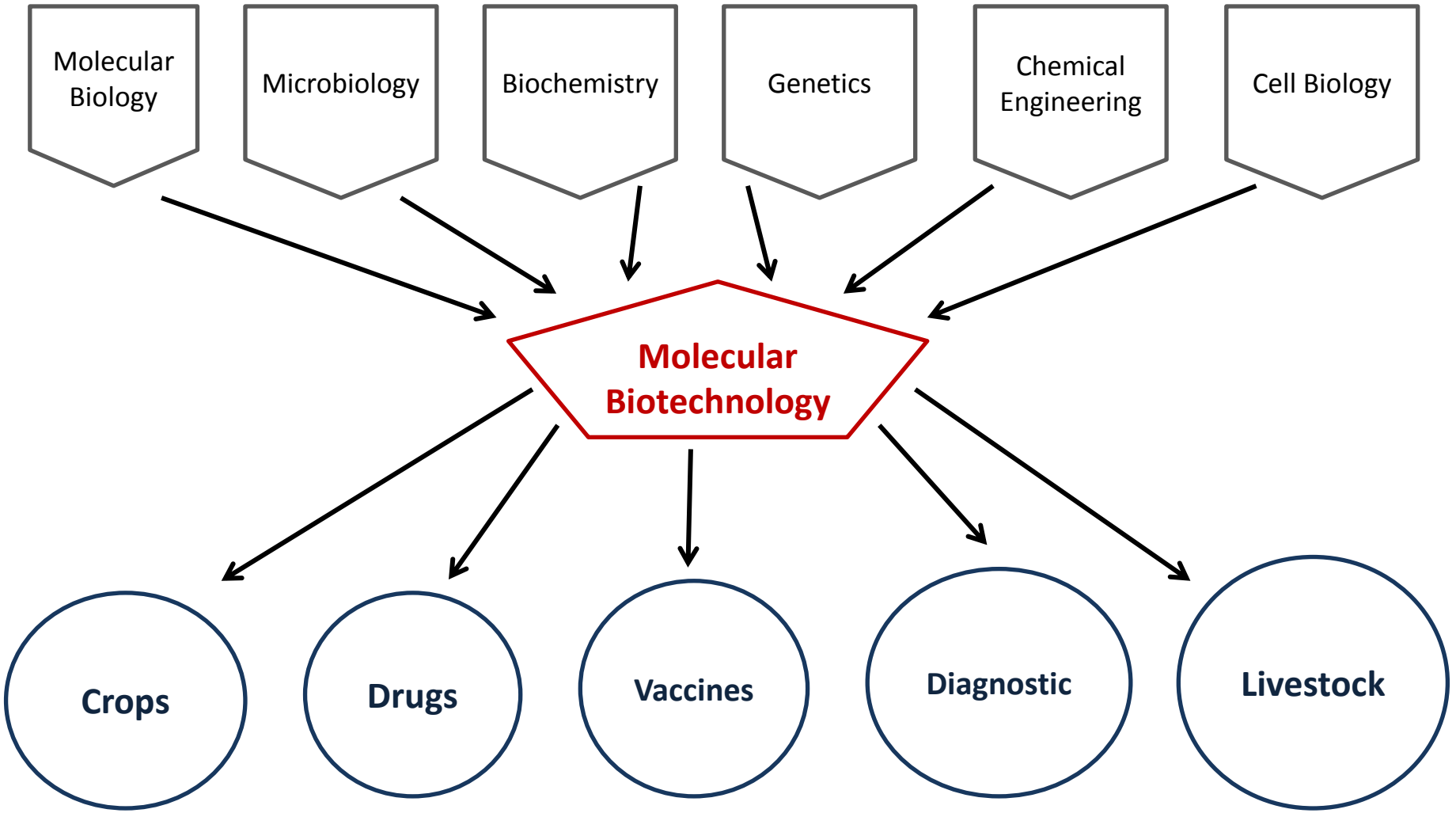
- Based on the ability of researcher to transfer specific unit of genetic information from one organism to another.

- Genes relies on the techniques of genetic engineering (recombinant DNA technology)

The objective of recombinant DNA Technology:

To produce a useful product or a commercial process.

Many scientific discplines contribute to molecular biotechnology

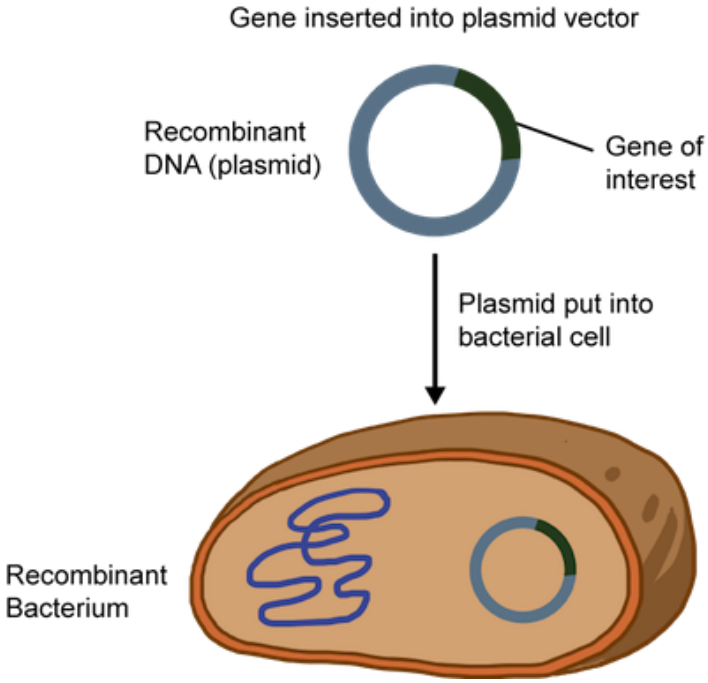


Recombinant DNA Technology:

Transferring genetic information (genes) from one generation to another.

To isolate specific genes and perpetuate them in host organism.

Providing rapid, efficient and powerful means for creating microorganisms with specific genetic attributes.



MILESTONE

Construction of Biologically Functional Bacterial Plasmids In Vitro

S. N. COHEN, A. C. Y. CHANG, H. W. BOYER, and R. B. HELLING

Proc. Natl. Acad. Sci. USA **70**:3240–3244, 1973

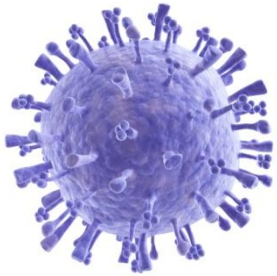
The landmark study of Cohen et al. established the foundation for recombinant DNA technology by showing how genetic information from different sources could be joined to create a novel, replicatable genetic structure. In this instance, the new genetic entities were derived from bacterial autonomously replicating extrachromosomal DNA structures called plasmids. In a previous study, Cohen and Chang (*Proc. Natl. Acad. Sci. USA* **70**:1293–1297, 1973) produced a small plasmid from a large naturally occurring plasmid by shearing the larger plasmid into smaller random pieces and introducing the mixture of pieces into a host cell, the bacterium *E. coli*. By chance, one of the fragments that was about 1/10 the size of the original plasmid was perpetuated as a functional plasmid. To overcome the randomness of this approach and make the genetic manipulation of plasmids more manageable, Cohen and his coworkers decided to use an enzyme (restriction endonuclease) that cuts a DNA molecule at a specific site and produces a short extension at each end. The extensions of the cut ends of a restriction endonuclease-treated DNA molecule can combine with the extensions of another DNA molecule that was cleaved with the same restriction endonuclease. Consequently, when DNA molecules from different sources are treated with the same restriction endonuclease and mixed together, new DNA combinations that never existed before can be formed. In this way, Cohen et al. not only introduced a gene from one plasmid into another plasmid but demonstrated that the introduced gene was biologically active. To their credit, these authors fully appreciated that their strategy was "potentially useful for insertion of specific sequences from prokaryotic or eukaryotic chromosomes or extrachromosomal DNA into independently replicating bacterial plasmids." In other words, any gene from any organism could theoretically be cloned into a plasmid which, after introduction into a host cell, would be maintained indefinitely and, perhaps, produce the protein encoded by the cloned gene. By demonstrating the feasibility of gene cloning, Cohen et al. provided the experimental basis for recombinant DNA technology; established that plasmids could act as vehicles (vectors) for maintaining cloned genes; motivated others to pursue research in this area that rapidly led to the development of more sophisticated vectors and gene cloning strategies; engendered concerns about the safety and ethics of this kind of research that, in turn, was responsible for the establishment of official guidelines and governmental agencies for conducting and regulating recombinant DNA research, respectively; and contributed to the formation of the molecular biotechnology industry.

1. Molecular Biotechnology Biological Systems

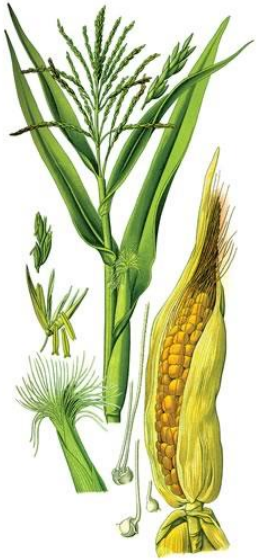
Biological systems:

1) Microorganisms

2) Insects



3) Plants



4) Mammalian cell lines

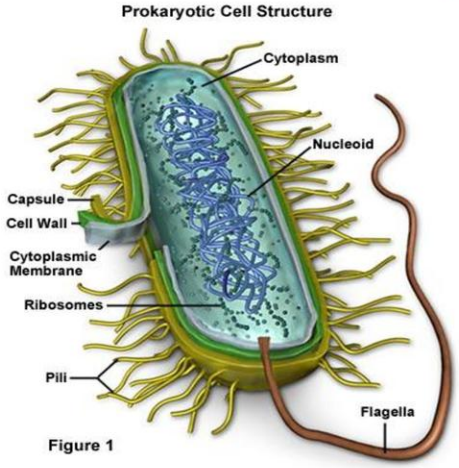
5) Mammalian viruses

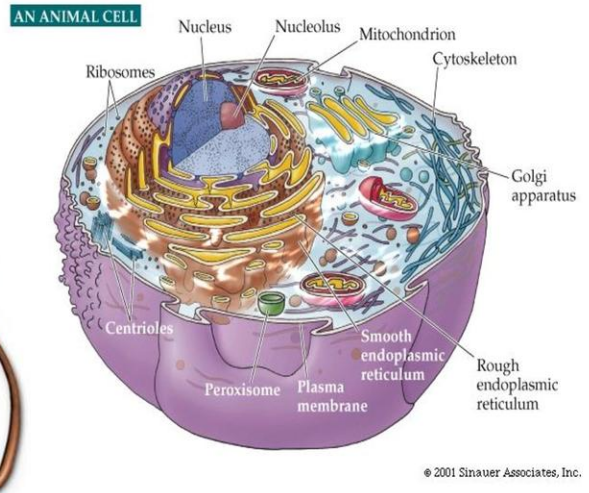
6) Mice

7) Domestic animals

Prokaryotic and Eukaryotic Organisms

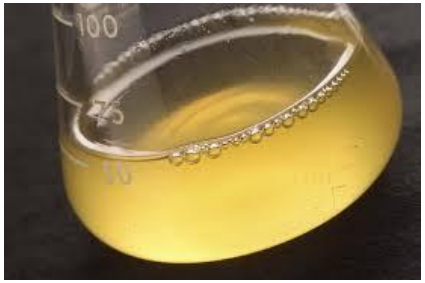
Prokaryotic	Eukaryotic
Absence of nucleus membrane	Presence of nucleus membrane
Absence of subcellular cytoplasmic organelles	Presence of subcellular cytoplasmic organelles
Cell wall contains peptidoglycan	Cell wall (if presents) contains chitin or cellulose





Escherichia coli

- Most studied organism in the world.
- A gram negative
- Non-pathogenic
- Short
- Motile
- Rod-shaped



- It can be grown in either the presence (aerobically) or absence (anaerobically) of oxygen.

- Located in intestines of humans but not normally in soil or water.

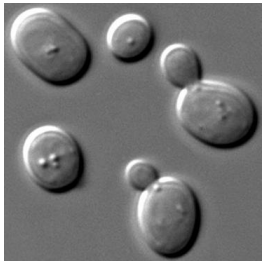
- Its ability to multiply by binary fission in the lab. on simple culture medium consisting ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, NH_4^+, Cl^-, HPO_4^{2-}, SO_4^{2-}), trace elements and a carbon source such as glucose has made it a favorite research organism.

- In complex liquid culture medium taht contains amoni acids, vitamins, salts, trace elements and a ccarbon source, the cell generation time at 37^0C for *E.coli* in the logarithmic phase of growth is about 22 minutes.

- Commercially there are lots of forms of genetically modified *E.coli.*

Saccharomyces cerevisiae

- non-pathological yeast
- Singelled-cell
- Eukaryotic version of *E.coli*
- Reproduces by budding off of a sibling cell from a parent cell



- Particular model organism for eukaryotes
- Similar cell cyle cells with humans, so they are very significant for cancer research
- First eukaryotic organism that has been sequenced

Eukaryotic Cells in Culture

- Insects
- Plants
- Mammalian cells

very similar approaches, procedures are used for growing

<u>In plants:</u>

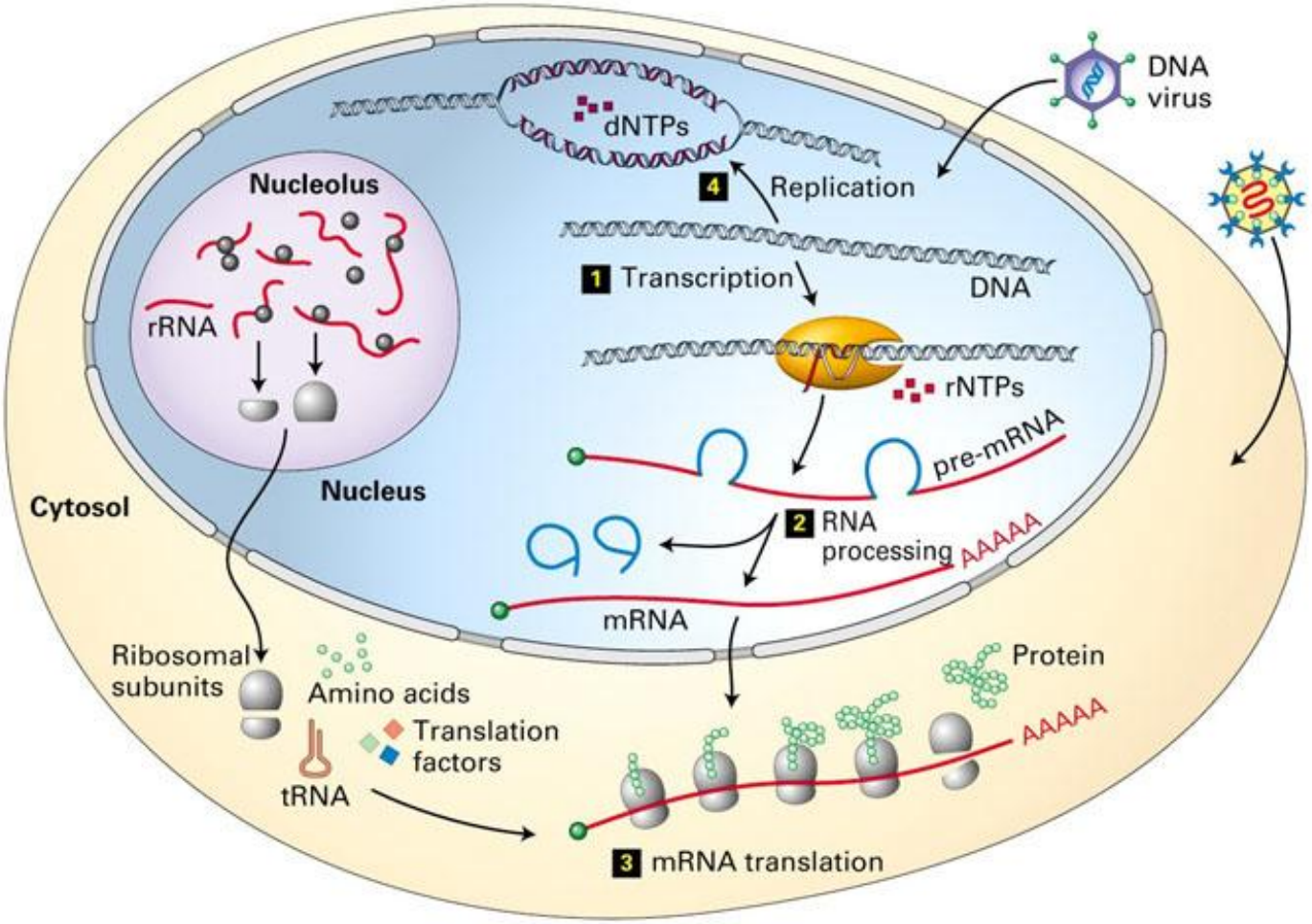
Additional enzymes are used to break the cell wall.

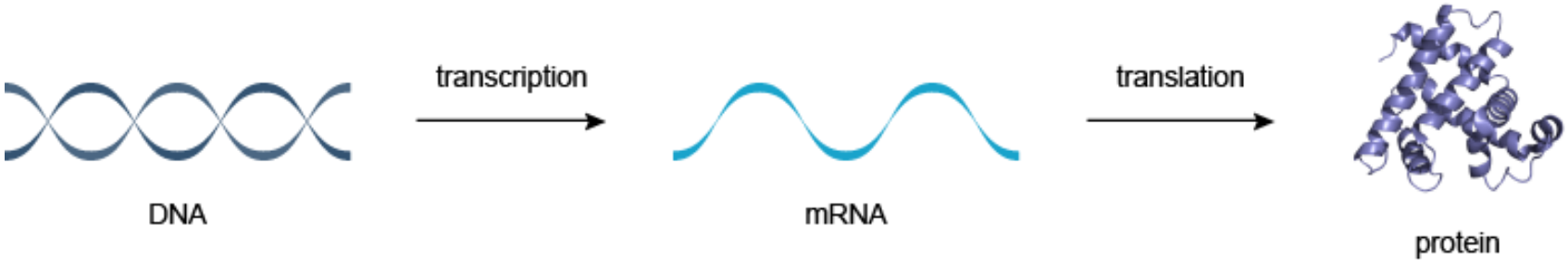
Extracellular material mechanically prevents cell division in vitro

Complex growth medium (amino acids, antibiotics, vitamins, salts, glucose and growth factors)

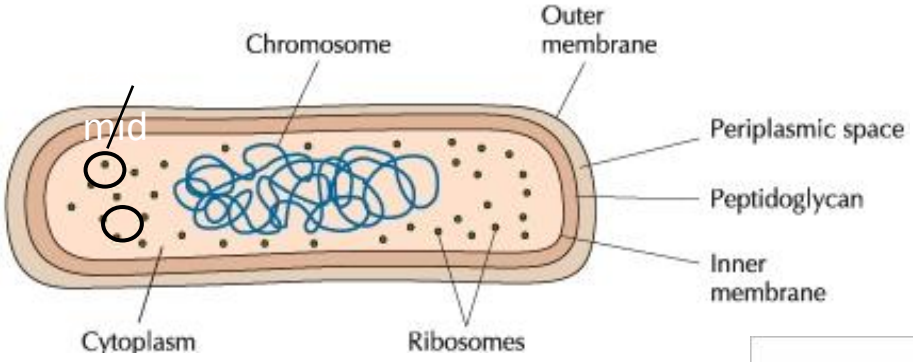
Some genetic changes can occure during their transfer to new mediums (especially at the first passage)

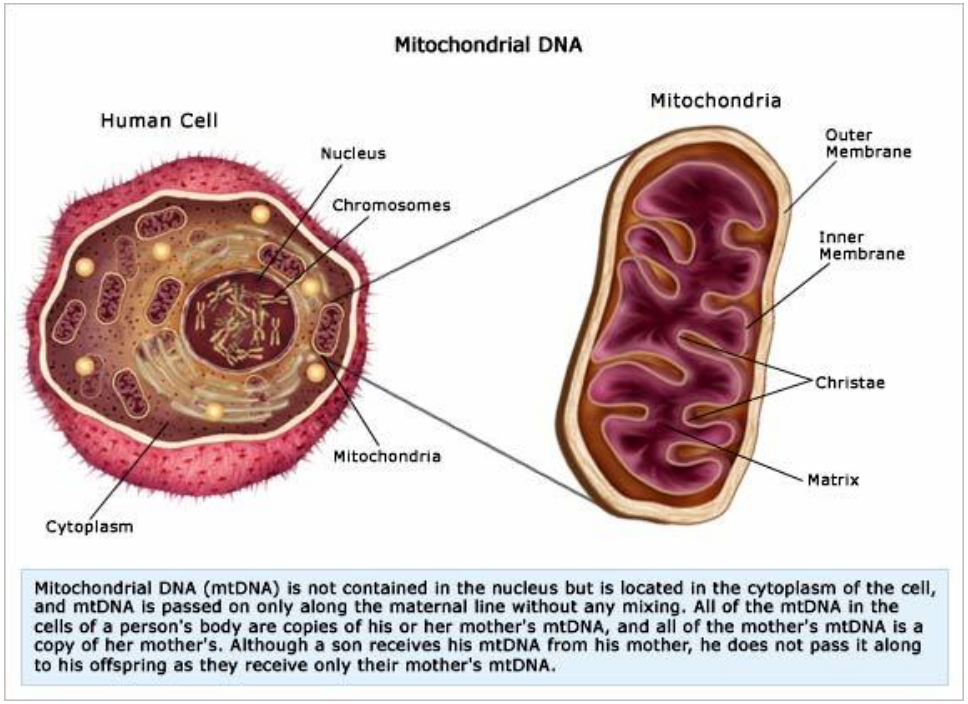
2. DNA, RNA and Protein Synthesis



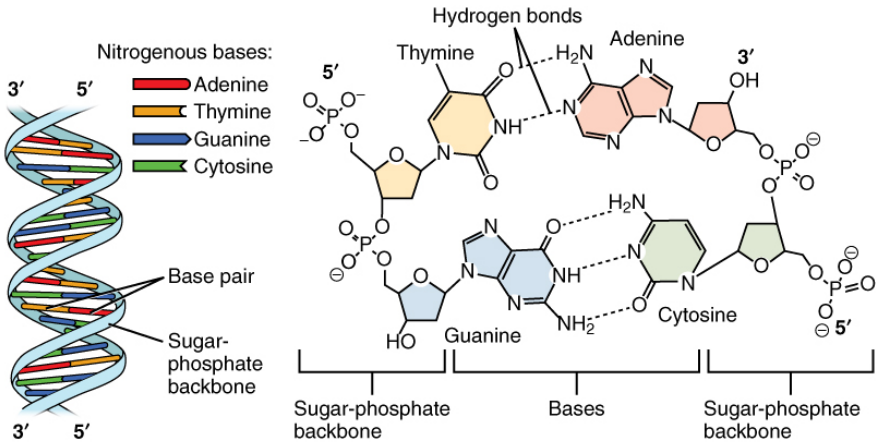


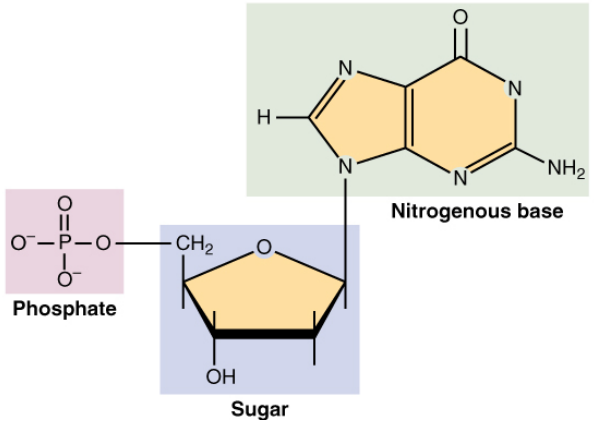
DNA is the genetic material of most organisms (from bacteria to humans)

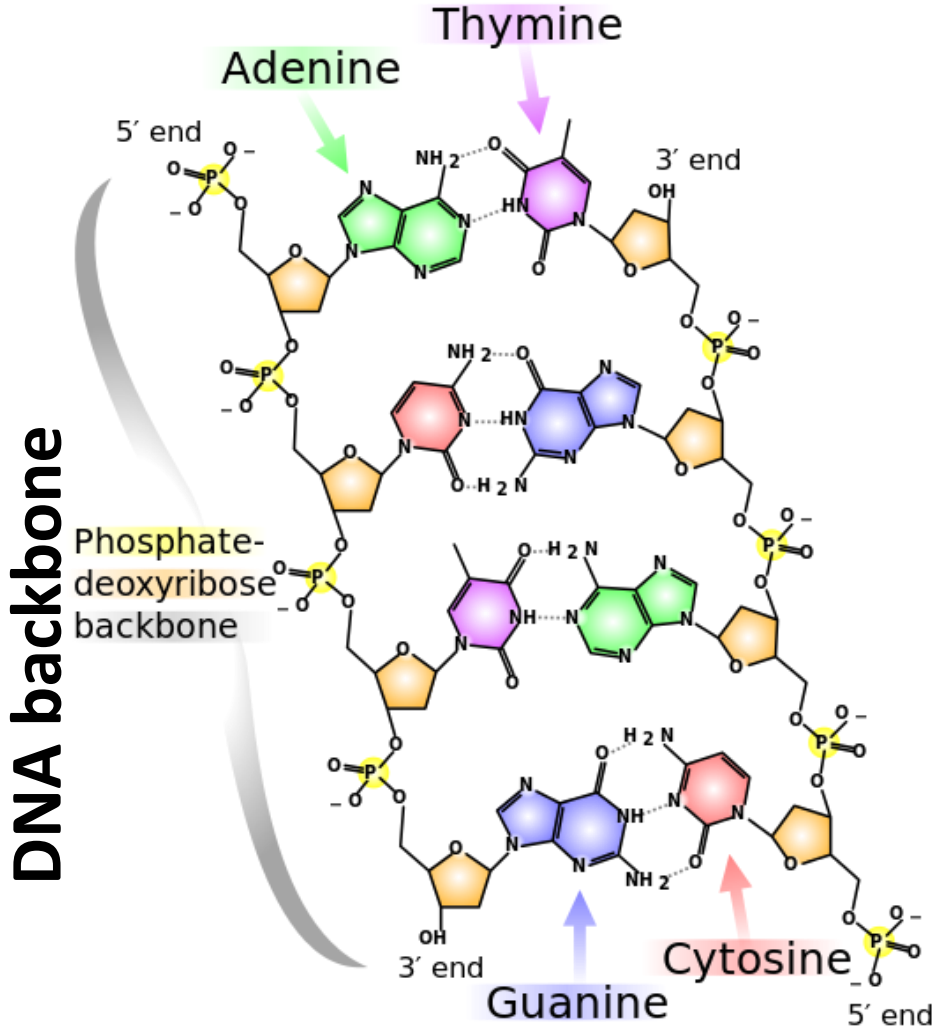




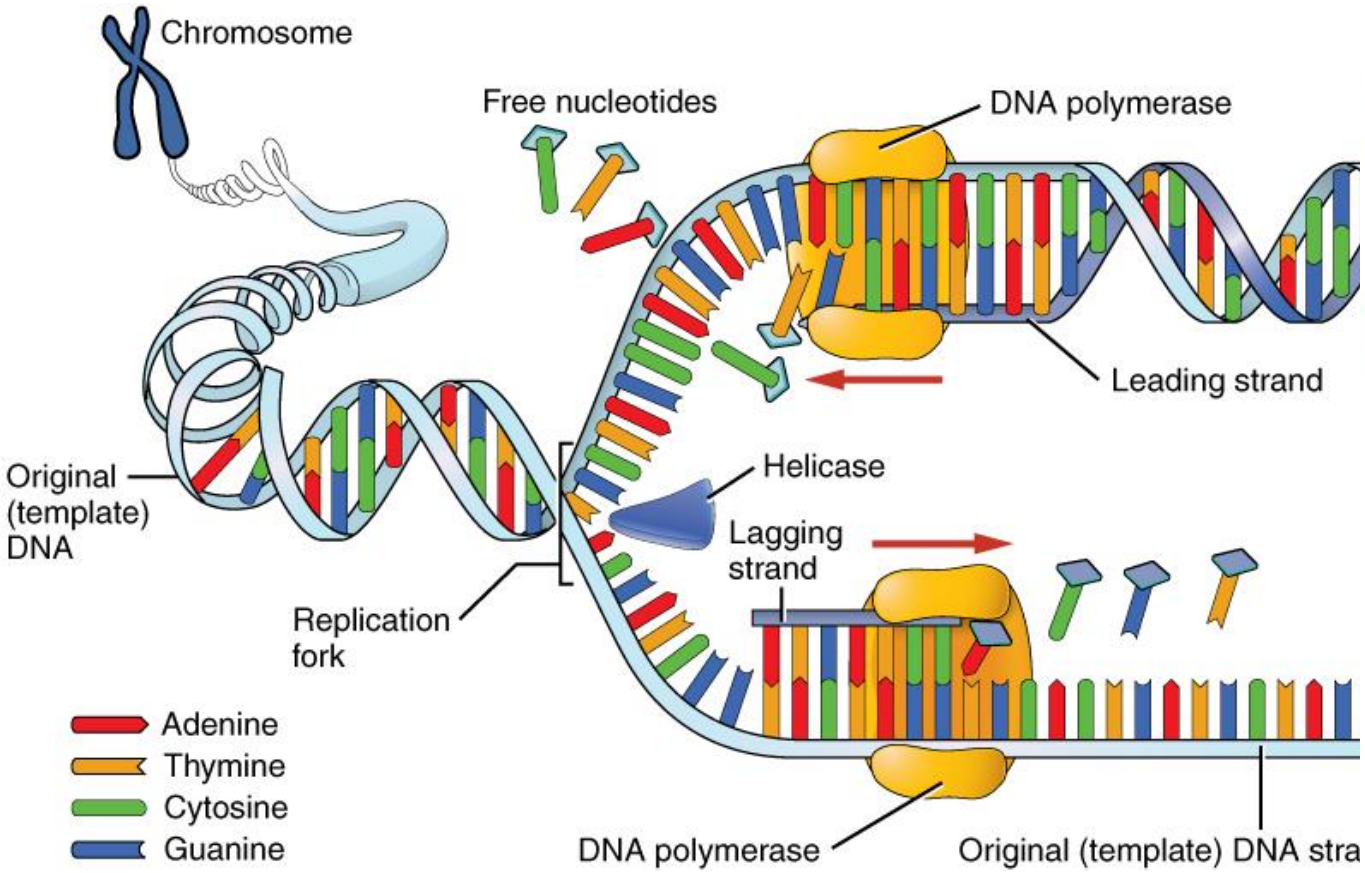
Structure of DNA

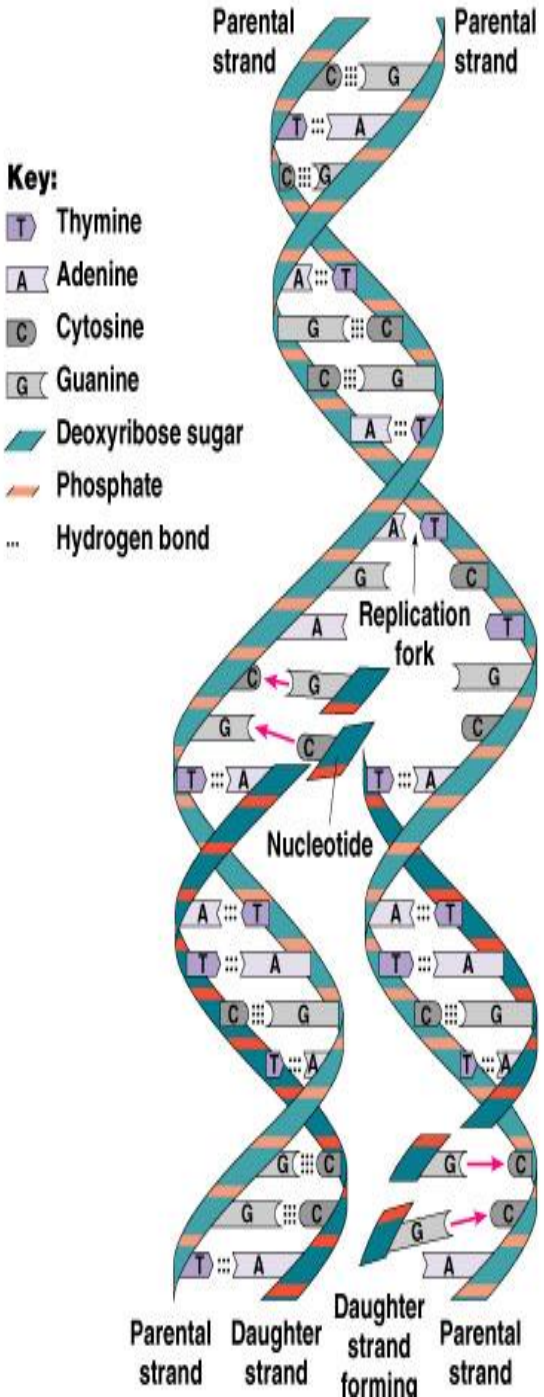




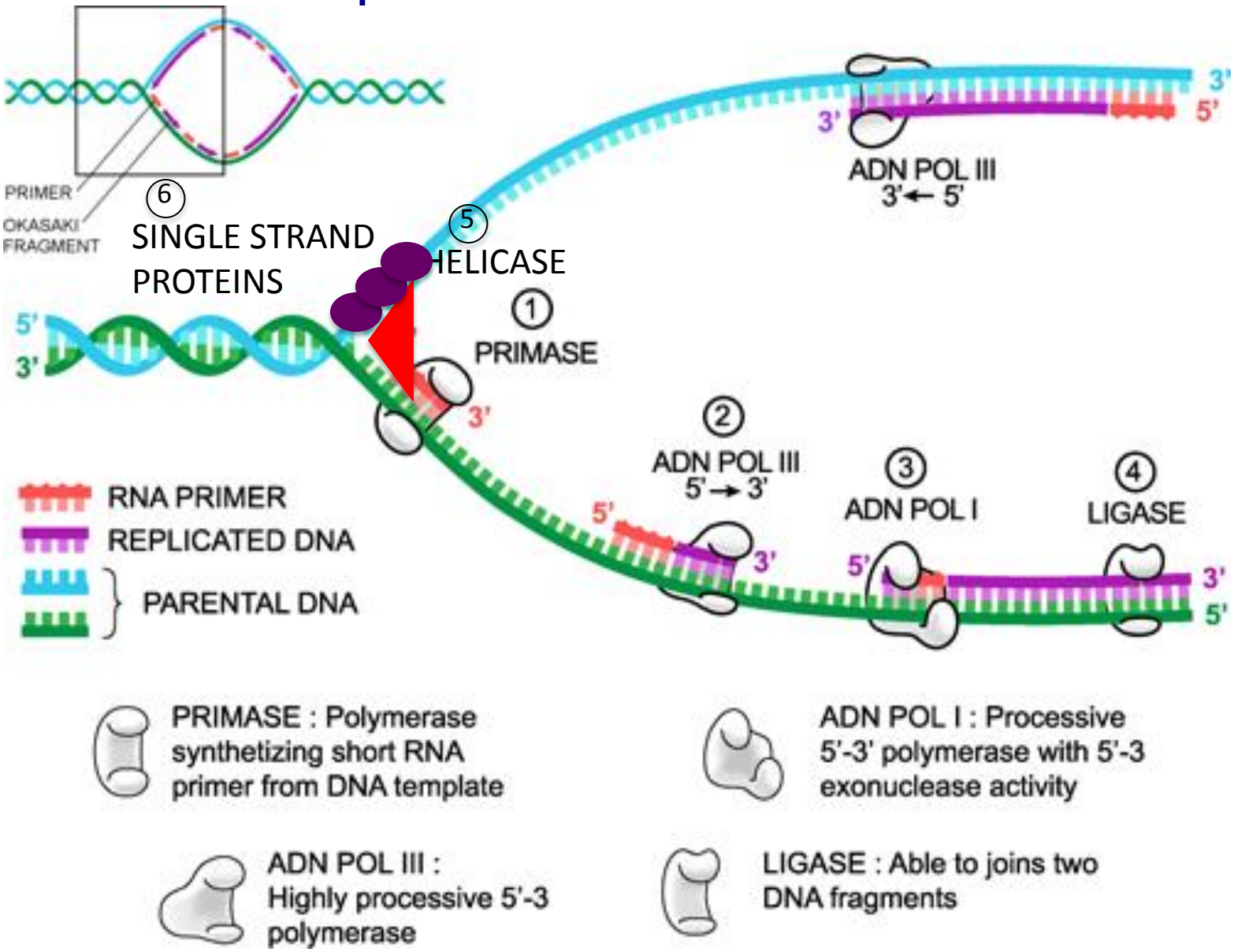


DNA replication

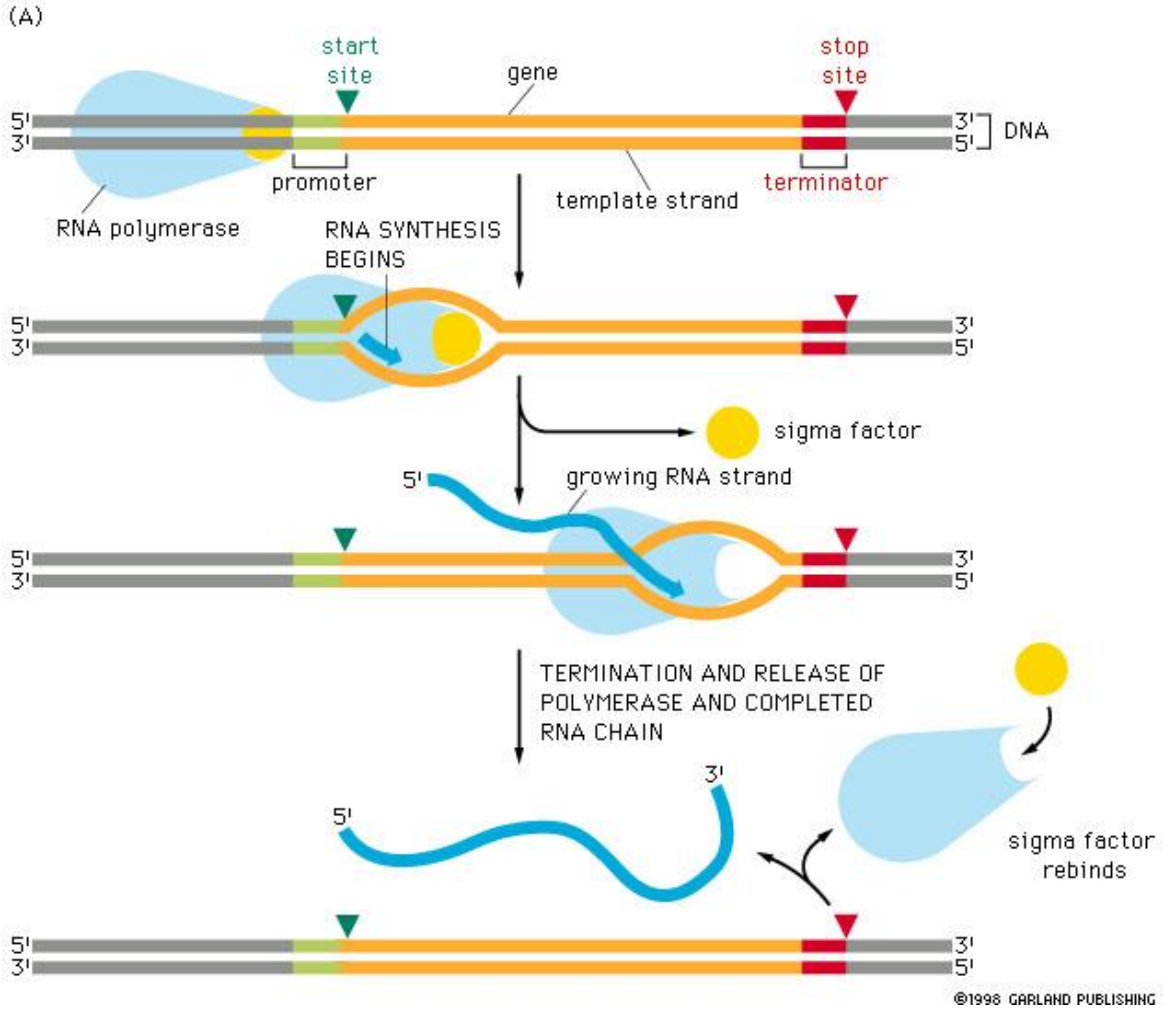


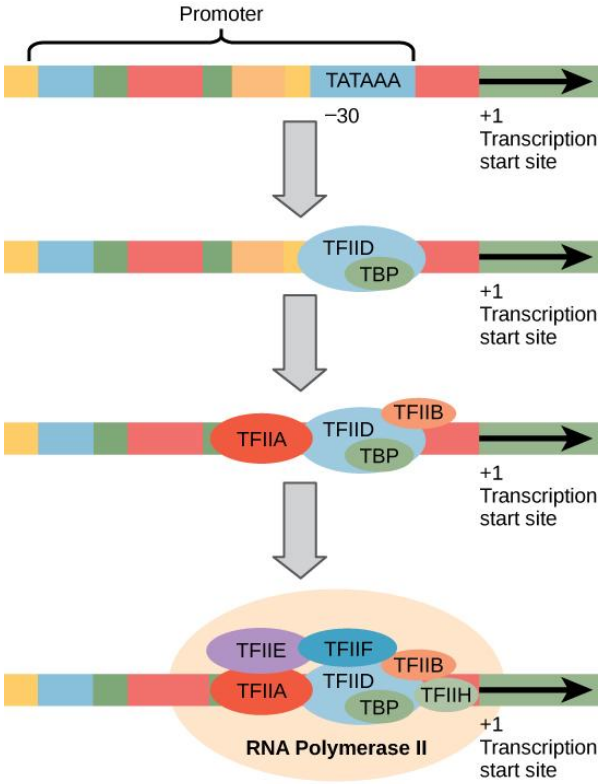


Enzymes in DNA replication:

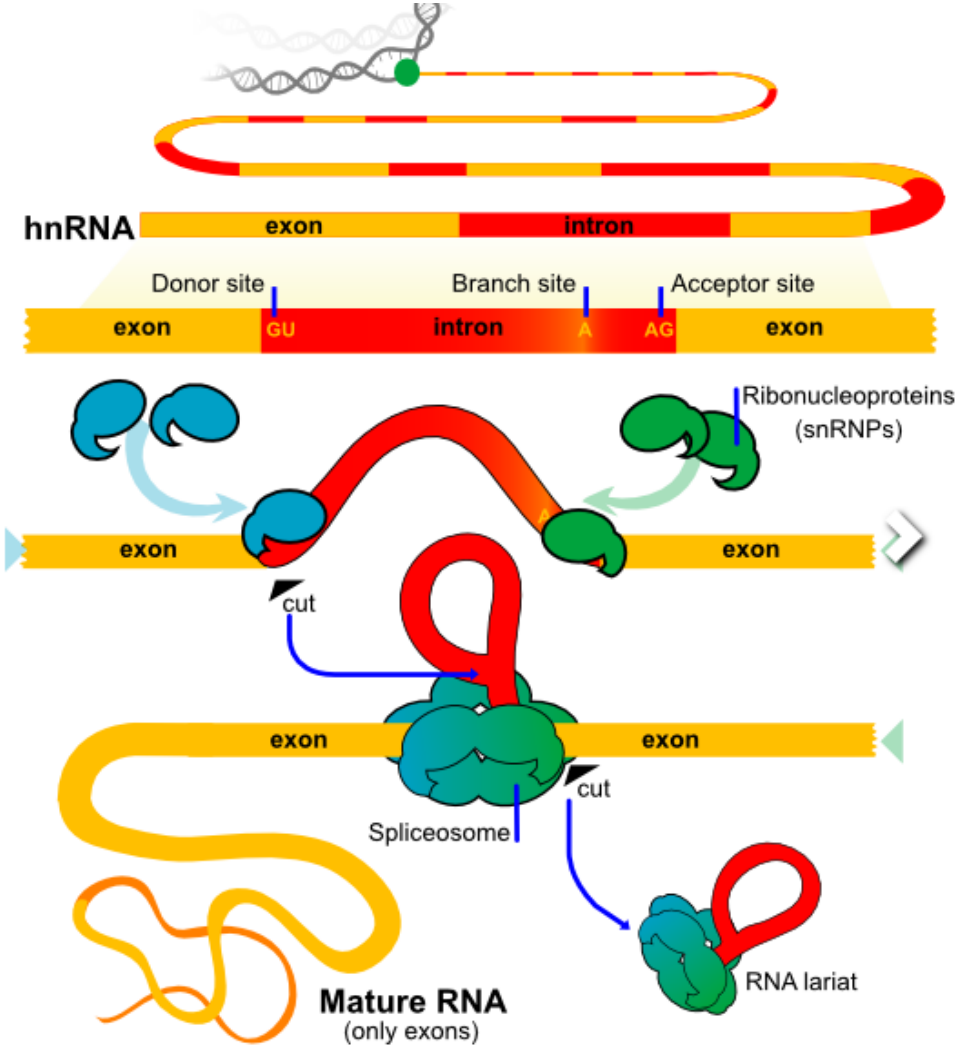


RNA Transcription

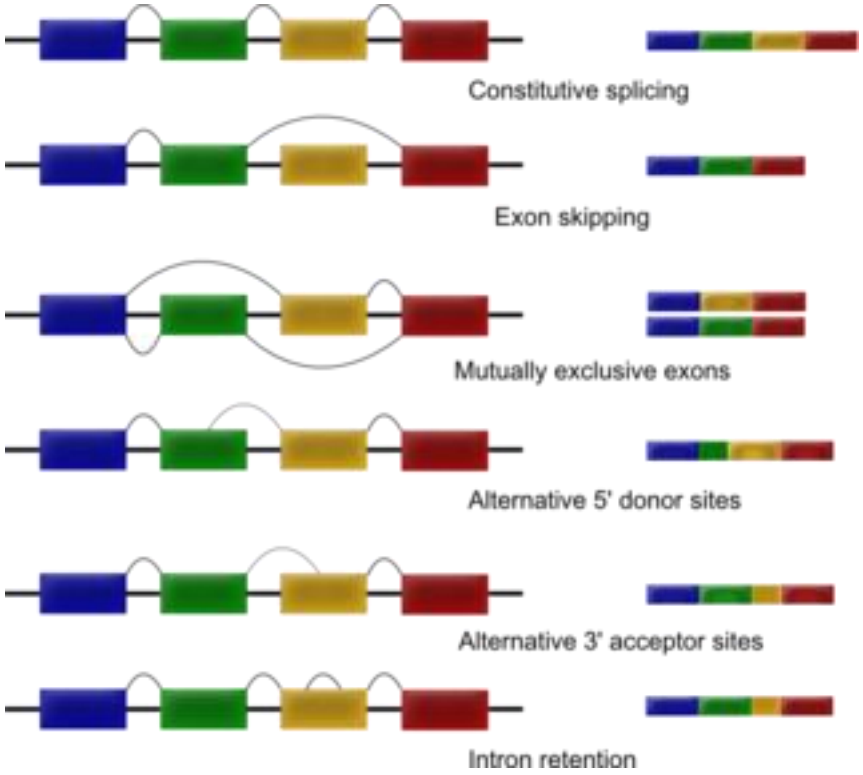


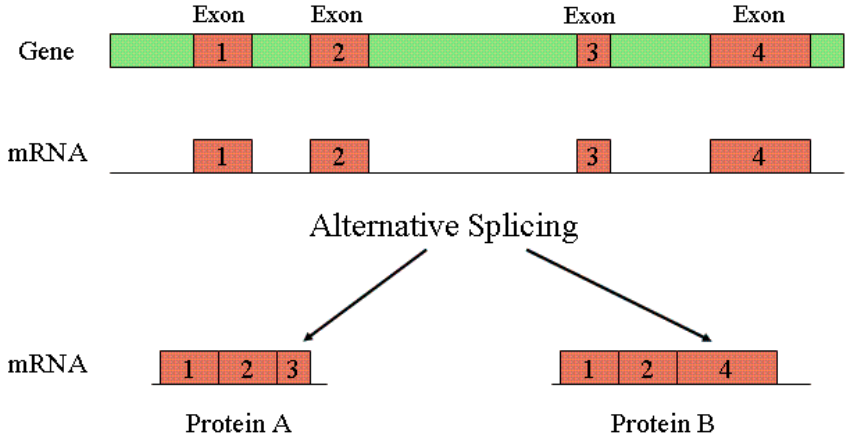


RNA maturation

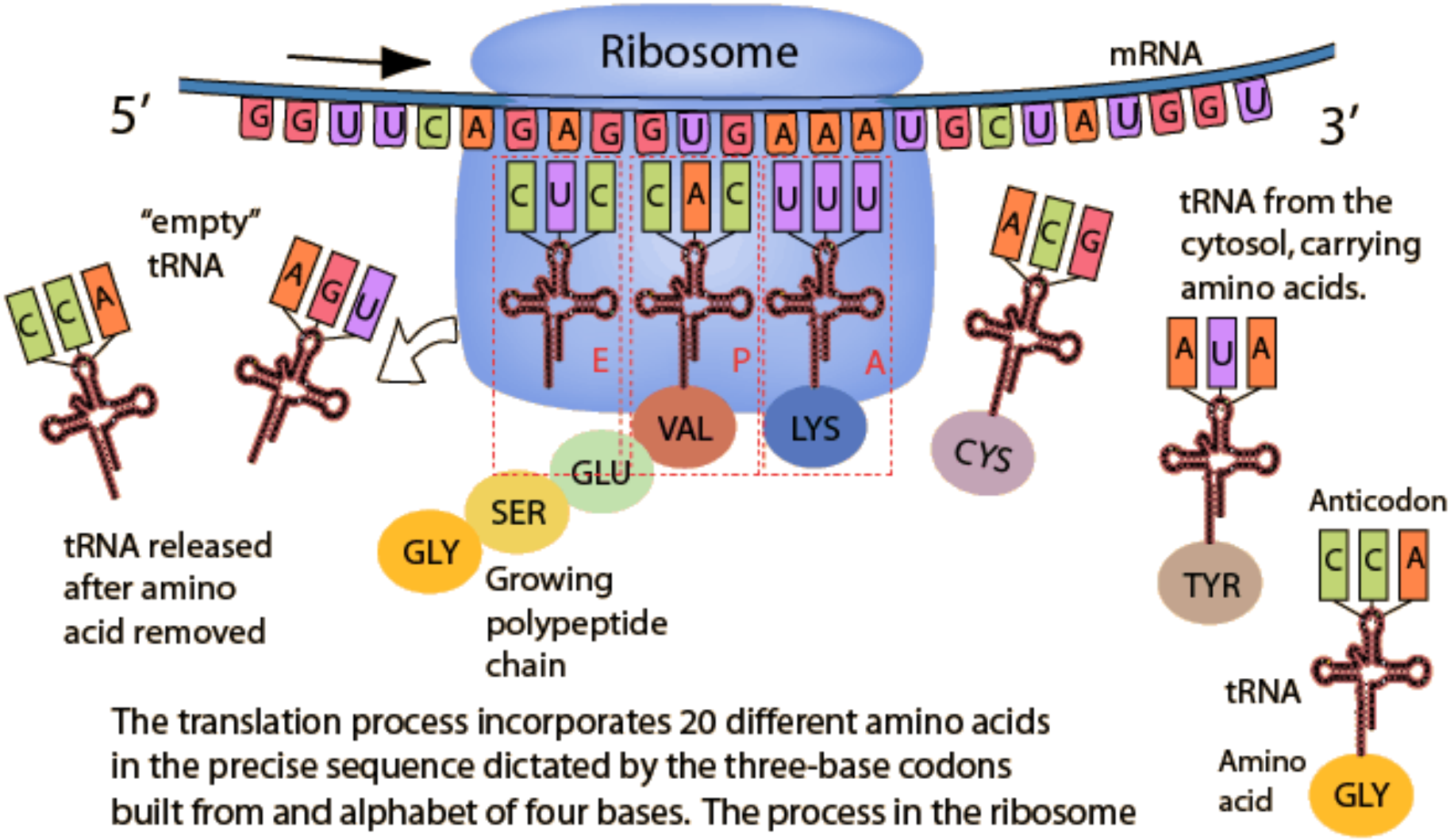


Alternative splicing





Translation: Protein synthesis

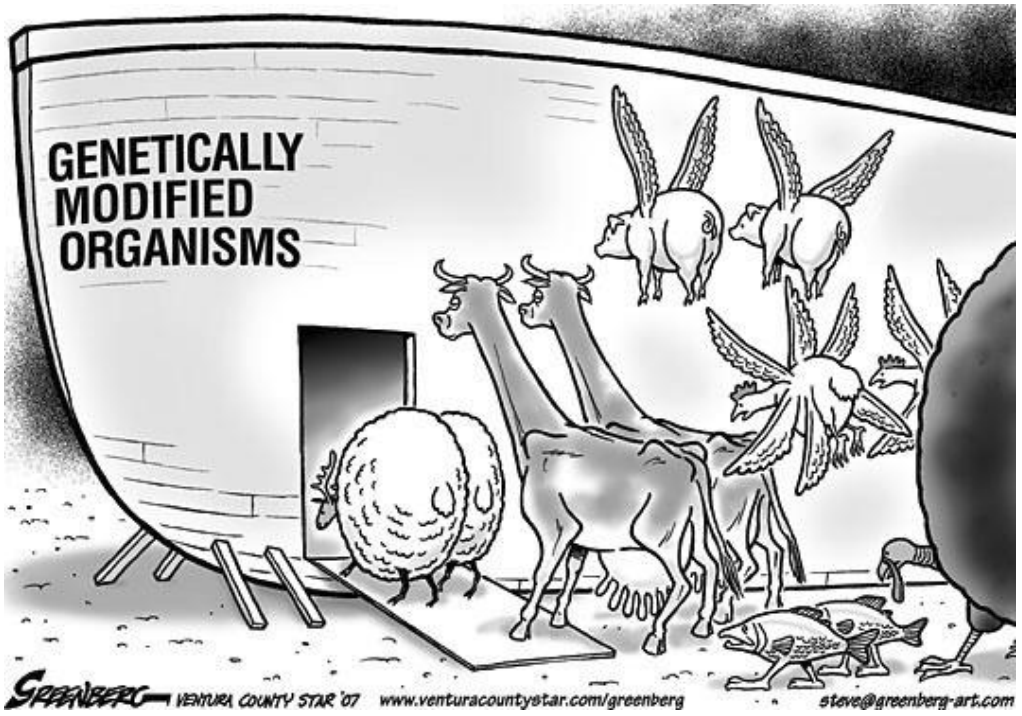


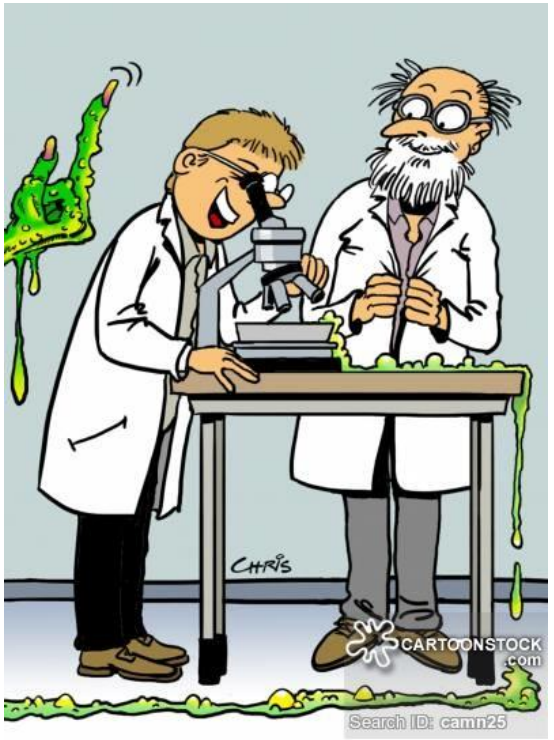
The translation process incorporates 20 different amino acids in the precise sequence dictated by the three-base codons built from and alphabet of four bases. The process in the ribosome builds the polypeptide chains tha will become proteins.

II. Recombinant DNA Technology

OR

How to MESS with DNA?





"Hurrah, we've created the world's first synthetic life form!!! Welcome Cynthia!"

What Does It Mean: “To Clone”?

Clone: a collection of molecules or cells, all identical to an original molecule or cell

- To "clone a gene" is to make many copies of it - for example, by replicating it in a culture of bacteria.
- Cloned gene can be a normal copy of a gene **(= “wild type”)**.
- Cloned gene can be an altered version of a gene **(= “mutant”)**.
- Recombinant DNA technology makes manipulating genes possible.

Recombinant DNA Technology

Introduction:

Current technology allow us to cut out a specific piece of DNA, produce a large number of copies, determine its nucleotide sequence, slightly alter it and then as a final step transfer it back into the cell.

Three goals:

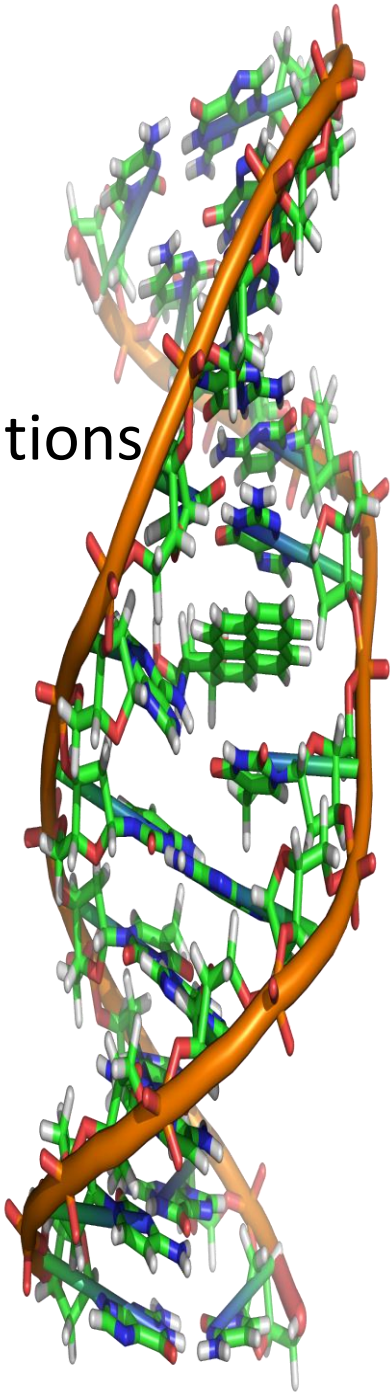
1) Eliminate undesirable phenotypic traits

2) Combine beneficial traits of two or more organisms

3) Create organisms that synthesize products humans need

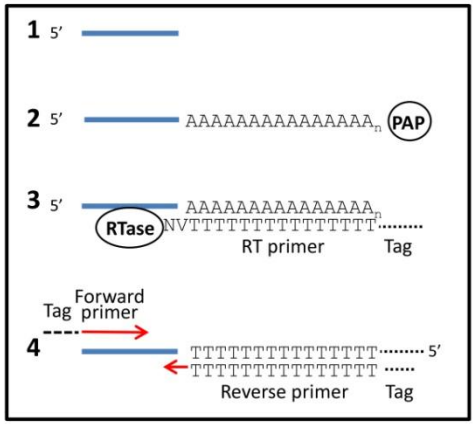
The Tools of Recombinant DNA Technology

- **Mutagens**
  - Physical and chemical agents that produce mutations
  - Scientists utilize mutagens to
    - Create changes in microbes' genomes to change phenotypes
    - Select for and culture cells with beneficial characteristics
  - Mutated genes alone can be isolated



The Tools of Recombinant DNA Technology

- **The Use of Reverse Transcriptase to Synthesize cDNA**
  - Isolated from retroviruses
  - Uses RNA template to transcribe molecule of cDNA
  - Easier to isolate mRNA molecule for desired protein first
  - mRNA of eukaryotes has **introns** removed
    - Allows cloning in prokaryotic cells

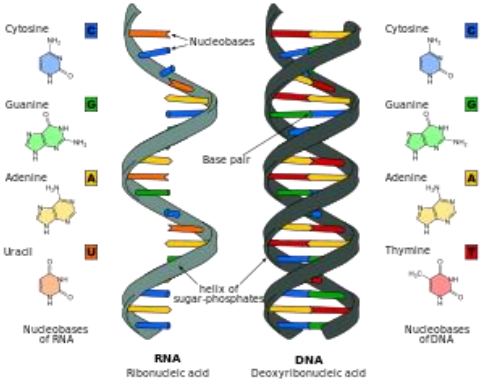


The Tools of Recombinant DNA Tehnology

- **Synthetic Nucleic Acids**

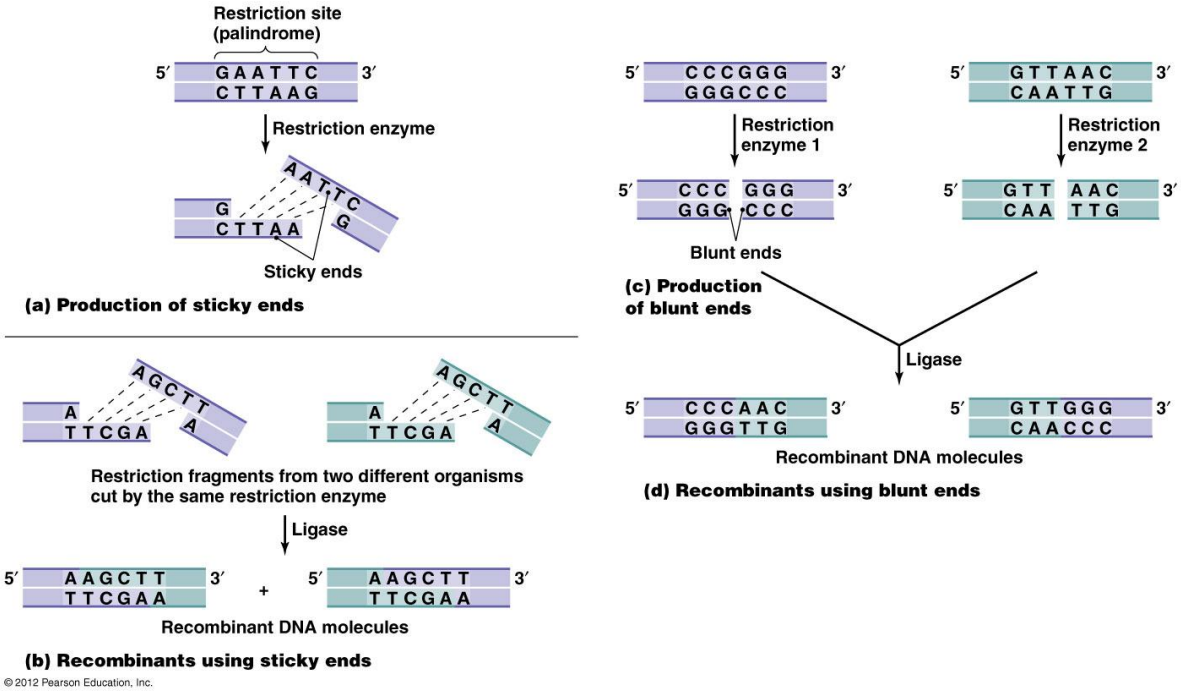
  – Molecules of DNA and RNA produced in cell-free solutions

  – Uses of synthetic nucleic acids
    - Elucidating the genetic code
    - Creating genes for specific proteins
    - Synthesizing DNA and RNA probes to locate specific sequences of nucleotides
    - Synthesizing antisense nucleic acid molecules



The Tools of Recombinant DNA Tehnology

- **Restriction Enzymes**
  - Bacterial enzymes that cut DNA molecules only at restriction sites
  - Categorized into two groups based on type of cut
    - Cuts with sticky ends
    - Cuts with blunt ends



The technology based on:

Bacteria contain extra-chromosomal molecules of DNA called **plasmids** which are circular.

Bacteria also produce enzymes called **restriction endonucleases** that cut DNA molecules at specific places into many smaller fragments called **restriction fragments.**

Each nuclei cuts DNA at a specific site defined by a sequence of bases in the DNA called **recognition site.**

A restriction enzyme cuts only double-helical segments that contain a particular sequence, and it makes its incisions only within that sequence **“recognition site”.**

Recombinant DNA Technology:

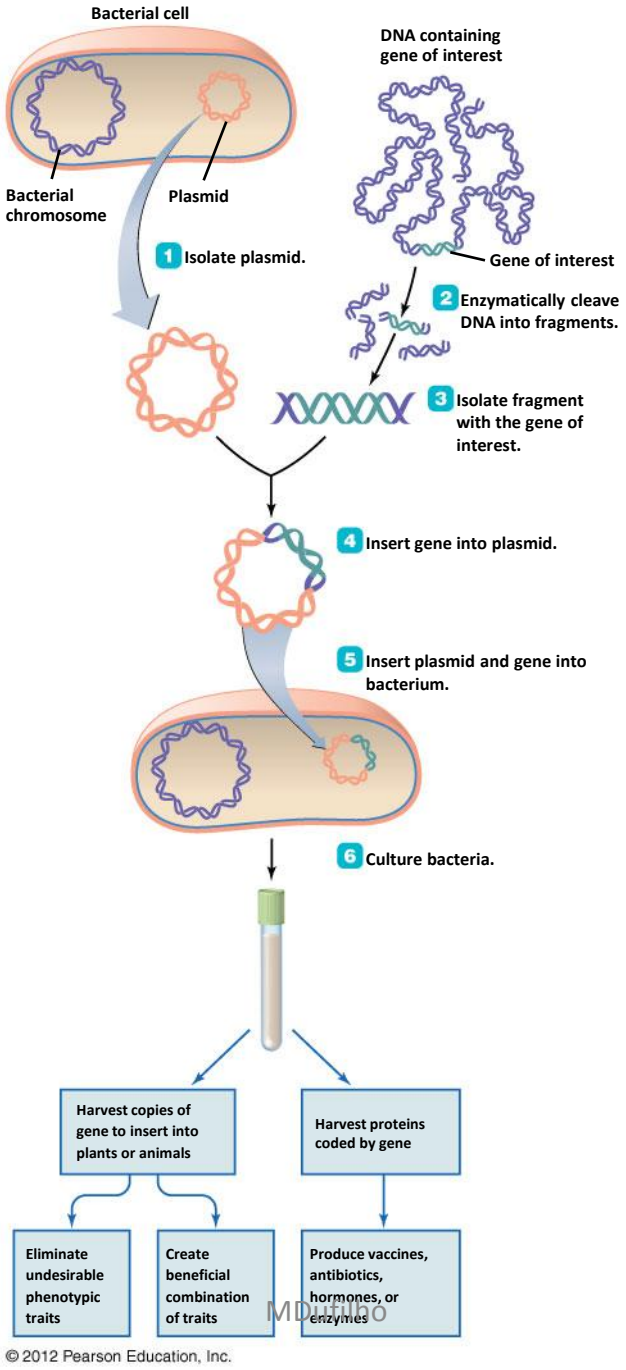
-DNA (cloned DNA, insert DNA, target DNA, foreign DNA) from a donor organism is extracted, enzymatically cleaved (cut, digested) and joined (ligated) to another DNA entity (cloning vector) to form a new, recombined DNA molecule (cloning-vector-insert DNA contruct)

- The cloning vector-insert DNA contruct is transferred into and maintained within host cell. T

-The introduction of DNA into a bacterial host cell is called transformation.

-Those host cells that take up the DNA construct (transformed cells) are identified and selected (seperated, isolated) from those that do not.

-If required, a DNA construct can be prepared to ensure that the protein product that is encoded by the cloned DNA sequence is produced by the host cell.



Summary of Recombinant DNA Technology process:

- It requires DNA extracion, purification and fragmentation.
- Fragmentation of DNA is done by specific REs and is followed by sorting and isolation of fragments containing a particular gene.
- The portion of the DNA is then coupled to a carrier molecule.
- The hybrid DNA is introduced into a chosen cell for reproduction and synthesis.

Restriction Enzymes

- Bacteria have learned to "restrict" the possibility of attack from foreign DNA by means of "restriction enzymes".
- Cut up "foreign" DNA that invades the cell.
- Type II and III restriction enzymes cleave DNA chains at selected sites.
- Enzymes may recognize 4, 6 or more bases in selecting sites for cleavage.
- An enzyme that recognizes a 6-base sequence is called a "six-base cutter".

Basics of type II Restriction Enzymes

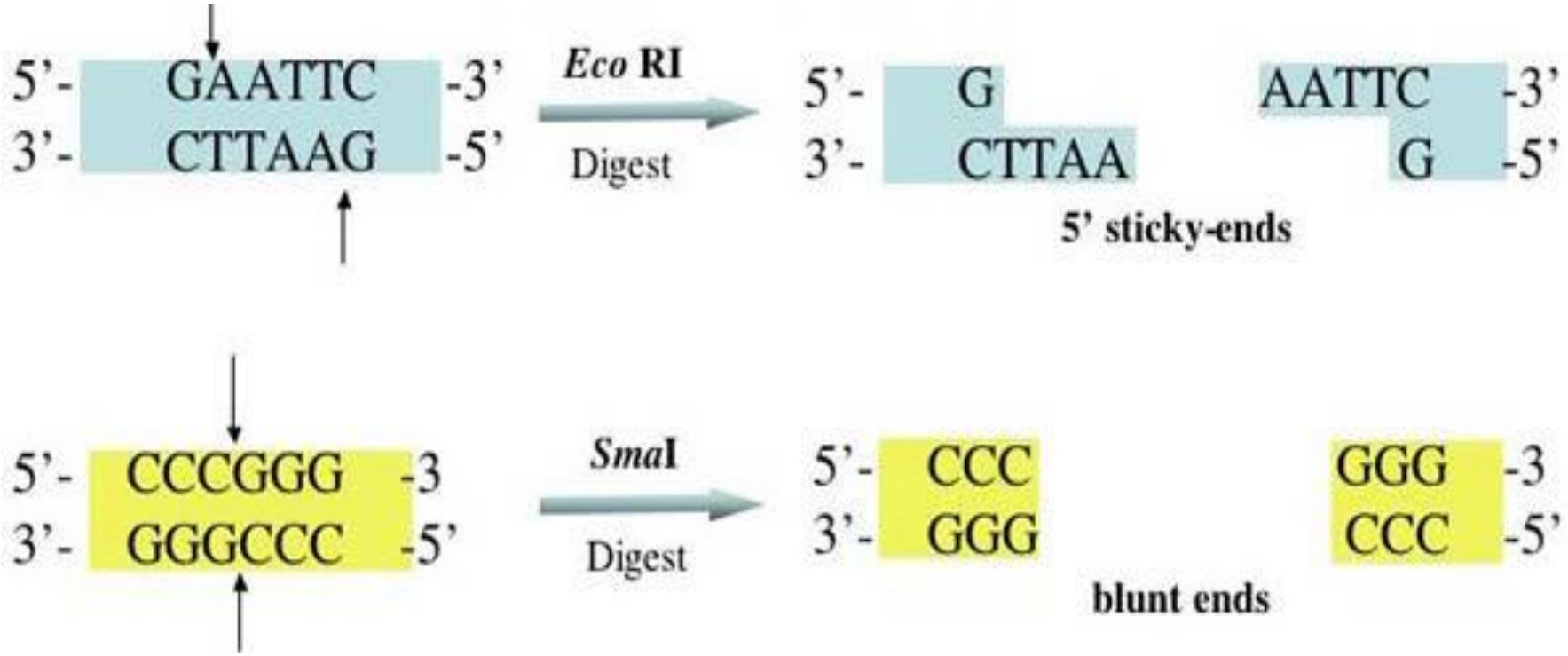
- **No** ATP requirement.

- Recognition sites in double stranded DNA have a 2-fold axis of symmetry – a **“palindrome”.**

- Cleavage can leave staggered or **"sticky"** ends or can produce "blunt” ends.

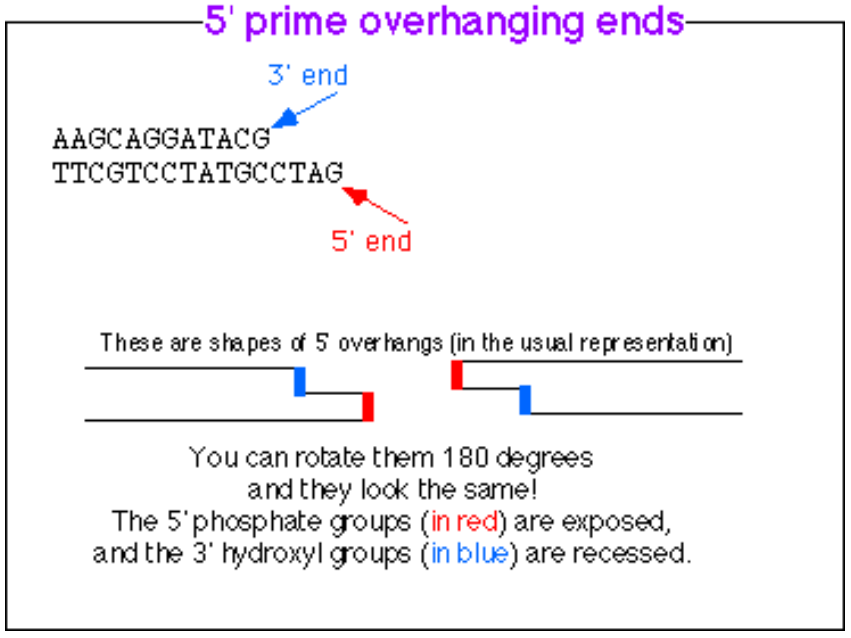
Sticky end and **blunt end** are the two possible configurations resulting from the breaking of double-stranded DNA.

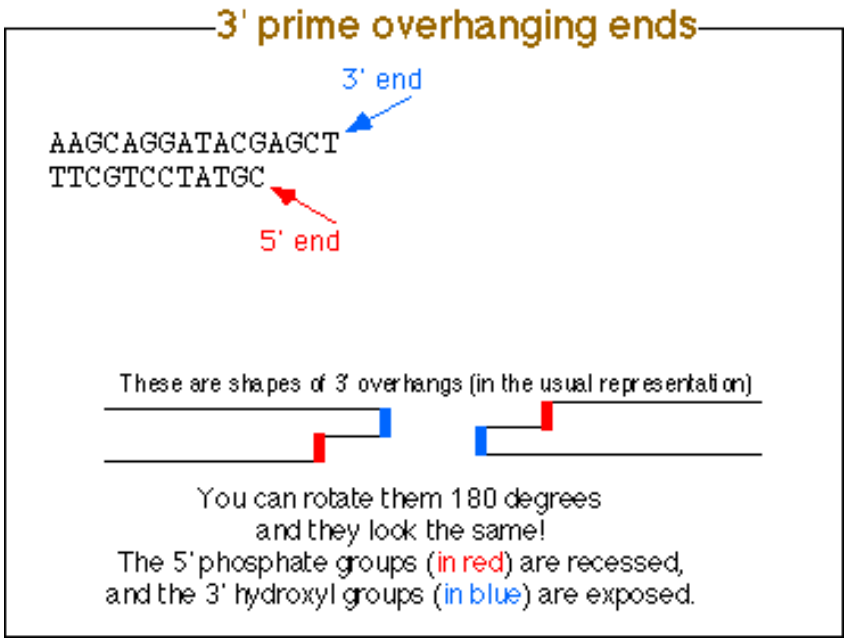
If two complementary strands of DNA are of equal length, then they will terminate in a **blunt end**.



If one strand extends beyond the complementary region, the the DNA is said to **possess an overhang**.

If an other DNA fragment exist with a complementary overhang, then these two overhangs will tend to associate with each other and each strand is said to **possess a sticky end**

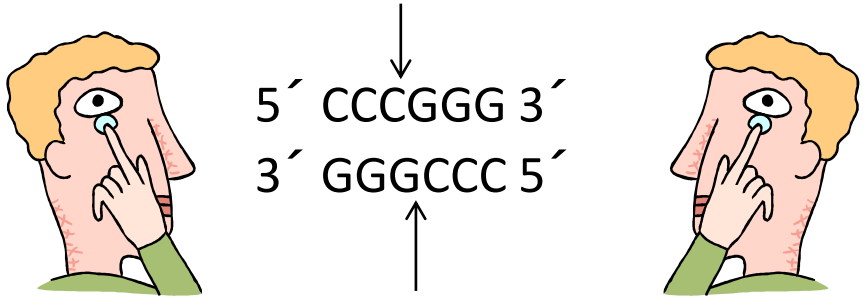




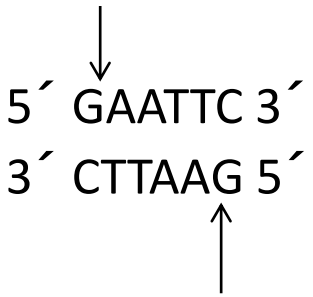
Recognition/Cleavage Sites of Type II Restriction Enzymes

Cuts usually occurs at a palindromic sequence

*Sma*I: produces blunt ends



*Eco*RI: produces sticky ends



Examples of Palindromes

Don't nod
Dogma: I am God
Never odd or even
Too bad – I hid a boot
Rats live on no evil star
No trace; not one carton
Was it Eliot's toilet I saw?
Murder for a jar of red rum
Some men interpret nine memos
Campus Motto: Bottoms up, Mac
Go deliver a dare, vile dog!
Madam, in Eden I'm Adam
Oozy rat in a sanitary zoo
Ah, Satan sees Natasha
Lisa Bonet ate no basil
Do geese see God?
God saw I was dog
Dennis sinned

Type II restriction enzyme nomenclature

Why the funny names?

- *Eco*RI – *Escherichia coli* strain R, 1st enzyme
- *Bam*HI – *Bacillus amyloliquefaciens* strain H, 1st enzyme
- *Dpn*I – *Diplococcus pneumoniae*, 1st enzyme
- *Hin*dIII – *Haemophilus influenzae*, strain D, 3rd enzyme
- *Bgl*II – *Bacillus globigii*, 2nd enzyme
- *Pst*I – *Providencia stuartii* 164, 1st enzyme
- *Sau*3AI – *Staphylococcus aureus* strain 3A, 1st enzyme
- *Kpn*I – *Klebsiella pneumoniae*, 1st enzyme

Results of Type II Digestion

- *Hin*dIII - leaves 5´ overhangs (“sticky”)

5’ --AAGCTT-- 3’ → 5’ --A AGCTT--3’

3’ --TTCGAA-- 5’ → 3’ –TTCGA A--5’

- *Kpn*I leaves 3´ overhangs (“sticky”)

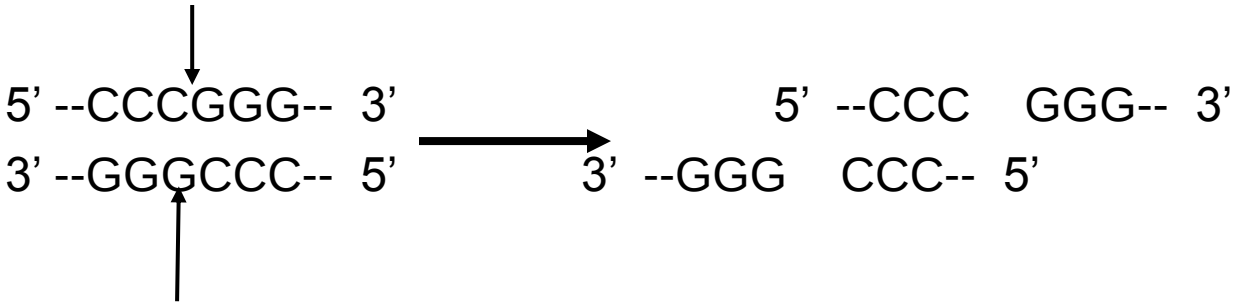
5’--GGTACC-- 3’ → 5’ –GGTAC C-- 3’

3’--CCATGG-- 5’ → 3’ –C CATGG-- 5’

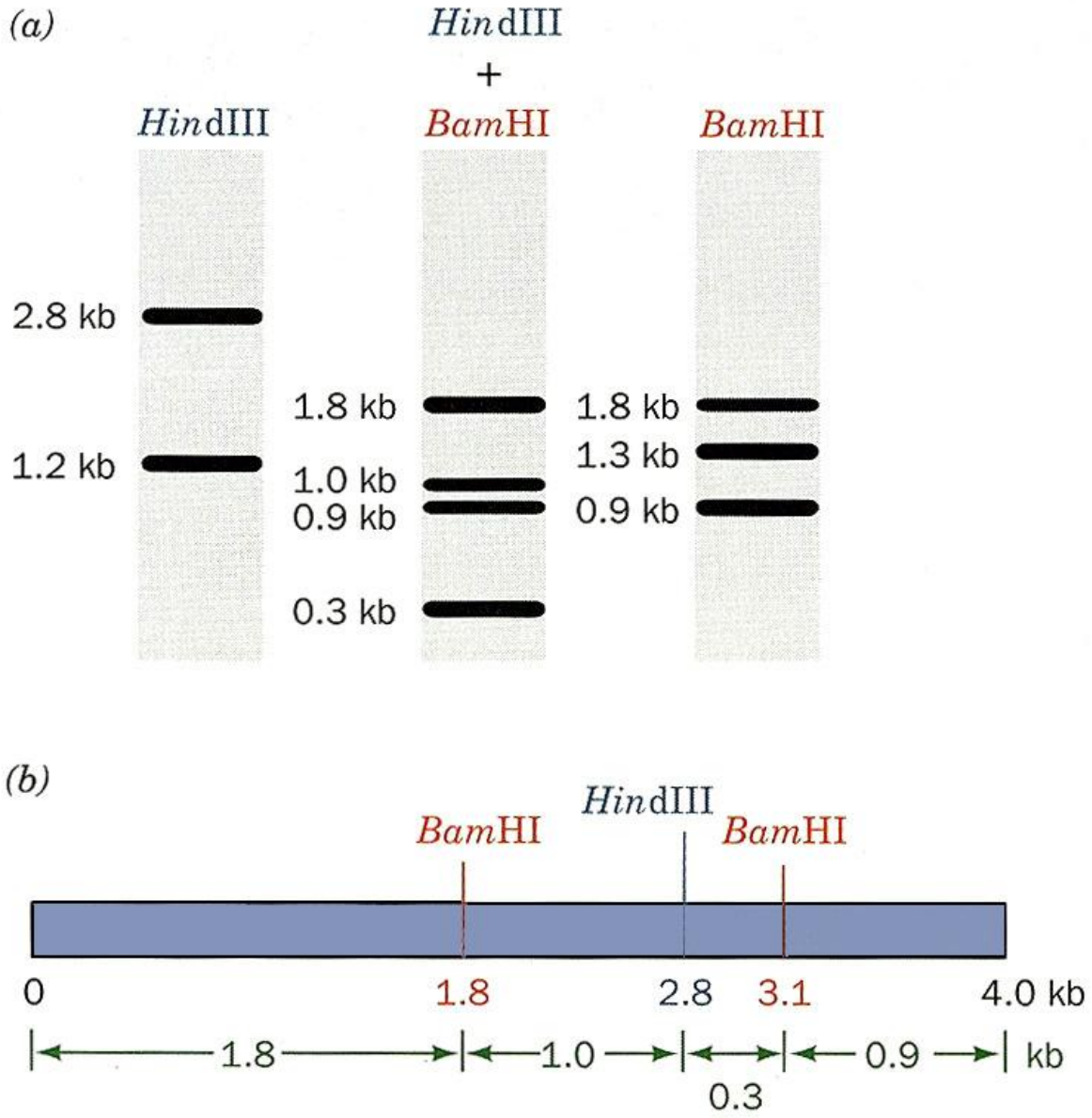
Results of Type II Digestion

- Enzymes that cut at same position on both strands leave "blunt" ends

- *Sma*I



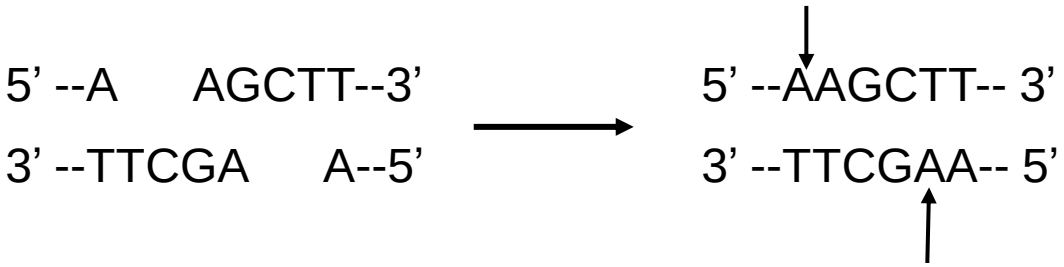
Restriction Endonucleases Cleave DNA at specific DNA sequences



DNA Ligase joins DNA fragments together

- Enzymes that cut with staggered cuts result in complementary ends that can be ligated together.

- *Hin*dIII - leaves 5’ overhangs (“sticky”)



- Sticky ends that are complementary (from digests with the same or different enzymes) can be ligated together.

- Sticky ends that are not complementary cannot be ligated together.

DNA Ligase can also join blunt ends

DNA fragments with blunt ends generated by different enzymes can be ligated together (with lower efficiency), but usually cannot be re-cut by either original restriction enzyme.

- Ligations that re-constitute a *Sma*I or *Dra*I site (CCCGGG or AAATTT) can be re-cut by *Sma*I or *Dra*I.

- Mixed ligation products (CCCTTT + AAAGGG) cannot be re-cut by *Sma*I or *Dra*I.

Can complementary Ends Be Ligated?

- *Bam*HI -G GATCC-
  -CCTAG G-
- *Bgl*II -A GATCT-
  -TCTAG A-
- Result -GGATCT-
  -CCTAGA-

No longer palindromic, so not cut by *Bam*HI or *Bgl*II

The Tools of Recombinant DNA Technology

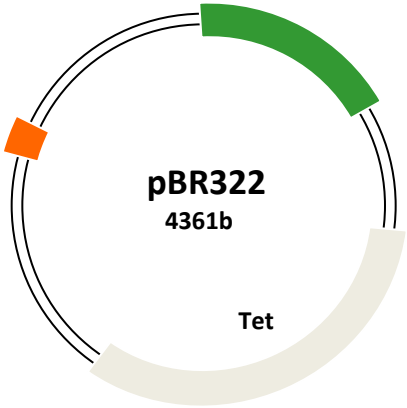
- **Vectors**

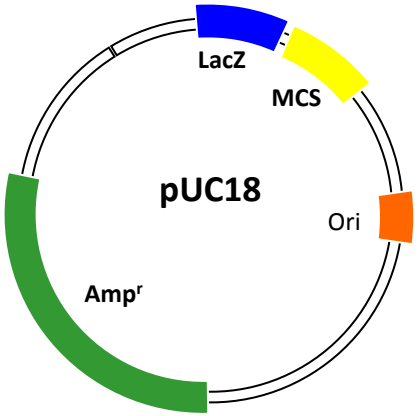
Types of expression systems

- Bacterial: plasmids, phages
- Yeast: expression vectors: plasmids, yeast artifical chromosomes (YACs)
- Insect cells: baculovirus, plasmids
- Mammalian:
  - viral expression vectors (gene therapy):
    - SV40
    - vaccinia virus
    - adenovirus
    - retrovirus
  - Stable cell lines (CHO, HEK293)

Plasmids – vehicles for cloning

- Plasmids are naturally occurring extra-chromosomal DNA molecules.
- Plasmids are circular, double-stranded DNA.
- Plasmids are the means by which antibiotic resistance is often transferred from one bacteria to another.
- Plasmids can be cleaved by restriction enzymes, leaving sticky or blunt ends.
- Artificial plasmids can be constructed by linking new DNA fragments to the sticky ends of plasmid.



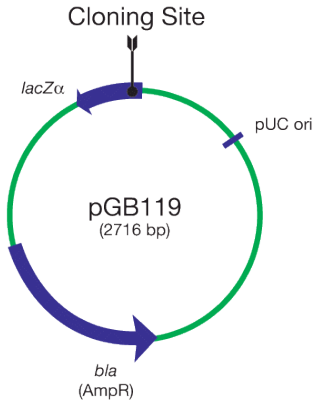


CLONING METHODOLOGY

- **Cut** the cloning vector with R.E. of choice, eg *Eco* RI
- **Cut** DNA of interest with same R.E. or R.E. yielding same sticky ends, e.g. *Bam* HI and *Sau* 3A
- **Mix** the restricted cloning vector and DNA of interest together.
- **Ligate** fragments together using **DNA ligase**
- Insert ligated DNA into host of choice - **transformation** of *E. Coli*
- Grow host cells under **restrictive conditions**, grow on plates containing an antibiotic

CLONING VECTORS

- Plasmids useful as cloning vectors must have:
  - An origin of replication.
  - A selectable marker (antibiotic resistance gene, such as amp^r and tet^r).
  - Multiple cloning site (MCS) (site where insertion of foreign DNA will not disrupt replication or inactivate essential markers).
  - Easy to purify away from host DNA.

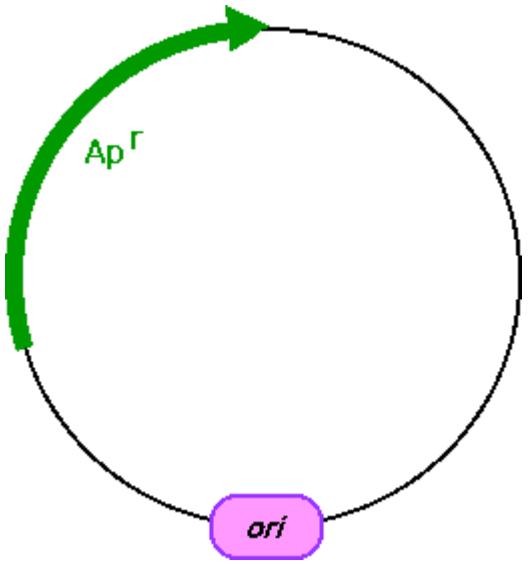


Why Plasmids are Good Cloning Vectors

- small size (easy to manipulate and isolate)
- circular (more stable)
- replication independent of host cell
- several copies may be present (facilitates replication)
- frequently have antibody resistance (detection easy)

SELECTIVE MARKER

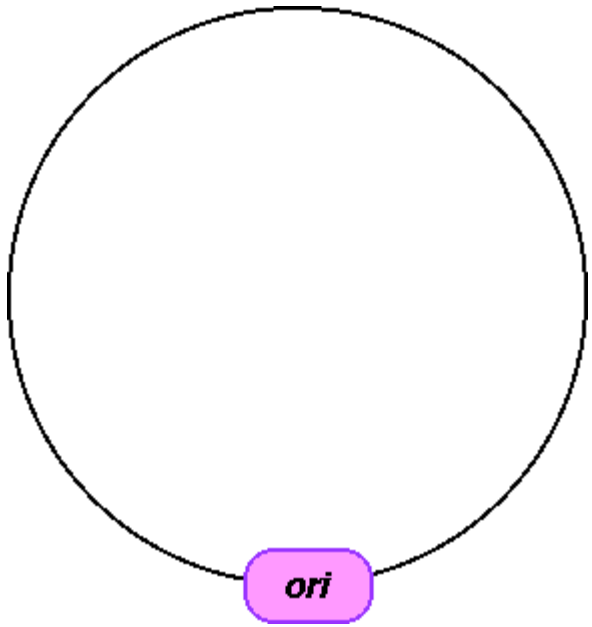
- **Selective marker** is required for maintenance of plasmid in the cell.
- Because of the presence of the selective marker the plasmid becomes useful for the cell.
- Under the selective conditions, only cells that contain plasmids with selectable marker can survive
- Genes that confer resistance to various antibiotics are used.
- Genes that make cells resistant to ampicillin, neomycin, or chloramphenicol are used



ORIGIN OF REPLICATION

- **Origin of replication** is a DNA segment recognized by the cellular DNA-replication enzymes.

- Without replication origin, **DNA cannot be replicated in the cell.**

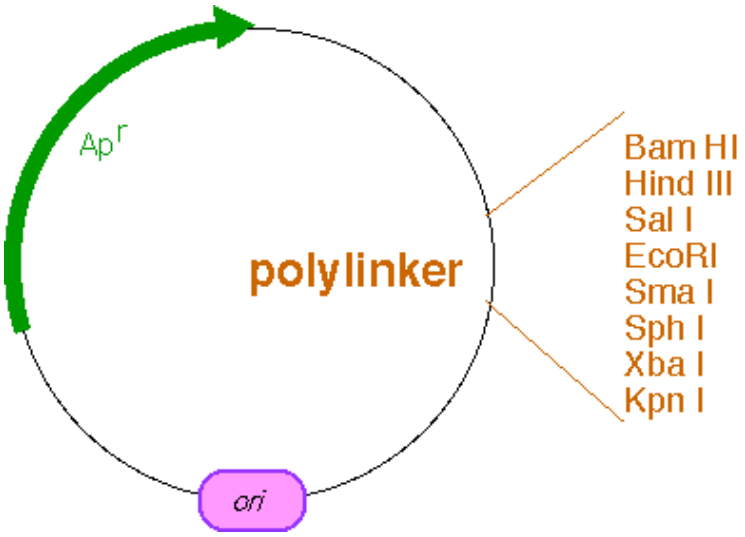


MULTIPLE CLONING SITE

- Many cloning vectors contain a **multiple cloning site** or **polylinker**: a DNA segment with several unique sites for restriction endo- nucleases located next to each other

- Restriction sites of the poly-linker are not present anywhere else in the plasmid.

- Cutting plasmids with one of the restriction enzymes that recognize a site in the poly-linker does not disrupt any of the essential features of the vector



TYPES OF CLONING VECTORS

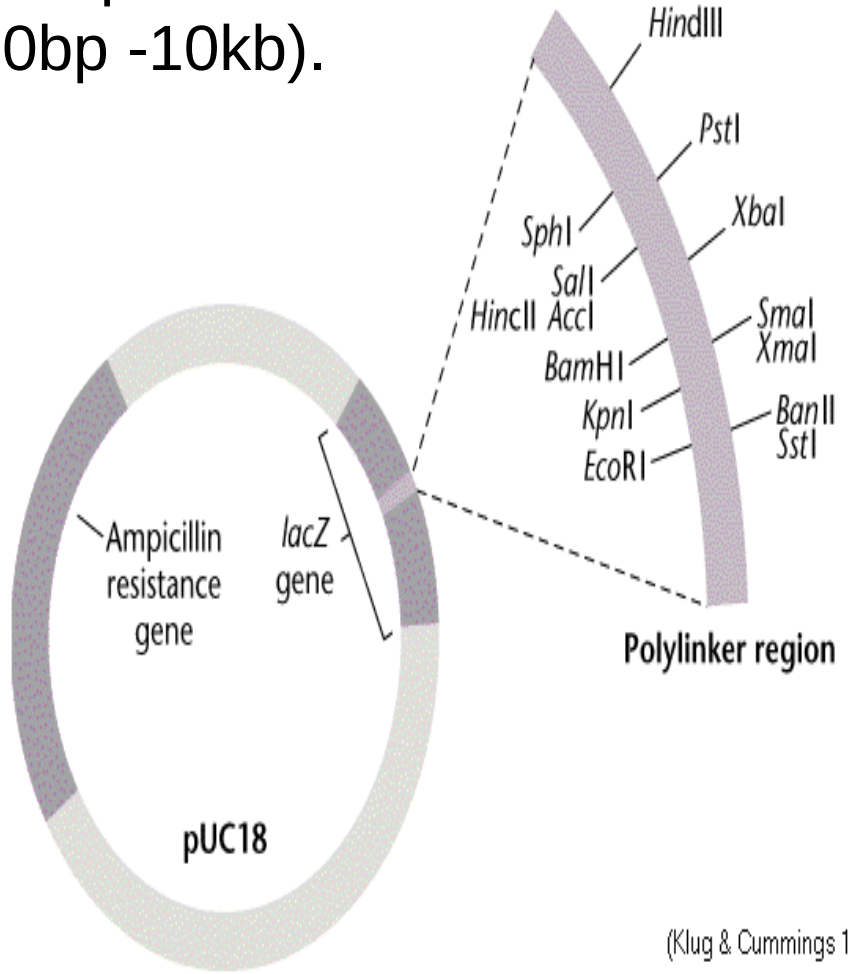
CLONING VECTORS

- Different types of cloning vectors are used for different types of cloning experiments.

- The vector is chosen according to the size and type of DNA to be cloned

PLASMID VECTORS

- Plasmid vectors are used to clone DNA ranging in size from several base pairs to several thousands of base pairs (100bp -10kb).



(Klug & Cummings 1997)

Disadvantages using plasmids

- Cannot accept large fragments
- Sizes range from 0- 10 kb
- Standard methods of transformation are inefficient

BACTERIOPHAGE LAMBDA

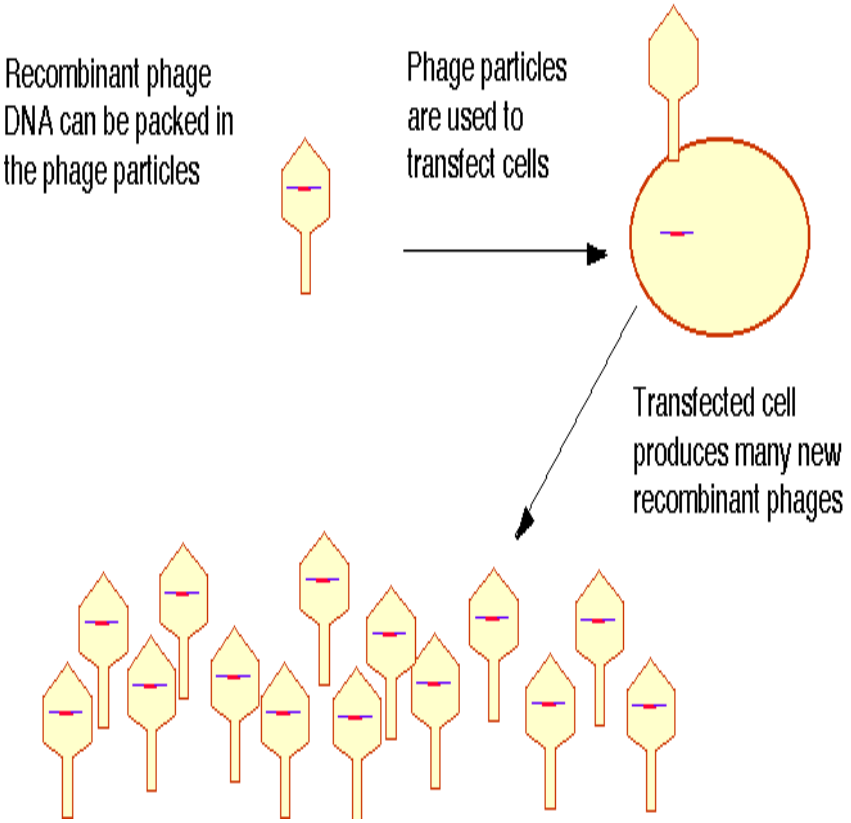
- **Phage lambda** is a **bacteriophage** or **phage**, i.e. bacterial virus, that uses *E. coli* as host.
- Its structure is that of a typical phage: **head, tail, tail fibres.**
- **Lambda viral genome:** 48.5 kb linear DNA with a 12 base ssDNA "sticky end" at both ends; these ends are complementary in sequence and can hybridize to each other (this is the **cos** site: **co**he**s**ive ends).
- **Infection:** lambda tail fibres adsorb to a cell surface receptor, the tail contracts, and the DNA is injected.
- The DNA circularizes at the **cos** site, and lambda begins its life cycle in the *E. coli* host.

BACTERIOPHAGE LAMBDA

Genome of bacteriophage lambda is approximately 45,000 base pairs long

Central portion (approximately 15,000 base pairs) of the lambda genome is not essential for replication of phage DNA and can be removed.

Left and right arms of the phage lambda can be ligated with a fragment of foreign DNA which must have the size of approximately 15,000 base pairs.

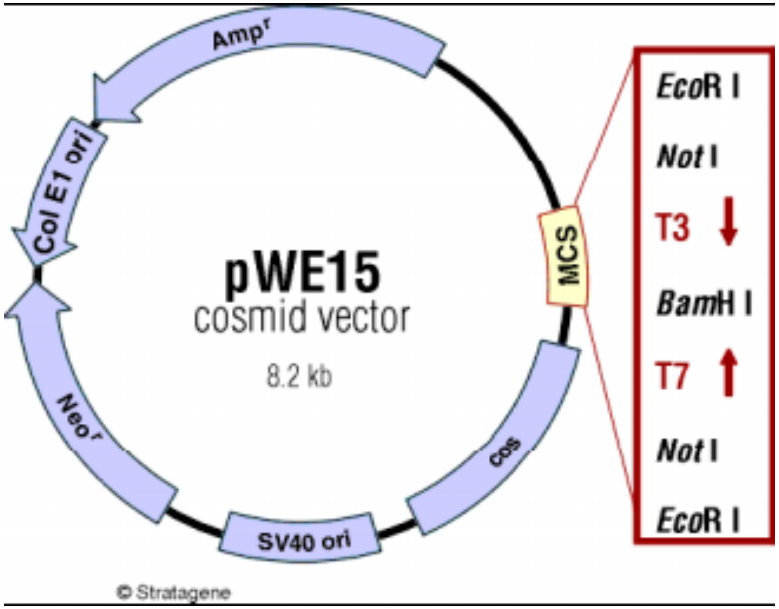


COSMID VECTOR

- **Purpose:**
  1. clone large inserts of DNA: size ~ 45 kb

- **Features:**
  Cosmids are Plasmids with one or two Lambda Cos sites.

- Presence of the Cos site permits *in vitro* packaging of cosmid DNA into Lambda particles



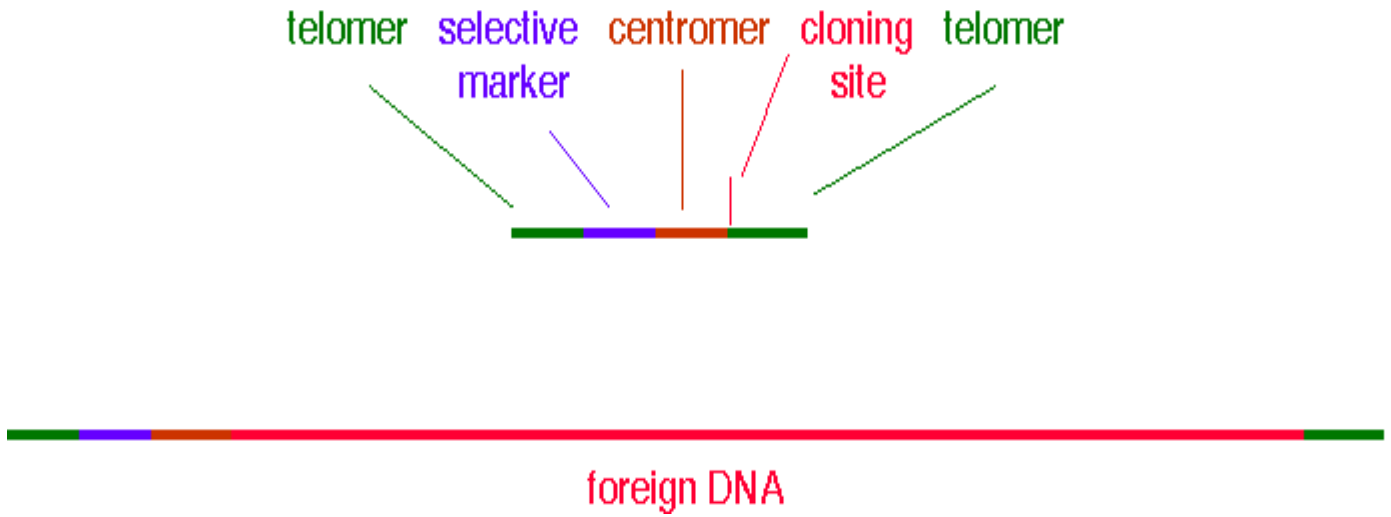
COSMID VECTOR

- Thus, have some advantages of Lambda as Cloning Vehicle:
- Strong selection for cloning of large inserts
- Infection process rather than transformation for entry of chimeric DNA into *E. coli* host
- Maintain Cosmids as phage particles in solution
- **But** Cosmids are Plasmids:

  Thus do NOT form plaques but rather cloning proceeds via *E. coli* colony formation

Yeast Artificial Chromosomes

Yeast artificial chromosomes are special linear DNA vectors that resemble normal yeast chromosome. YACs contain telomers that stabilize chromosome ends, centromer, that ensures chromosome partitioning between two daughter cells and a selective marker gene. YAC vectors can carry hundreds of thousands of base pairs of foreign DNA.



Yeast Artificial Chromosomes

Purpose:

- Cloning vehicles that propagate in eukaryotic cell hosts as eukaryotic Chromosomes

- Clone **very** large inserts of DNA: 100 kb - 10 Mb

Features:

YAC cloning vehicles are plasmids
Final chimeric DNA is a linear DNA molecule with telomeric ends: **Artificial Chromosome**

Additional features:

- Often have a selection for an insert

- YAC cloning vehicles often have a bacterial origin of DNA replication (**ori**) and a selection marker for propogation of the YAC through bacteria.

- The YAC can use both yeast and bacteria as a host

PACs vs. BACs

- **PACs - P1-derived Artificial Chromosomes**
- ***E. coli* bacteriophage P1 is similar to phage lambda in that it can exist in *E. coli* in a prophage state.**
- **Exists in the *E. coli* cell as a plasmid, NOT integrated into the *E. coli* chromosome.**
- **P1 cloning vehicles have been constructed that permit cloning of large DNA fragments- few hundred kb of DNA**
- **Cloning and propogation of the chimeric DNA as a P1 plasmid inside *E. coli* cells**

- **BACs - Bacterial Artificial Chromosomes**
- **These chimeric DNA molecules use a naturally-occurring low-copy number bacterial plasmid origin of replication, such as that of F-plasmid in *E. coli*.**
- **Can be cloned as a plasmid in a bacterial host, and its natural stability generally permits cloning of large pieces of insert DNA, i.e. up to a few hundred kb of DNA.**

RETROVIRAL VECTORS

- Retroviral vectors are used to introduce new or altered genes into the genomes of human and animal cells.
- Retroviruses are RNA viruses.
- The viral RNA is converted into DNA by the viral reverse transcriptase and then is efficiently integrated into the host genome
- Any foreign or mutated host gene introduced into the retroviral genome will be integrated into the host chromosome and can reside there practically indefinitely.
- Retroviral vectors are widely used to study oncogenes and other human genes.

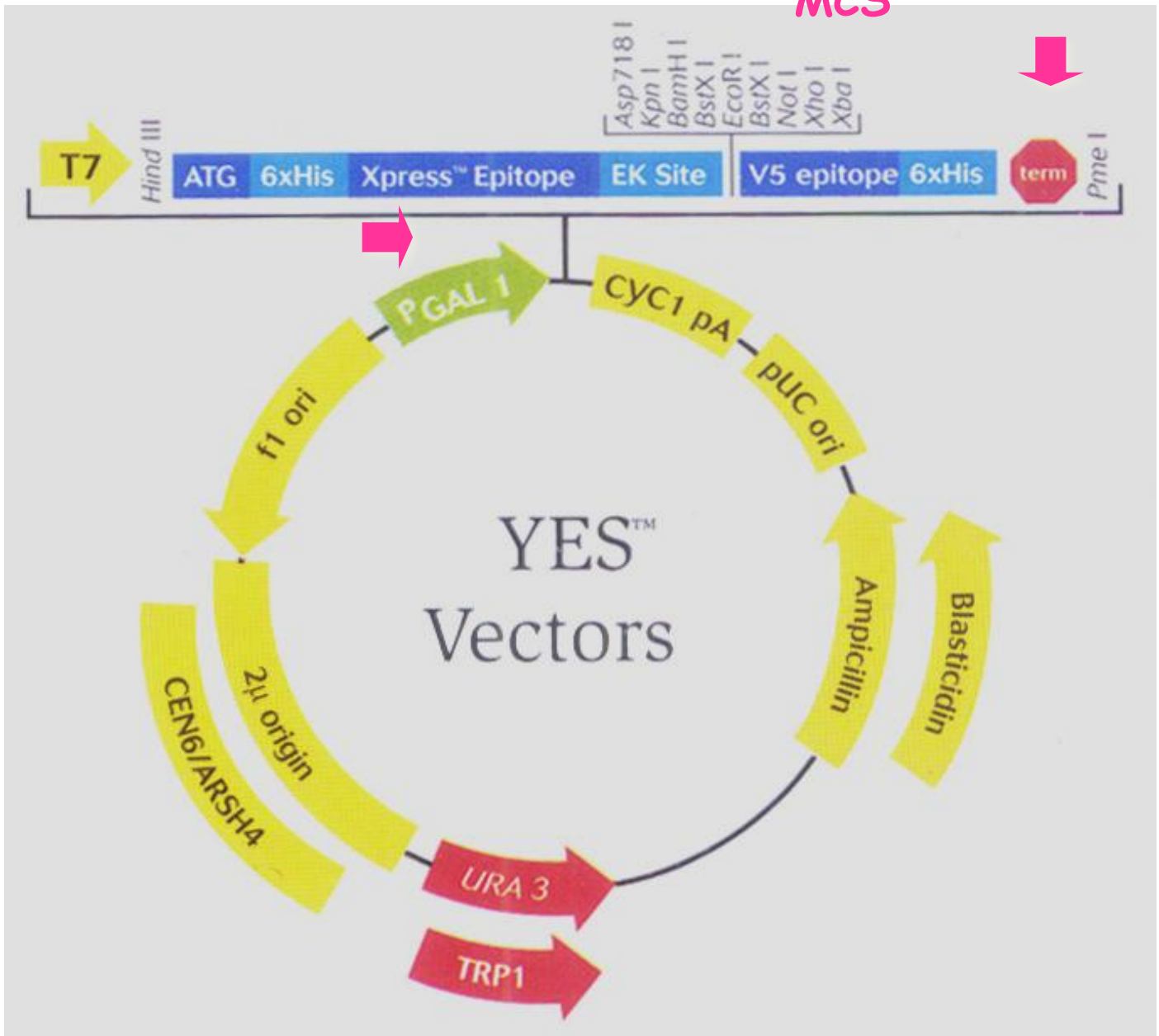
Yeast episomal plasmids (YEps)

Vectors for the cloning and expression of genes in *Saccharomyces cerevisiae*.

1. Based on 2 micron (2m) plasmid which is 6 kb in length.
- One origin
- Two genes involved in replication
- A site-specific recombination protein FLP, homologous to l Int.

2. Normally replicate as plasmids, and may integrate into the yeast genome.

A YEp vector


MCS
Asp718 I
Kpn I
BamH I
BstX I
EcoR I
BstX I
Not I
Xho I
Xba I
T7
Hind III
ATG
6xHis
Xpress™ Epitope
EK Site
V5 epitope
6xHis
term
Pme I
PGAL 1
CYC1 pA
pUC ori
f1 ori
YES™
Vectors
Ampicillin
Blasticidin
2μ origin
CEN6/ARSH4
URA 3
TRP1

Baculovirus

1. Infects insect cells

2. The strong promoter expressing polyhedrin protein can be used to over-express foreign genes engineered. Thus, large quantities of proteins can be produced in infected insect cells.

3. Insect expression system is an important eukaryotic expression system.

Mammalian viral vectors

1. SV40: 5.2 kb, can pack DNA fragment similar to phage l.

2. Retroviruss:

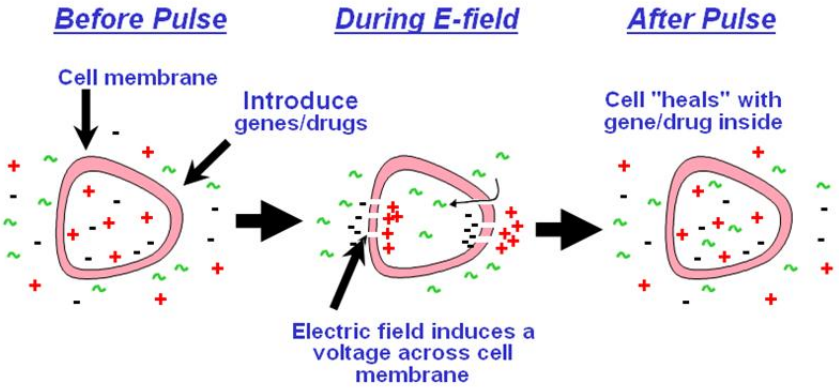
- single-stranded RNA genome, which copy to dsDNA after infection.
- Have some strong promoters for gene expression
- Gene therapy

Transformation and Antibiotic Selection

- Transformation is the genetic alteration of a cell resulting from the introduction, uptake and expression of foreign DNA.

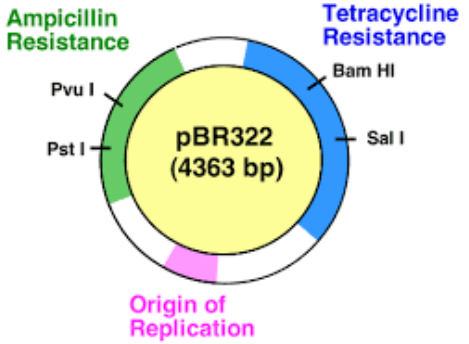
- There are more aggressive techniques for inserting foreign DNA into eukaryotic cells. Ex: through **electroporation**

- **Electroporation** involves applying a brief (milliseconds) pulse high voltage electricity to create tiny holes in the bacterial cell wall that allow DNA to enter.



Plasmids and Antibiotic Resistance

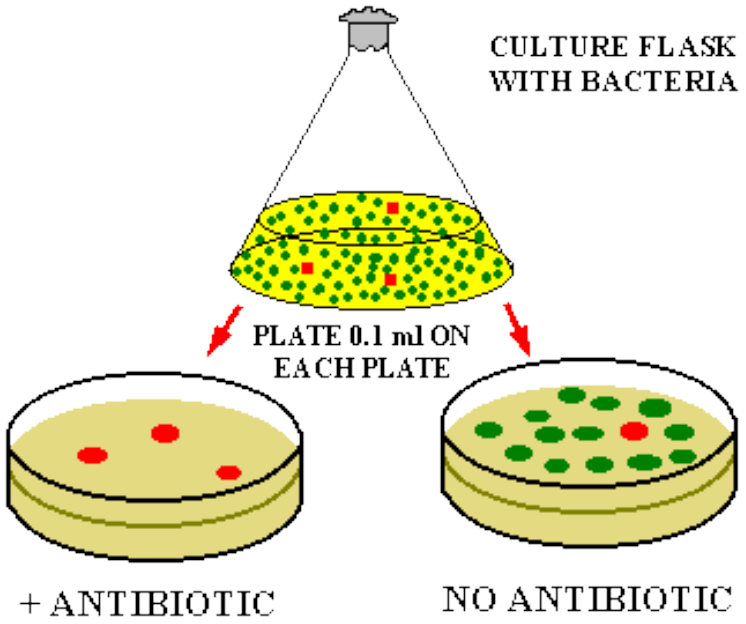
- Plasmids can use to amplify **a gene of interest**.
- A plasmid containing resistance to an antibiotic (usually Ampicillin) or Tetracycline, is used as a vector.
- The gene of interest (resistant to Ampicillin) is inserted into the vector plasmid and this newly contructed plasmid is then put into *E.coli* that is sensitive to Ampiciline.
- The bacteria are then spread over the plate that contains ampiciline.

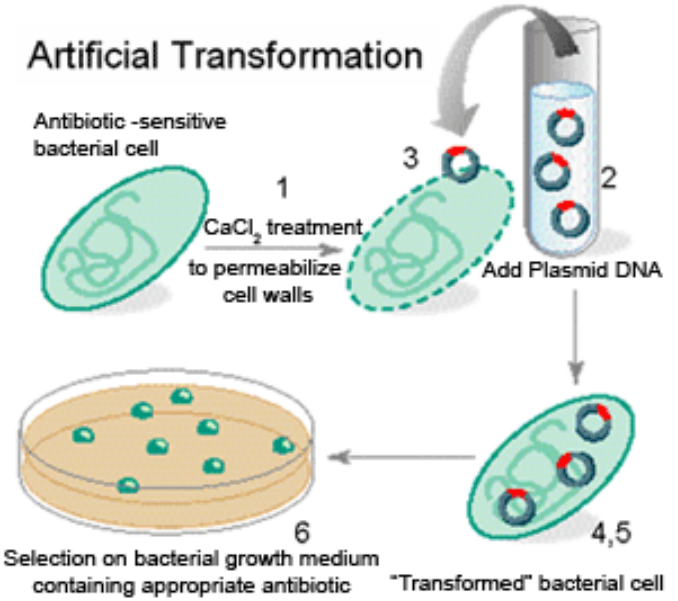


- The ampicillin provides a selective pressure because only bacteria that have acquired the plasmid can grow on the plate.

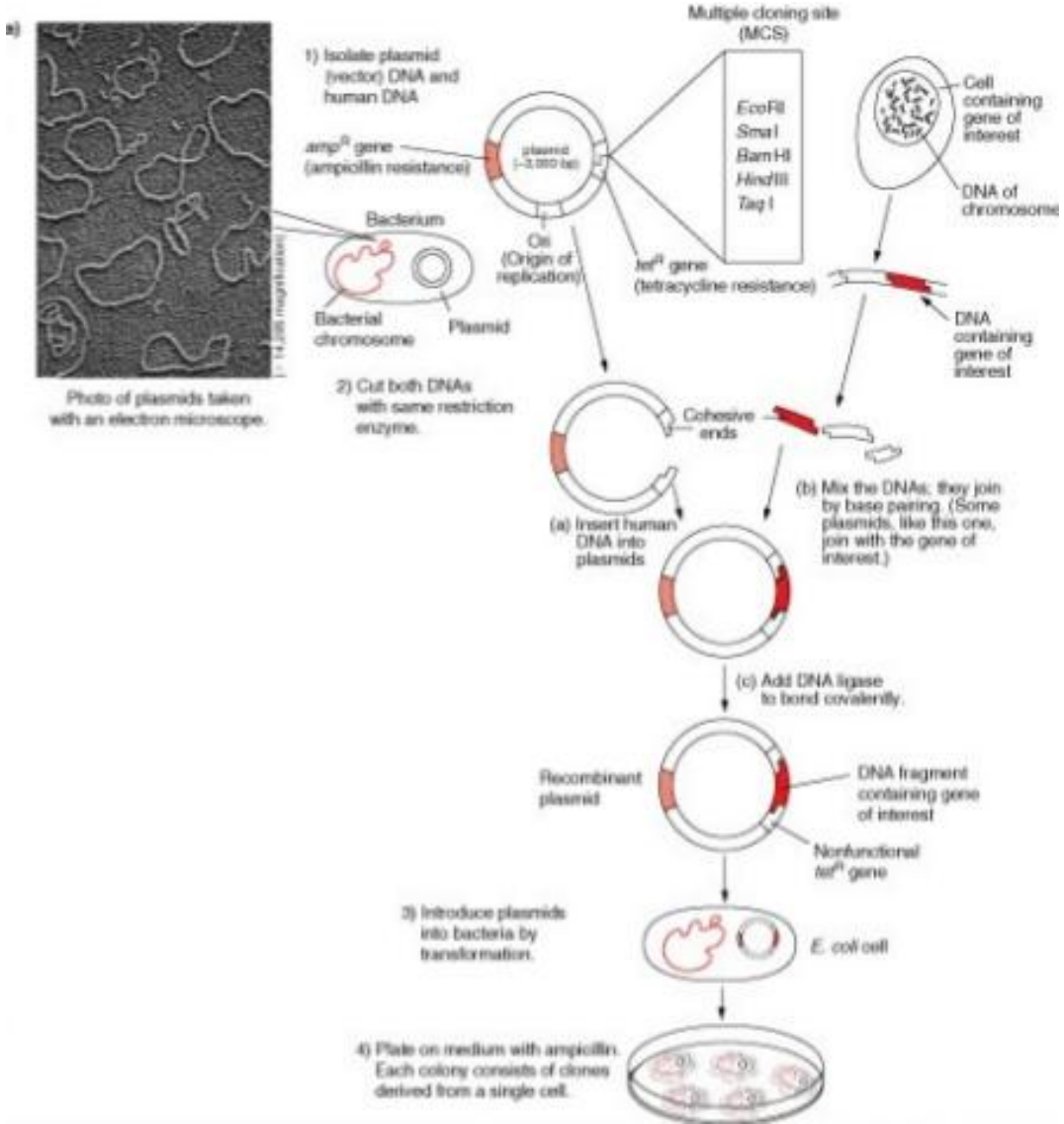
- Those bacteria which do not acquire the plasmid with the inserted gene of interest will die.

- As long as the bacteria grow in ampicIllin, it will need the plasmid to suriveve and it will contunially replicate it, along with the gene of interest that has been inserted to the plasmid.





Selecting A Gene in A Plasmid and Antibiotic Selection



THE *lac* OPERON

The control of gene expression

- Each cell in the human contains all the genetic material for the growth and development of a human
- Some of these genes will be need to be expressed all the time
- These are the genes that are involved in of vital biochemical processes such as respiration
- Other genes are not expressed all the time
- They are switched on/ off at need

Operons

- An operon is a group of genes that are **transcribed at the same time**.
- They usually control an important biochemical process.
- They are **only found in prokaryotes**.

Jacob, Monod & Lwoff

The *lac* Operon

- The *lac* operon consists of **three genes** each involved in processing the sugar lactose
- One of them is the gene for the enzyme **β-galactosidase**
- This enzyme hydrolyses lactose into glucose and galactose

Adapting to the environment

- *E. coli* can use either glucose, which is a monosaccharide, or lactose, which is a disaccharide

- However, lactose needs to be hydrolysed (digested) first

- So the bacterium prefers to use glucose when it can

Four situations are possible

1. When glucose is **present** and lactose is **absent** the E. coli does **not** produce β-galactosidase.

2. When glucose is **present** and lactose is **present** the E. coli does **not** produce β-galactosidase.

3. When glucose is **absent** and lactose is **absent** the E. coli does **not** produce β-galactosidase.

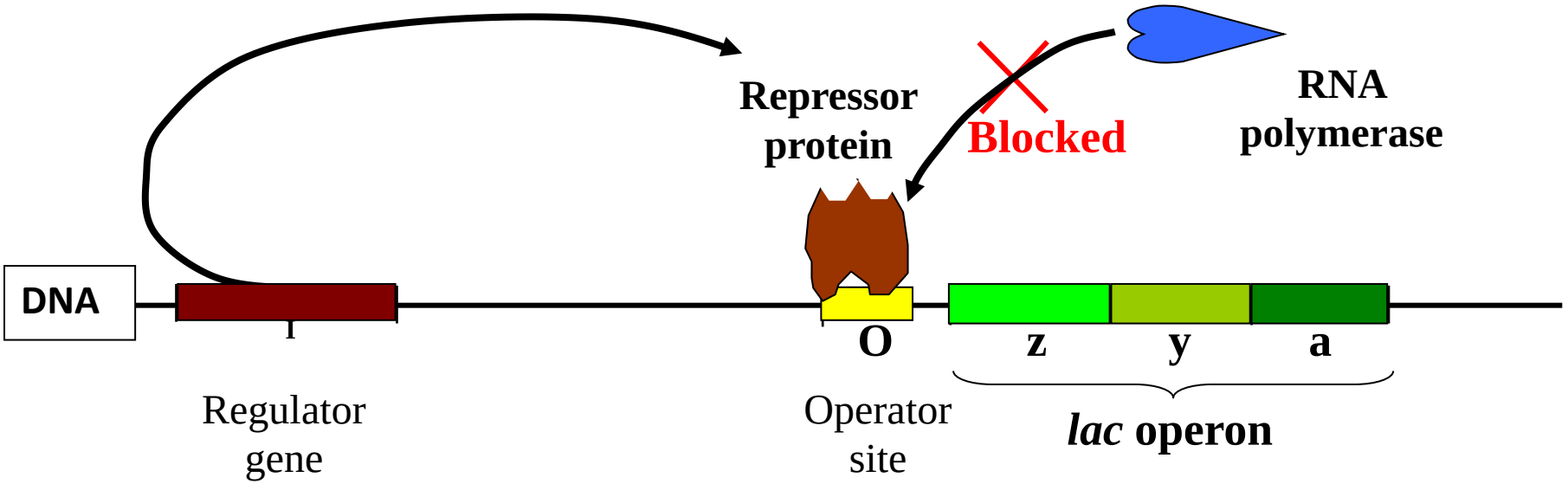
4. When glucose is **absent** and lactose is **present** the E. coli **does** produce β-galactosidase

The control of the *lac* operon

1. When lactose is absent

- A repressor protein is continuously synthesised. It sits on a sequence of DNA just in front of the *lac* operon, the **Operator site**

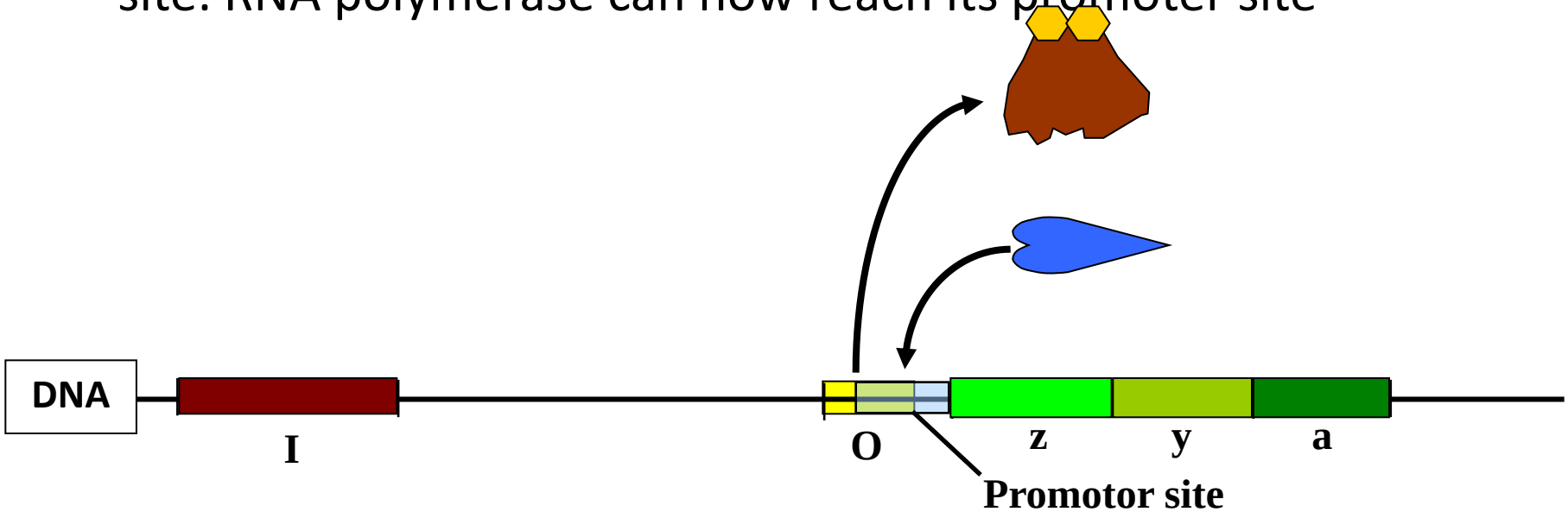
- The **repressor protein** blocks the **Promoter site** where the RNA polymerase settles before it starts transcribing



2. When lactose is present

- A small amount of a sugar allolactose is formed within the bacterial cell. This fits onto the repressor protein at another active site **(allosteric site)**

- This causes the repressor protein to change its shape **(a conformational change).** It can no longer sit on the operator site. RNA polymerase can now reach its promoter site

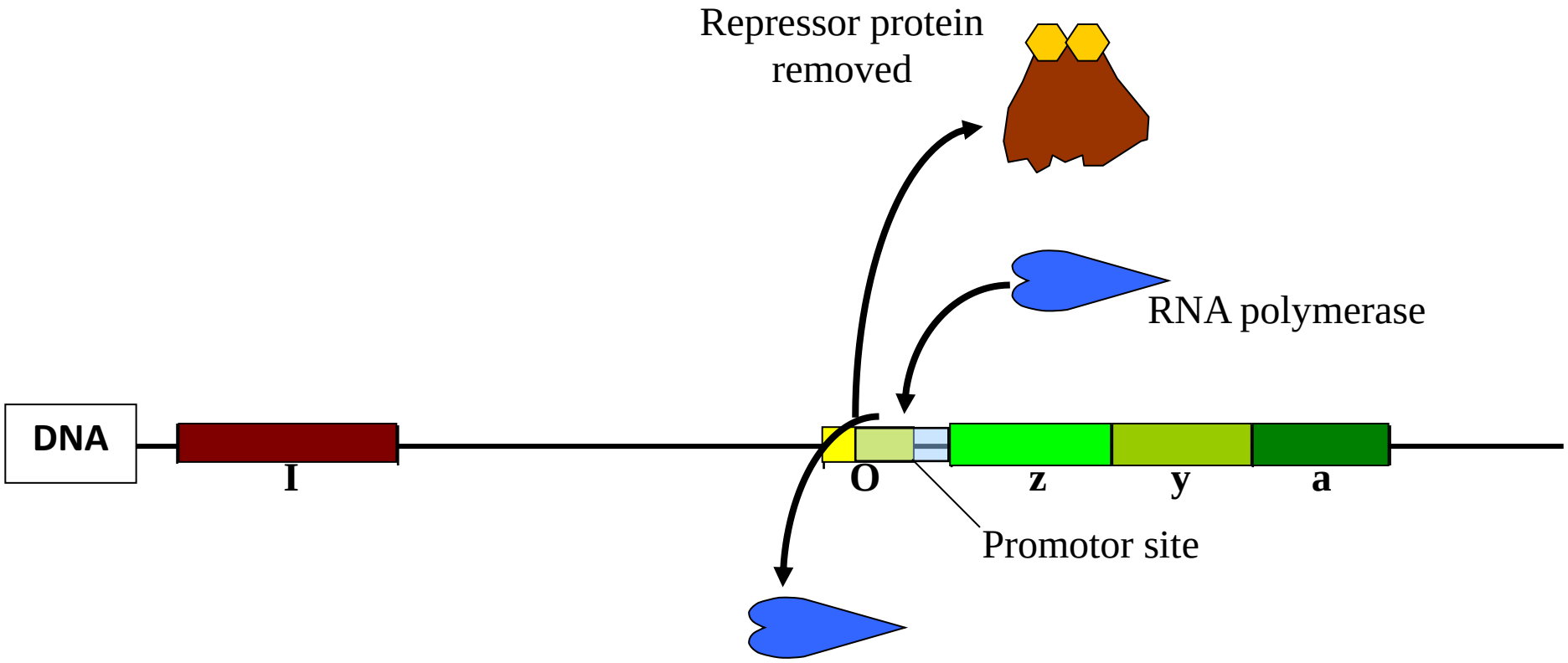


3. When both glucose and lactose are present

- This explains how the *lac* operon is transcribed only when lactose is present.

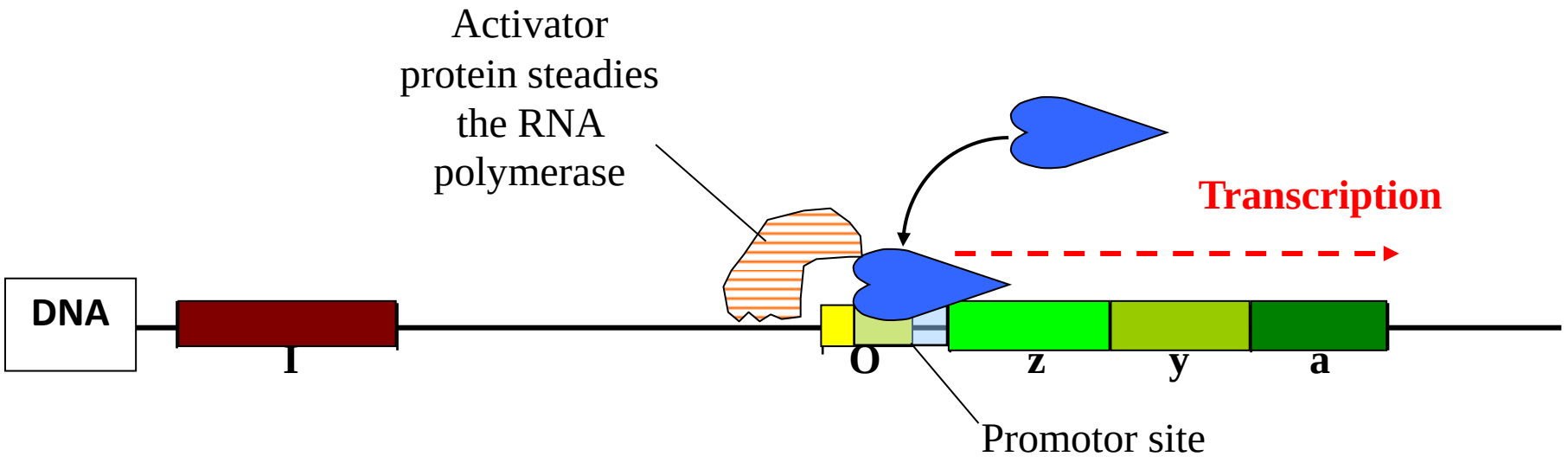
- BUT….. this does not explain why the operon is not transcribed when both glucose and lactose are present.

When glucose and lactose are present RNA polymerase can sit on the promoter site but it is unstable and it keeps falling off



4. When glucose is absent and lactose is present

- Another protein is needed, an **activator protein**. This stabilises RNA polymerase.
- The activator protein only works when glucose is absent
- In this way *E. coli* only makes enzymes to metabolise other sugars in the absence of glucose



Summary

Carbohydrates	Activator protein	Repressor protein	RNA polymerase	*lac* Operon
+ GLUCOSE + LACTOSE	Not bound to DNA	**Lifted off operator site**	Keeps falling off promoter site	**No transcription**
+ GLUCOSE - LACTOSE	Not bound to DNA	Bound to operator site	Blocked by the repressor	**No transcription**
- GLUCOSE - LACTOSE	**Bound to DNA**	Bound to operator site	Blocked by the repressor	**No transcription**
- GLUCOSE + LACTOSE	**Bound to DNA**	**Lifted off operator site**	**Sits on the promoter site**	**Transcription**

BLUE / WHITE SCREENING

- **Colony Selection**: finding the rare bacterium with **recombinant DNA**
- Only *E. coli* cells with resistant plasmids grow on antibiotic medium
- Only plasmids with functional ***lacZ*** gene can grow on

  Xgal
  lacZ(+) => **blue** colonies

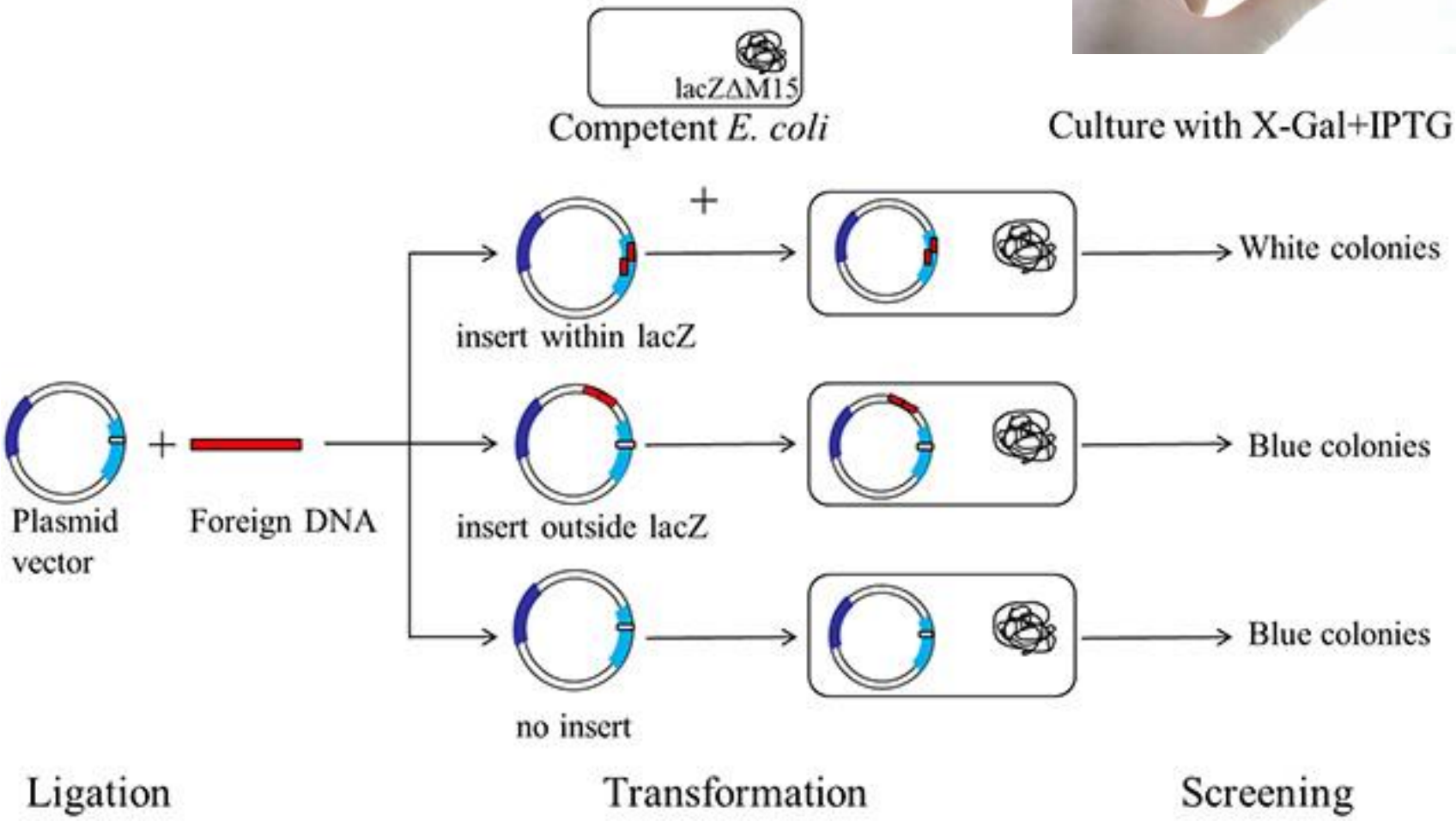
  lacZ functional => polylinker intact => *nothing inserted****, no clone***

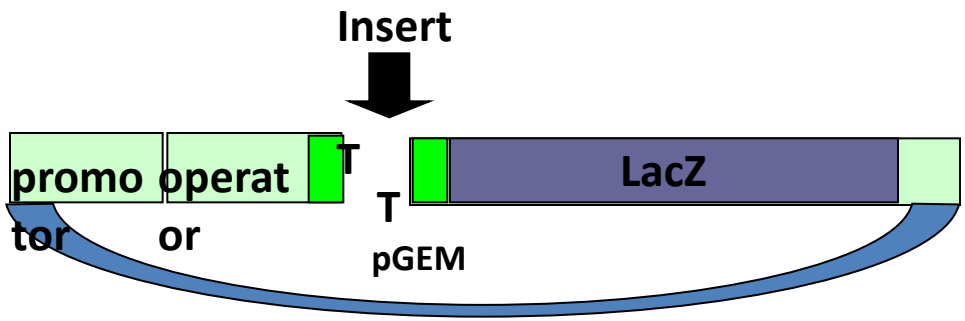
  lacZ(-) => **white** colonies polylinker *disrupted* => *successful insertion* & ***recombination***!

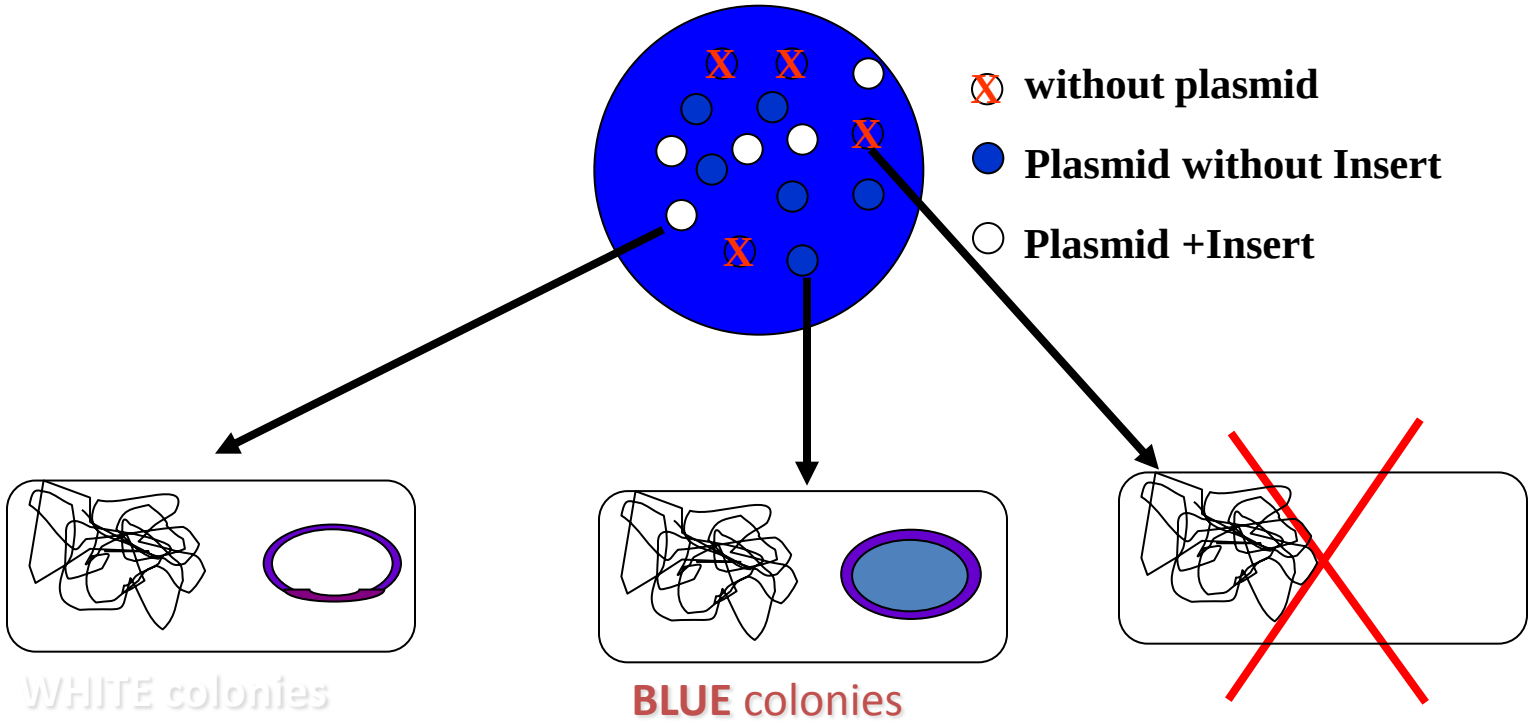
α -complementation

- The portion of the *lacZ* gene encoding the first 146 amino acids (the α -fragment) are on the plasmid
- The remainder of the *lacZ* gene is found on the chromosome of the host.
- If the α -fragment of the *lacZ* gene on the plasmid is intact (that is, you have a non-recombinant plasmid), these two fragments of the *lacZ* gene (one on the plasmid and the other on the chromosome) complement each other and will produce a functional β -galactosidase enzyme.

blue-white screening



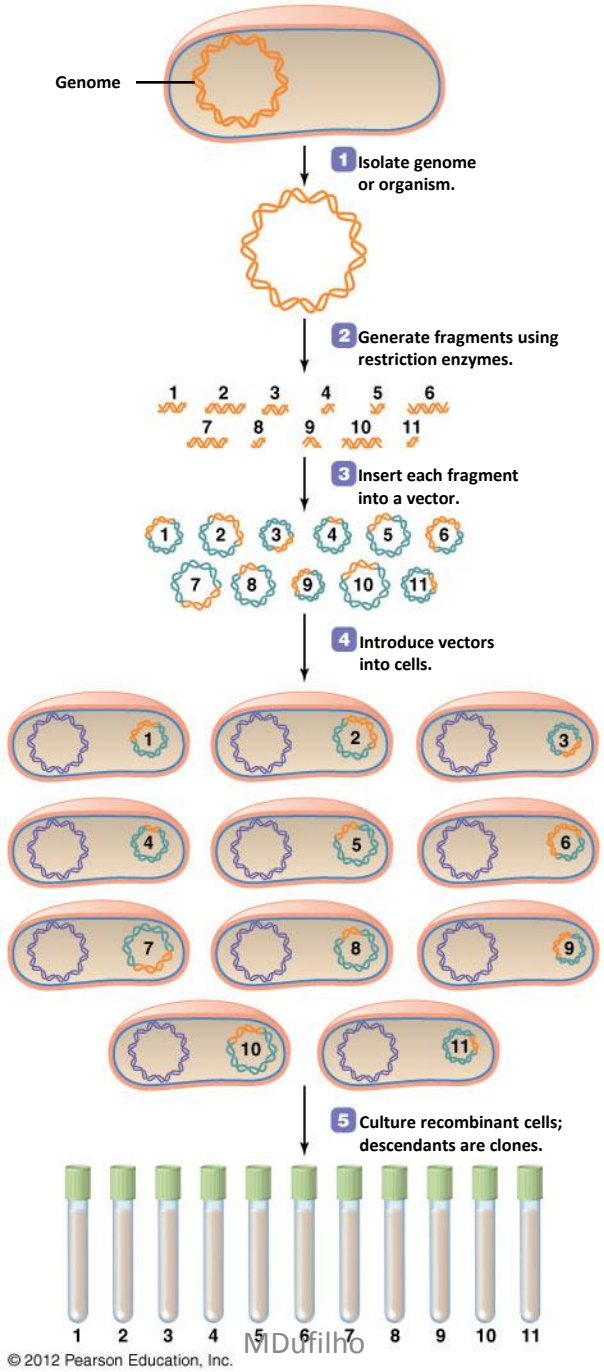

Insert
promo
tor
operat
or
T
T
LacZ
pGEM


without plasmid
Plasmid without Insert
Plasmid +Insert
WHITE colonies
BLUE colonies

The Tools of Recombinant DNA Tehnology

- **Gene Libraries**
  - A collection of bacterial or phage clones
    - Each clone in library often contains one gene of an organism's genome
  - Library may contain all genes of a single chromosome
  - Library may contain set of cDNA complementary to mRNA

Production of a gene library-overview



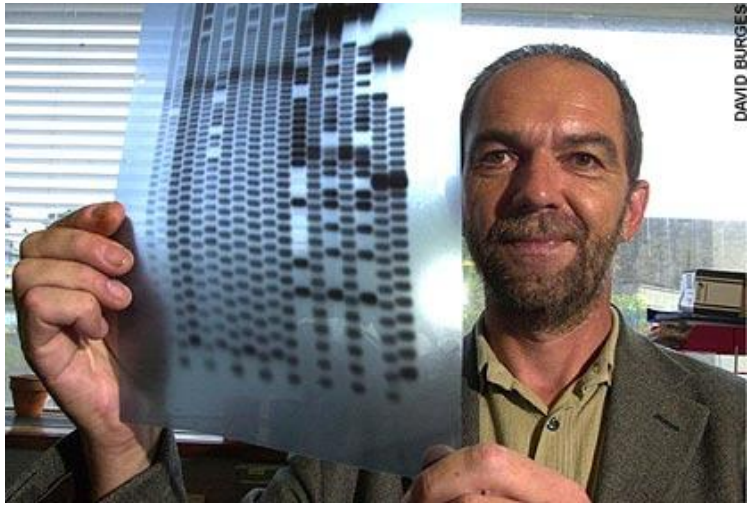
Techniques of Recombinant DNA Technology:

- **Multiplying DNA *in vitro*: The Polymerase Chain Reaction (PCR)**
- **Selecting a Clone of Recombinant Cells**
- **Separating DNA Molecules: Gel Electrophoresis and the Southern Blot**
- **DNA Microarrays**
- **Inserting DNA into Cells**
  - Goal of DNA technology is insertion of DNA into cell
  - Natural methods
    - Transformation
    - Transduction
    - Conjugation
  - Artificial methods
    - Electroporation
    - Protoplast fusion
    - Injection: gene gun and microinjection

Applications of Recombinant DNA Technology:

- **Genetic Mapping**
- **Locating Genes**
- **Environmental Studies**
- **Pharmaceutical and Therapeutic Applications**

- Protein synthesis
- Vaccines
- Genetic screening
- DNA fingerprinting
- Gene therapy
- Medical diagnosis
- Xenotransplants



DNA fingerprinting

The Ethics and Safety of Recombinant DNA Technology

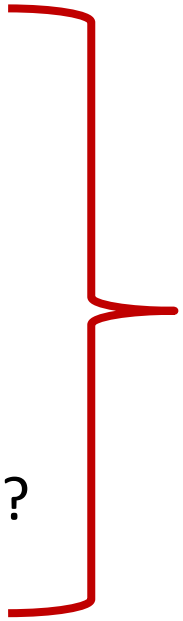
- Supremacist view: humans are of greater value than animals
- Long-term effects of transgenic manipulations are unknown
- Unforeseen problems arise from every new technology and procedure
- Natural genetic transfer could deliver genes from transgenic plants and animals into other organisms
- Transgenic organisms could trigger allergies or cause harmless organisms to become pathogenic

- **Studies have not shown any risks to human health or environment**

- **Standards imposed on labs involved in recombinant DNA technology**

- **Can create biological weapons using same technology**

  - Routine screenings?
  - Who should pay?
  - Genetic privacy rights?
  - Profits from genetically altered organisms?
  - Required genetic screening?
  - Forced correction of “genetic abnormalities”?



Ethical Issues