Lecture 24 (11/13/20)

TODAY

•Reading: Ch9; 328-332
Ch25; 990-995, 1005-1012

•Problems: Ch9 (study-guide: applying); 1,2
Ch9 (study-guide: facts); 7,8
Ch25 (text); 1-3,5-7,9,10,13-16
Ch25 (study-guide: applying); 1,4
Ch25 (study-guide: facts); 3,4,6

NEXT

• Reading: Ch26; 1035-1038
Ch27; 1077-1085, 1092-1096

• Problems: Ch26 (text); 1,2,5,6,12
Ch26 (study-guide: applying); 1
Ch8 (study-guide: facts); 1,3,5
Ch27 (text); 6,7,9
Ch27 (study-guide: facts); 1,3,5

Nucleic Acids

A. Recombinant DNA: Biochemical Basis of Biotechnology
   1. Restriction enzymes, DNA ligase
   2. Vectors and Inserts to make recombinant DNA (rDNA)
      a. Inserts
         i. cDNA
         ii. Genomic
      b. Vectors
   3. Transformation of hosts
   4. Selection of transformants
      a. Selectable marker/gene
      b. Distinguish empty plasmids
         i. Loss of resistance
         ii. Reporter gene
   5. Expression
      a. Special vectors
      b. Fusion proteins
         i. purification
         ii. labeling
   6. Site-directed mutagenesis
B. Replication
   1. Polymerases
   2. Fidelity
   3. Sequence determination

Recombinant DNA and Biotechnology

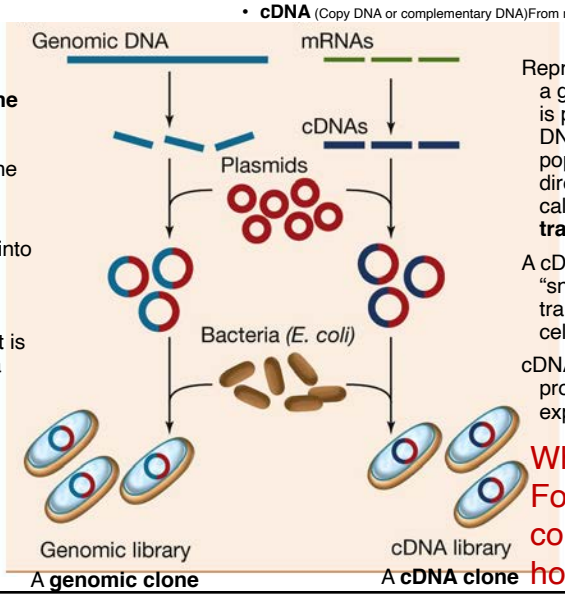
Vectors and Inserts

DNA fragments used for molecular cloning come from two sources:

- **Genomic DNA**
- **cDNA** (Copy DNA or complementary DNA)From reverse transcription of mRNA

A **genomic clone** contains the gene(s) as a fragment of the genome of an organism.

The DNA is cut into fragments by restriction enzymes, and each fragment is inserted into a vector.



A **genomic clone** A **cDNA clone**

Represents an mRNA from a given cell/tissue. cDNA is produced by making a DNA copy of the mRNA population using the RNA-directed DNA polymerase called, **reverse transcriptase**.

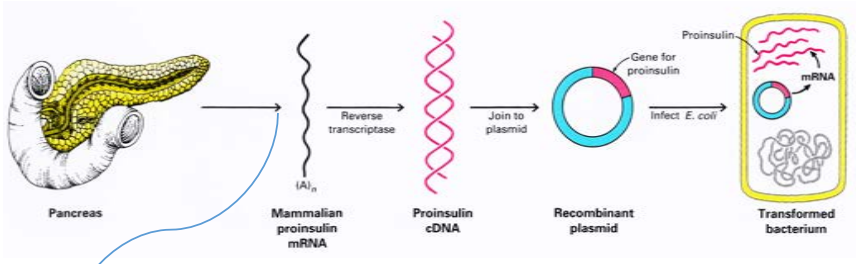
A cDNA library is a "snapshot" of the transcription pattern of the cell.

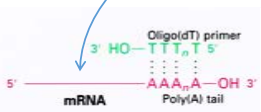
cDNA clones are used to provide the ORF for expressing the protein

Where does this Foreign DNA come from and how is it purified?

Recombinant DNA and Biotechnology

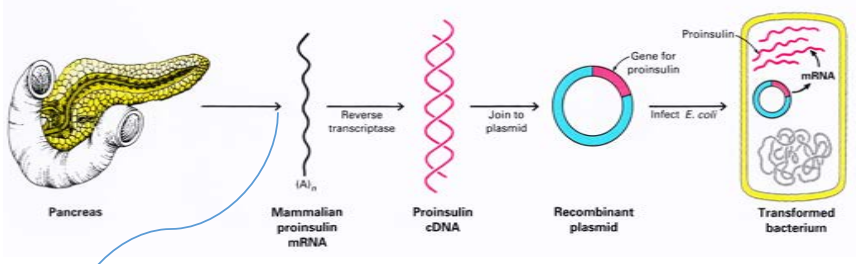
Vectors and Inserts: cDNA Cloning

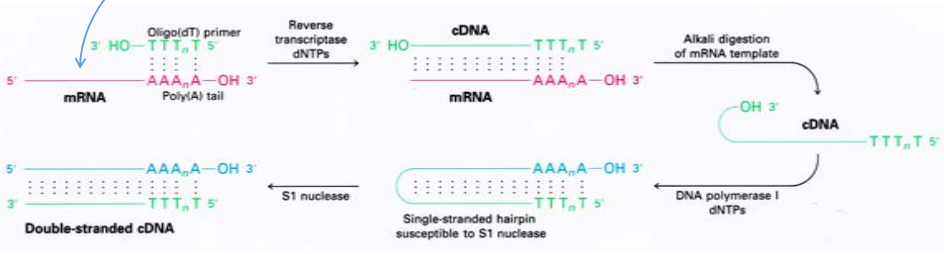




Recombinant DNA and Biotechnology

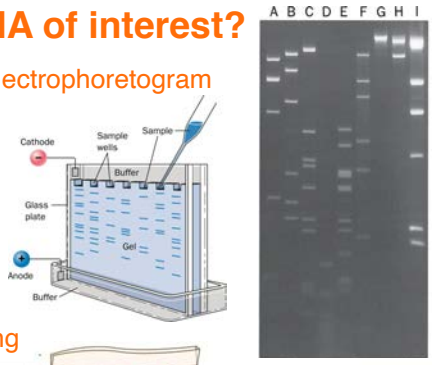
Vectors and Inserts: cDNA Cloning



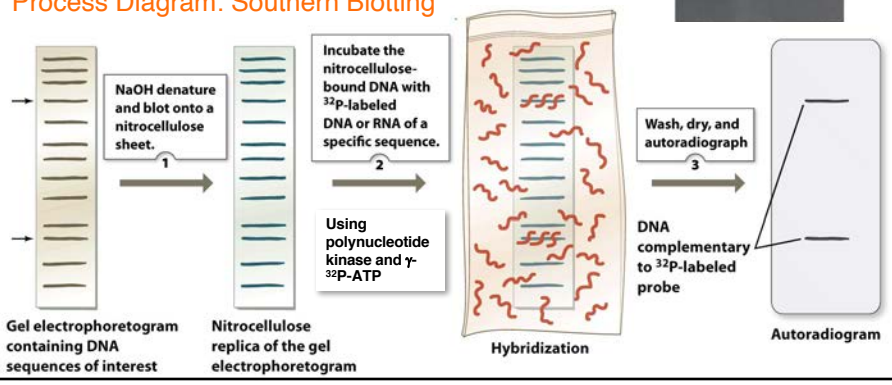


How do you find your DNA of interest?

Restriction Digest Electrophoretogram



Process Diagram: Southern Blotting

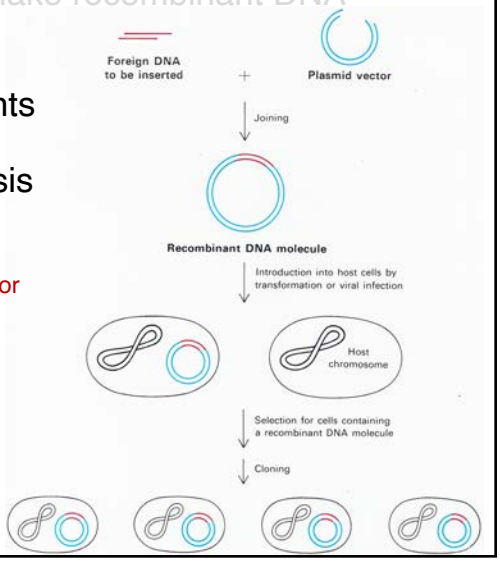


Recombinant DNA and Biotechnology

- Biochemical Basis of Biotechnology
  - Restriction enzymes, DNA ligase
  - Vectors and Inserts to make recombinant DNA (rDNA)
  - Transformation of hosts
  - Selection of transformants
  - Expression
  - Site-directed mutagenesis

All vectors have 3 things:

1. Autonomous replication ability
2. Selection for hosts that contain vector
3. Site for insertion of rDNA



Viruses as vectors (bacteriophage or retroviruses).

- Viruses can be altered to attenuate some detrimental genes in the virus (e.g., those that kill the host).
- Viruses can be altered to carry recombinant DNA into cells

Recombinant DNA and Biotechnology

- Biochemical Basis of Biotechnology
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  - Transformation of hosts

Transformation: Recombinant DNA is cloned by inserting it into host cells (**transfection** if host cells are from an animal). Second key discovery in biotechnology.

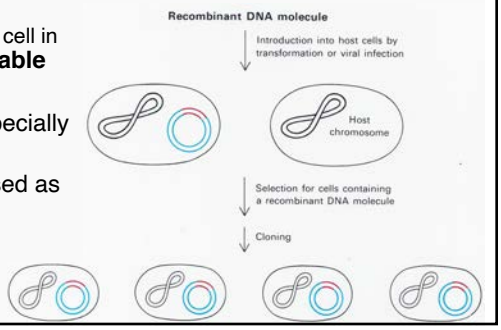
Usually only a few cells are transformed (1 cell in 10,000). Reason for the need for a **selectable marker**.

The first host cells used were bacteria, especially *E. coli*.

Yeasts (*Saccharomyces*) are commonly used as eukaryotic hosts.

Inserting the recombinant DNA into a cell:

- Cells may be treated with chemicals to make plasma membranes more permeable—DNA diffuses into cells.
- *Electroporation*—a short electric shock creates temporary pores in membranes, and DNA shoots to the + end and can enter cells.



Recombinant DNA and Biotechnology

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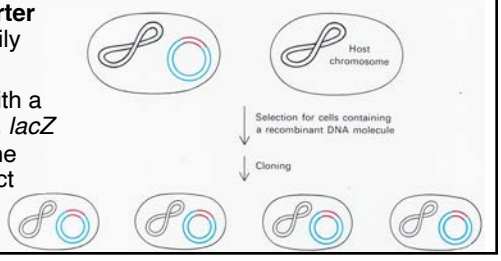
- Use of antibiotic resistance gene (e.g., ampicilin resistance) on a plasmid
- For viral vectors, use of "infected" phenotype.
- Use of "selectable markers" to detect either insertion into the vector or incorporation into the host. Some of these are a type of **reporter gene**—a gene whose expression is easily observed.
- Many plasmids contain the *lacZ* gene with a multiple cloning site within its sequence. *lacZ* codes for an enzyme that can convert the substrate X-Gal into a bright blue product

Plasmid Cloning Video

How can you distinguish between recDNA vectors and "empty" vectors?



Recombinant DNA and Biotechnology

DNA Cloning

Introduce DNA into Organism

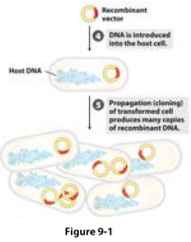
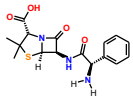


Figure 9-1

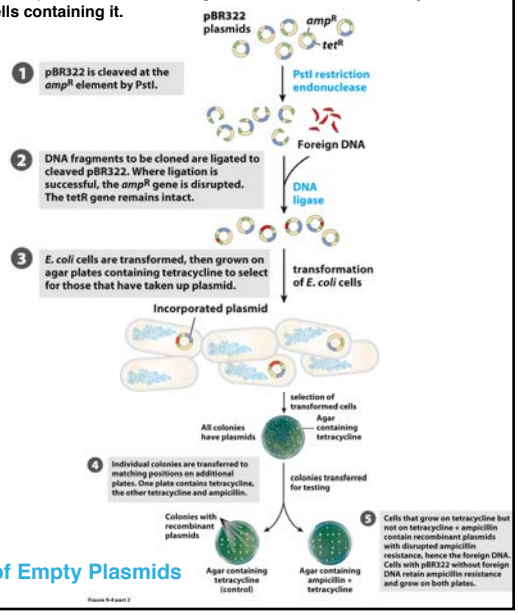
Antibiotic Selection

- Antibiotics, such as penicillin and ampicillin, kill bacteria.
- Plasmids can carry genes that give a host bacterium a resistance against antibiotics.
- Allows growth (selection) of bacteria that have taken up the plasmid



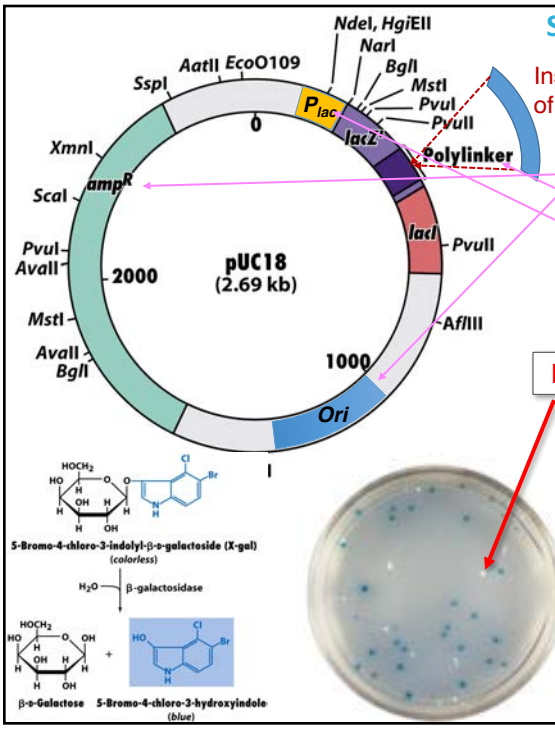
Ampicillin

Use of pBR322 to clone foreign DNA in *E. coli* and identify cells containing it.



Identification of Empty Plasmids

Selection using Reporter Gene



Insert ORF of interest

- Origin of replication
- Antibiotic resistance gene
- Multiple cloning site
- Promoter for transcription, translation (host specific)

Recombinant DNA Clone

(–) Insert – Makes β-Galactosidase

(+) Insert – Doesn't make β-Galactosidase

DNA taken up by *amp*^{s}, *lacZ*$^-$ *E. coli*	Phenotype for ampicillin	Phenotype for *lacZ*
None	Sensitive	~~White~~ N/A
Foreign DNA	Sensitive	~~White~~ N/A
Plasmid	Resistant	Blue
Recombinant DNA	Resistant	White

Recombinant DNA and Biotechnology

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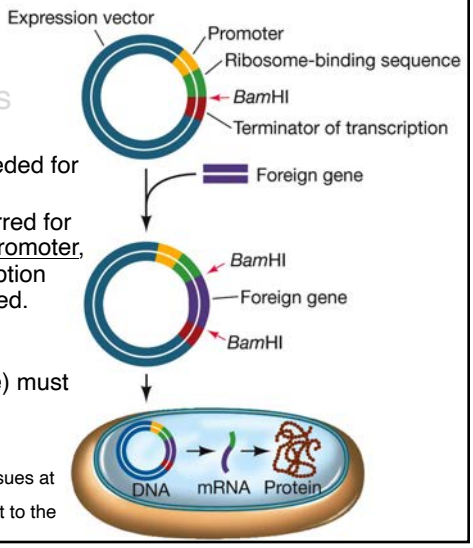
  - Expression

Expression vectors include sequences needed for expression of a *transgene* in a host cell.

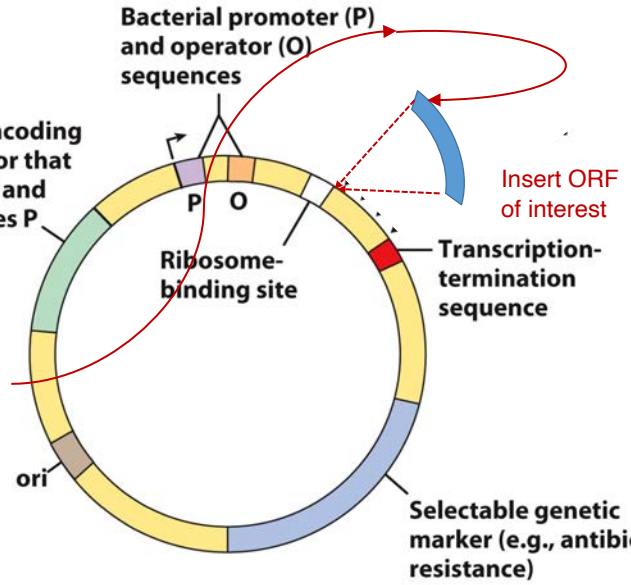
- For prokaryote host, which are preferred for making large amounts: A **bacterial** promoter, ribosome binding site, and a transcription termination signal, must all be included.
- For eukaryote (mostly yeast): The **eukaryotic** promoter/enhancer and terminator (poly-A addition signal/site) must be included.

These can also be:
- *Inducible promoters* which respond to a specific signal
- *Tissue-specific promoters* expressed only in certain tissues at certain times
- *Signal sequences*—e.g., a signal to secrete the product to the extracellular medium



Recombinant DNA and Biotechnology

Typical Expression Vector



Can you use such an expression system to help in purification?

Figure 9-7

Recombinant DNA and Biotechnology

Purification of Recombinant Proteins

- Purification of natural proteins is difficult.
- Recombinant proteins can be tagged for purification.
- The tag binds to the affinity resin, binding the protein of interest to a purification column.

TABLE 9-3 Commonly Used Protein Tags

Tag protein/peptide	Molecular mass (kDa)	Immobilized ligand
Protein A	59	Fc portion of IgG
$(His)_6$	0.8	Ni^{2+}
Glutathione-*S*-transferase (GST)	26	Glutathione
Maltose-binding protein	41	Maltose
β-Galactosidase	116	*p*-Aminophenyl-*β*-D-thiogalactoside (TPEG)
Chitin-binding domain	5.7	Chitin

Recall:

Affinity Chromatography

Basis = function

Biotechnology (recombinant DNA technology) has revolutionized protein purification.

At the level of the DNA sequence, the DNA sequence encoding such binding proteins or "tags" can be "fused" to the sequence encoding YFP. In this way, a chimeric protein is produced that has the binding function, which allows the use of affinity chromatography.

Common "tags" are:
- Maltose-binding protein
- Chitin-binding protein
- Glutathione-S-transferase (GST)
- His-His-His-His-His-His

Column beads have attached:
- Maltose
- Chitin
- Glutathione (γ-Glu-Cys-Gly)
- Ni-chelate

Recombinant DNA and Biotechnology

Purification of Recombinant Proteins

Can you use such an expression system to help visualize proteins INSIDE of cells?

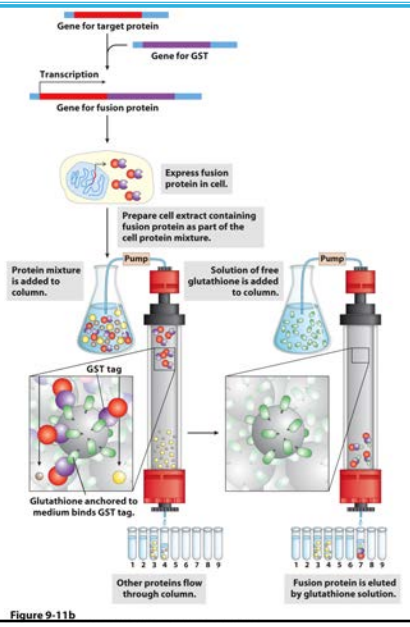
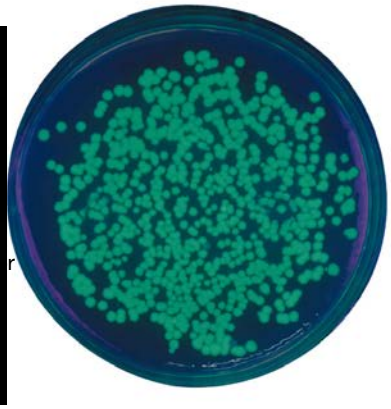


Figure 9-11b

Recombinant DNA and Biotechnology

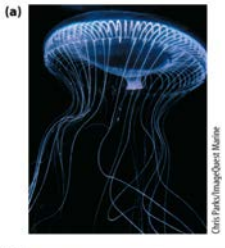
Expression

- *EXAMPLE*: Green fluorescent protein, which normally occurs in a jellyfish, emits visible light when exposed to UV light. The gene for this protein has been isolated and incorporated into vectors as a reporter gene



Recombinant DNA and Biotechnology

GFP–Tagged Protein Localization



Plasmid -binding protein

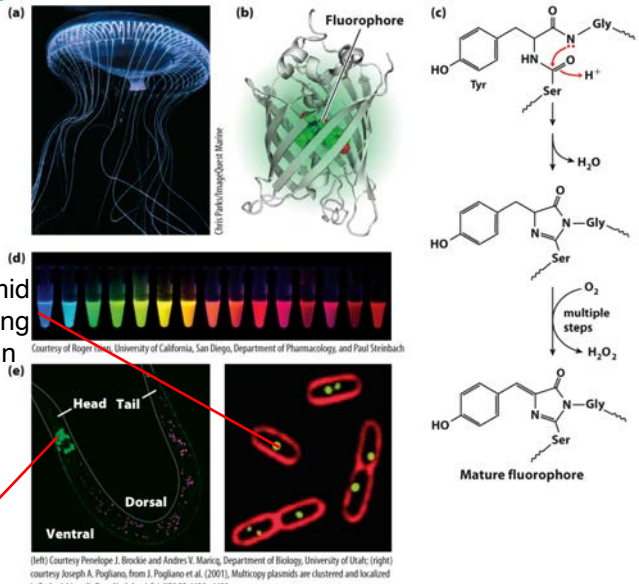
Glu receptor in round worm

Figure 9-16

http://olympus.magnet.fsu.edu/primer/techniques/confocal/applications/fpcolorpalette.html

Recombinant DNA and Biotechnology

GFP–Tagged Protein Localization



Plasmid -binding protein

Glu receptor in round worm

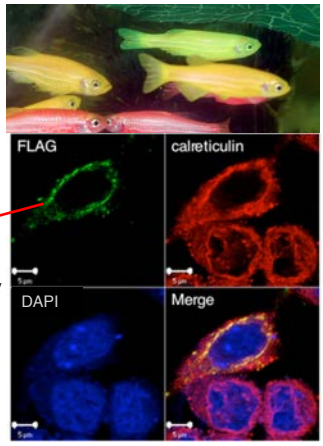
Figure 9-16

http://olympus.magnet.fsu.edu/primer/techniques/confocal/applications/fpcolorpalette.html

Recombinant DNA and Biotechnology

YES! Fluorescence Can Be Used to Determine Protein Location *In Vivo*

- Green fluorescent protein (GFP)
  - use recombinant DNA technologies to attach GFP to protein of interest
  - visualize with a fluorescent microscope
- Immunofluorescence
  - tag protein with primary antibody and detect with secondary antibody containing fluorescent tag
  - Protein can also be fused to a short epitope (e.g., myc-tag [EQKLISEEDL], HA-tag [YPYDVPDYA] or Flag-tag [DYKDDDDK]), and the primary antibody detecting the epitope can be fluorescently labeled.



Example of Flag-tagged Ceramide Synthase, in which an expression plasmid for the fusion protein was transfected into SW480 cells (lymphoma). The counter stains are fluorescent dyes specific for DNA (4',6-diamidino-2-phenylindole, DAPI) and the ER (calreticulin).

Recombinant DNA and Biotechnology

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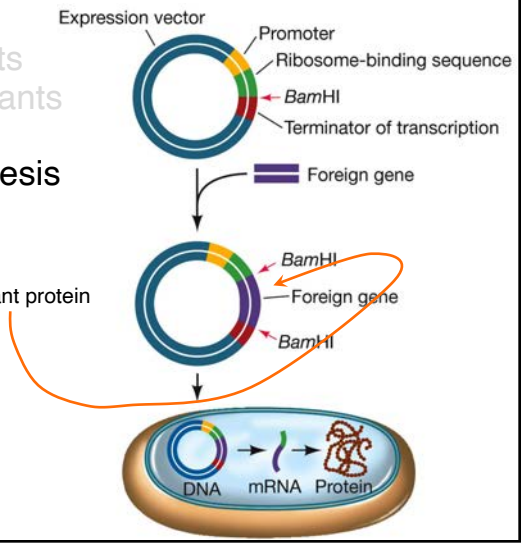
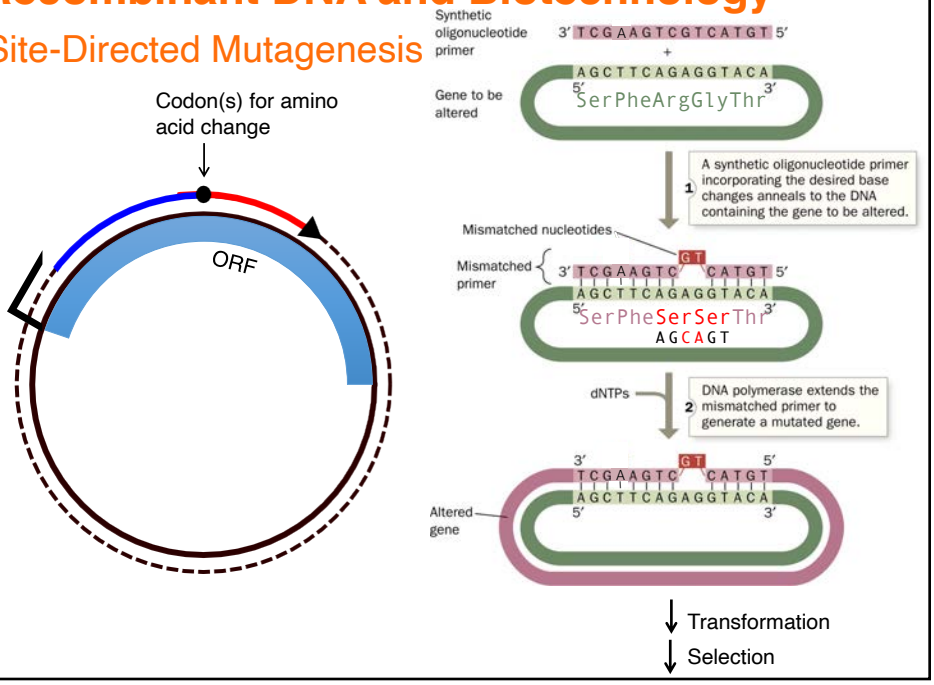
Make a specific mutation to make a variant protein



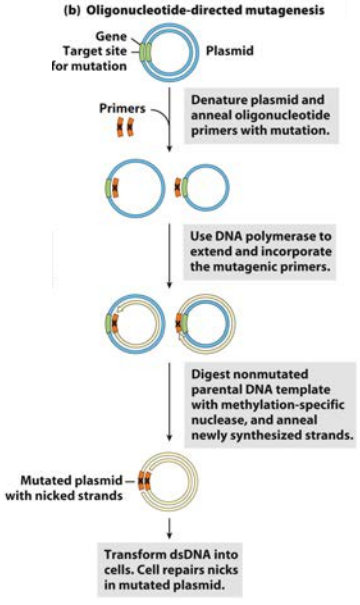
Recombinant DNA and Biotechnology

Site-Directed Mutagenesis

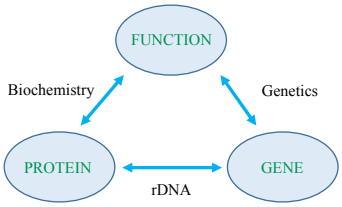


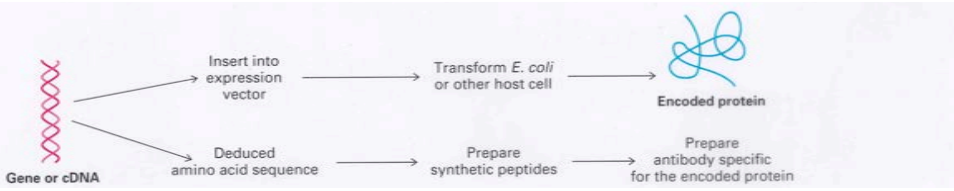
Recombinant DNA and Biotechnology

Site-Directed Mutagenesis



Recombinant DNA and Biotechnology





Recombinant DNA and Biotechnology