CHAPTER 14 LECTURE NOTES: RECOMBINANT DNA TECHNOLOGY

I. General Info

A. Landmarks in modern genetics

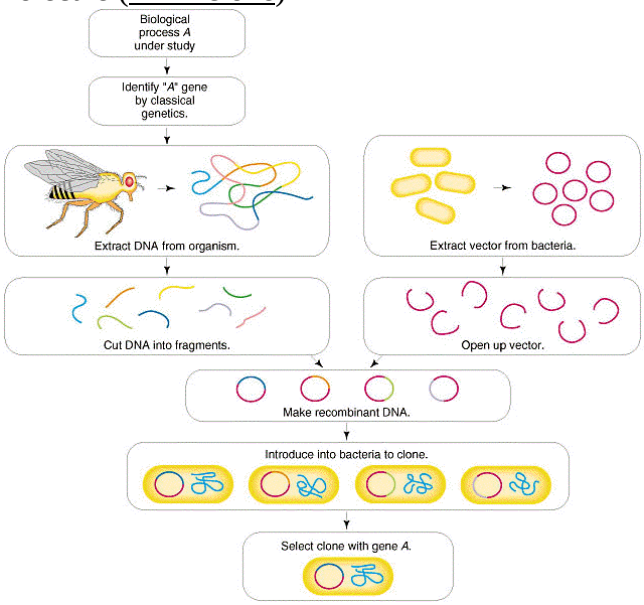
1. Rediscovery of Mendel's work
2. Chromosomal theory of inheritance
3. DNA as the genetic material
4. Recombinant DNA technology development and applications

B. Recombinant DNA refers to the creation of new combinations of DNA segments that are not found together in nature. The isolation and manipulation of genes allows for more precise genetic analysis as well as practical applications in medicine, agriculture, and industry.

C. Fundamental changes in our society are occurring as a result of genetic engineering.

II. Making recombinant DNA

Overview: Isolate DNA → Cut with restriction enzymes → Ligate into cloning vector → transform recombinant DNA molecule into host cell → each transformed cell will divide many, many times to form a colony of millions of cells, each of which carries the recombinant DNA molecule (DNA clone)



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A. Isolating DNA

1. Crude isolation of donor (foreign) DNA is accomplished by isolating cells → disrupting lipid membranes with detergents → destroying proteins with phenol or proteases → degrading RNAs with RNase → leaving DNA at the end

2. Crude isolation of plasmid vector DNA is accomplished by an alkaline lysis procedure or by boiling cells which removes bacterial chromosomal DNA from plasmid DNA.
3. To get purer DNA from either (1) or (2), crude DNA is
   a) Fractionated on a $CsCl_2$ gradient
   b) Precipitated with ethanol
   c) Poured over a resin column that specifically binds DNA

B. Cutting DNA

1. DNA can be cut into large fragments by mechanical shearing.
2. Restriction enzymes are the scissors of molecular genetics. Restriction enzymes (RE) are endonucleases that will recognize specific nucleotide sequences in the DNA and break the DNA chain at those points. A variety of RE have been isolated and are commercially available. Most cut at specific palindromic sites in the DNA (sequence that is the same on both antiparallel DNA strands). These cuts can be a staggered which generate "sticky or overhanging ends" or a blunt which generate flush ends.

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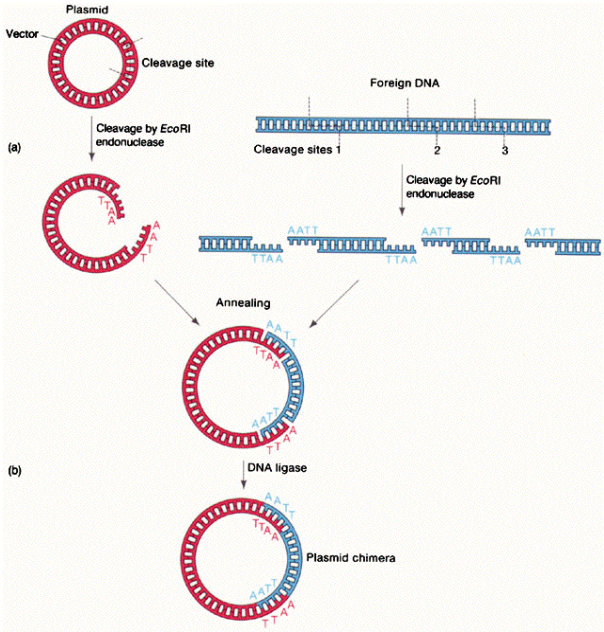
5'--G|AATTC--3'          5'--G          AATTC--3'
3'--CTTAA|G--5'    →     3'--CTTAA            G--5'
```

C. Joining DNA

Once you have isolated and cut the donor and vector DNAs, they must be joined together. The DNAs are mixed together in a tube. If both have been cut with the same RE, the ends will match up because they are sticky. DNA ligase is the glue of molecular genetics that holds the ends of the DNAs together. DNA ligase creates a phosophodiester bond between two DNA ends.



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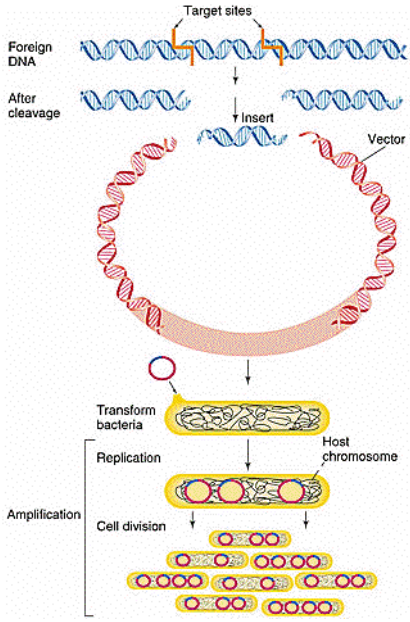
D. Amplifying the recombinant DNA

To recover large amounts of the recombinant DNA molecule, it must be amplified. This is accomplished by <u>transforming</u> the recombinant DNA into a bacterial host strain. (The cells are treated with $CaCl_2$ → DNA is added → Cells are heat shocked at 42 C → DNA goes into cell by a somewhat unknown mechanism.) Once in a cell, the recombinant DNA will be replicated. When the cell divides, the replicated recombinant molecules go to both daughter cells which themselves will divide later. Thus, the DNA is amplified.



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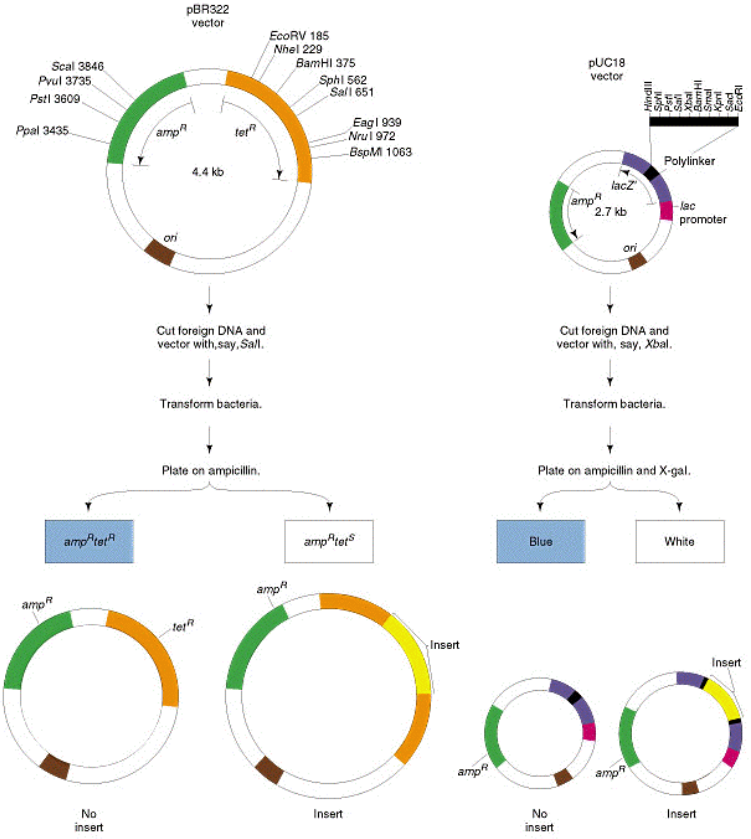
<u>DNA clone</u> = A section of DNA that has been inserted into a vector molecule and then replicated in a host cell to form many copies.

E. Vectors

1. Requirements for a cloning vector
    a) Should be capable of replicating in host cell
    b) Should have convenient RE sites for inserting DNA of interest
    c) Should have a selectable marker to indicate which host cells received recombinant DNA molecule
    d) Should be small and easy to isolate

2. Bacterial plasmids are small, circular DNA molecules that are separate from the rest of the chromosome. They replicate independently of the bacterial chromosome. Useful for cloning DNA inserts less that 20 kb (kilobase pairs). Inserts larger than 20 kb are lost easily in the bacterial cell.



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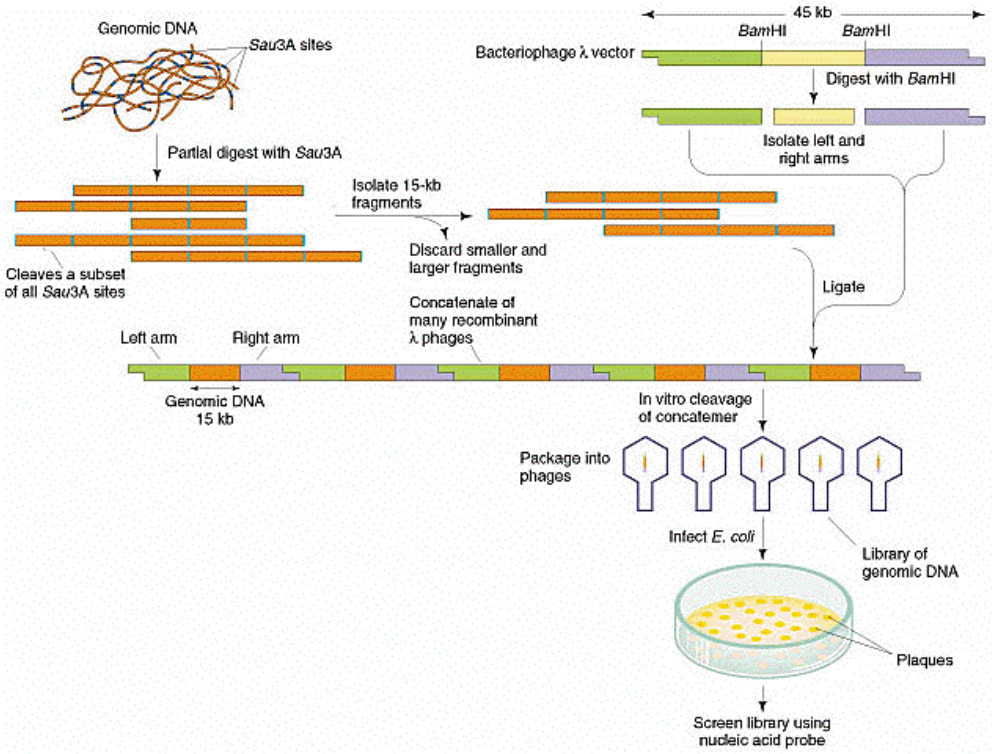
3. Bacteriophage lambda (45 kb) contains a central region of 15 kb that is not required for replication or formation of progeny phage in *E. coli*. Thus, lambda can be used as a cloning vector by replacing the central 15 kb with 10-15 kb of foreign DNA. This is done as follows: mix RE cut donor DNA and lambda DNA in test tube → ligate → use *in vitro* packaging mix that will assemble progeny phage carrying the foreign DNA → infect *E. coli* with the phage to amplify



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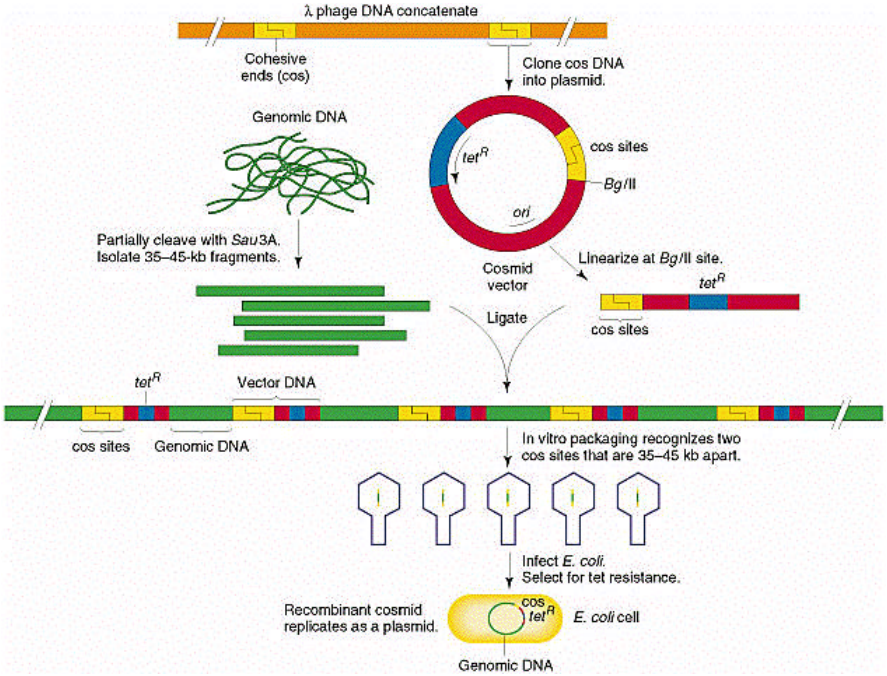
4. Cosmids are hybrids of phages and plasmids that can carry DNA fragments up to 45 kb. They can replicate like plasmids but can be packaged like phage lambda.



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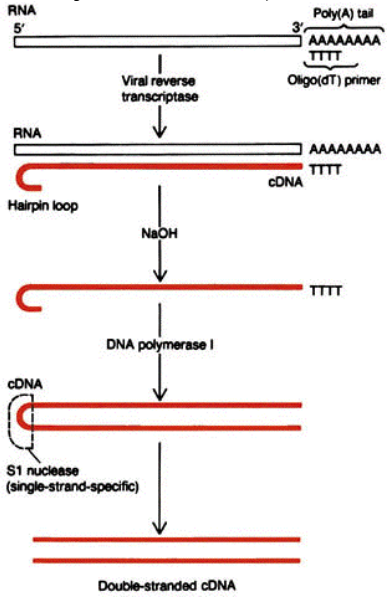
5. Expression vectors are vectors that carry host signals that facilitate the transcription and translation of an inserted gene. They are very useful for expressing eukaryotic genes in bacteria.
6. Yeast artificial chromosomes (YACS) are yeast vectors that have been engineered to contain a centromere, telomere, origin of replication, and a selectable marker. They can carry up to 1,000 kb of DNA. Since they are maintained in yeast (a eukaryote), they are useful for cloning eukaryotic genes that contain introns. Also, eukaryotic genes are more easily expressed in a eukaryotic host such as yeast.
7. Bacterial artificial chromosomes (BACS) are bacterial plasmids derived from the F plasmid. They are capable of carrying up to 300 kb of DNA.

III. Making a Library

A. Libraries are collection of DNA clones in a certain vector. The goal is to have each gene represented in the library at least once.

B. Types- categorized by

1. Source of vector DNA
2. Source of donor DNA
   a) Genomic - made from RE DNA fragments of total genomic DNA
   b) Chromosome – made from RE DNA fragments of one chromosome isolated via flow cytometry or pulsed field gel electrophoresis
   c) cDNA (complementary DNA) – made from DNA synthesized from mRNA (no need to worry about introns)



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IV. Identification of the specific gene of interest in the library

A. Probing for the gene

1. DNA probe

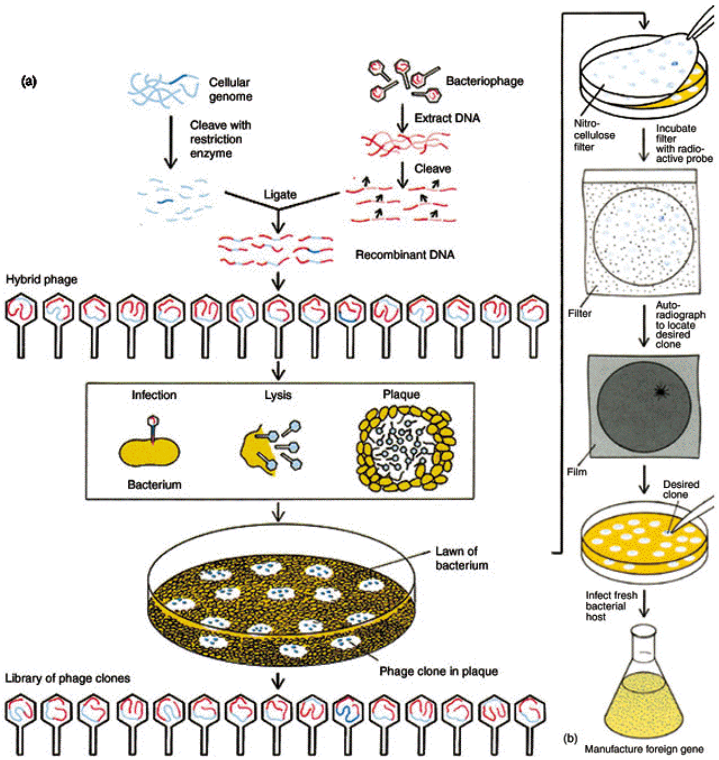
a) DNA probes are based on the fact that a denatured (heated or chemically treated to become single stranded) DNA molecule will hybridize (bind) to sequences that match or are similar to it.

b) Where does the probe DNA come from?

(1) cDNA from highly expressed mRNA from a tissue

(2) homologous gene from a related organism

(3) DNA obtained from "reverse genetics" (protein → DNA): If you have the protein product of the gene in which you are interested….. sequence part of the protein → synthesize a short (>20 nucleotides) DNA probe based the protein sequence using the genetic code → use as your probe



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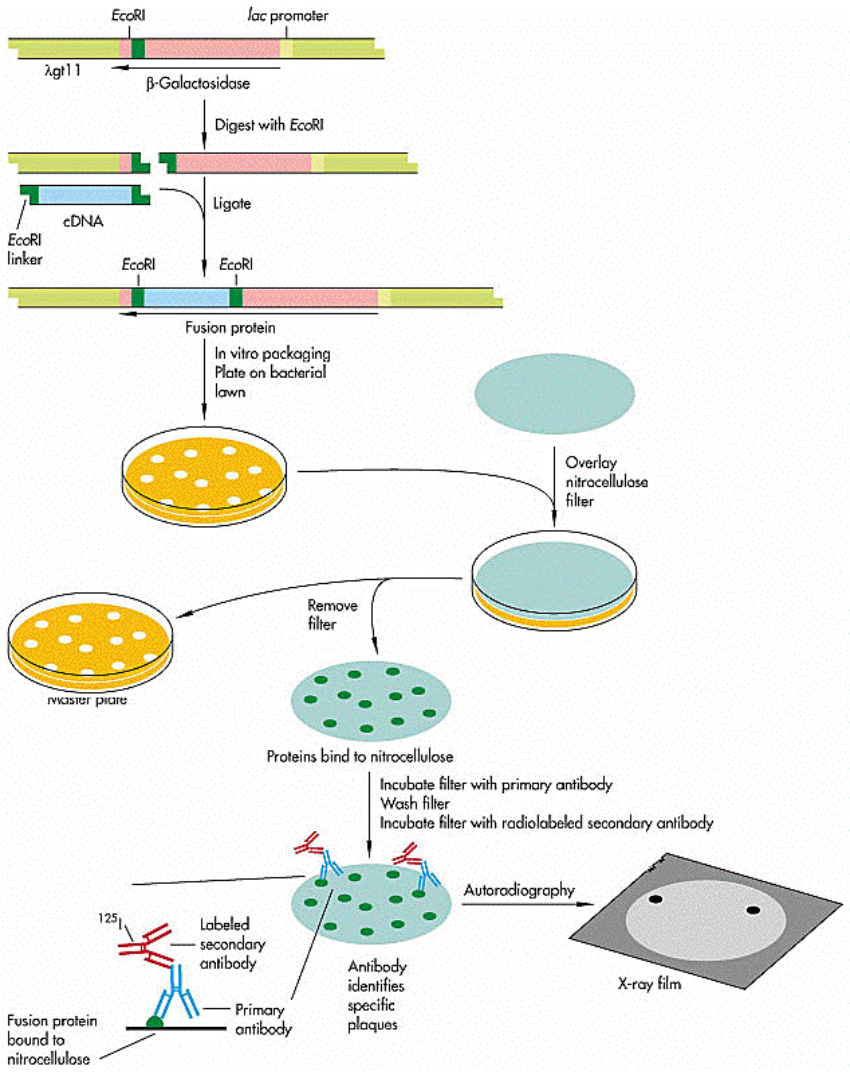
2. Protein probe – If you have the protein product of the gene of interest, make an antibody against it → use the antibody to protein of interest is used to screen the library for the clone that is expressing the gene that codes for the protein



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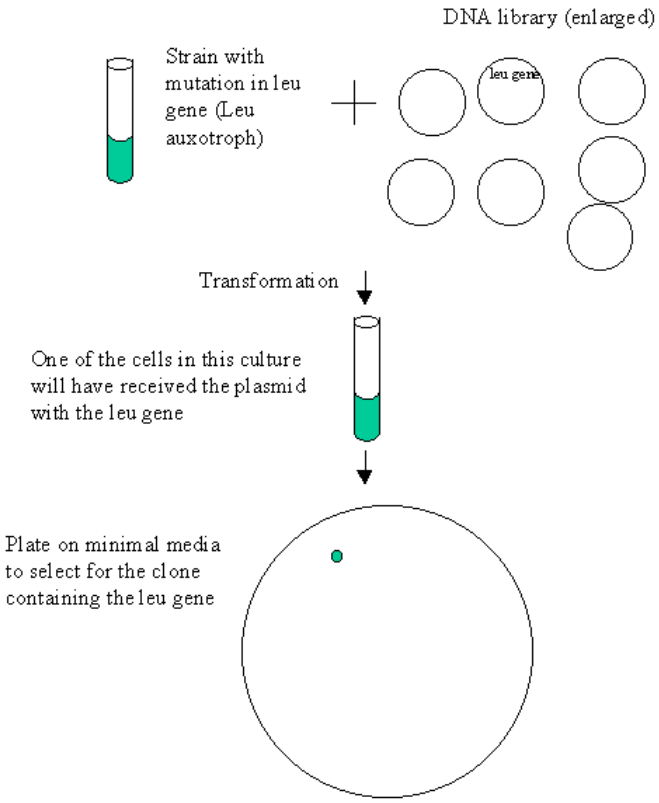
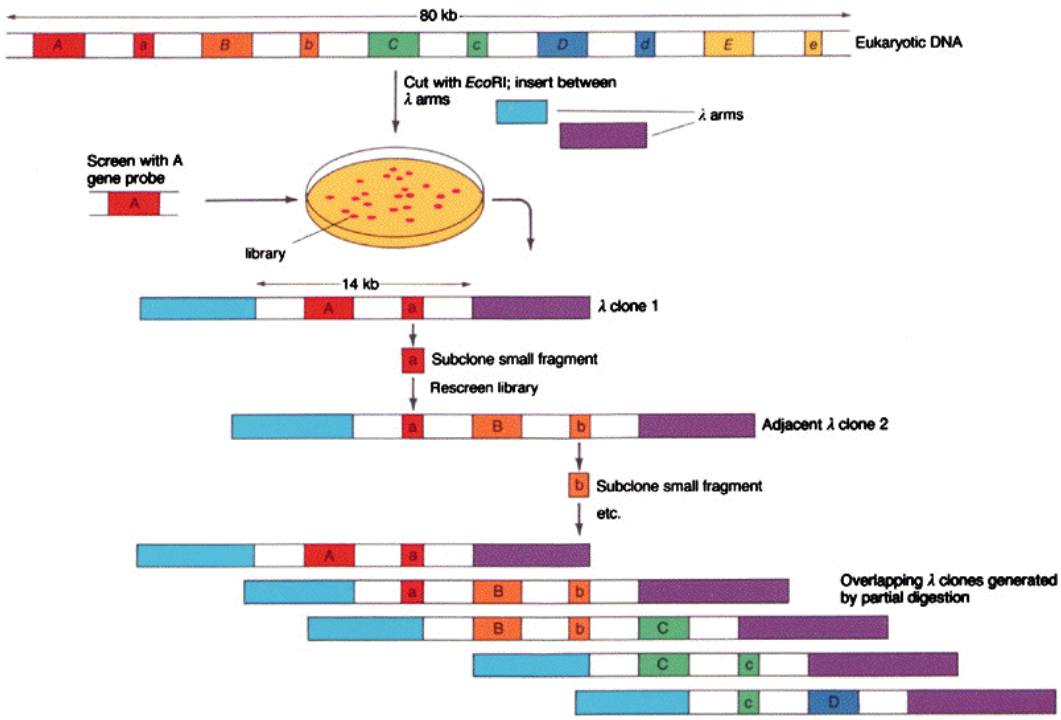
B. Complementation

Clones can be detected based on their ability to confer a missing function on a mutant.



C. Positional cloning is any method of cloning that makes use of information about a gene's chromosomal location in order to clone it.

You know that your gene of interest (gene X) is linked to gene A, for which you have a probe: Using a library of overlapping RE fragments → Isolate a clone (clone 1) containing A → RE analysis of clone 1→ use end of clone 1 as a probe to isolate a new clone (clone 2) → RE analysis of clone 2 → use end of clone 2 as a probe to isolate a new clone → etc → until you get to gene X



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D. Tagging

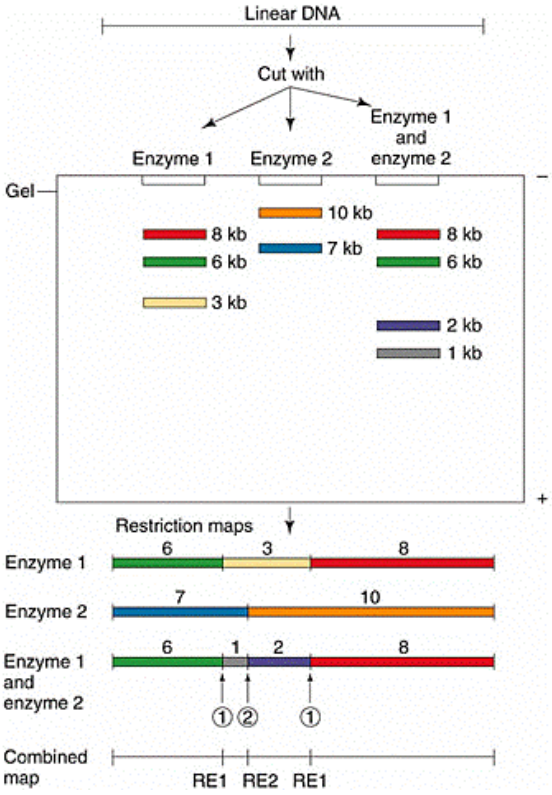
Use a gene (tag) to which you have a probe to mark your gene of interest by inserting that gene into your gene of interest

For example, you are interested in cloning genes that are important for iron transport….
Use transposon (jumping gene) to hop randomly into the chromosome → Screen for those organisms that are affected in iron transport → cross putative tagged iron transport mutants with tester to verify that the mutant phenotype segregates with the tag → make library of the DNA from tagged mutant → select or probe for the tag (and therefore your gene)

V. Analysis of cloned genes

A. Gel electrophoresis – DNA fragments of different sizes can be separated by an electrical field applied to a "gel". The negatively charged DNA migrates away from the negative electrode and to the positive electrode. The smaller the fragment the faster it migrates.

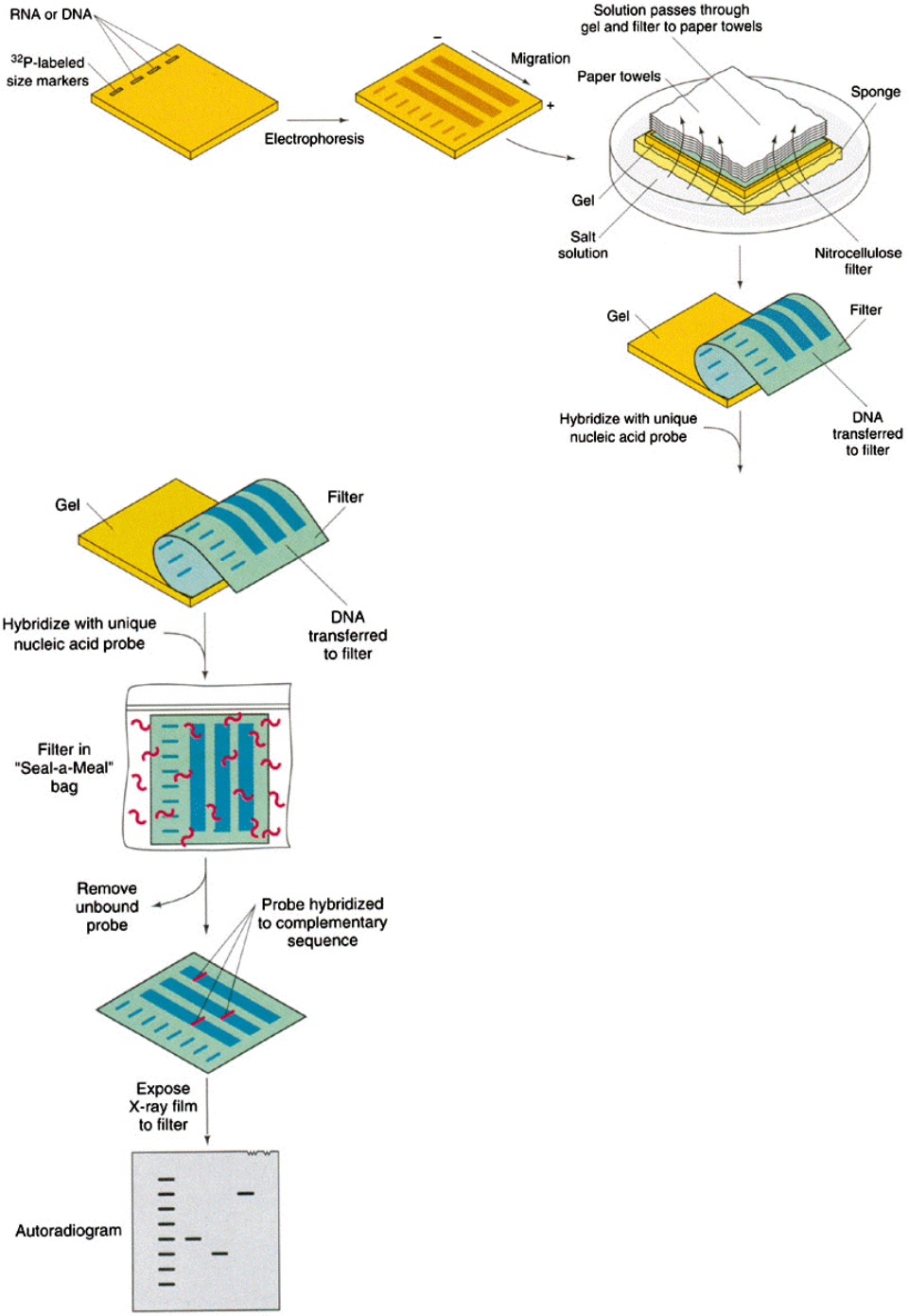
B. Restriction enzyme mapping – Frequently it is important to have a restriction enzyme site map of a cloned gene for further manipulations of the gene. This is accomplished by digestion of the gene singly with several enzymes and then in combinations. The fragments are subjected to gel electrophoresis to separate the fragments by size and the sites are deduced based on the sizes of the fragments.



In this example, digestion with Enzyme 1 shows that there are two restriction sites for this enzyme, but does not reveal whether the 3 kb segment is in the middle or on the end of the digested sequence, which is 17 kb long. Combined digestion by both enzyme 1 and enzyme 2 leaves the 6 and 8 kb segments intact but cleaves the 3 kb segment, showing that enzyme 2 cuts within this enzyme 1 fragment. If the 3 kb section were on the outside of the fragment being studied, digestion by enzyme 2 alone would yield a 1 or 2 kb fragment. Since this is not the case, of the three restriction fragments produced by enzyme 1, the 3 kb fragment must lie in the middle. That the RE2 site lies closer to the 6 kb section can be inferred from the 7 and 10 kb lengths of the enzyme 2 digestion.

C. Southern Blot

1. A Southern allows the detection of a gene of interest by probing DNA fragments that have been separated by electrophoresis with a "labeled" probe.



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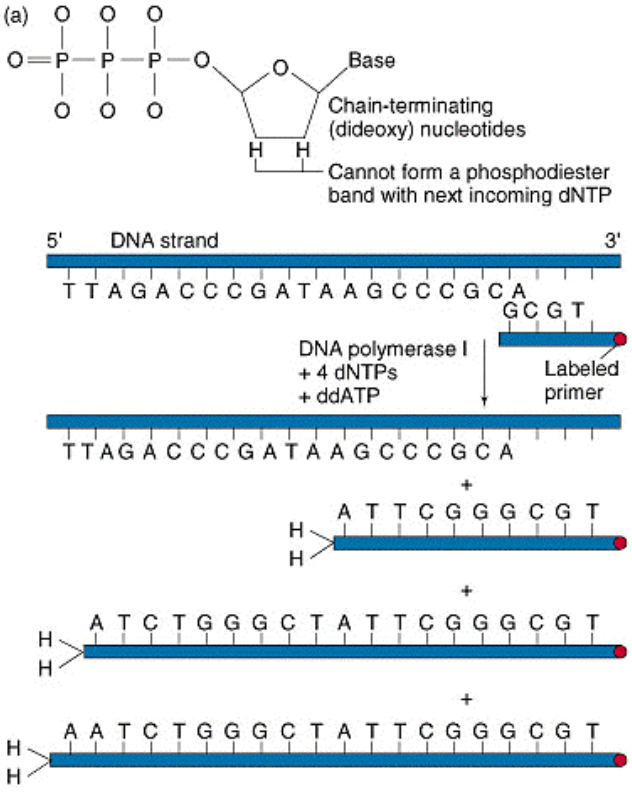
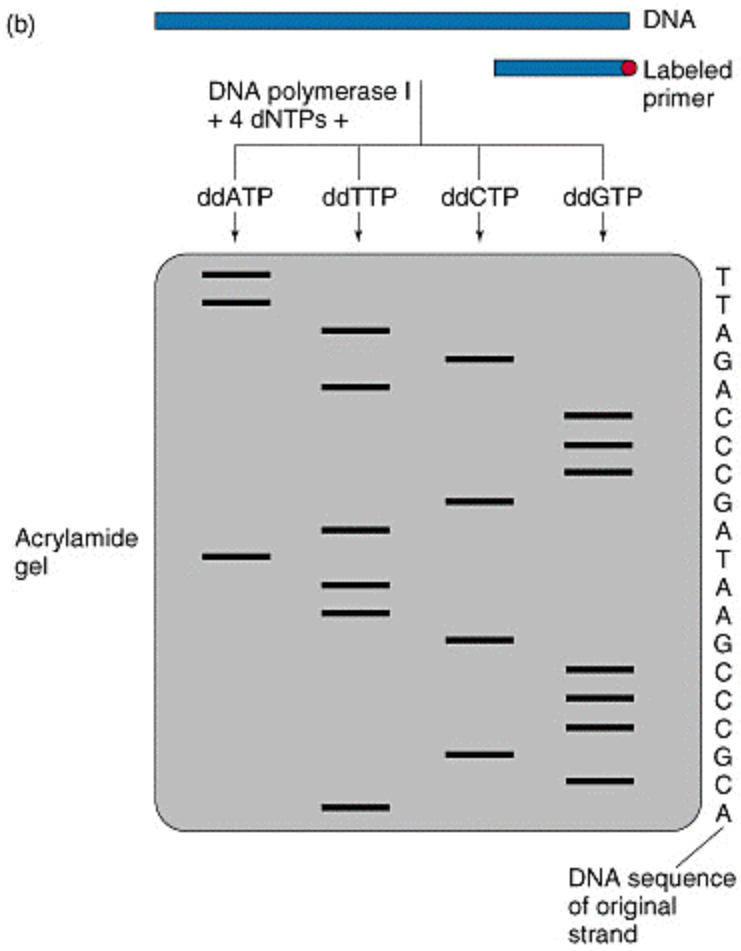
2. Northern Blot (probe RNA on a gel with a DNA probe)
3. Western Blot (probe proteins on a gel with an antibody)

D. DNA sequencing of a gene

1. Maxam-Gilbert base destruction method – bases of a DNA molecule are selectively destroyed – not used very much anymore because reagents are highly toxic and very dangerous
2. Sanger dideoxy method – Gene to be sequenced is used as a template for the synthesis of new DNA strands, each randomly terminating due to the incorporation of a chain terminating dideoxynucleotide in 4 different reaction tubes. This produces a population of molecules, each terminating at a different site. Running the products in each tube on a gel allows the determination of where each chain terminating dideoxynucleotide was incorporated. The DNA is visualized because the DNA primer to start the reaction is radioactive or some of the dNTPs are radioactive.





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This procedure is now automated so that a computer reads the sequence. Instead of using radioactive primers, the primers are labeled with different color fluorescent dye for each reaction.

E. PCR (polymerase chain reaction) – Allows the isolation of a specific segment of DNA from a small DNA (or cell sample) using DNA primers at the ends of the segment of interest.