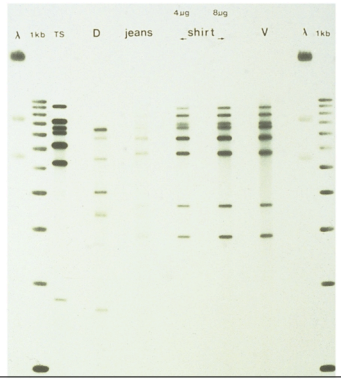
Chapter 19

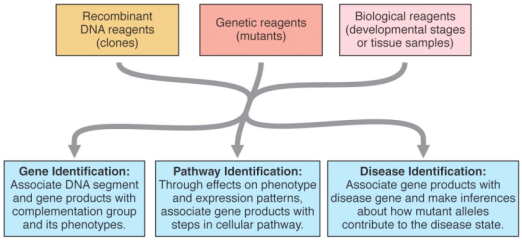
Recombinant DNA and Genetic Engineering

Genetic manipulation

Crime scene



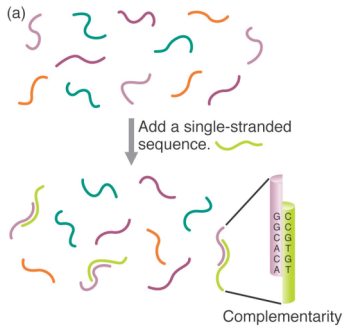
Application of recombinant DNA technology



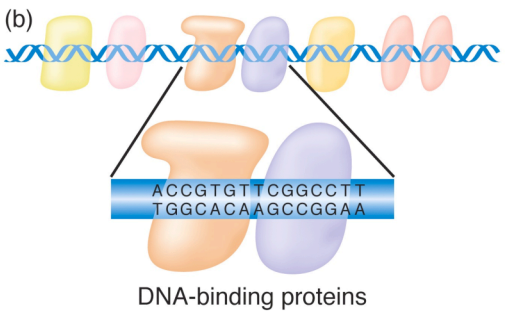
Recombinant DNA technology

- Active -- mid-1970s
- Genetic engineering - problems in biology, medicine, and agriculture
- Genomics - genome at the molecular level

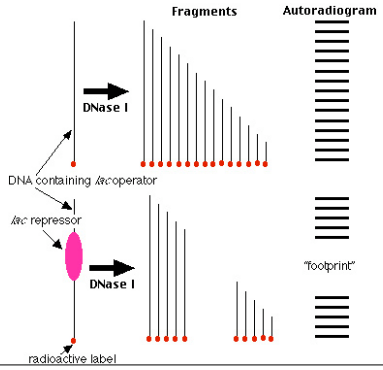
Complementarity



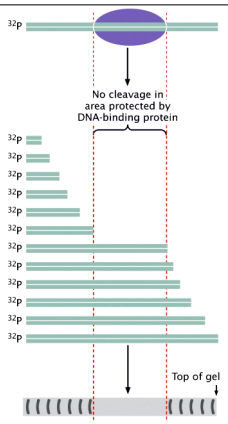
DNA-Binding proteins (Footprinting)



DNA footprinting



DNA footprinting

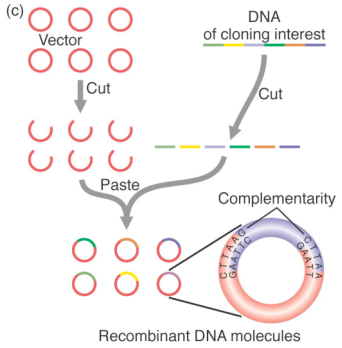


Generating recombinant DNA (1)

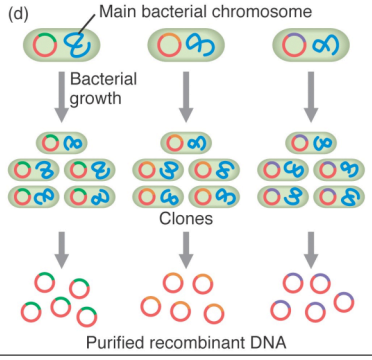
- DNA sources
  - genomic DNA from chromosomes
    - usually too large to clone directly
  - cDNA (complementary DNA) derived by action of reverse transcriptase from (usually) mRNA template
  - chemically synthesized oligonucleotides
- Digestion of DNA by restriction enzyme
  - recognize palindromic double-stranded sequences
  - produce complementary "sticky" ends
  - dozens of such enzymes
  - sticky ends can be ligated under appropriate conditions

5'-G^AATTC-3'
3'-CTTAA^G-5'

Recombinant DNA molecules



Cloning recombinants



Cloning

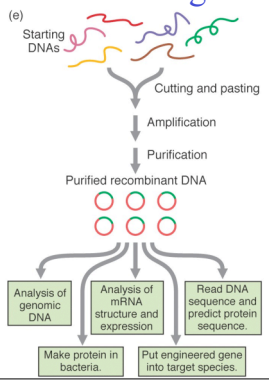
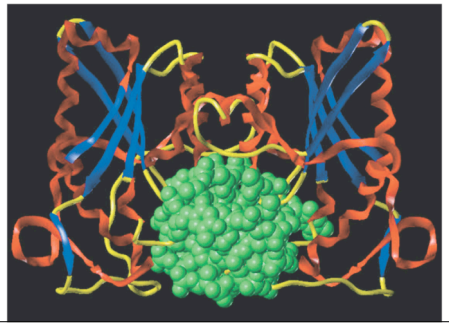
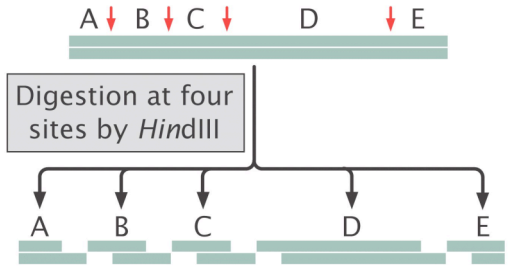


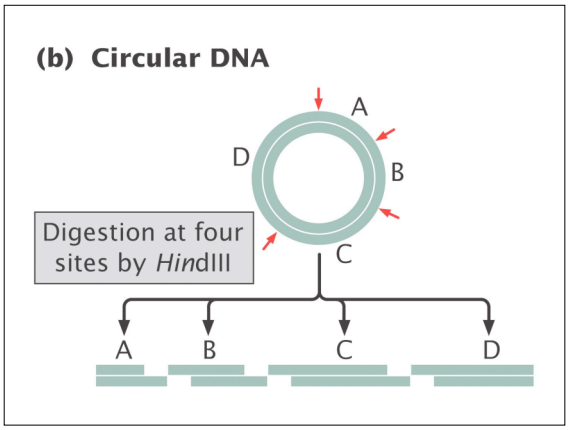
Table 18.1 Types of restriction enzymes

Type	Activity of Enzyme	ATP Required	Cleavage Site
I	Cleavage and methylation	Yes	Random sites distant from recognition site
II	Cleavage only	No	Within recognition site
III	Cleavage and methylation	Yes	Random sites near recognition site

BamHI restriction enzyme







7 base recognition enzymes

Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a) *Eco*RI	*Escherichia coli*	5′ —G—A—A—T—T—C— / —C—T—T—A—A—G— 5′	—G / A—A—T—T—C— ; —C—T—T—A—A / G— ; 5′ sticky ends
*Pst*I	*Providencia stuartii*	5′ —C—T—G—C—A—G— / —G—A—C—G—T—C— 5′	—C—T—G—C—A / G— ; —G / A—C—G—T—C— ; 3′ sticky ends
*Sma*I	*Serratia marcescens*	5′ —C—C—C—G—G—G— / —G—G—G—C—C—C— 5′	—C—C—C / G—G—G— ; —G—G—G / C—C—C— ; Blunt ends

6 base recognition enzymes

Some restriction enzymes

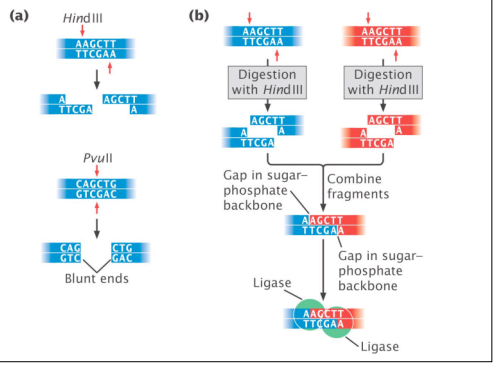
Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(b) *Hin*dIII	*Haemophilus influenzae*	5′ —A—A—G—C—T—T— / —T—T—C—G—A—A— 5′	—A / A—G—C—T—T— ; —T—T—C—G—A / A— ; 5′ sticky ends
*Bam*I	*Bacillus amyloliquefaciens*	5′ —G—G—A—T—C—C— / —C—C—T—A—G—G— 5′	—G / G—A—T—C—C— ; —C—C—T—A—G / G— ; 5′ sticky ends
*Bgl*II	*Bacillus globiggi*	5′ —A—G—A—T—C—T— / —T—C—T—A—G—A— 5′	—A / G—A—T—C—T— ; —T—C—T—A—G / A— ; 5′ sticky ends

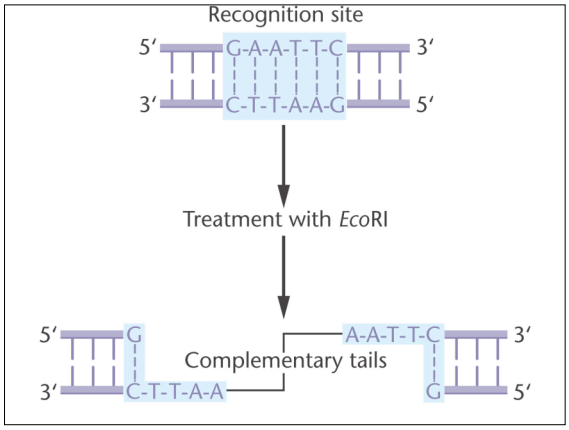
4 base recognition enzymes

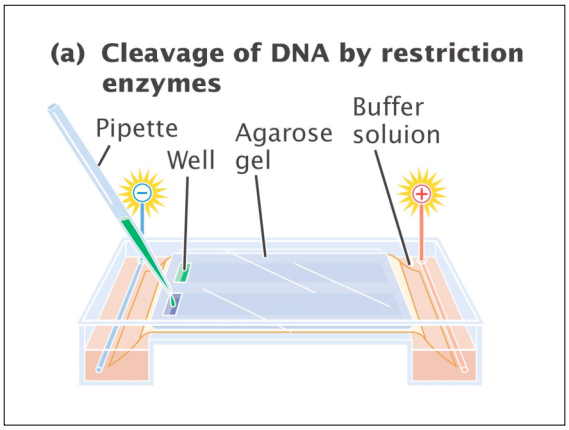
Some restriction enzymes

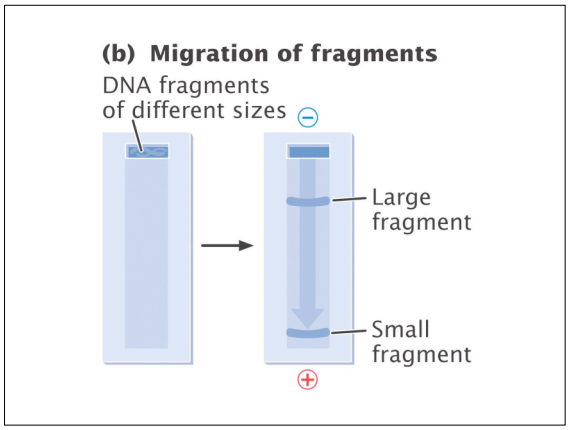
Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(c) *Hae*III	*Haemophilus aegyptius*	5′ —G—G—C—C— / —C—C—G—G— 5′	—G—G / C—C— ; —C—C / G—G— ; Blunt ends
*Hpa*II	*Haemophilus parainfluenzae*	5′ —C—C—G—G— / —G—G—C—C— 5′	—C / C—G—G— ; —G—G—C / C— ; 5′ sticky ends

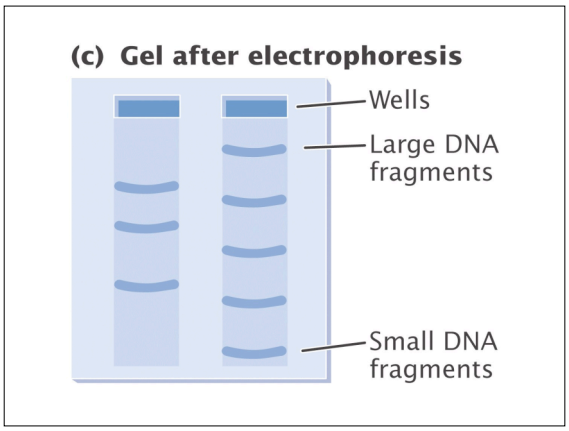
Cohesive or sticky ends

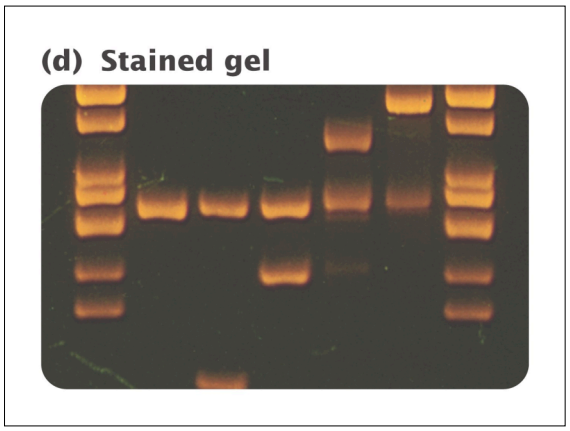


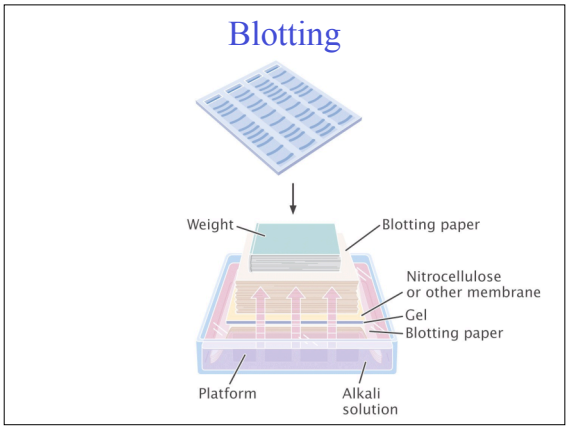


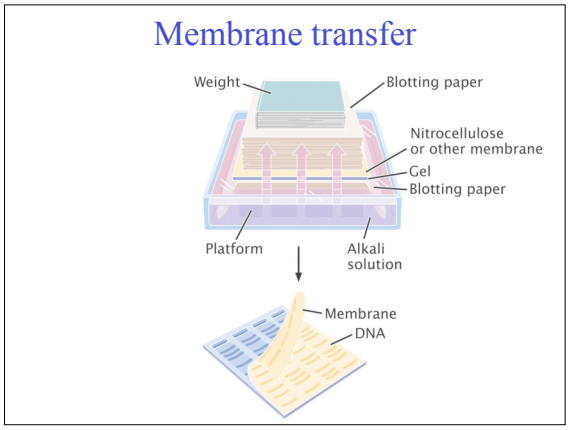

(a) Cleavage of DNA by restriction enzymes
Pipette
Well
Agarose gel
Buffer soluion


(b) Migration of fragments
DNA fragments of different sizes
Large fragment
Small fragment

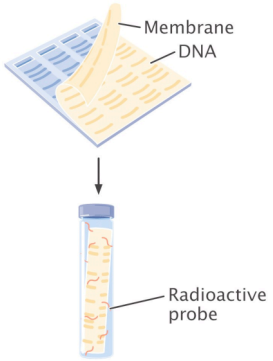

(c) Gel after electrophoresis
Wells
Large DNA fragments
Small DNA fragments


(d) Stained gel

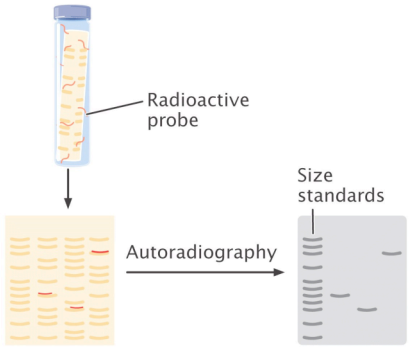

Blotting
Weight
Blotting paper
Nitrocellulose or other membrane
Gel
Blotting paper
Platform
Alkali solution


Membrane transfer
Weight
Blotting paper
Nitrocellulose or other membrane
Gel
Blotting paper
Platform
Alkali solution
Membrane
DNA

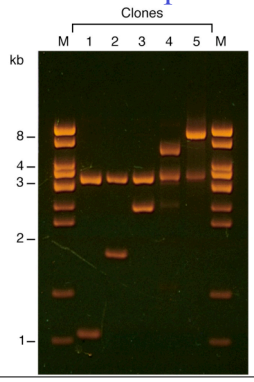
Radiolabeled probe



Hybridization/Autoradiography



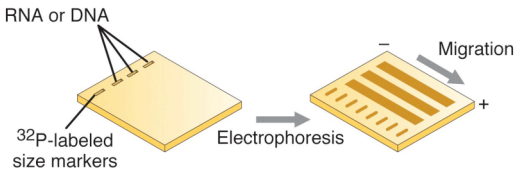
DNA electrophoresis



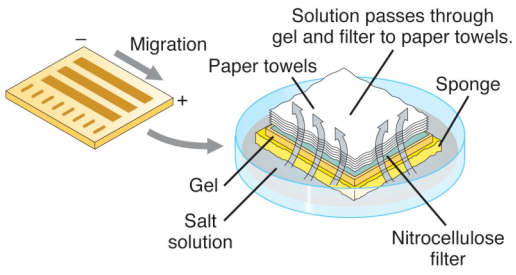
Probing for polynucleotide in mixture

- From mixture of restriction fragments, mixture of mRNA, etc.
- Nucleic acids are electrophoresed, blotted onto membrane, and membrane probed
  - Southern blot: separation of DNA molecules
    - can identify size of restriction fragment containing gene sequence of interest
    - such DNA can be cut from gel and cloned
  - Northern blot: separation of RNA molecules
    - can be used to determine conditions for gene transcription

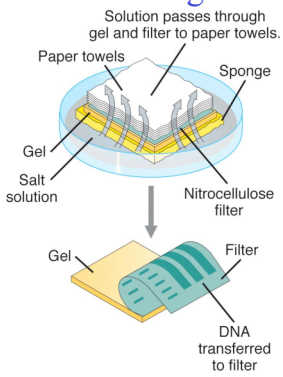
Blotting



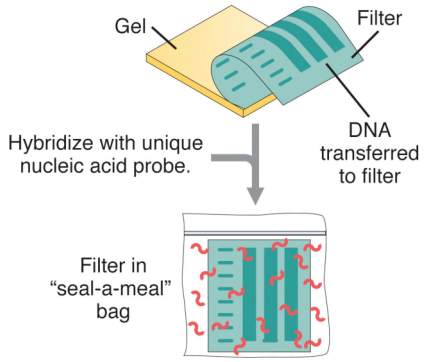
Blotting



Blotting



Blotting



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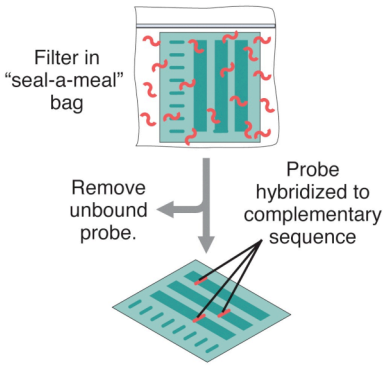
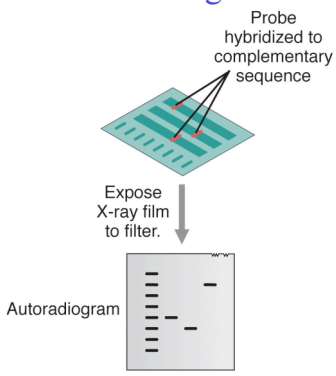
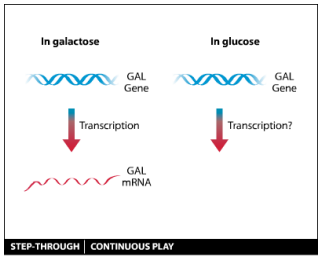


Blotting



Blotting video



Generating recombinant DNA (2)

- Insertion into vector
  - cloning vectors permit replication of inserted DNA
  - include plasmids, vectors, artificial chromosomes
  - complementary restriction ends joined by DNA ligase
  - multiple fragments can be joined
- Transformation into expression system
  - bacterial cell, e.g., *E. coli*
  - eukaryotic cell, e.g., yeast

Cloning vectors

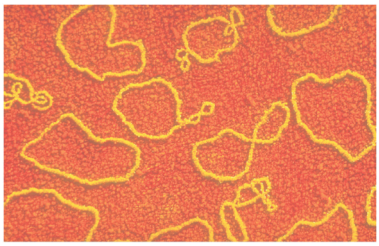
Table 18.3 Comparison of plasmids, phage lambda vectors, and cosmids

Cloning Vector	Size of DNA That Can Be Cloned	Method of Propagation	Introduction to Bacteria
Plasmid	As large as 15 kb	Plasmid replication	Transformation
Phage lambda	As large as 23 kb	Phage reproduction	Phage infection
Cosmid	As large as 44 kb	Plasmid reproduction	Phage infection

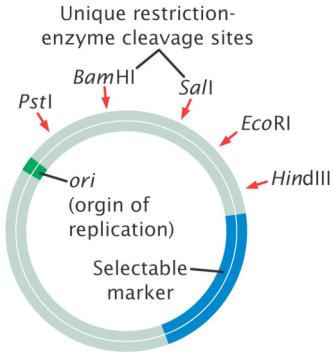
Note: 1 kb = 1000 bp

Cloning vectors

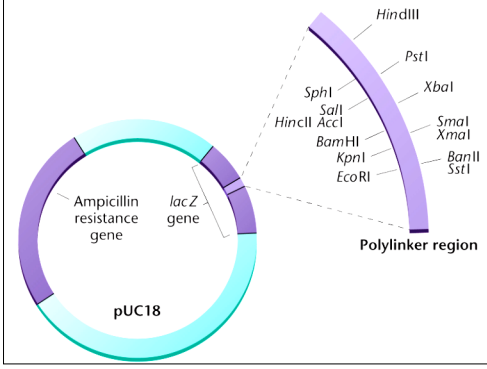
- Plasmid--
  - extrachromosomal double-stranded DNA
  - replicates autonomously



Parts of plasmid



pUC18



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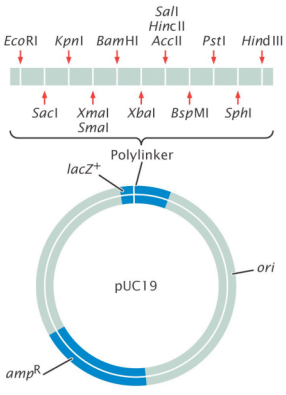
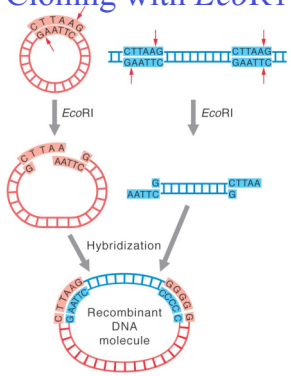
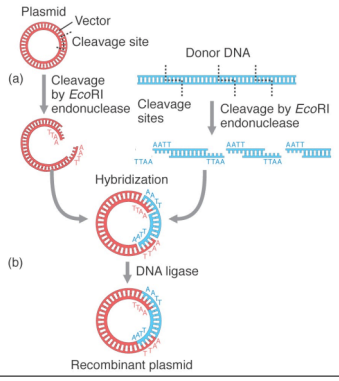


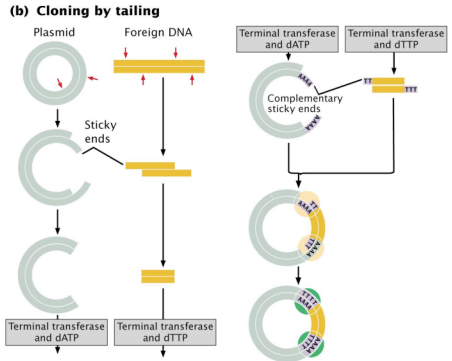
Cloning with *Eco*R1



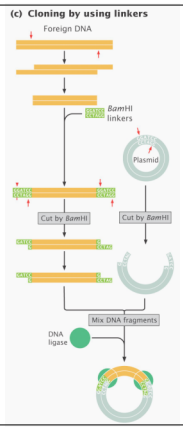
Cutting and gluing the donor



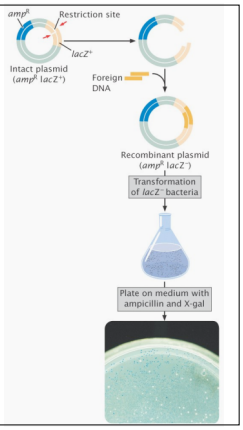
Cloning by tailing

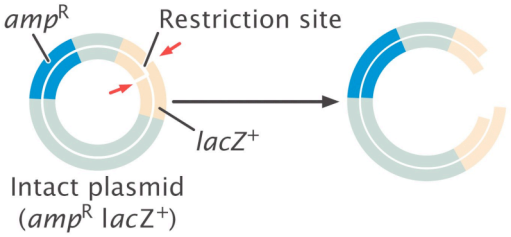


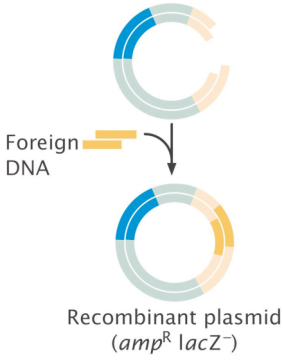
Cloning using linkers

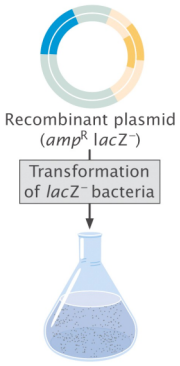


Blue/white selection





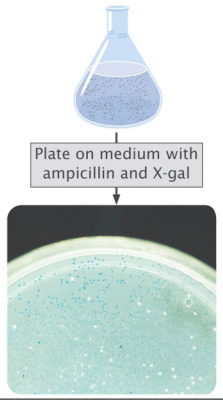




Blue colonies are *Amp*^{R} and *LacZ*$^+$

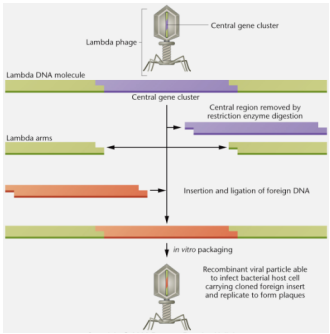
White colonies are *Amp*^{R} and *LacZ*$^-$

Clear or no growth are *Amp*^{S} or no plasmid



lambda (λ) phage

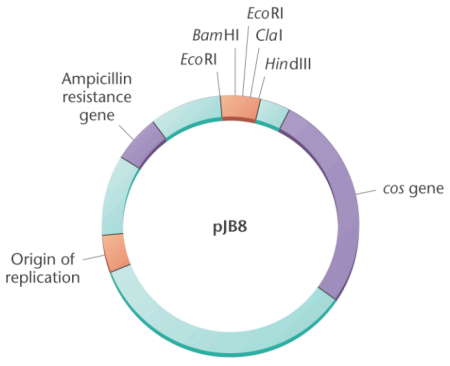
- Highly efficient
  - The central third of lambda (λ) phage is not essential
  - can be replaced with foreign DNA
  - Small fragments will not be packaged into λ
    - Phage genome needs to be 40-50 kb
  - *Cos* sites are required for packaging



Cosmids

- combining parts of λ phage and parts of plasmids
- contain the *cos* sites of lambda,
  - necessary for packaging of phage DNA
- cosmids replicate as plasmids
- Up to 44 kb of foreign DNA

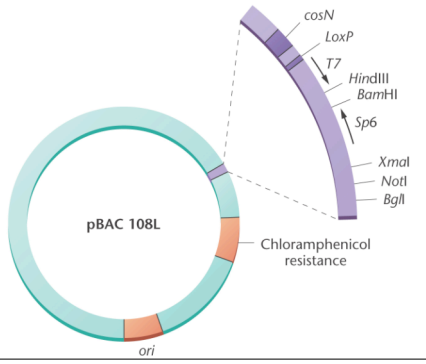
Cosmid



Bacterial artificial chromosomes (BACs)

- Bacterial artificial chromosomes (BACs)
- based on F factor and can carry up to 300 kb of inserted DNA

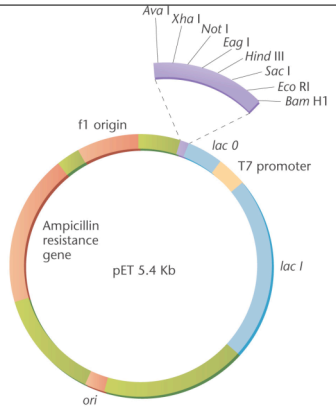
BACs



Expression vectors

- engineered to express a gene of interest
- produce large quantities of protein
  - i.e. insulin
- Bacterial promoter is required
  - *lac* promoter
    - Sequences that control transcription initiation, termination & ribosome binding site
    - Results in a fusion protein

Expression vector



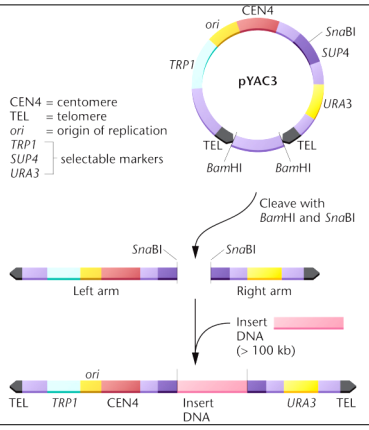
Yeast artificial chromosomes (YACs)

- Yeast artificial chromosomes (YACs) can contain 100–1000 kb of inserted DNA
- Yeast also have posttranslational modification processes
  - ORI - ensures replication
  - Telomeres - chromosome stability
  - centromere - attachment of mitotic spindle fibers

TABLE 19.1 RECOMBINANT PROTEINS SYNTHESIZED IN YEAST CELLS

Hepatitis B virus surface protein
Malaria parasite protein
Epidermal growth factor
Platelet-derived growth factor
α_1-antitrypsin
Clotting factor XIIIA

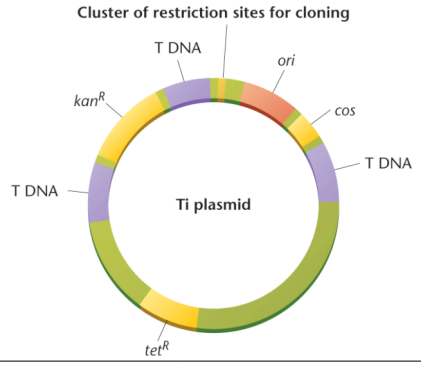
YACs



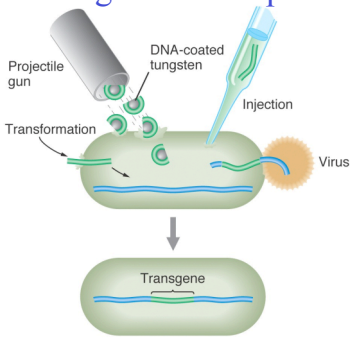
Ti plasmid

- *Agrobacterium tumefaciens*
- used to transform plant cells
- T-DNA containing foreign DNA

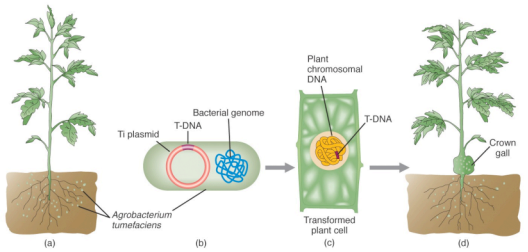
Ti plasmid



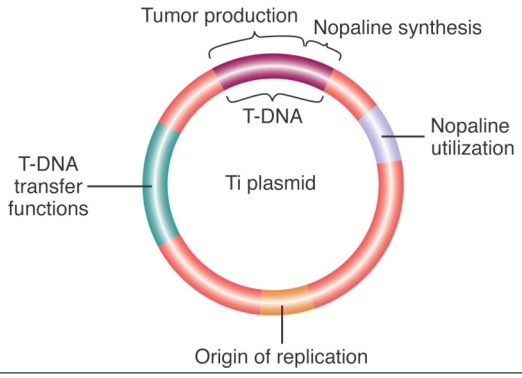
Getting DNA into plants



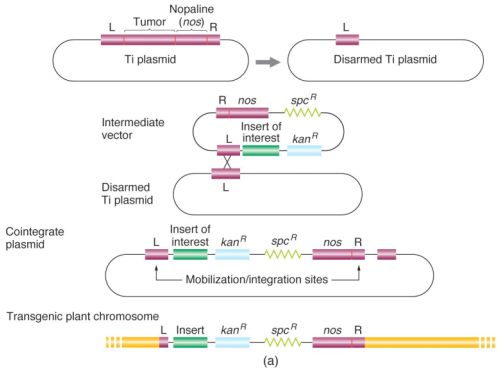
Ti plasmid/crown gall



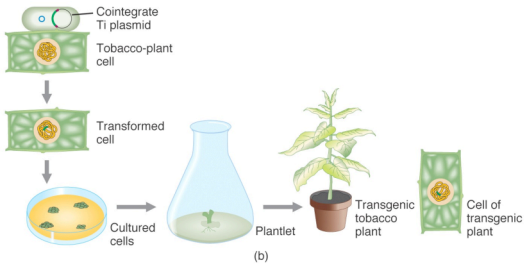
Ti plasmid



Stripped down and integrated plasmids



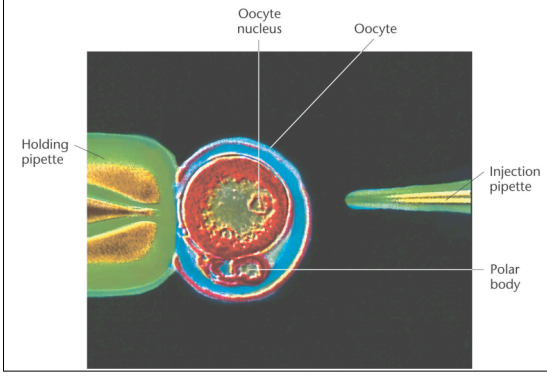
Plant tissue culture



Mammalian cells

- DNA can be transferred to mammalian cells
  - endocytosis and
  - encapsulation liposomes followed by fusion with cell membranes.
- Transgenic mice can be produced by transferring YACs by microinjection into the nucleus of a mouse oocyte
- Avian and mouse retroviruses

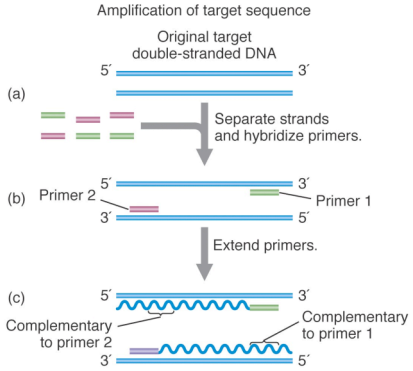
Micropipetting

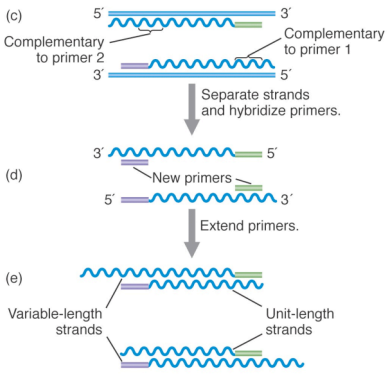


Polymerase chain reaction (PCR)

- Must know sequences flanking desired region
- No cloning procedures necessary
- Principle: DNA made in one amplification cycle is used as template in subsequent cycle
  - heat denaturation to yield single-stranded DNA
  - annealing of primers (oligonucleotides) to single-stranded DNA
  - extension of primers by thermostable DNA polymerase
- Highly sensitive, requiring as little as one copy of single-stranded DNA as initial template

Polymerase chain reaction (PCR)





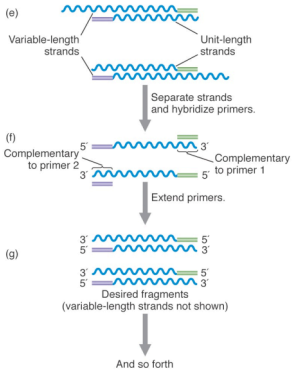
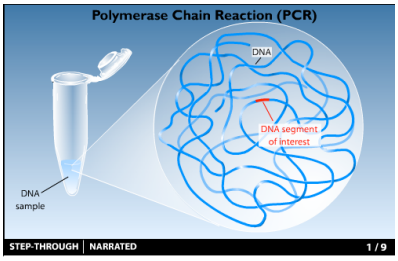


Table 18.5 Number of copies of DNA fragment in PCR amplification

Number of PCR Cycles (n)	Number of Double-Stranded Copies of Original DNA (2^n)
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
20	1,048,576
30	1,073,741,824

Polymerase chain reaction (PCR)



A sample of chromosomal DNA, also called genomic DNA, can be used as the starting material for polymerase chain reaction (PCR). With PCR, an investigator can amplify a single copy of a DNA segment into billions of identical copies.

Genomic and cDNA libraries

- Consist of collections of DNA molecules
- Genomic library consists of fragments of genome
  - often constructed from partial restriction digests
  - typically multifold representation of inserts
  - contain introns and regulatory sequences
- cDNA libraries consist of DNA derived from mRNA population of cell types or tissue
  - limited to transcribed genes
  - introns and flanking regulatory sequences absent

Clones needed

- The number of clones in a library needed to give a certain probability of containing all genomic sequences

$$N = \frac{\ln(1-P)}{\ln(1-f)}$$

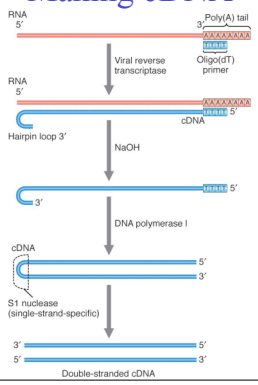
  - N is the number of required clones
  - P is the probability of recovering a sequence
  - and f is the fraction of the genome in each clone

How many clones are needed?

- 99% probability of getting the DNA
- 17 kb is average insert size for a phage
- Human genome is 3.0 x 10^6 kb

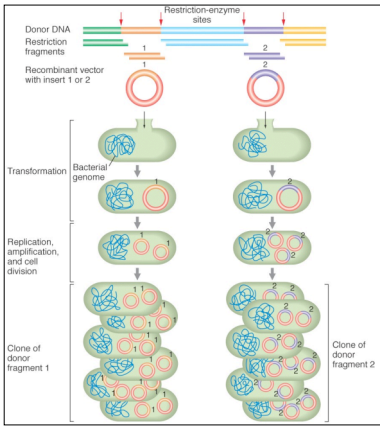
- How many clones are needed using a plasmid?
  - 5 kb average insert

Making cDNA



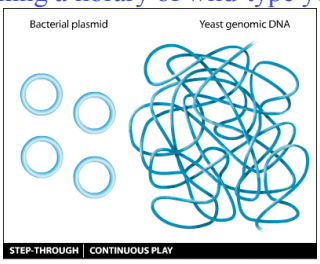
cDNA

- Reverse transcriptase PCR (RT-PCR)
  - generate cDNA from mRNA
  - a single-stranded cDNA copy of the mRNAs using reverse transcriptase
  - Treatment with RNAase H
  - PCR to copy the single-stranded DNA into double-stranded DNA.

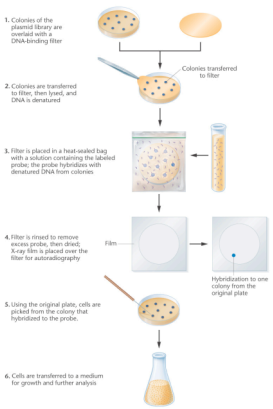


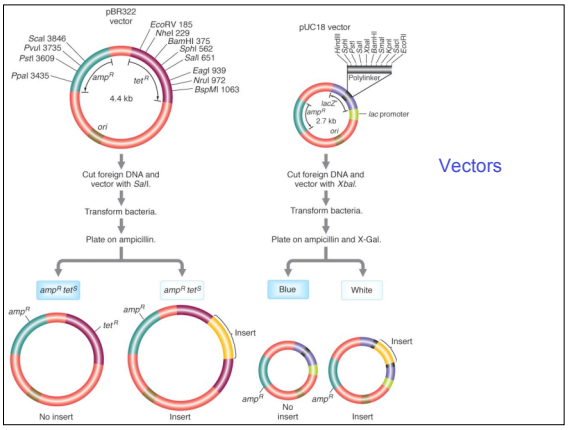
Amplification of clones

Making a library of wild-type yeast

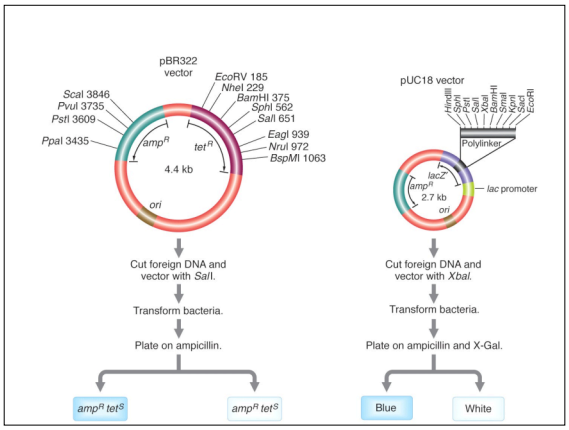


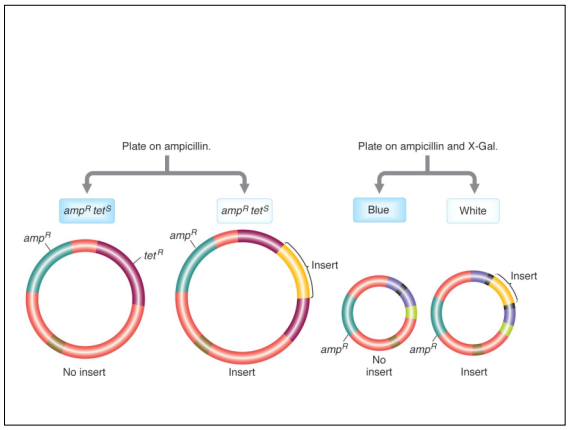
Screening a library

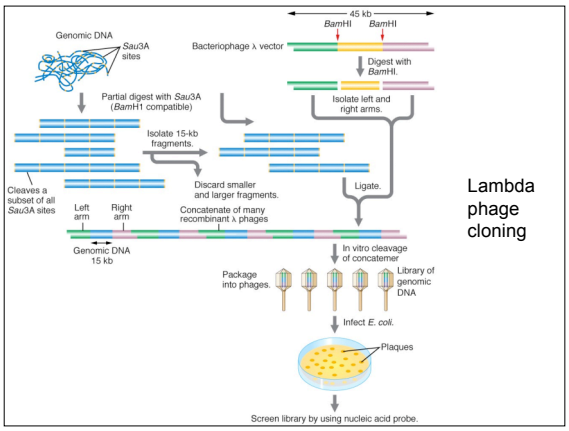




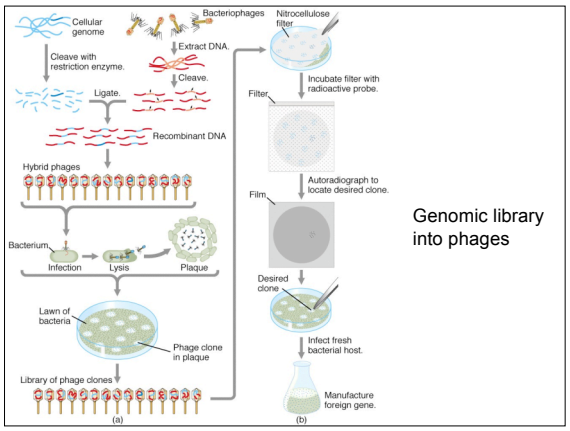
Vectors



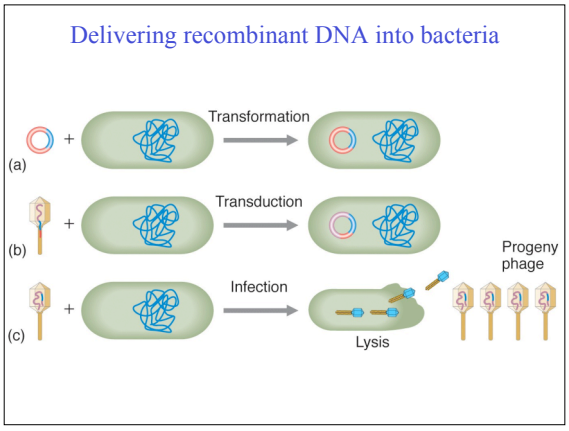


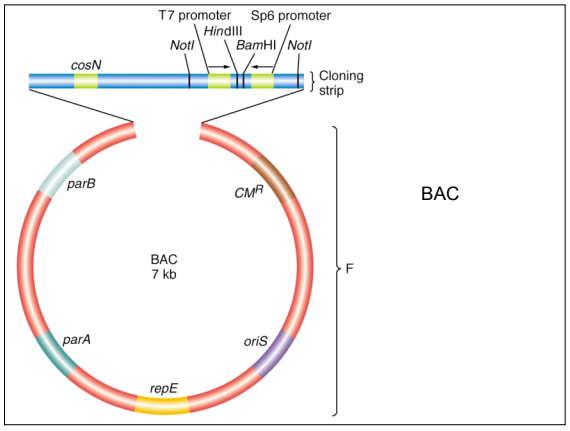


Lambda phage cloning



Genomic library into phages





BAC

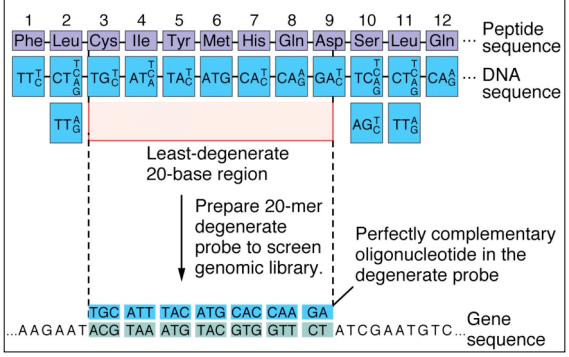
Identifying DNA molecules

- It is often a challenge to identify desired gene in library of thousands of clones
- Using nucleotide probes
  - principle based on base-pair complementarity
  - colonies or phage plaques are transferred to membrane, lysed and DNA is denatured
  - probe is applied to membrane
    - labeled with radioactive isotope or fluorescent dye
    - probe forms double helix with complementary DNA
  - hybrid DNA is identified in autoradiogram or by exposure to exciting wavelength of light

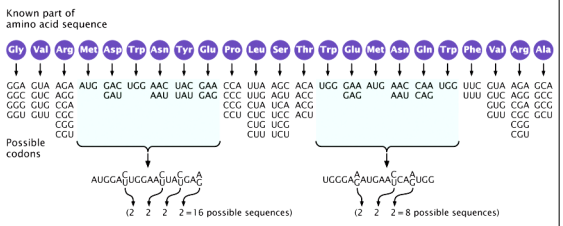
Nucleotide probes

- Multiple possible sources
  - previously cloned genomic DNA or cDNA from another species, tissue, etc.
  - PCR-amplified DNA
  - synthetic oligonucleotide
    - reverse translated from amino acid sequence, if known
    - synthesized by machine
  - RNA, such as rRNA or tRNA
- Hybridization of probe to complement is sensitive to temperature and salt

Finding the gene from the protein



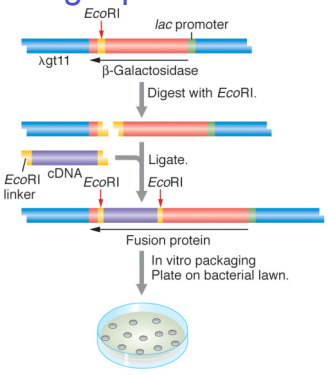
Designing a probe



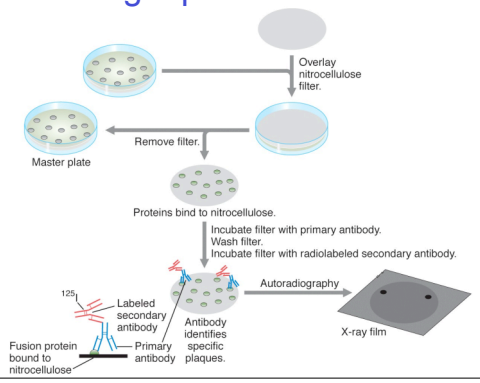
Probes for finding proteins

- Cloned genes, particularly cDNA, can be expressed and protein product detected
- cDNA is inserted into expression vector, designed to express insert at high level
- Membrane is laid over surface of colonies induced to express inserts
  - expressed proteins bind to membrane
- Membrane is probed with antibody against protein

Finding a protein of interest



Finding a protein of interest



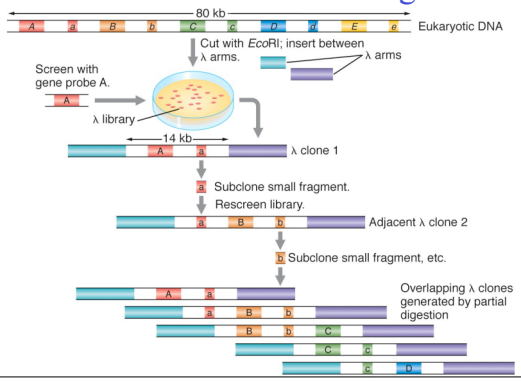
Functional complementation

- Also called mutant rescue
- Takes advantage of ability to perform transformation in many species
- Library is prepared from wild-type recombinant donor DNA
- Cells expressing recessive mutant gene are transformed with library
- Transformants (often obtained by selection) are examined for wild-type expression
- Wild-type allele is recovered from transformants, taking advantage of vector

Positional cloning

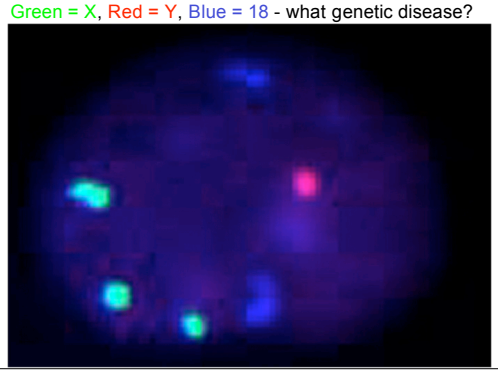
- Based on detailed genetic map, e.g., restriction map of chromosome or genome
- Also called chromosome walking
  - cloned landmark is used as probe to screen library for inserts that extend from the landmark and include DNA not in landmark clone
  - process repeated using newly isolated DNA
  - eventually obtain gene of interest

Chromosome walking

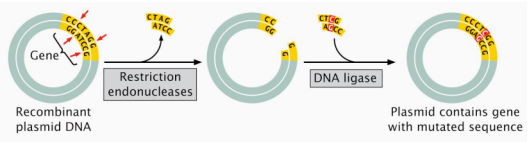


FISH

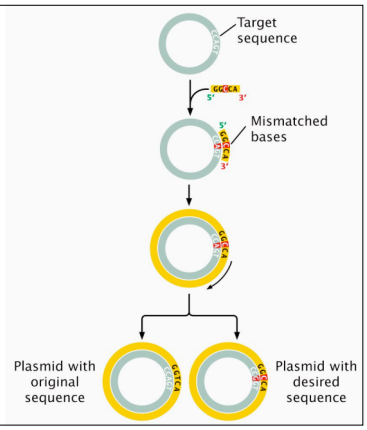
Green = X, Red = Y, Blue = 18 - what genetic disease?



Site directed mutagenis



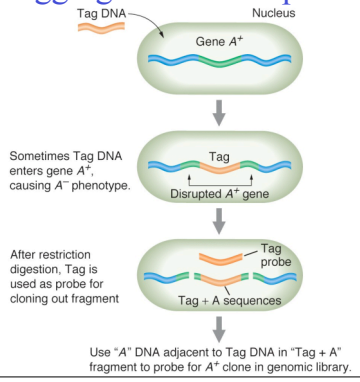
Oligonucleotide -directed mutagenesis

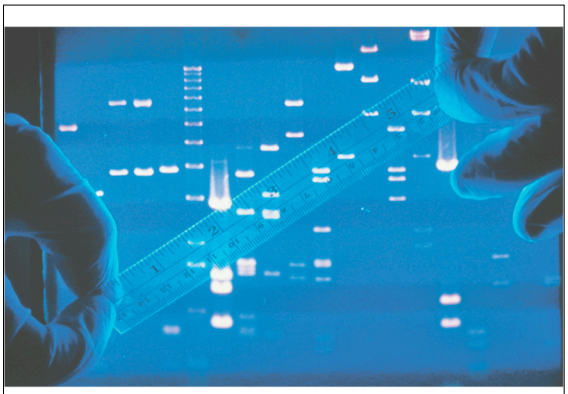


Gene tagging

- DNA, such as virus or transposon, is introduced into cells
  - acts as mutagen, randomly inserting into chromosomes as a "tag"
  - tag disrupts gene function, resulting in mutant phenotype
- Tag can be used to isolate flanking sequences (gene), e.g., through PCR, screening of genomic library, etc.

Tagging with a transposon





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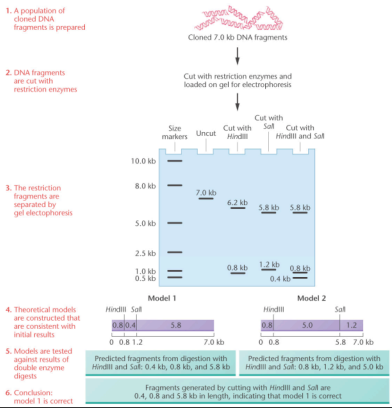
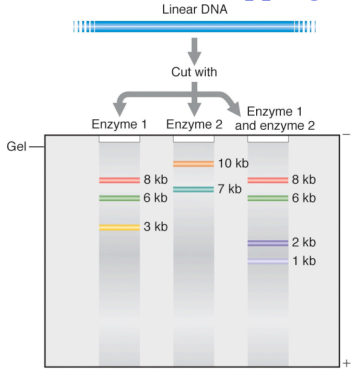
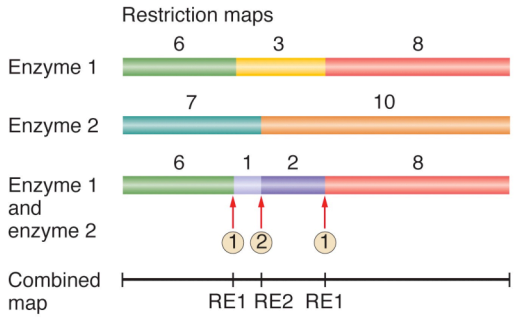


Restriction mapping



Restriction mapping



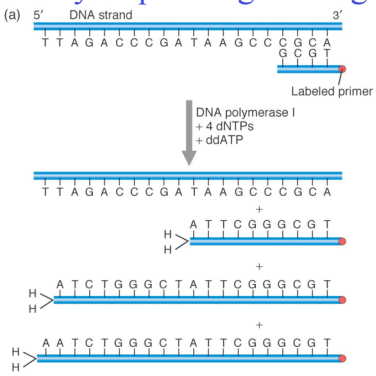
Sequencing of DNA

- Takes advantage of base-pair complementarity
- Dideoxy sequencing (Sanger method) is most commonly used
- General method
  - denature target DNA to form single-stranded DNA
  - hybridize primer to DNA
  - extend primer in mixture containing one or more dideoxynucleotides (ddNTP + dNTPs)
  - resolve resulting DNA fragments by electrophoresis
- Analyze sequence results, look for open reading frame
  - usually computer-assisted

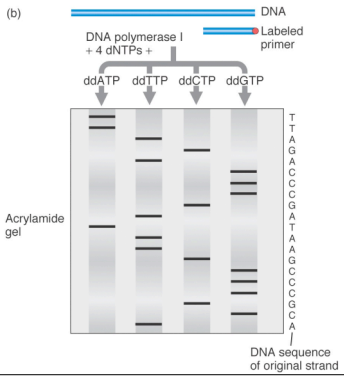
Dideoxynucleotides

- Hydroxyl group on 3' carbon of deoxyribose is replaced by hydrogen (hence dideoxy)
- Incapable of being extended by DNA polymerase; result in chain termination
- Sequencing mixture contains all dNTPs and one or more ddNTPs
  - random incorporation of ddNTP halts DNA synthesis
  - for given template, all possible fragments differing in length by single nucleotide are formed, resolvable by polyacrylamide gel electrophoresis
  - reaction can be labeled by radioactive ATP or by fluorescently tagged ddNTPs

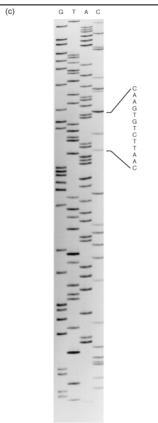
Dideoxy sequencing - "Sanger"



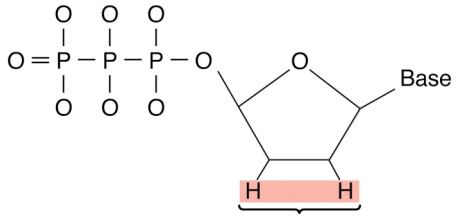
sequencing



Autoradiograph

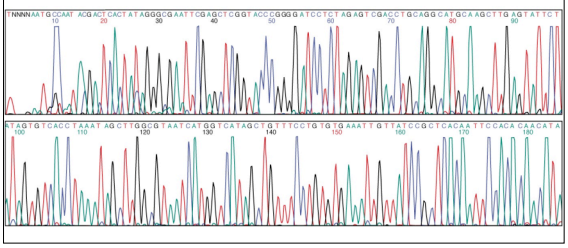


2',3'- dideoxynucleotides

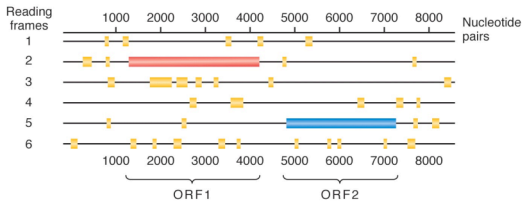


Cannot form a phosphodiester bond with next incoming dNTP

Fluorescent tags

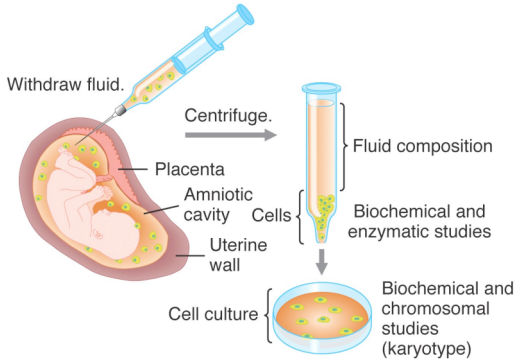


Computer analysis (ORF)

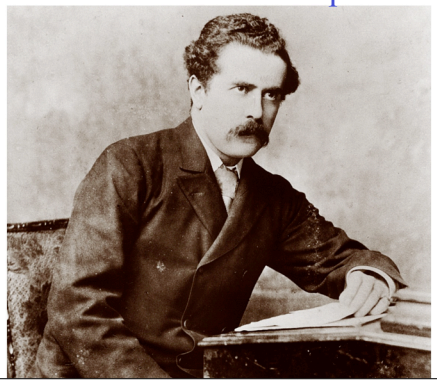


Applications

- Early detection of disease-associated alleles
  - fetal cells obtained by amniocentesis or chorionic villus sampling
  - combinations of PCR, restriction digestion, Southern blotting, and direct sequencing can identify homozygotes as well as heterozygotes
  - can identify SNPs, single nucleotide polymorphisms
- Genetic engineering
  - use of recombinant DNA technology to alter genotype of organism
  - engineered genes called transgenes are used to construct **transgenic organisms**
  - numerous applications in addition to study of genes

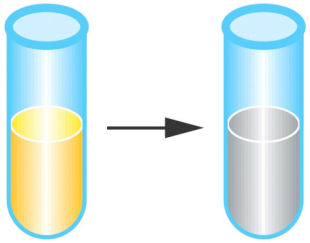


Archibald Garrod - alkaptonuria



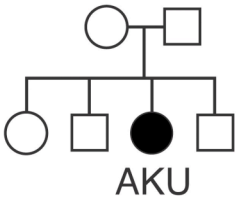
Homogentisic acid (homogetisate 1,2-dioxygenase

1. Black urine disease



Inherited

2. Mendelian recessive



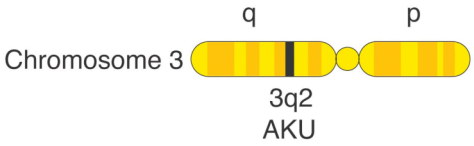
Homegentisate 1,2 dioxygenase HGO

3. Proposed enzyme deficiency

Homogentisic acid
↓ Enzyme ~~HGO~~
Maleylacetoacetic acid

1992 -mapped to the long arm, band 2

4. AKU gene mapped



1995-

5. HGO gene isolated from fungus *Aspergillus*

1996 - 52% similarity

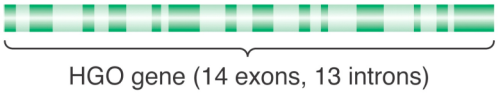
6. *Aspergillus* HGO finds human HGO cDNA.

Northern blot to find mRNA

7. HGO as probe finds mRNA in liver.

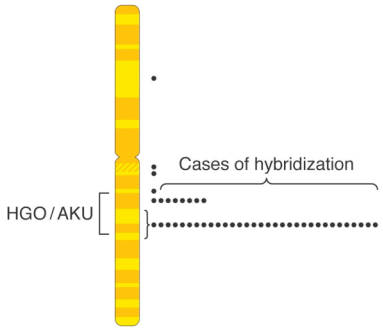
cDNA to find gene in genomic library

8. cDNA finds gene in λ genomic library.



Hybridization to chromosome 3

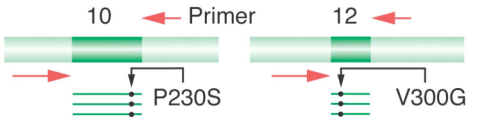
9. HGO clone hybridizes to 3q2.



PCR to find mutant sites in the gene

(proline-serine/valine-glycine substitutions)

10. PCR of exons 10 and 12 find mutant sites.



11. Inheritance of mutations

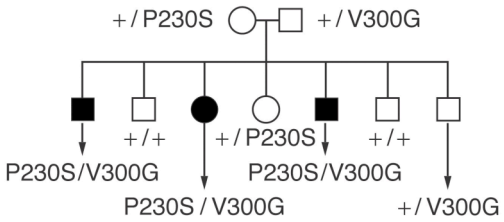


TABLE 8-1 Some Common Genetic Diseases

Inborn Errors of Metabolism	*Approximate Incidence among Live Births*
1. Cystic fibrosis	1/1600 Caucasians
2. Duchenne muscular dystrophy	1/3000 boys (X linked)
3. Gaucher disease (defective glucocerebrosidase)	1/2500 Ashkenazi Jews; 1/75,000 others
4. Tay-Sachs disease (defective hexosaminidase A)	1/3500 Ashkenazi Jews; 1/35,000 others
5. Essential pentosuria (a benign condition)	1/2000 Ashkenazi Jews; 1/50,000 others
6. Classic hemophilia (defective clotting factor VIII)	1/10,000 boys (X linked)
7. Phenylketonuria (defective phenylalanine hydroxylase)	1/5000 Celtic Irish; 1/15,000 others
8. Cystinuria (mutated gene unknown)	1/15,000
9. Metachromatic leukodystrophy (defective arylsulfatase A)	1/40,000
10. Galactosemia (defective galactose 1-phosphate uridyl transferase)	1/40,000
Hemoglobinopathies	*Approximate Incidence among Live Births*
1. Sickle-cell anemia (defective β-globin chain)	1/400 U.S. blacks. In some West African populations, the frequency of heterozygotes is 40%.
2. β-Thalassemia (defective β-globin chain)	1/400 among some Mediterranean populations

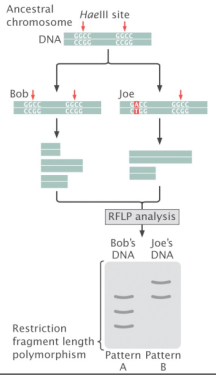
Note: Although a vast majority of more than 500 recognized recessive genetic diseases are extremely rare, in combination they constitute an enormous burden of human suffering. As is consistent with Mendelian mutations, the incidence of some of these diseases is much higher in certain racial groups than in others.

SOURCE: J. D. Watson, M. Gilman, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Scientific American Books. © 1992 by J. D. Watson, M. Gilman, J. Witkowski, and M. Zoller.

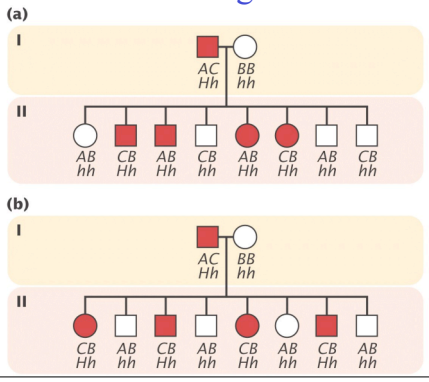
Sickle cell anemia

Type of Hb	Amino acid sequence Nucleotide sequence
A	—Pro—Glu—Glu— —CCT—GAG—GAG— *Mst*II
S	—Pro—Val—Glu— —CCT—GTG—GAG

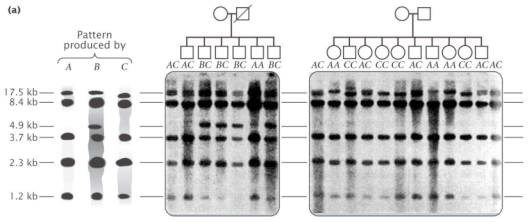
RFLPs



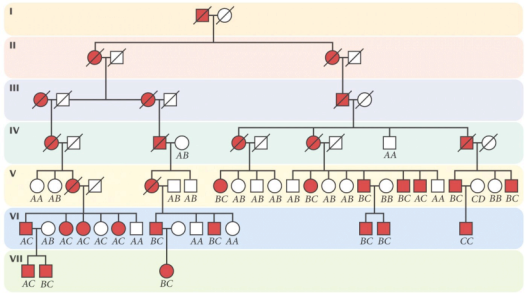
Linkage



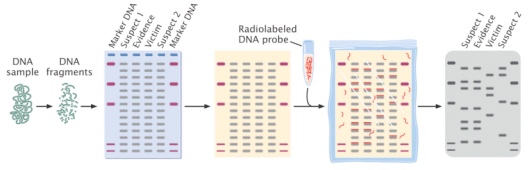
Huntington's disease RFLPs

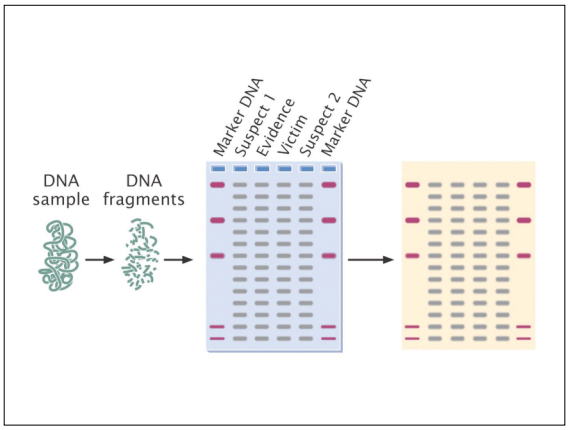


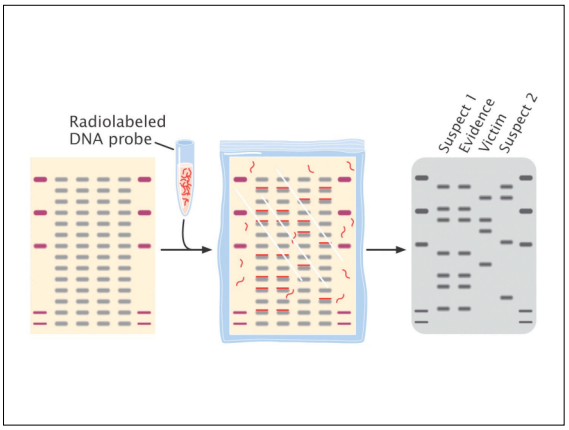
Huntington's disease RFLPs



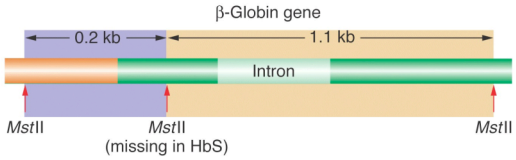
DNA fingerprinting



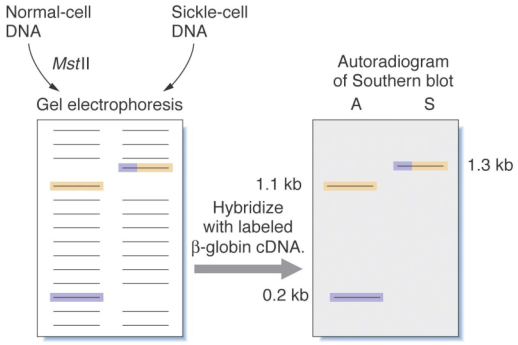




*Mst*II site missing in sickle cell

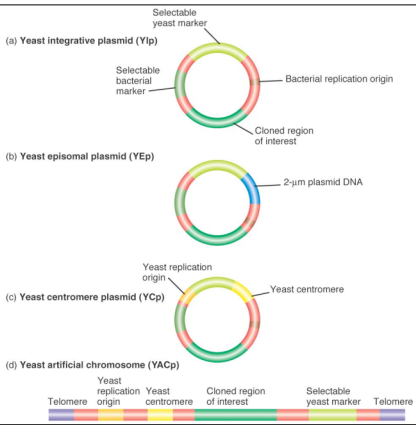


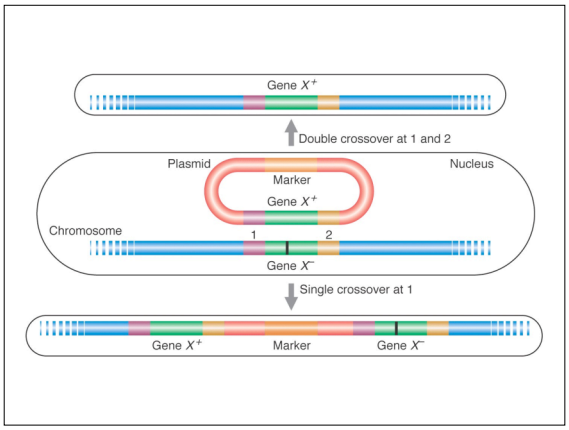
Southern blot analysis



Genetic engineering: fungi

- Mainly the yeast *Saccharomyces cerevisiae*
- Integrative plasmids (YIps)
  - derived from bacterial plasmids
  - have yeast sequences that facilitate homologous recombination with yeast chromosomes
- Autonomously replicating vectors, e.g., shuttle vectors with both bacterial and yeast origins of replication
- Yeast artificial chromosomes (YACs)
  - derived from plasmid with centromere and origin of replication
  - widely used for cloning large genomes

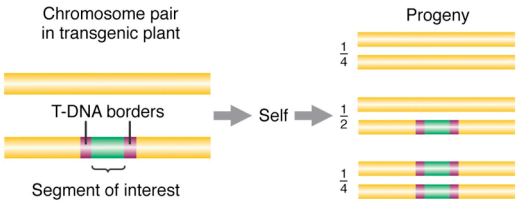




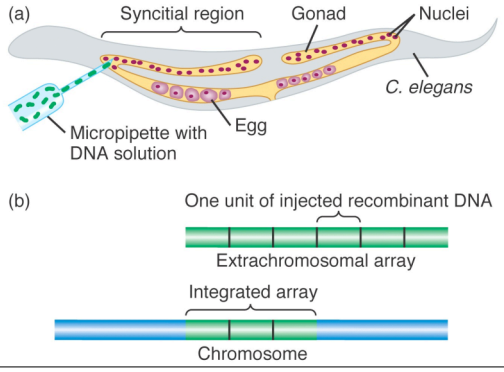
Genetic engineering: plants

- Considerable agricultural importance
- Considerable controversy regarding health and environmental safety
  - recombinant plants often referred to as GMOs, genetically modified organisms
- Two major methods for transformation
  - Ti plasmid from *Agrobacterium tumefaciens*
    - upon infection of plant with bacteria containing recombinant Ti plasmids, plasmids are transferred and inserted into host plant genome
    - plasmid itself is genetically modified to include polylinker (multiple restriction sites) and drug resistance genes
  - gene gun to inject DNA-coated micropellets into cells

DNA insert inherited by Mendelian patterns



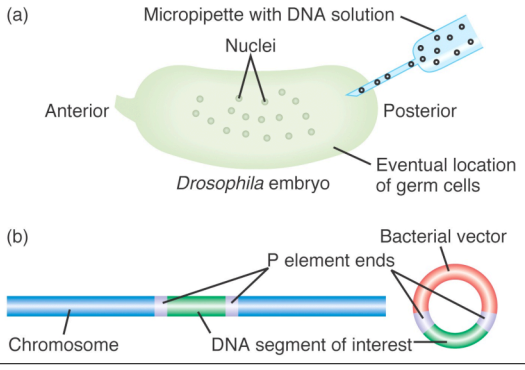
Microinjection in animal cells



Genetic engineering: animals

- Numerous model systems and applications
- *D. melanogaster*
  - transformation using plasmids derived from P transposable elements
    - one recombinant containing ends of P element needed for insertion flanking the cloned DNA
    - one containing P transposase to allow integration
  - injected into posterior pole of syncitial egg
  - recombinant vector integrates into host chromosomes
  - genes recovered in F_1 progeny of injected individuals

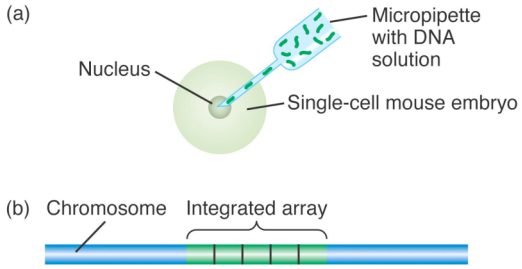
Transposable element in *Drosophila*

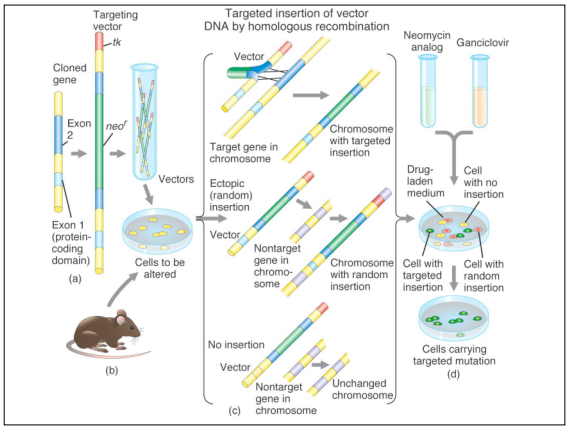


Genetic engineering: mice

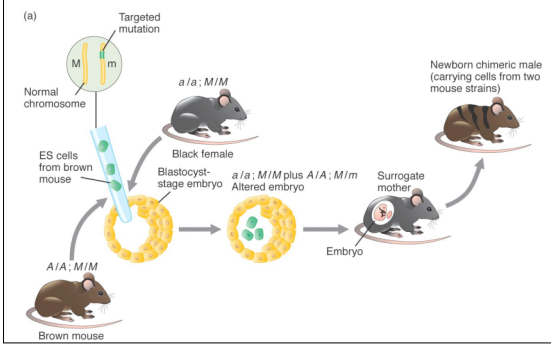
- Technology developed for mice is potentially applicable to humans
- Ectopic insertions and gene targeting
  - pros and cons to each method
    - e.g., ectopic insertion may place gene in chromosomal location where its expression is affected, phenomenon called position effect
  - targeted gene replacement is common tool
    - gene knockout (KO) replaces active gene in entire organism with inactive version
    - KO often prepared using genetically modified embryonic stem cells

Microinjection and targeted integration in mice

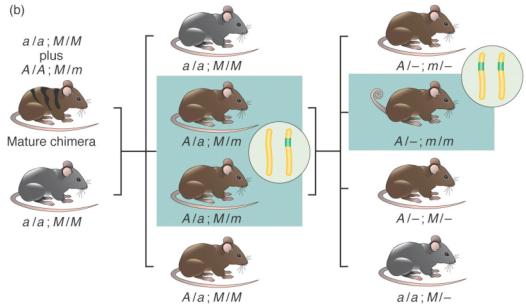




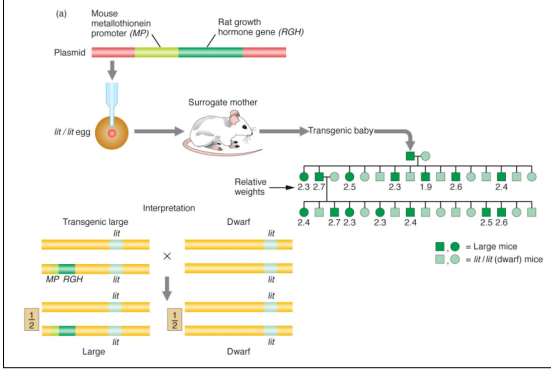
Knockout mice / chimeras



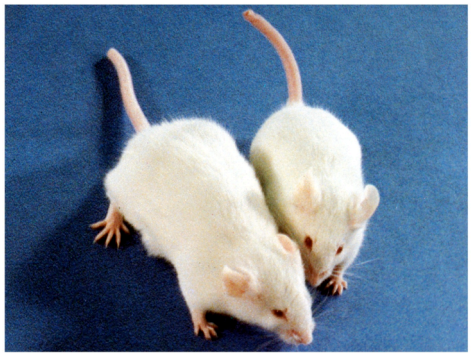
Knockout mice



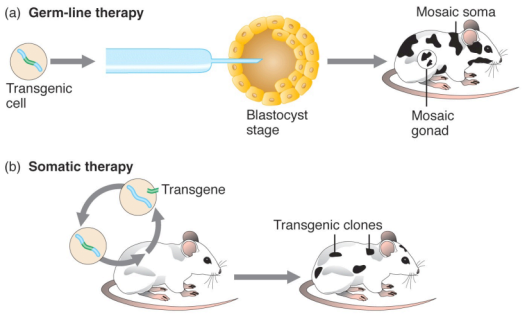
Mouse gene therapy



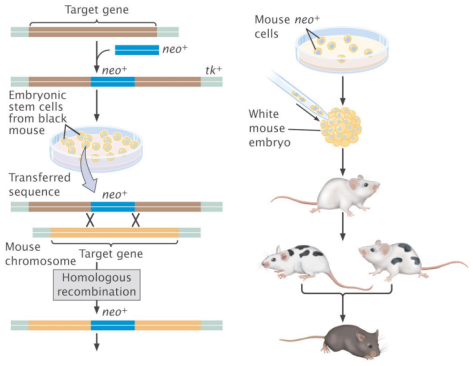
(b)



Gene therapy



Knockout mice



Genetic engineering: humans

- Gene replacement therapy or gene therapy
- Many technical and ethical issues
  - implications for gene pool for germ-line gene therapy
  - what traits constitute disease rather than just a characteristic
  - risk versus benefit
- Germ-line gene therapy currently impractical
- Somatic gene therapy in clinical trials

Overview

- Recombinant DNA technology exploits features of genes, gene expression, and DNA enzymology to create novel DNA molecules for study.
- Foreign DNA is spliced into a vector for amplification, producing a clone of the inserted DNA.
- Restriction endonucleases cut DNA at specific target sites.
- Polymerase chain reaction (PCR) can be used for specific DNA amplification.
- Labeled single-stranded DNA or RNA can be used as a probe to identify molecules containing its base-pair complement.
- Virtually any nucleotide sequence, including restriction sites can be mapped.
- DNA can be sequenced.
- Transgenes can be constructed and expressed in foreign hosts.