

Lecture 6:

DNA Technology

Part A:

- How DNA molecules are analyzed
 - Restriction enzymes
 - Gel electrophoresis
 - DNA sequencing
 - DNA Hybridization
 - Polymerase Chain Reaction (PCR)

Alberts *et al*, Chap 10

How DNA Molecules Are Analyzed

Bacterial enzymes: restriction nucleases -

Nuclease: catalyzes hydrolysis of phosphodiester bond between nucleotides at particular sites determined by a short sequence of nucleotides

-> specific DNA fragments from a genome

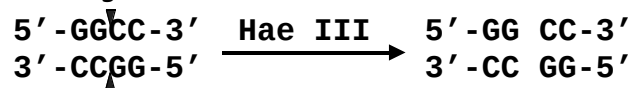
Look at how these DNA fragments are produced, separated, how the order of nucleotides in such DNA fragments are determined

Restriction nucleases cut DNA molecules at specific nucleotide sequences

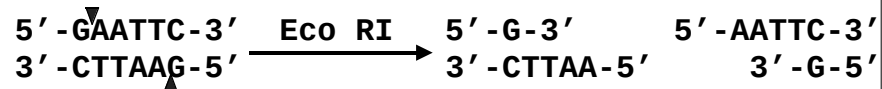
- Found in bacteria degrading DNA that was experimentally introduced into them (Transformation).
 - Thus restricting transfer of DNA between certain strains of bacteria -> restriction nucleases
 - Bacteria's own DNA protected by chem modification of these seqs
- Different bacterial species contain different restriction nucleases
 - each cutting at a different, specific sequence of nucleotides

Examples of restriction enzymes and their cleavage sites:

Cleavage site



Blunt ends



Flanking ends

- Restriction Nuclease synonymous with Restriction Enzymes (RE)
- Target sequences are often palindromic (symmetrical around a certain point)
- Restriction nucleases are usually obtained from bacteria
- Their names reflect their origins - E.g. Eco RI comes from *Escherichia coli*

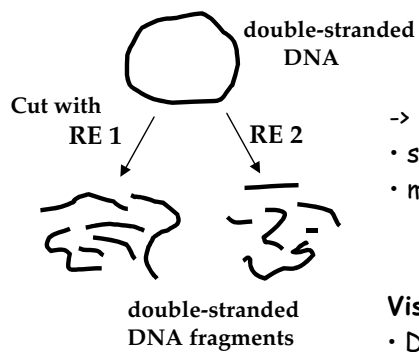
- Target seqs of RE vary in frequency with which they will occur in DNA.
- Average size of DNA fragments produced by different RE varies.
- Thus can cleave a long DNA molecule into the fragment sizes suitable for each particular application.

Example:

Hae III $\begin{array}{l} 5' - \text{GGCC} - 3' \\ 3' - \text{CCGG} - 5' \end{array}$ Cuts seq of 4 bps in length so expect seq occur by chance approximately once every 256 nuc pairs (1 in 4⁴)

Eco RI $\begin{array}{l} 5' - \text{GAATTC} - 3' \\ 3' - \text{CTTAAG} - 5' \end{array}$ Target seq is 6 nucs in length, expect to cleave DNA on average once every 4,096 nuc pairs (1 in 4⁶)

Separation of DNA Fragments using Gel Electrophoresis

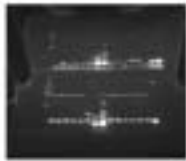
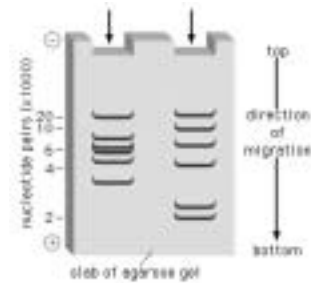


Separation according to size

- Fragments separated by gel electrophoresis
- separation according to size: smaller move faster
- movement influenced by electric field
 - move to positive electrode
- *DNA is negatively charged ($-PO_4^-$)

Visualization:

- DNA intercalating dye: ethidium bromide (EtBr)
 - fluoresces under UV light
- Autoradiography.....



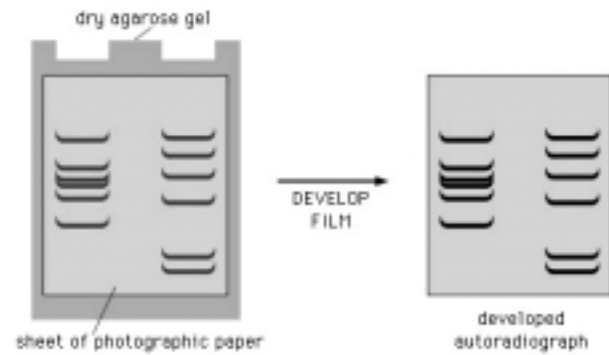
Agarose: contains network of pores

DNA fragment is isolated

→ cut gel section

Fig 10.5

Note: Intercalating agents are hydrophobic & planar fitting b/w DNA bps



Autoradiography: used in sequencing

DNA is "labeled" with radioisotope ^{32}P by substituting ^{32}P for some of the nonradioactive P atoms of DNA

e.g. replicating bacterium/virus in presence of ^{32}P

The β particles emitted from ^{32}P will expose photographic film, so a sheet of film is placed on top of the agarose gel

Develop film → shows the position of all the DNA bands

Why do this? Radioactivity gives a stronger signal than visual methods.

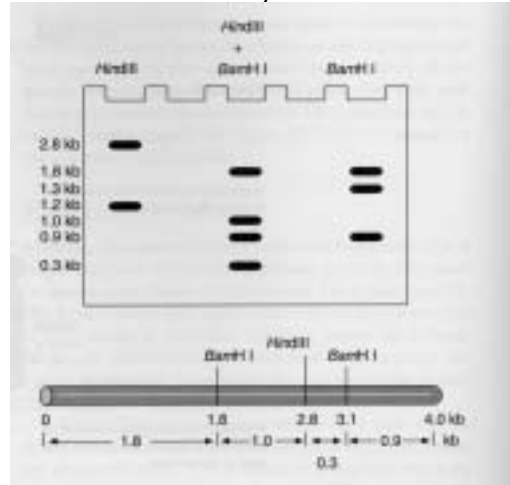
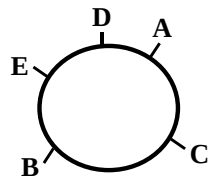
Construction of Physical maps of small DNA regions

- characterizes a stretch of DNA by charting the landmarks along it
- RE cleavage sites!

How ?

Compare sizes of DNA fragments produced from a particular region of DNA after treatment (digestion) with different combinations of REs.

Physical map (Restriction Map) is constructed showing location of each cutting site.



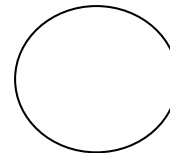
Try this....

Restriction enzyme A cleaves a 3kb plasmid resulting in two fragments, 1kb and 2kb.

Restriction enzyme B digests this same plasmid into one fragment of 3kb.

When used together, restriction enzyme A and B digest this plasmid resulting in three fragments of 1.0 kb, 1.2kb and 0.8 kb in size.

Draw the restriction map of this plasmid for these restriction enzymes.



Restriction map: sites where REs can cut depends on nuc seq of DNA
 -> so reveals differences between even closely related DNA molecules

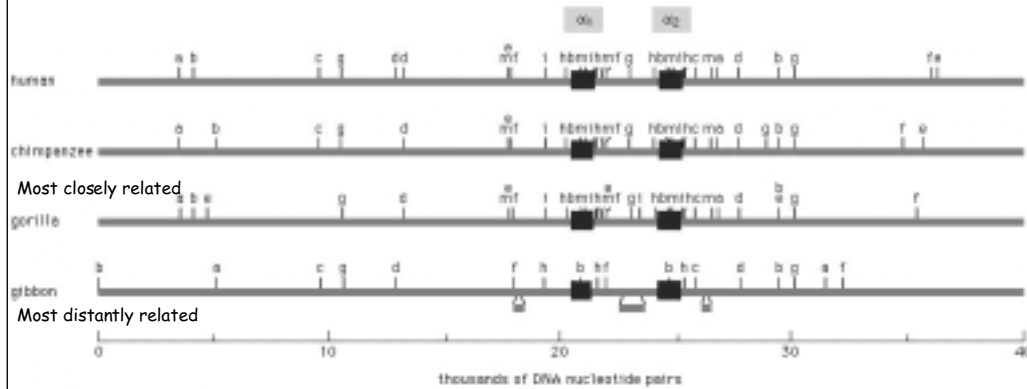


Fig 10.4

Comparison of restriction maps of DNA (—)containing α -globin genes (■)
 Comparing humans to 3 primates
 Letters (a,b,c,etc) denote sites cut by different REs

Average Fragment Sizes Generated by Endonuclease Cleavage....

Enzyme	Sequence	E. coli	Mycobacterium tuberculosis	Saccharomyces cerevisiae	Drosophila melanogaster	Mus musculus	Homo sapiens
Apa I	GGGCCC	68,000	30,000	15,000	13,000	5,000	5,000

However, if nucleotides are randomly distributed, you would get a 6 bp cut every 4096 (4^6) nucleotides

E.g of commercially available restriction enzyme.....

EcoR I

#R0101S 10,000 units \$50 (USA)
 #R0101L 50,000 units \$200 (USA)
 for high (5X) concentration, order #R0101T (10,000 units) or #R0101M (50,000 units)

5' ... G^A A T T C ... 3'
 3' ... C T T A A^G ... 5'

Source: An E. coli strain that carries the cloned EcoR I gene from E. coli RY 13 (R. N. Yoshimori)

Reaction Buffer: (Supplied with enzyme) NEBuffer EcoR I

50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl₂, 0.025% Triton X-100 (pH 7.5 @ 25°C). Incubate at 37°C.

Ligation and Recutting: After 100-fold overdigestion with EcoR I, > 95% of the DNA fragments can be ligated and recut.

Concentration: 20,000 and 100,000 units/ml. Assayed on lambda DNA.

Storage Conditions: 300 mM NaCl, 10 mM KPO₄ (pH 7.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.15% Triton X-100, 200 µg/ml BSA, and 50% glycerol. Store at -20°C.

Diluent Compatibility: Diluent C

Heat Inactivation: 65°C for 20 minutes.

Note: Conditions of low ionic strength, high enzyme concentration, glycerol concentration >5%, or pH >8.0 may result in star activity.

You used to have to make your own enzymes, now you buy them.

DNA Sequencing

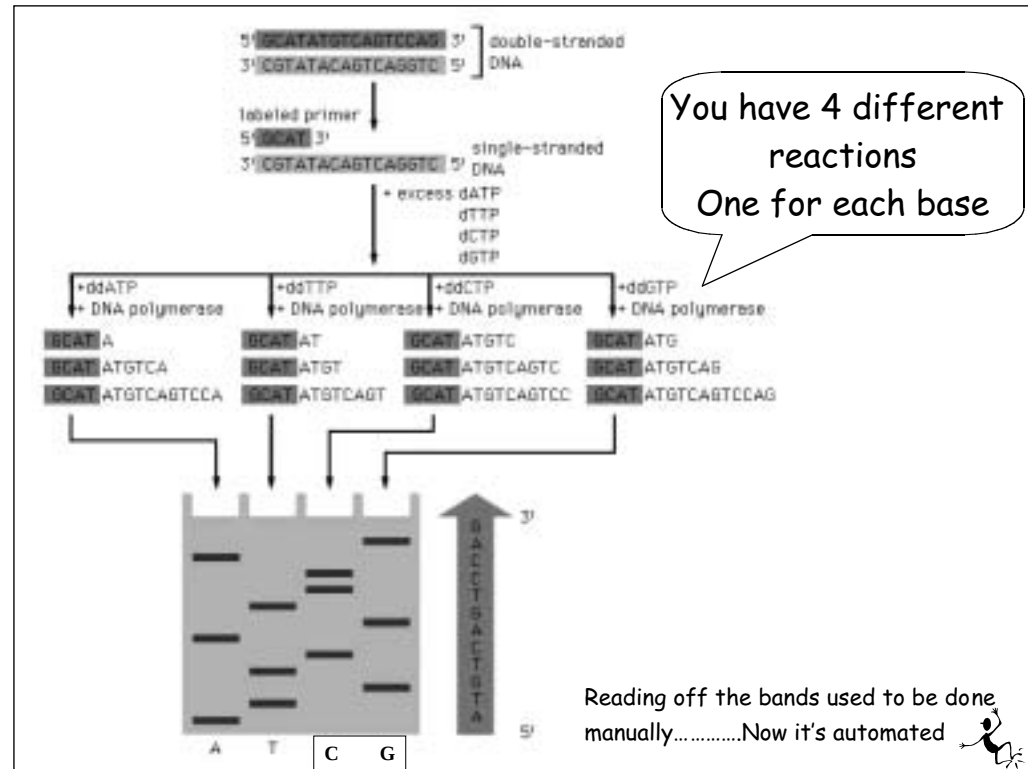
Some notes:

- Ultimate physical map of a DNA: its complete nucleotide sequence
- Methods developed in late 1970's
- Uses DNA polymerase to partially copy DNA fragment to be sequenced
- New DNA strands terminate when a given nucleotide is reached
- Produces collection of different DNA copies that terminate at each position in the original DNA, thus differ in length and separated based on length by gel electrophoresis. The nucleotide sequence of the original DNA can be determined by the order of these DNAs in the gel

The enzymatic method of sequencing DNA

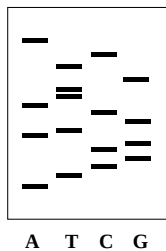
- Need 4 termination mixes of ddNTP's
- Also regular dNTP's
- DNA single stranded

- Other requirements:
 - Labeled DNA primer
 - DNA polymerase



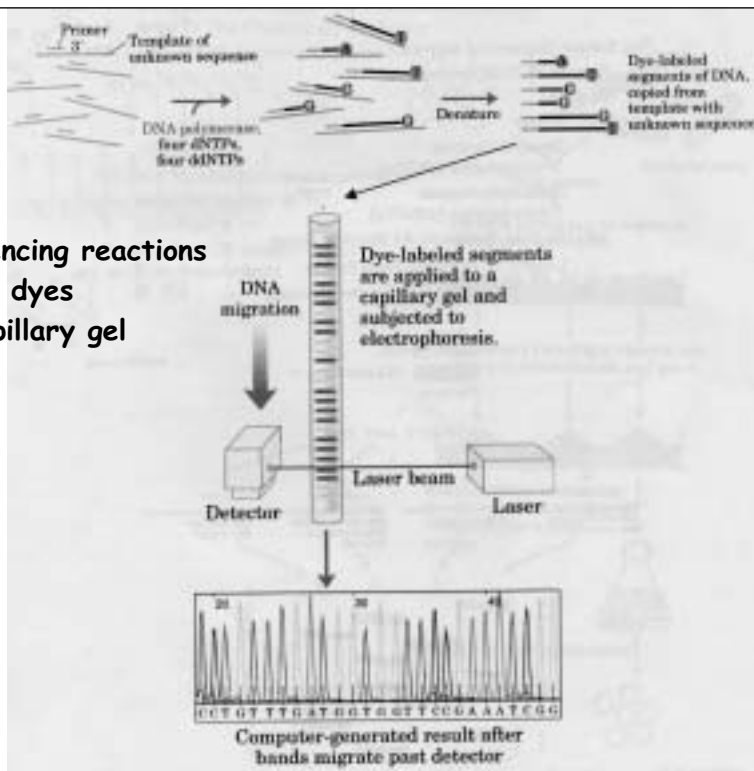
Try this....

1. What is the sequence of this cDNA cloned from a mouse?
2. What protein sequence does this sequence translate to?



Outline of Sequencing Method:

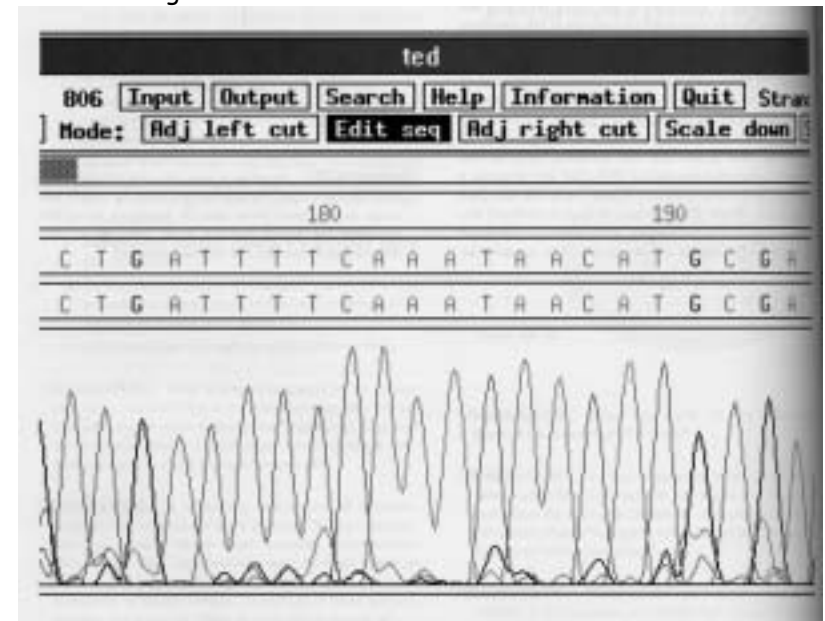
- Double stranded DNA is separated into single strands.
- One strand is used as template.
- Four separate chain-terminating ddNTPs mixes.
- Each reaction produces a set of DNA copies that terminate at different points in the seq.
- Products of four reactions are separated by electrophoresis in 4 lanes (A, T, C, G).
- The fragments are detected by a label (radioactive or fluorescent) incorporated either into primer or into one of the dNTPs used to extend the DNA chain.
- In each lane the bands represent fragments terminated at a given nucleotide (e.g A) but at different positions in the DNA.
- By reading off the bands in order from the bottom of the gel & working across all lanes, can determine the DNA seq of the newly synthesized strand.
- Sequence is identical to the 5' → 3' strand of the original double stranded DNA.



Automated sequencing reactions using fluorescent dyes
Separation in capillary gel

Representation of Results:

- Electropherogram
- String of nucleotides



Genomes: sequence specifies all possible RNA & protein molecules used to make the organism -> info to speed up future research of all kinds

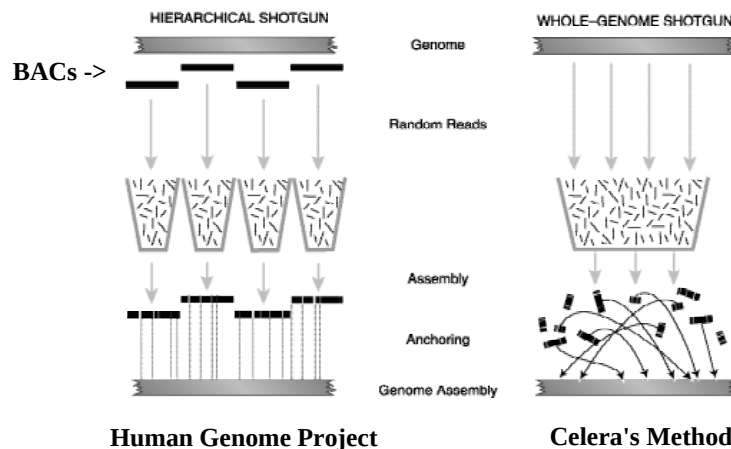
Databases of DNA seq info are huge

-> need advanced software to organize & analyze

e.g University of Wisconsin GCG package

Bioinformatics: term used to describe the development & use of such software to analyze biological data

Human genome is distributed over 23 pairs of chromosomes



Scientists are still battling over whether Celera's sequencing approach is superior to the public consortium's.

Now if the gene is isolated.....

.....Questions to answer:

- From which chromosome?
- Where on the chromosome is it located?
- In which cells is the gene transcribed?
- Do other organisms have similar genes?
- Test a DNA sample for mutations in the gene suspected of causing inherited disease?

Nucleic Acid Hybridization

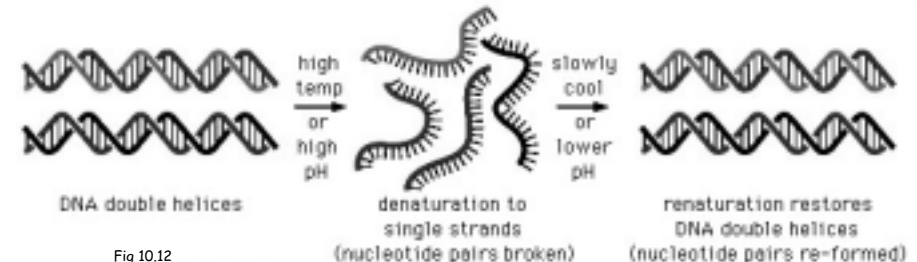
Know DNA forms Watson-Crick base pairs -> double helix

DNA strand pairs highly selectively with complementary (nuc seq) strand.
Two strands held together by weak H bonds.

H bonds can be broken by heating the DNA to around 90°C, or extremes pH
-> 2 strands are separated without breaking covalent bonds linking nucleotides together within each strand.

Reversing process: decreasing temp to body temp, or bringing pH back to neutral
-> complementary strands reform double helix (re-anneal)
- Process called renaturation, hybridization

So ability of ss nucleic acid molecule to form double helix with a molecule complementary to it is a tool to detect specific nuc. seqs in both DNA & RNA



Examples of uses of Nucleic Acid Hybridization:

DNA Hybridization Requirements:

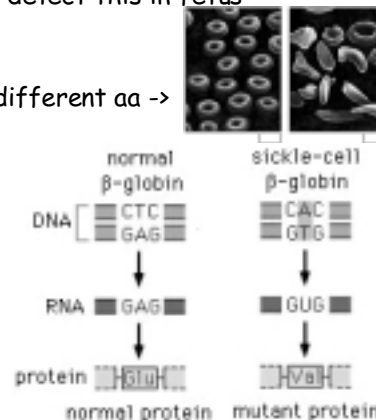
- DNA probe: short single strand (ss) DNA 10-1000 nucleotides long
- Oligonucleotides: short single-stranded DNA chemically synthesized

DNA Hybridization Used In Prenatal Diagnosis of Genetic Diseases:

> 3000 different human genetic diseases caused by mutations in single genes.

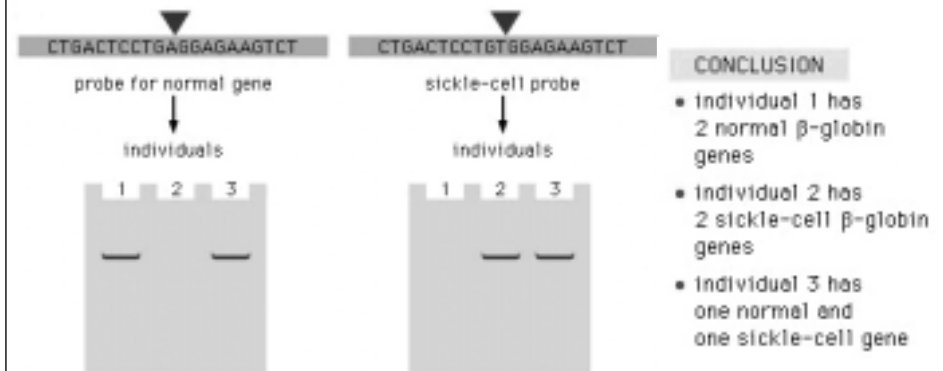
Recessive mutation: not evident phenotypically unless person inherits 2 defective copies of the gene (one from each parent) - need to detect this in fetus
Need sample from fetus.

E.g Sickle cell anemia: single nuc mutation A->T --> different aa -> defective β -globin gene; recessive



Basic procedure:

- DNA sample from fetal cells is treated with REs
- All fragments are electrophoresed through gel
- Then gel is treated with DNA probe that detects only the restriction fragment carrying β -globin gene.
- Two oligo-probes: one to normal sequence & one to mutant sequence
- Probes are labeled with either radioactive isotopes or with a fluorescent dye incorporated when manufactured



Method to visualize the hybridization: Southern Blotting

Digestion of DNA molecule:

- DNA + REs → double strand (ds) DNA fragments.
- Fragments are separated by electrophoresis.

Transfer of DNA onto NCF (in order to keep DNA firmly in place):

- Gel supported on sponge in bath of alkali solution.
- Sheet of nitrocellulose paper/filter (NCF) is laid over gel.
- Separated fragments transferred to NCF by blotting.

as the buffer is sucked through the gel and NCF by paper towels stacked on top of NCF.

- Buffer denatures DNA and transfers ss fragments from gel to NCF where they adhere firmly.

Hybridization:

- NCF is placed in sealed plastic bag + radioactively labeled DNA probe specific for required DNA seq. Incubate.
- NCF is removed from bag, washed to remove free probe.
- Autoradiography: visualize bands: DNA hybridized with labeled probe

Southern Blot Hybridization

Gel Electrophoresis:

Separates DNA fragments based on size

Southern Blot Hybridization:

Probes for specific DNA fragments

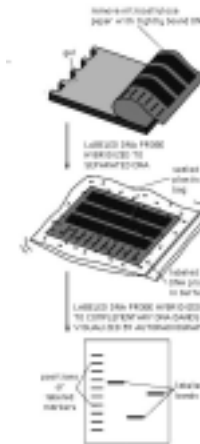
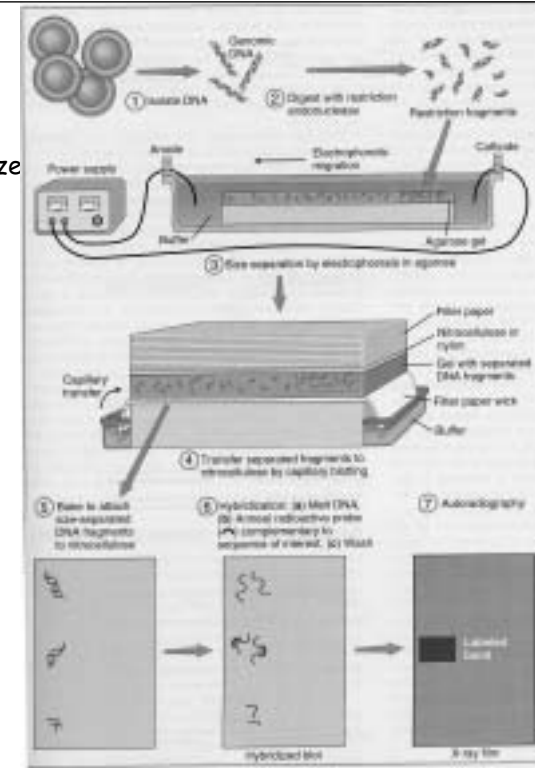


Fig 10.14



Northern Blotting:

Detection of specific RNA sequences

- mRNA molecules are electrophoresed through gel
- Probe is usually single strand DNA

Another Example: Detection of individuals susceptible to disease

Inheritance of abnormal copies of a DNA mismatch repair gene

→ cannot repair mistakes

E.g. increased risk of colon cancer

→ Patients need to take protective measures

In Situ Hybridization

Locates nucleic acid sequences (DNA or RNA) in cells or on chromosomes

Latin, *in situ* "in place"

Chromosomes: briefly exposed to very high pH to separate double strand (Fluorescent probe)

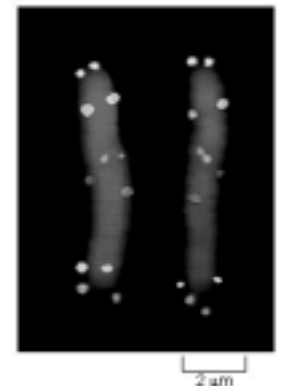
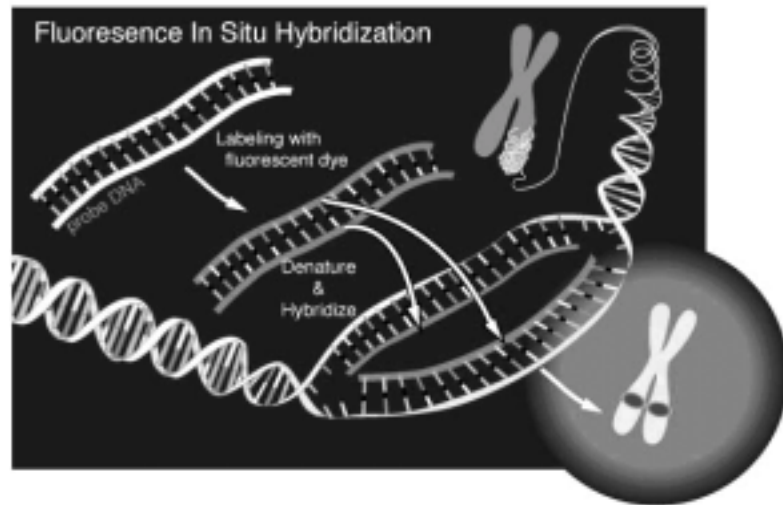


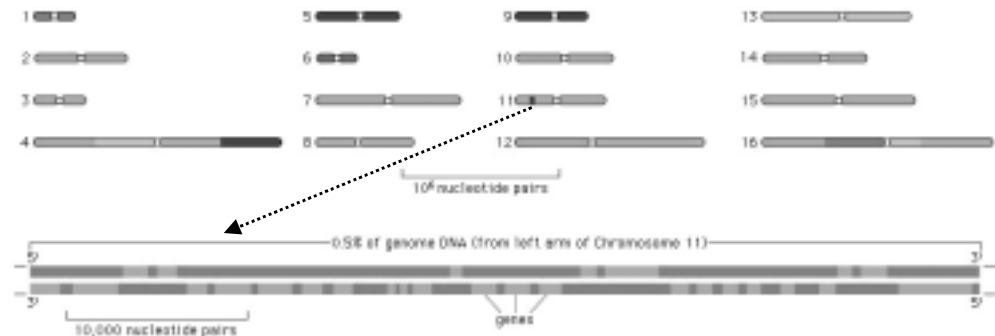
Fig 10.10 Human chromosome 5 in metaphase of mitosis.
Probe → 2 dots on each chromosome because already replicated DNA

Fluorescence In Situ Hybridization (FISH):



Example of Complexity:

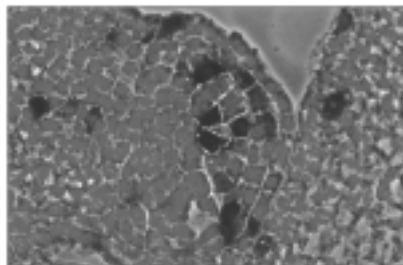
Representation of complete nucleotide sequence of *S. cerevisiae* (budding yeast)
Involved many scientists from all parts of the world to seq genome



Note: high density of genes; genes transcribed from different strands
About 6200 genes in genome -> 12,147,813 bps long!!!

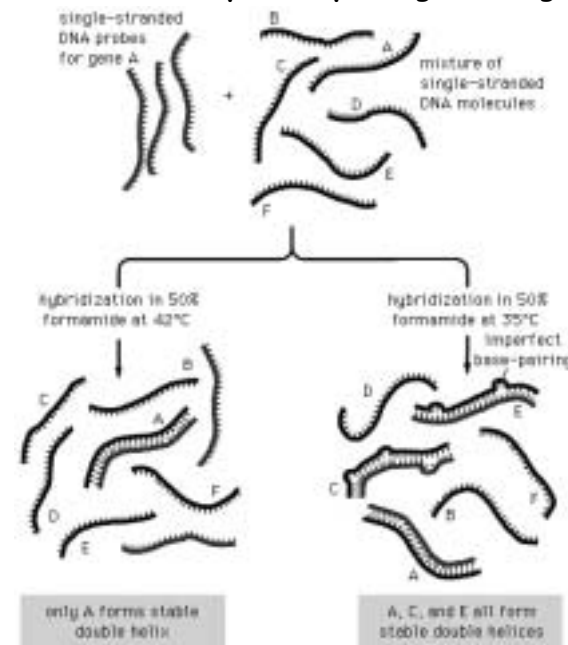
Study of cells

-> info on gene expression (mRNA) without disturbing cell content too much



Dark blue stain indicates mRNA expression for protein X
Cells treated with DNA probe to protein X mRNA
Probe linked to enzyme that produces dark blue reaction product
Light blue stain: DNA stained with dye DAPI

Use of hybridization to identify genes of the same family or even another species (probing human genome with mouse gene).....



Different hybridization conditions allow imperfect match between probe and DNA to form stable double helix

What if limited material to work with?

What if searching for a sequence of DNA in a large piece of DNA, such as genomic DNA?

Polymerase Chain Reaction

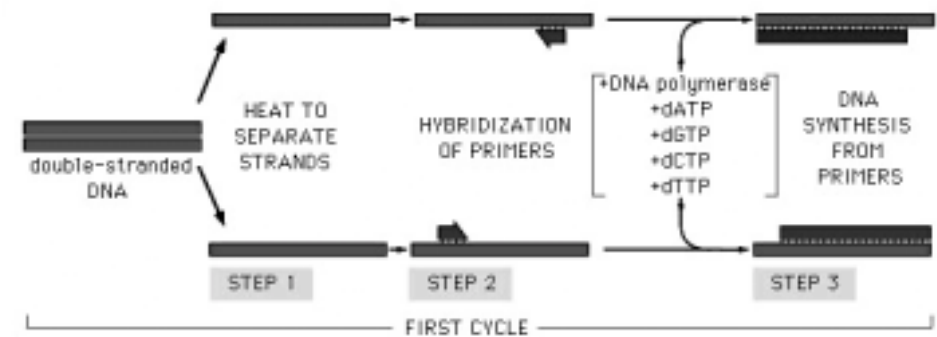
Used to amplify selected DNA sequences

Need: Template (DNA or RNA), primers, DNA polymerase, dNTPs

Basic method:

- ds DNA → ss by heating
- Primers hybridize at lower temp
- Nucleotides are added by DNA polymerase → new strand
- Increase temp → ss DNA
- Cycle continues

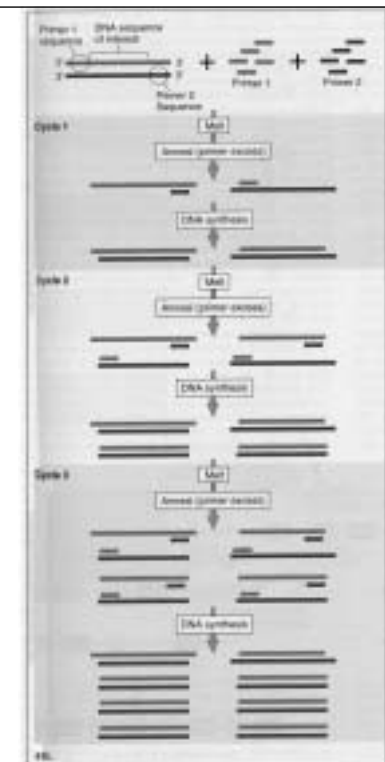
Fig 10.27



- Each cycle doubles amount of DNA synthesized in previous cycle....

Polymerase Chain Reaction

Used to amplify the number of copies of a specific sequence of DNA for either preparative or analytical purposes



PCR:
used to clone directly a DNA fragment
(make identical copies)

E.g. gene from cell

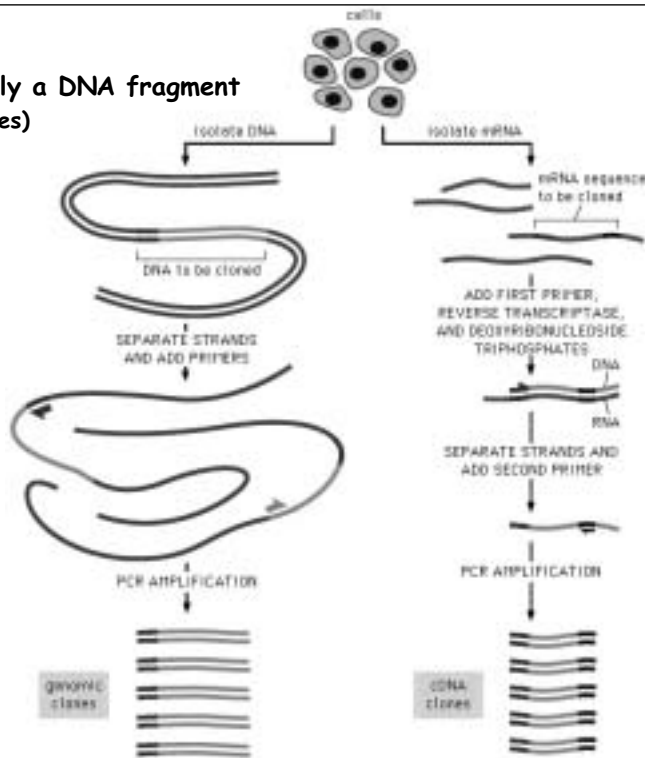


Fig 10.28

PCR: used to detect viral infection at early stage.....

- very sensitive method of detection

Primer: short seq to viral genome

Sample: blood

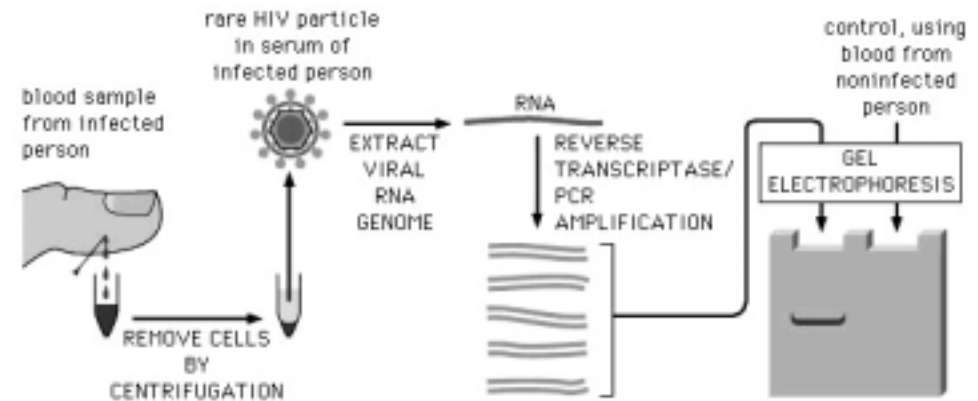


Fig 10.29

PCR: used in forensic medicine

--> DNA fingerprint at crime scene

Primer: carefully selected
pair of primers of known
highly variable parts of
human genome

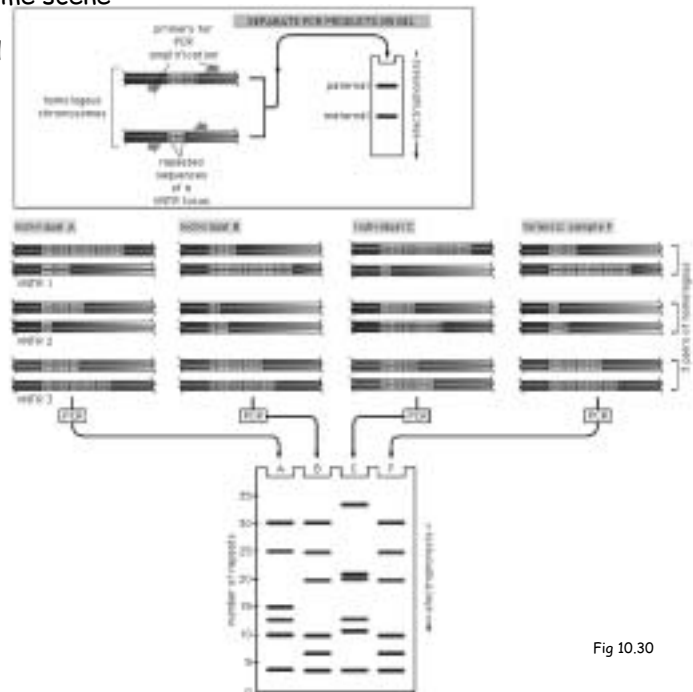


Fig 10.30

**PCR movie
and
Cycle Sequencing movie**

Lecture 6: Part B

DNA Technology

Part B:

- How DNA molecules are analyzed
 - Restriction enzymes; Gel electrophoresis; DNA sequencing
 - DNA Hybridization; Polymerase Chain Reaction (PCR)
- Bacterial Genetics as a means for studying DNA
 - Why & How
 - Viruses vs. Bacteria
- DNA cloning
- DNA Engineering
 - New DNA molecules
 - Transgenic technology

Alberts et al, Chap 10, 6

Bacterial Genetics

Bacterial cells duplicate quickly: 20-25 mins
24hrs → more descendants than humans on earth!

E. coli genome:

single (haploid) circular DNA of $\sim 4.6 \times 10^6$ bps encoding $\sim 4,000$ proteins

Need the right growth conditions: media/medium

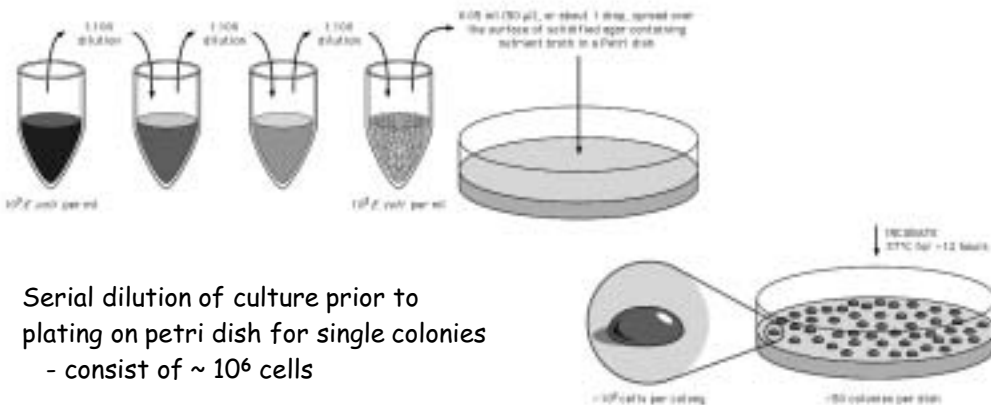
media contains: amino acids, sugars, salts; aerated (O_2) temp $37^\circ C$

Media: liquid form "broth": cell growth indicated by turbidity

solid form "agar": cell growth seen as single colonies visible to the eye

agar: inert polysaccharide from seaweed, solidifies at room temp

Colonies of bacteria



Mutations in bacteria can be selected by a change in environmental conditions

Culture medium + Antibiotic Rifampicin (binds RNA pol, prevents transcription)

Bacterial Culture A + rifampicin → death Rifampicin Sensitive

Bacteria Culture B + rifampicin → growth Rifampicin Resistant

Colony from Bacterial Culture A

→ cultured in liquid media

→ Growth!

.....rare mutants grow

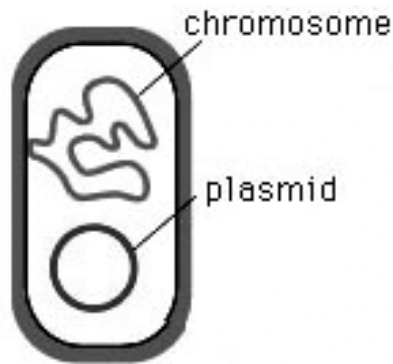
Every large bacterial population contains the predominant genetic type (e.g. wildtype) And a pool of mutant bacteria providing genetic variants.

→ Over time mutations are created/selected in response to environmental conditions

→ Under selective pressure any rare individuals better able to survive will proliferate (grow)

Bacterial plasmids:

- small, circular, double-stranded DNA molecules
- separate from the larger bacterial chromosome
- contain own replication origins -> can replicate independently



Bacterial Cells can Acquire genes from other Bacteria

Transfer of genetic material by **bacterial mating: conjugation** of two cells

Not all bacteria can initiate mating: ability is by genes in **plasmids**

Only a few plasmids can initiate mating and DNA transfer

e.g. *E. coli* - F (fertility) plasmid initiates conjugation

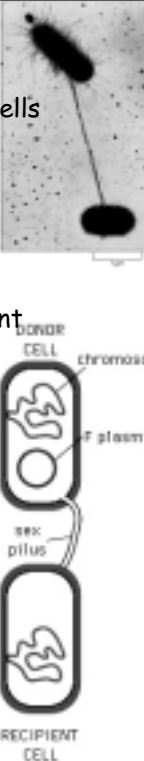
with bacterium (recipient) lacking F plasmid

Transfer of F plasmid DNA via cytoplasmic bridge (pilus) to recipient (genes to make pilus are found in F plasmid)

Transfer of small variety of genes: most concerned with mating

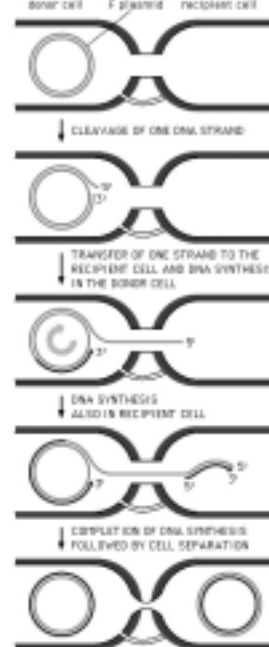
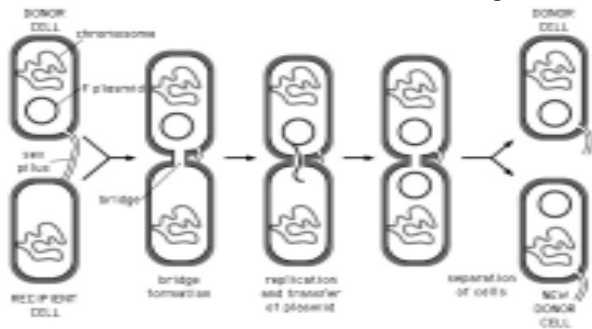
BUT F plasmid can become part of bacterial chromosomal DNA

via integration -> during mating transfer much of chromosomal DNA



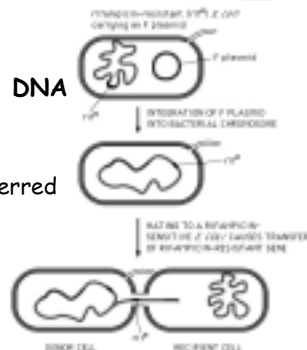
Bacterial Mating

Synthesis & transfer of F plasmid DNA by "rolling circle replication" (donor plasmid rotates)



Transfer of chromosomal DNA by F plasmid

- rarely complete copy of chromosomal DNA is transferred



- **Antibiotic resistance:** Bacteria carry plasmids (similar to F plasmid) encoding proteins that inactivate antibiotics

- Many different plasmids have been isolated from bacteria:

- concern from a medical point of view

But powerful research tool for recombinant DNA technology !!!!

E. coli Strain L -> mutation in gene for enz required to synthesize Leucine

E. coli Strain M -> mutation in gene for enz required to synthesize Methionine

Strain L + Strain M -> incubation over time in media without L & M -> cell growth

----> New *E. coli* strain able to synthesize both L & M

How?

Transfer of DNA by mating

Bacteria can take up DNA from their surroundings:

-> Bacterial Transformation

Transformation: Process by which DNA molecules are taken up by bacterial cells
To transform its genetic make-up

Requirements: permeable cell membrane; or physically permeabilize membrane

E.g *Bacillus subtilis* (soil bacterium) - cell membrane allows DNA uptake
E. coli - can not but we can force it to electrically or chemically!

Disadvantage: DNA exposed to environmental elements - nucleases etc
-> DNA damage

Great research tool !!!

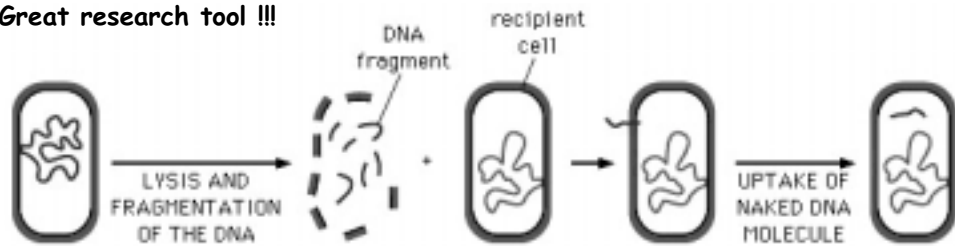
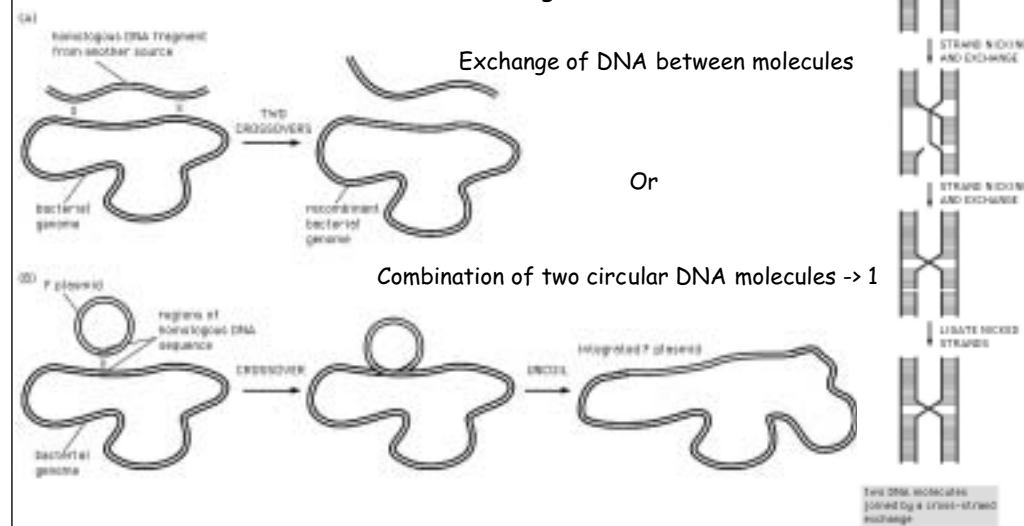


Fig 10.19

Now once cell has taken up the DNA it incorporates it in its own genome by **homologous recombination**

- 2 double-stranded DNA molecules with homologous (very similar) regions align
- regions cross over involving nicking of double strands & rejoining ends to get intact double helix
- no loss of nucleotides at site of exchange



Transfer of Bacterial Genes by Bacterial Viruses

Bacteriophages: viruses that attack bacteria

- uses the bacterial machinery to replicate & synthesize viral coat proteins
- Lethal to bacterium
- Can initially be dormant in cell

Life cycle of a hypothetical virus
Note: genetic material can be DNA or RNA

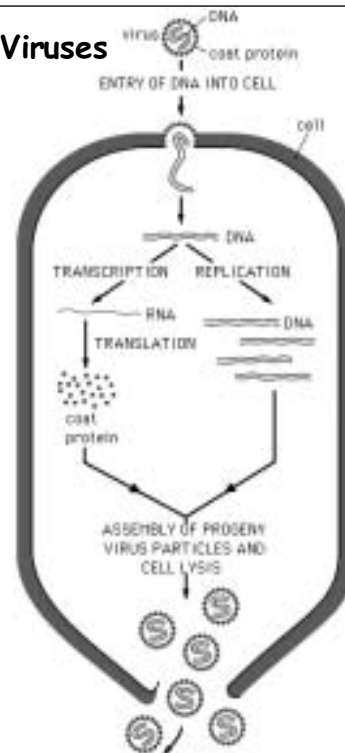
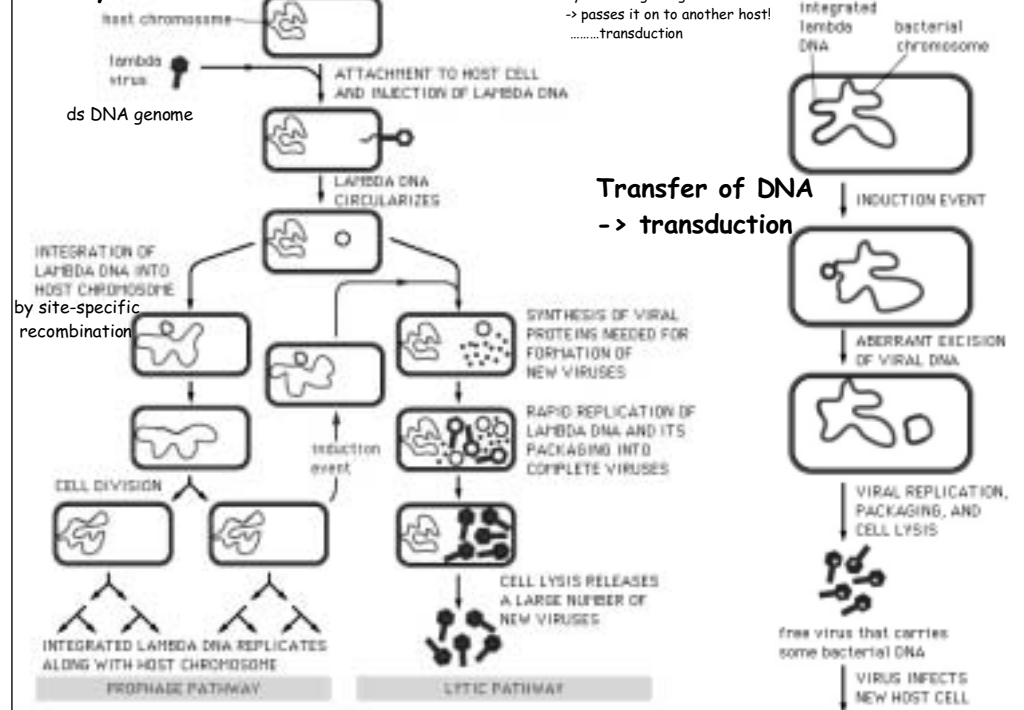


Fig 6.37

Life cycle of λ



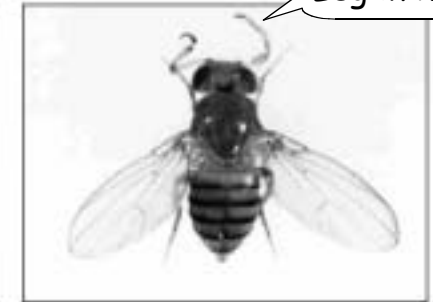
excision of λ DNA from bacterial chromosome by reversing integration BUT can take some of host DNA with it
-> passes it on to another host!
.....transduction

**Transfer of DNA
-> transduction**

Transposable Elements

- **Transposable elements:** DNA that can move from place to place within the chromosome by transposition, hence named **Transposons**
- Variable in length (100's - 1000's)
- A bacterial cell may contain many different transposons per cell
- Transposons move within DNA by recombination enzymes (**transposases**) encoded by the transposable element
- Transposable elements can be advantageous to cell: some carry promoter
- Homologous recombination between transposons → integration of plasmid into cell genome
- Transposons can "jump" from bacterial genome into plasmid
→ which can be transferred to other bacteria by conjugation

DNA rearrangement by transposable elements can have dramatic effects to the organism....



Leg in head

Drosophila melanogaster Fruit fly

Viruses

Virus:

- Infectious particle
- Made up of genes enclosed by protective coat made of protein
- Requires host to replicate

Viral Reproduction:

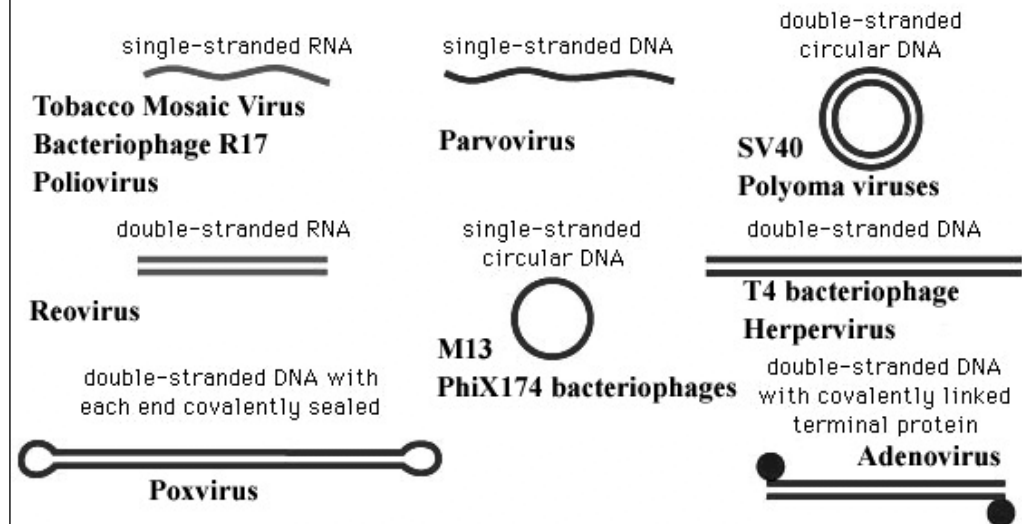
enters cytosol, uses cell machinery to replicate (ribosomes, polymerases)
lyses cell → gains access to nearby cells
lytic effect: visualized as cell damage e.g cold sores by herpes simplex virus (HSV)

Viral genomes can be DNA or RNA, single-stranded or double-stranded
- limited amount of genetic material packed into protein shell
~ 3 - several 100's genes

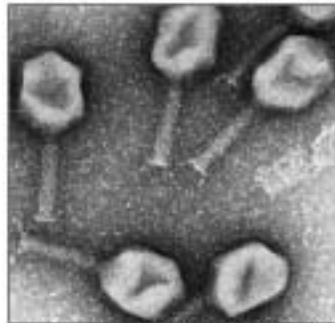
Viral protein coats: variable

- single polypeptide chain - many different proteins

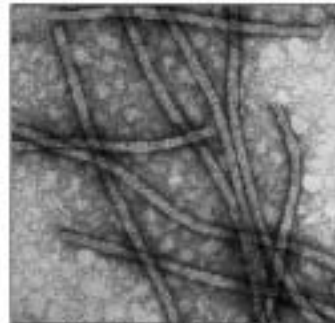
Viral genomes



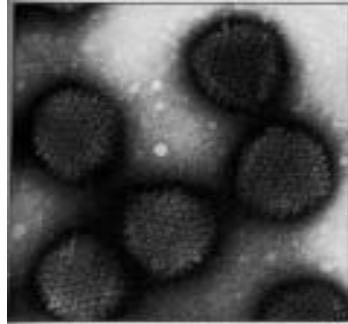
Viral Protein Coats



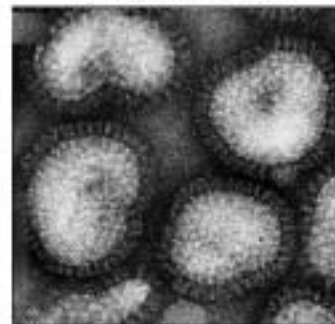
T4: DNA virus that infects *E. coli*



Potato virus X : RNA

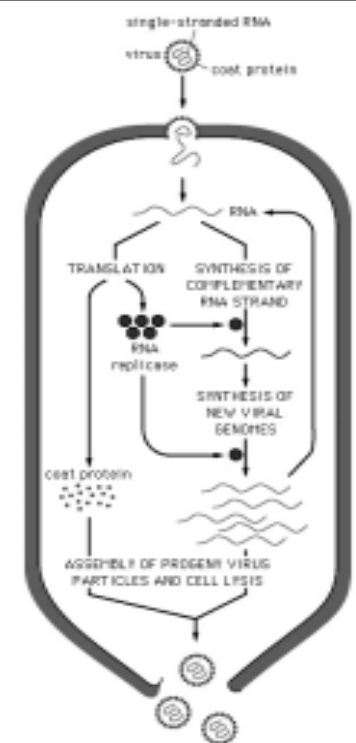
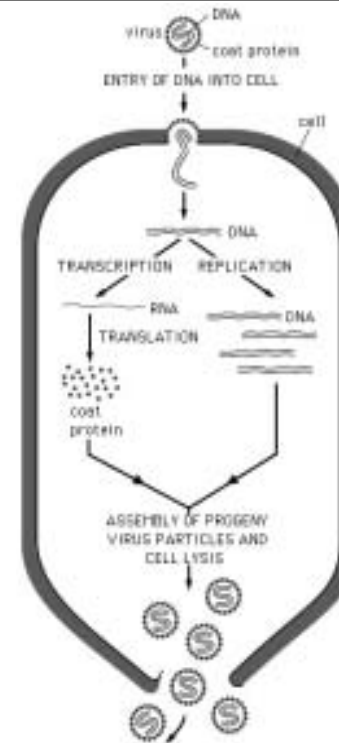


Adenovirus: DNA



Influenza virus : RNA

Fig 6.38



Viruses that can cause cancer How? They carry cancer causing genes: **oncogenes**

Table 9-2 Some Oncogenes Originally Identified Through Their Presence in Retroviruses That Cause Cells to Become Cancerous

Oncogene	Proto-oncogene Function	Source of Virus	Virus-induced Tumor
<i>abl</i>	protein kinase (tyrosine)	mouse	pre-B-cell leukemia
		cat	sarcoma
<i>erb-B</i>	epidermal growth factor (EGF) receptor	chicken	erythroleukemia; fibrosarcoma
<i>src</i>	protein kinase (tyrosine)	cat/chicken	sarcoma
<i>fos</i>	macrophage colony-stimulating factor (M-CSF) receptor	cat	sarcoma
<i>fos, jun</i>	associate to form a gene regulatory protein	mouse	osteosarcoma
		chicken	fibrosarcoma
<i>myb</i>	gene regulatory protein	chicken	sarcoma; myelocytoma carcinoma
<i>ras</i>	protein kinase (serine/threonine)	chicken/mouse	sarcoma
<i>H-ras</i>	GTP-binding protein	rat	sarcoma; erythroleukemia
<i>ret</i>	gene regulatory protein	turkey	reticuloendotheliosis
<i>sis</i>	platelet-derived growth factor, B chain	monkey	sarcoma
<i>src</i>	protein kinase (tyrosine)	chicken	sarcoma

The *src*, *ras*, *myb*, and *ret* proto-oncogenes encode proteins normally located in the nucleus, where they regulate gene transcription. The *erb-B* and *sis* proto-oncogenes encode proteins located on the outer membrane of cells that receive signals from outside the cell and transmit that information to the inside of the cell (see Chapter 15). *src*, *ras*, *ret*, and *sis* proto-oncogenes encode proteins located in the cytoplasm that are components of signaling pathways (Chapter 15). The *src* proto-oncogene encodes a secreted protein that signals other cells to divide.

Genetic variation in Bacteria :

- Replication errors
- Mutation in response to environment (adaptation)
- Mixing of genes from diff. individuals - Transfer of DNA (plasmid, chromosomal)
 - Replacement of DNA by recombination

These mechanisms of genetic variability may explain the emergence of disease-causing bacteria becoming resistant to antibiotics

Why don't the same antibiotics work for the same symptoms?
Why take a course of antibiotics?

Genetic Variation in Eucaryotic Cells:

- Errors in Replication, Transcription, Translation
- Recombination (crossover) - duplication of intron, exon
- Exon Shuffling
- Transposons - DNA; RNA: retrotransposons; unique to eucaryotes
 - (name derived from retrovirus due to similar mechanism of replication)
 - gene transfer from viruses

Analysis & manipulation of bacteria has led to the development of a variety of tools to study genomes of more complex organisms : eucaryotic cells

DNA Cloning

DNA cloning:

1. The act of making identical copies of a DNA molecule
2. The isolation of a particular stretch of DNA (often a particular gene)
 - Requires making many identical copies of the DNA of interest

DNA technology:

Depends on fragmenting long pieces of DNA
and

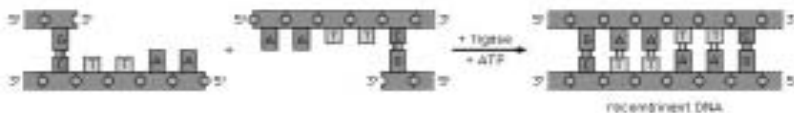
Ability to join these fragments in new combinations

-> Enzyme: DNA ligase! (ref. DNA replication and repair!)

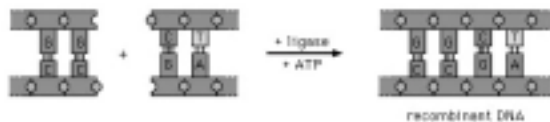
DNA the same chemical structure regardless of organism

The formation of recombinant DNA molecules *in vitro*

Joining of two complementary flanking ends:



Joining of two blunt ends:



Joining of a flanking end with a blunt end:

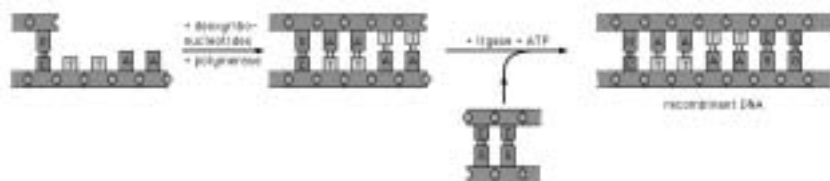


Fig 10.18

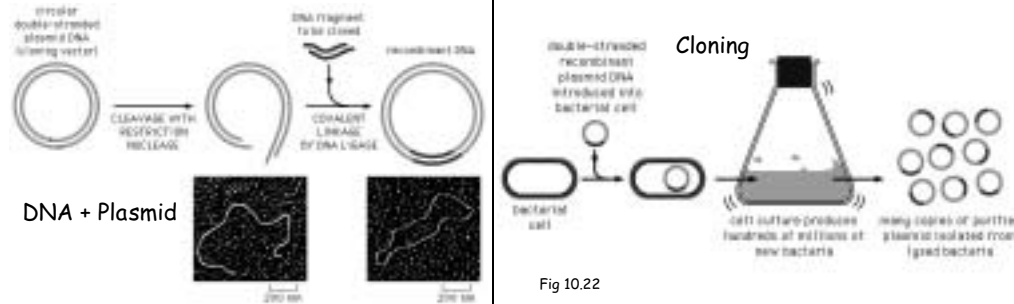
Cloning Using Bacterial Plasmids: for multiple copies of DNA

- Introduce DNA to be copied into rapidly dividing bacterium to make many copies of DNA.
- Bacterial/viral plasmid is used as a carrier (vector) of the DNA

Plasmid:

- small circular DNA molecule
- several 1000 nucs in length
- can replicate inside bacterium independent of bacterial chromosome
 - contains replication origin
- Cutting site for RE: to open and insert foreign DNA
- Contains gene for selectable property to identify bacteria that have taken up recombinant plasmid e.g. antibiotic resistance

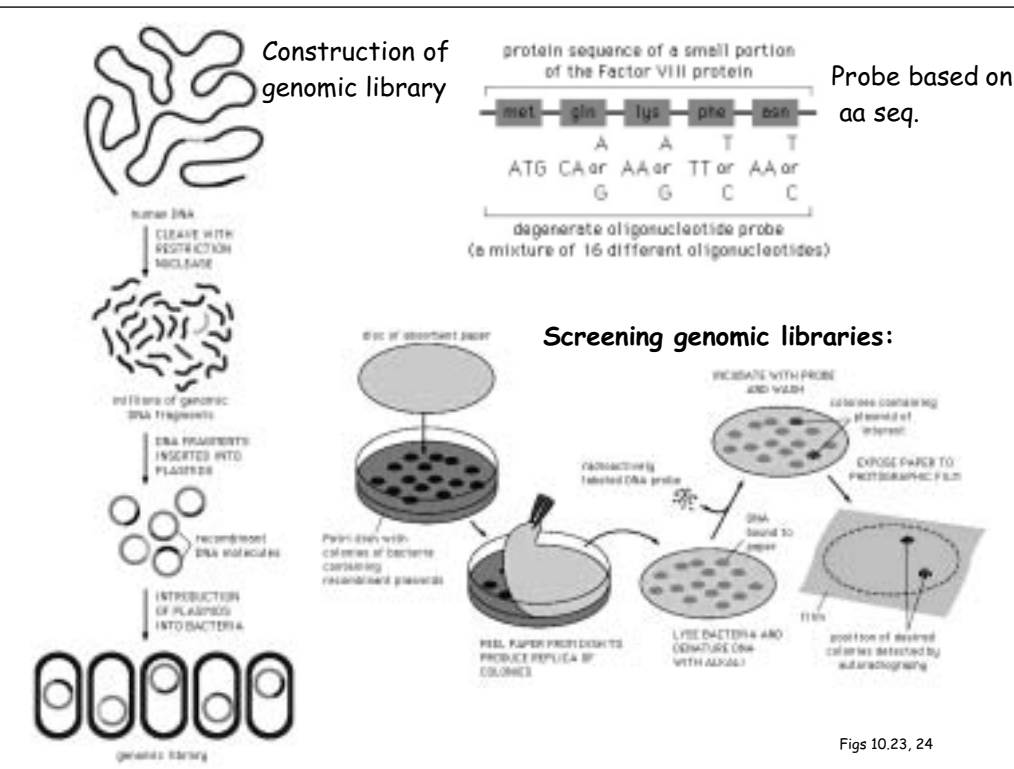
- Cut plasmid & DNA at specific site(s) (note ends)
- DNA to be cloned is covalently inserted into plasmid using DNA ligase
 - > Recombinant DNA molecule
- Recombinant DNA is introduced into bacterium (usually *E. coli*) by transformation
 - condition to ensure 1 plasmid per bacterium
- Grow bacterium in nutrient broth for a day (doubles every 30 mins)
 - > many copies of recombinant DNA
- Lyse bacterial cells
- Purify small plasmid DNA from cell content
- Remove DNA fragment by
 - cutting with RE originally used & separation by electrophoresis



Cloning of normal gene coding for protein & piecing together of its coding sequence
Coding seq then used to produce large amounts of protein

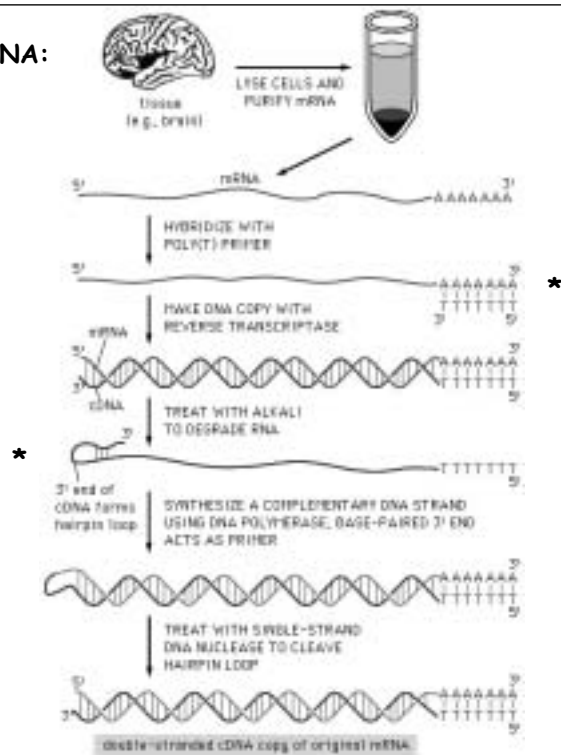
- break up total genomic DNA into smaller pieces: mechanical shear or REs
- each fragment inserted into plasmid used to transform bacteria
- Collection of (cloned) DNA fragments in bacteria called DNA library (genomic)
- "plate out" culture on petri dish with selective medium for uptake of plasmid
 - > million colonies each with one particular stretch of genomic DNA

Probe, by hybridization, library for rare bacterial clone containing DNA of interest



-> complete coding sequence

Synthesis of cDNA:



Figs 10.25

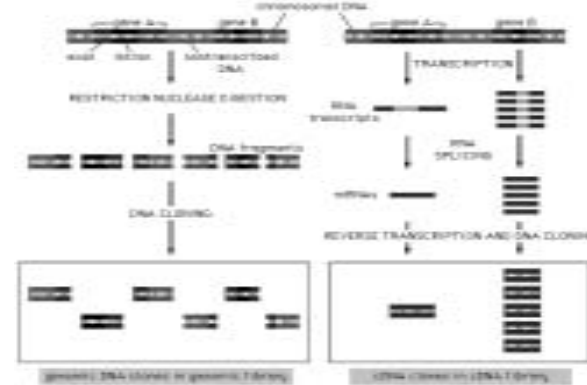
Differences between genomic DNA clones & cDNA clones:

Genomic clones: random sample of all DNA seqs in organisms genome

cDNA clones: contain coding seqs; **only genes transcribed into mRNA**

- different cells → different sets of mRNA molecules
→ different cDNA library for different tissue
- gene expression changes during development ***

Use cDNA if want to determine amino acid seq of prot from the DNA
or, if want to produce protein in bulk by expressing cloned gene in bacterial or yeast cell



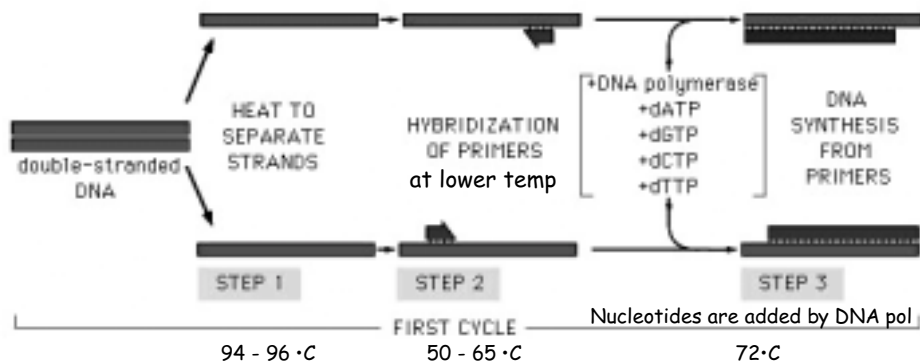
Figs 10.26

Polymerase Chain Reaction

Used to amplify selected DNA seqs; Searching for a sequence of DNA in a large piece of DNA, such as genomic DNA?

Need: Template (DNA or RNA), primers, DNA polymerase, dNTPs

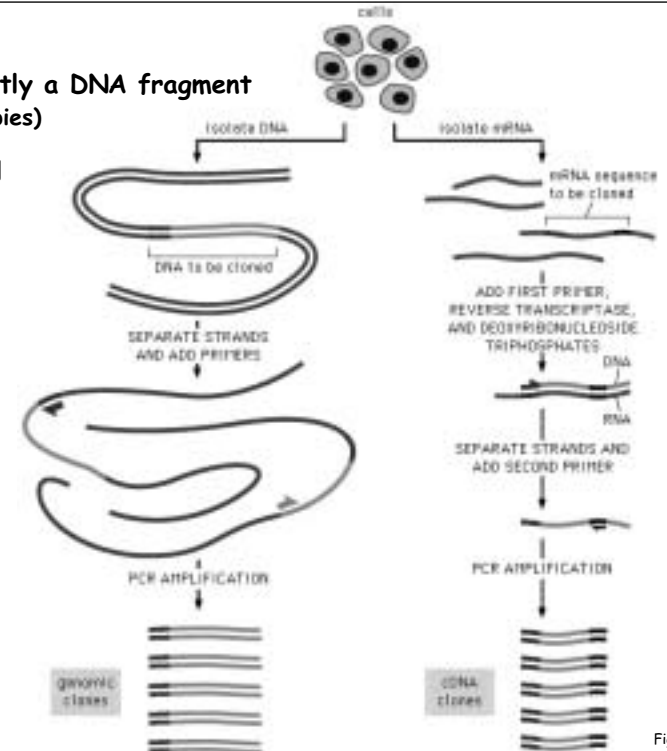
- Basic method:
- ds DNA → ss by heating
 - Primers hybridize at lower temp
 - Nucleotides are added by DNA polymerase → new strand
 - Increase temp → ss DNA and cycle continues



PCR:

used to clone directly a DNA fragment
(make identical copies)

E.g. gene from cell

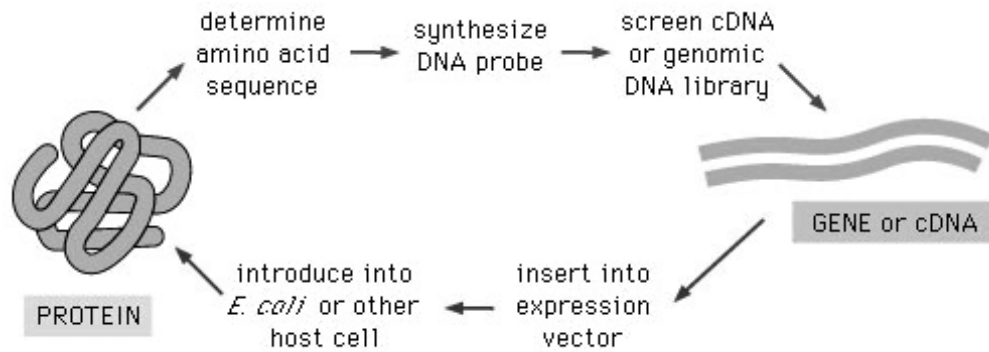


Figs 10.28

DNA Engineering

Technology to study the functions of genes, RNA molecules and proteins

Knowledge of molecular biology of cells makes it possible to experimentally move from gene to protein & from protein to gene

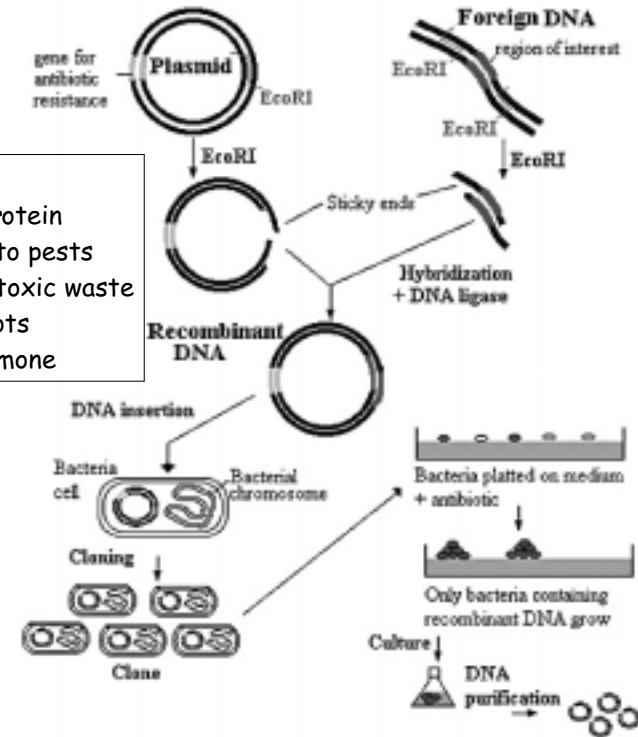


Genetic Engineering: Cloning

Cloning into a plasmid:

Uses:

Making lots of a particular protein
 Agriculture - gene resistant to pests
 Industry - bacteria clean up toxic waste
 Medicine - dissolves blood clots
 - human growth hormone



Construction of novel DNA molecules:

Recombination DNA molecules

- used in disease treatment: drug development

Serial DNA cloning to splice together different DNA fragments from different genes

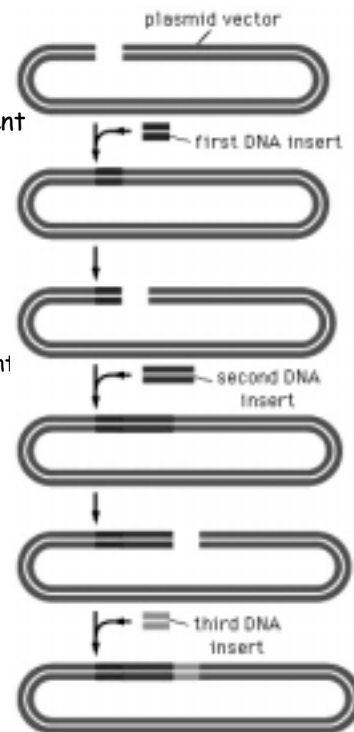


Fig 10.31

Expression of rare cellular proteins in large amounts:

Protein coding DNA seq cloned into expression vector and introduced into cells

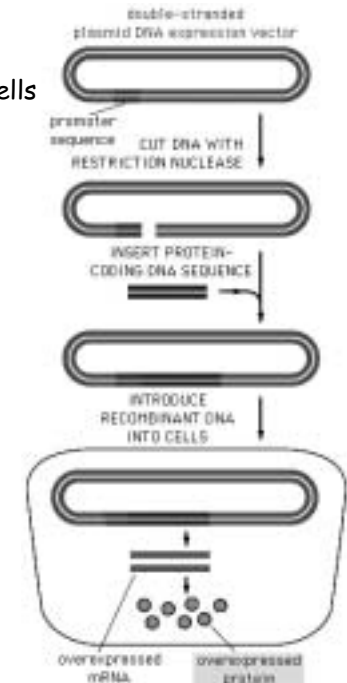
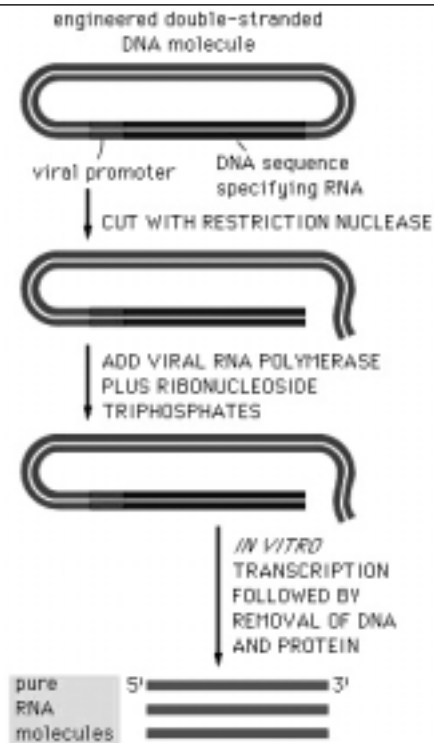


Fig 10.32

Production of large amount of RNA:

Uses viral RNA polymerase



Study the function of a gene using mutant gene

-> Transgenic gene

Change protein that gene codes by just a few amino acids

Method: site-directed mutagenesis

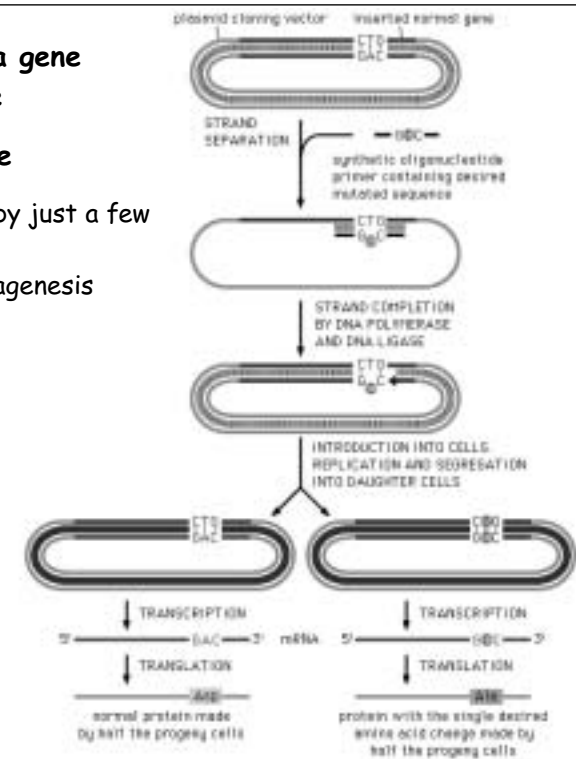


Fig 10.36

Study the function of a gene using organism

-> Transgenic organism

Ultimate test of the function of a gene is to add mutated gene
Or knockout gene of interest !!!

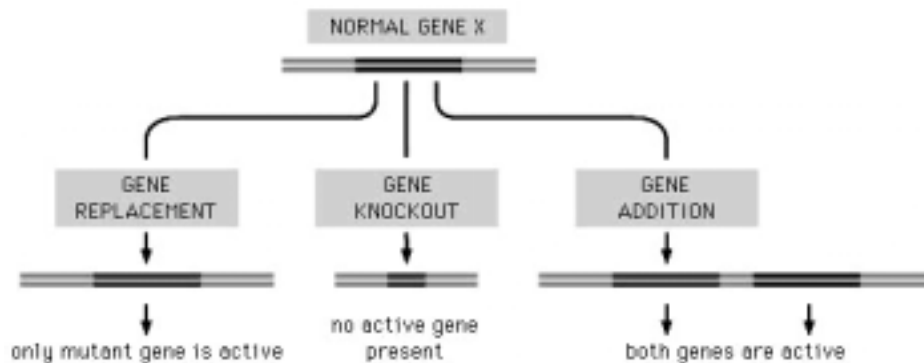


Fig 10.37

Transgenic animals carry engineered genes:

Altered gene is first introduced to embryonic stem (ES) cells in culture

These are then injected into very early Mouse embryo

Cells are incorporated into growing embryo

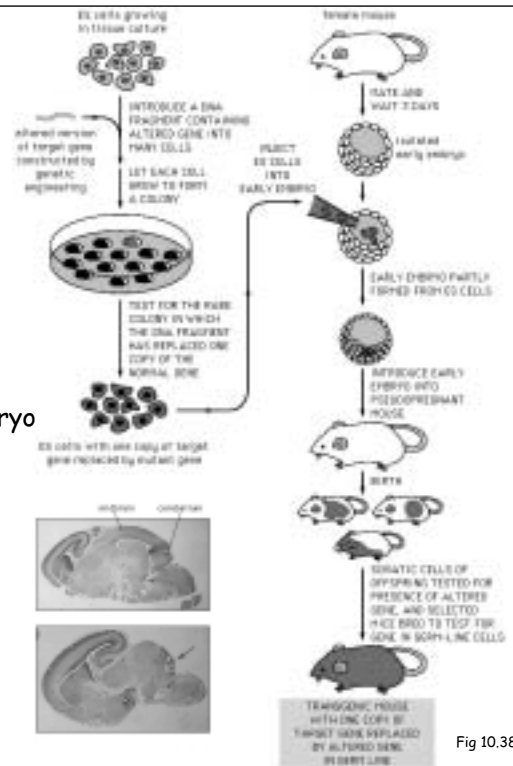


Fig 10.38

Recombinant DNA technology

Recombinant DNA technology has led to ...

- Discovery of new classes of genes
- New ways to study & engineer proteins
- Efficient mass production of engineered proteins
- New ways to study & engineer cells, organisms

Emerged in the 1970's

- Laboratories of Paul Berg, Herbert Boyer, Stanley Cohen
- Synthesis of *Genetics, Biochem., Cell Biology, Physical Chemistry*

Allowed one to make many identical copies of specific DNA molecules

- DNA cloning
- Required to study & manipulate specific chromosome segments
 - previously impossible from large chromosomes
- Today DNA is easiest biological molecule to manipulate

Basically:

Restriction nucleases - cleave DNA at specific seqs

Hybridization methods - many uses!

These are the nails, hammers, pliers, saw and other tools used to build something.....

