Chapter 20: Biotechnology: The DNA Toolbox

- Sequencing of the human genome was completed by 2007
- DNA sequencing has depended on advances in technology, starting with making recombinant DNA
  - In **recombinant DNA**, nucleotide sequences from two different sources, often two species, are combined *in vitro* into the same DNA molecule
- Methods for making recombinant DNA are central to **genetic engineering**, the direct manipulation of genes for practical purposes
- DNA technology has revolutionized **biotechnology**, the manipulation of organisms or their genetic components to make useful products
- An example of DNA technology is the microarray, a measurement of gene expression of thousands of different genes

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Chapter 20: Biotechnology: The DNA Toolbox

- Gene Cloning - Use of plasmids and restriction enzymes
- Genomic and cDNA libraries
- PCR – amplifying DNA!
- Techniques for the analysis of DNA
  - Gel electrophoresis, Southern blotting, Restriction fragment analysis, DNA sequencing
- Techniques for the analysis of gene expression
  - Northern blotting, RT-PCR, *In vitro* hybridization, Microarrays
- Techniques for the analysis of gene function
  - *In vitro* mutagenesis, RNAi
- Stem cells
- Cloning of organisms and gene therapy

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DNA cloning yields multiple copies of a gene or other DNA segment

- To work directly with specific genes, scientists prepare gene-sized pieces of DNA in identical copies, a process called ***DNA cloning***
- Most methods for cloning pieces of DNA in the laboratory share general features, such as the use of bacteria and their plasmids
- **Plasmids** are small circular DNA molecules that replicate separately from the bacterial chromosome
- Cloned genes are useful for making copies of a particular gene and producing a protein product in the laboratory
- **Gene cloning** involves using bacteria to make multiple copies of a gene
  - Foreign DNA is inserted into a plasmid, and the recombinant plasmid is inserted into a bacterial cell
  - Reproduction in the bacterial cell results in cloning of the plasmid including the foreign DNA and yields the production of multiple copies of a single gene

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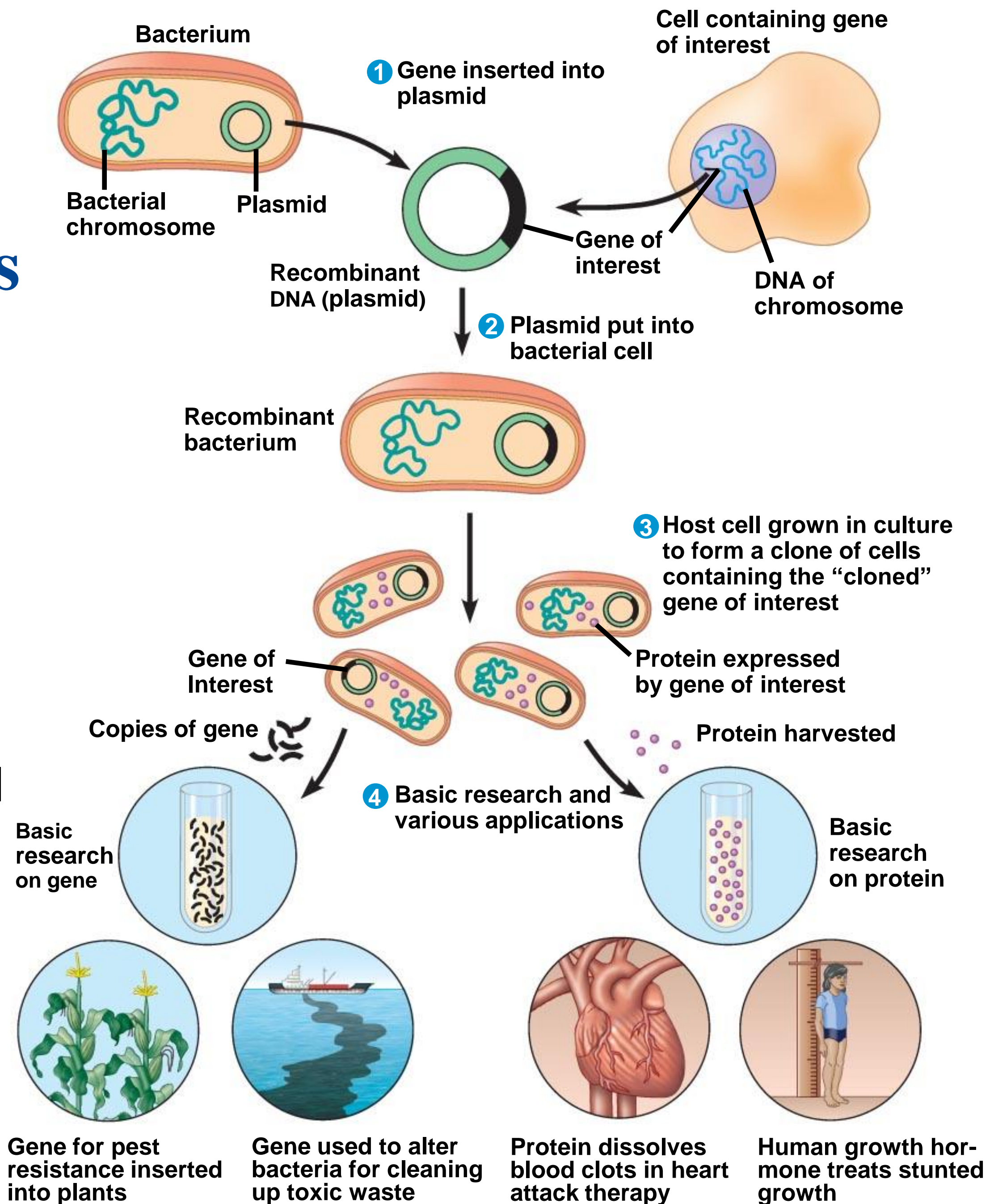
A preview of gene cloning and some uses of cloned genes



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- In gene cloning, the original plasmid is called a **cloning vector**

- A cloning vector is a DNA molecule that can carry foreign DNA into a host cell and replicate there

Using Restriction Enzymes to Make Recombinant DNA

- Bacterial **restriction enzymes** cut DNA molecules at specific DNA sequences called **restriction sites**
- A restriction enzyme usually makes many cuts, yielding **restriction fragments**
  - They are the ‘immune system’ of bacteria – they function to digest bacteriophage DNA that enters the cell.
  - Thus, they protect them from bacteriophage infections
- The most useful restriction enzymes cut DNA in a staggered way, producing fragments with “**sticky ends**” that bond with complementary sticky ends of other fragments
- **DNA ligase** is an enzyme that seals the bonds between restriction fragments and is then used to attach 2 pieces of DNA together

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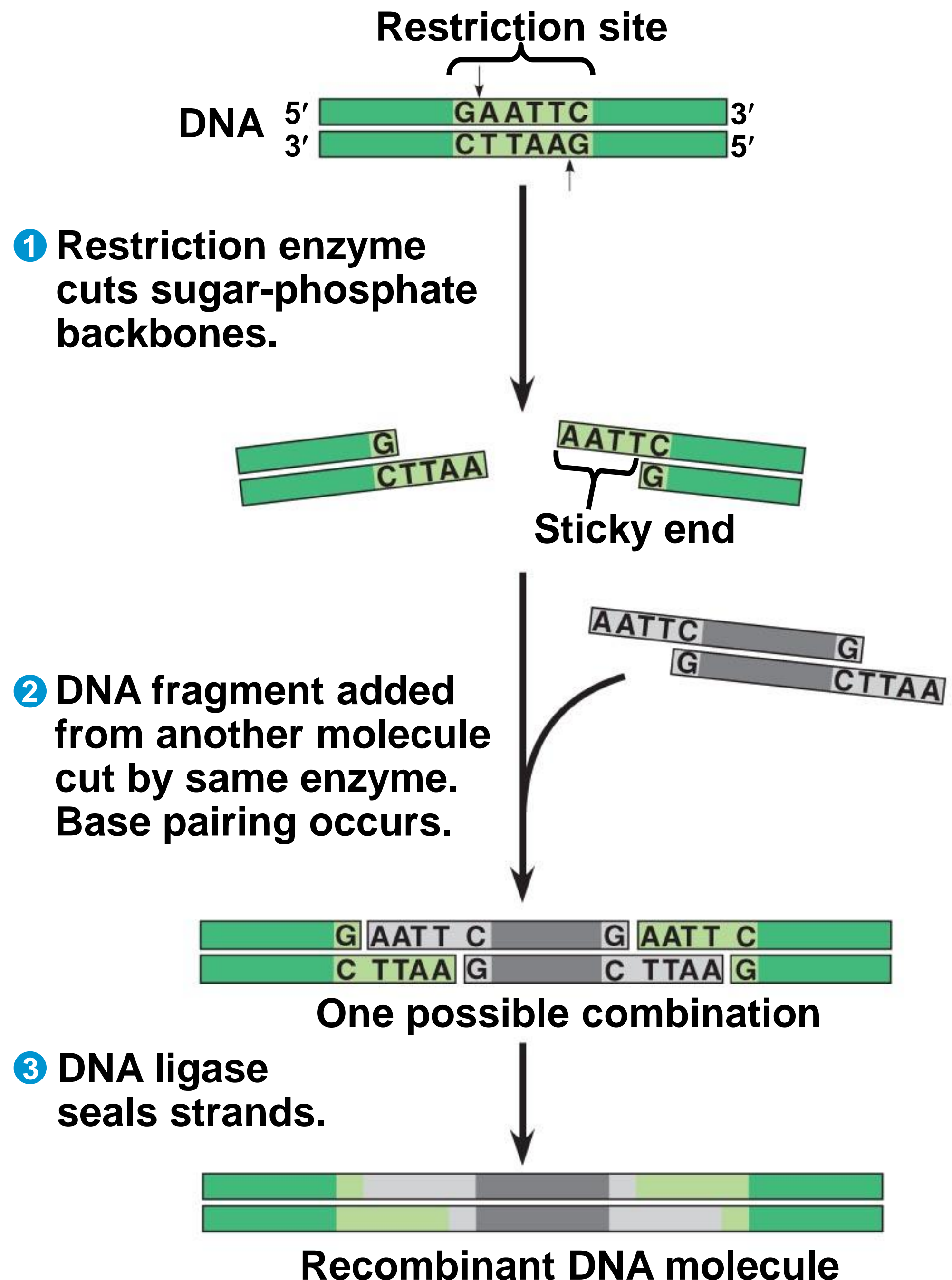
Using a restriction enzyme and DNA ligase to make recombinant DNA



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Cloning a Eukaryotic Gene in a Bacterial Plasmid

- Several steps are required to clone the hummingbird β-globin gene in a bacterial plasmid:
  - The hummingbird genomic DNA and a bacterial plasmid are isolated
  - Both are digested with the same restriction enzyme
  - The fragments are mixed, and DNA ligase is added to bond the fragment sticky ends
  - Some recombinant plasmids now contain hummingbird DNA
  - The DNA mixture is added to bacteria that have been genetically engineered to accept it
  - The bacteria are plated on a type of agar that selects for the bacteria with recombinant plasmids
  - This results in the cloning of many hummingbird DNA fragments, including the β-globin gene

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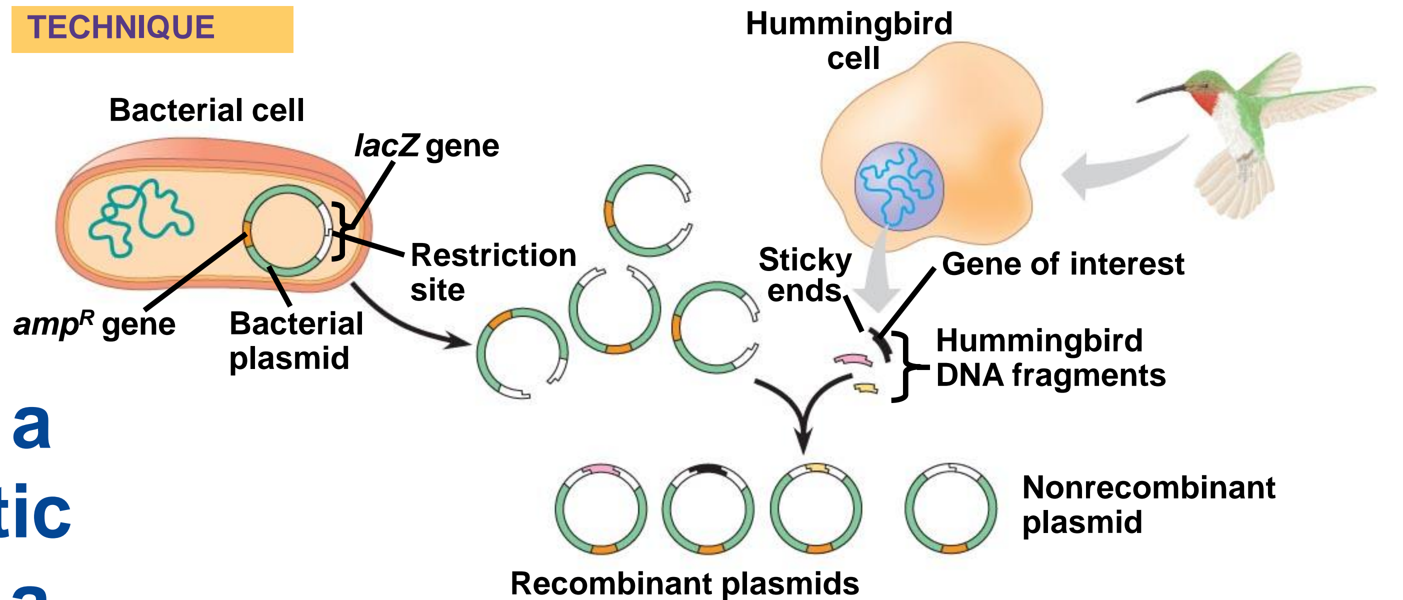
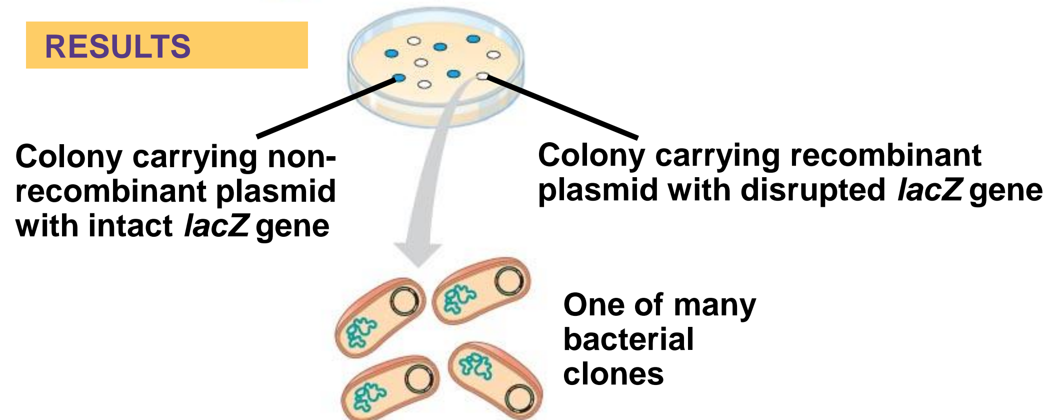
Cloning a Eukaryotic Gene in a Bacterial Plasmid





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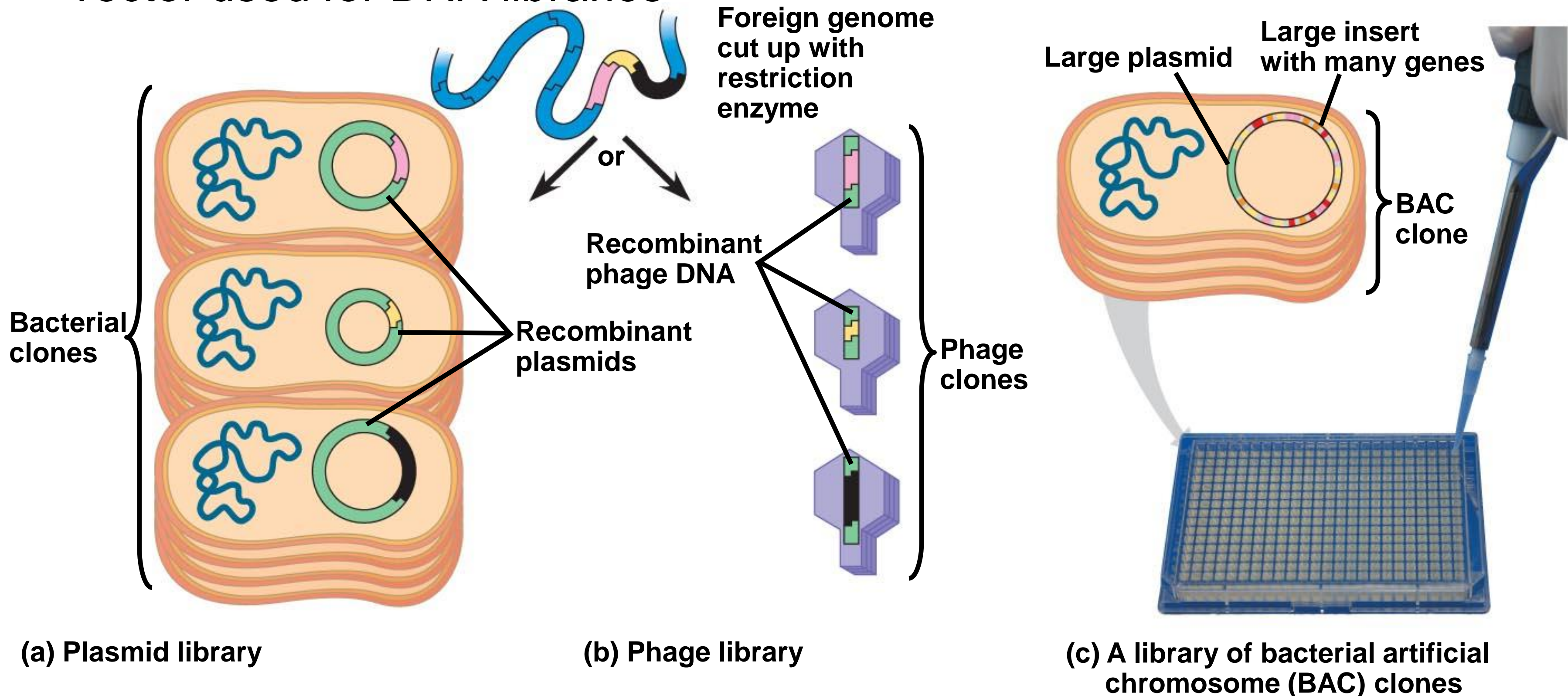
Storing Cloned Genes in DNA Libraries

- A **genomic library** that is made using bacteria is the collection of recombinant vector clones produced by cloning DNA fragments from an entire genome
  - A genomic library that is made using bacteriophages is stored as a collection of phage clones
- A **bacterial artificial chromosome (BAC)** is a large plasmid that has been trimmed down and can carry a large DNA insert and is a 2nd type of vector used for DNA libraries



(a) Plasmid library
(b) Phage library
(c) A library of bacterial artificial chromosome (BAC) clones

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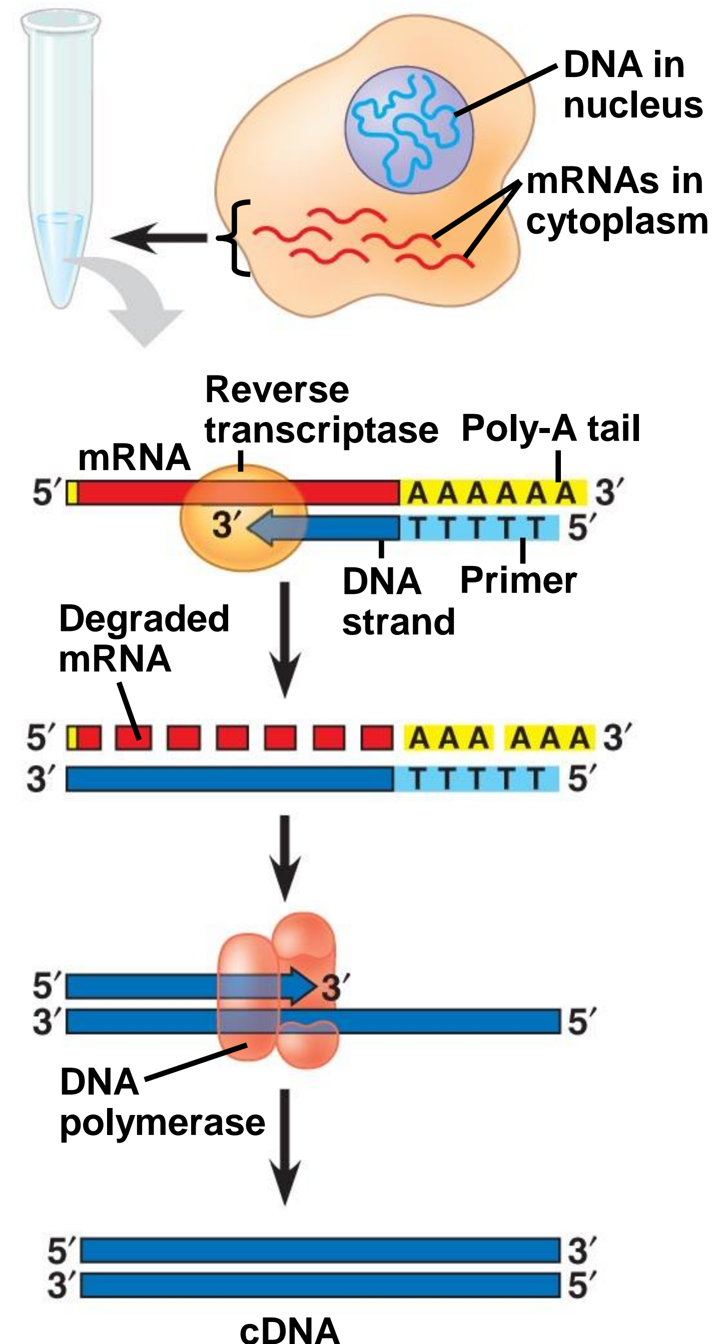
Storing Cloned Genes in DNA Libraries

- A **complementary DNA (cDNA)** library is made by cloning DNA made *in vitro* by reverse transcription (making a DNA copy of mRNA) of all the mRNA produced by a particular cell
- A **cDNA library** represents only part of the genome—only the subset of genes transcribed into mRNA in the original cells
- cDNA libraries lack introns, promoters, enhancers, and non-coding DNA (which are all present in genomic libraries



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Screening a Library for Clones Carrying a Gene of Interest – Identifying the proper clone!

- A clone carrying the gene of interest can be identified with a **nucleic acid probe** having a sequence complementary to the gene
- This process is called **nucleic acid hybridization**
- A probe can be synthesized that is complementary to the gene of interest
- For example, if the desired gene is

5′ ···GGCTAACTTAGC··· 3′

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  – Then we would synthesize this probe

3′ CCGATTGAATCG 5′

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- The DNA probe can be used to screen a large number of clones simultaneously for the gene of interest and the clone can then be cultured (grown in the lab)

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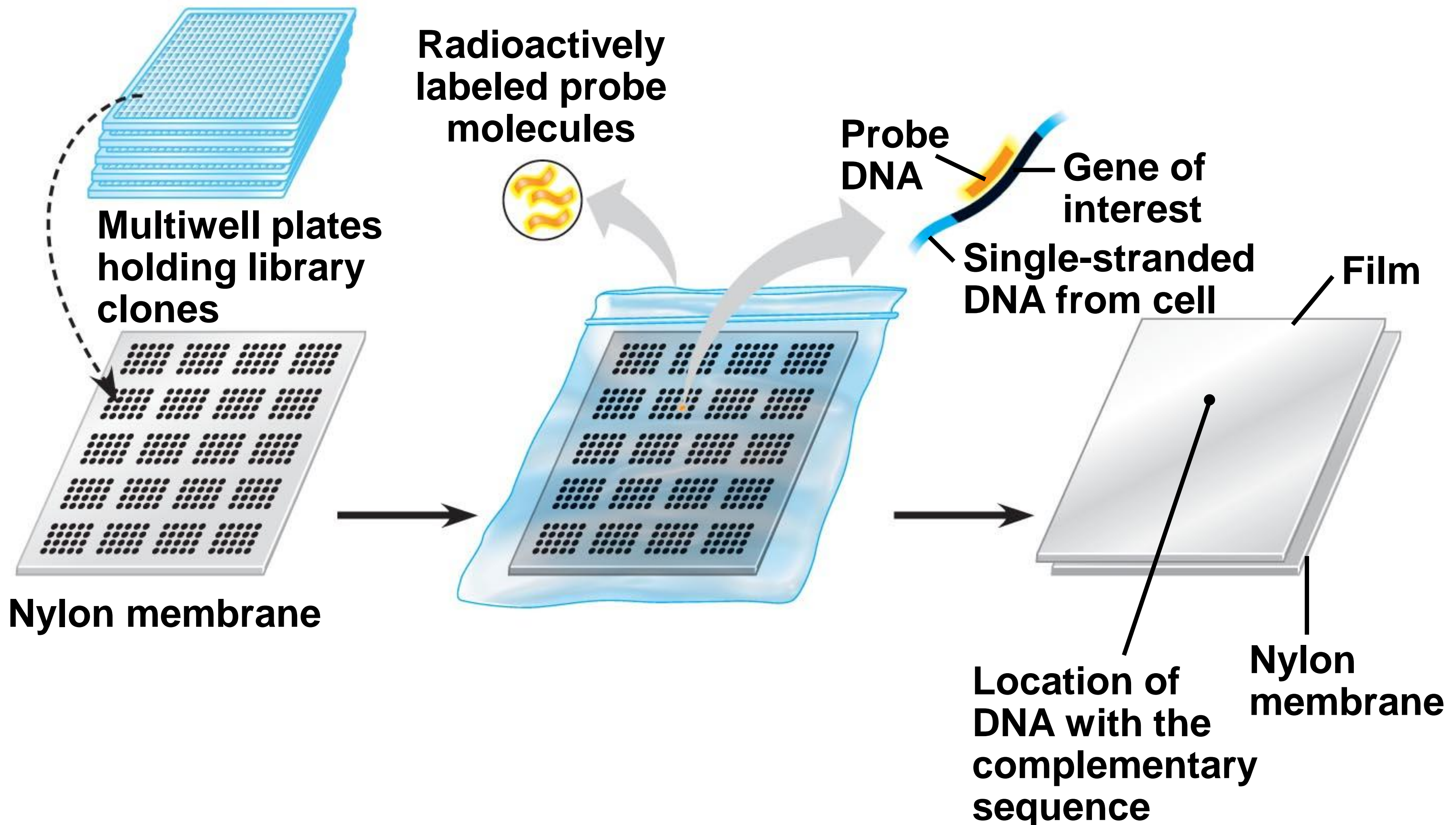
Detecting a specific DNA sequence by hybridizing with a nucleic acid probe



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Expressing Cloned Eukaryotic Genes

- After a gene has been cloned, its protein product can be produced in larger amounts for research
- Cloned genes can be expressed as protein in either bacterial or eukaryotic cells
- Several technical difficulties hinder expression of cloned eukaryotic genes in bacterial host cells
  - To overcome differences in promoters and other DNA control sequences, scientists usually employ an **expression vector**, a cloning vector that contains a highly active prokaryotic promoter
- The use of cultured eukaryotic cells as host cells and **yeast artificial chromosomes (YACs)** as vectors helps avoid gene expression problems
- YACs behave normally in mitosis and can carry more DNA than a plasmid
- Eukaryotic hosts can provide the post-translational modifications that many proteins require

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How do you get the recombinant DNA into the cells?

- One method of introducing recombinant DNA into eukaryotic cells is **electroporation**, applying a brief electrical pulse to create temporary holes in plasma membranes
- Alternatively, scientists can inject DNA into cells using microscopically thin needles
- Once inside the cell, the DNA is incorporated into the cell’s DNA by natural genetic recombination
- For prokaryotes, electroporation is one method of introducing the plasmid into the cells
- The second method is known as heat shock, briefly exposing the bacteria to a temperature of 42°C (they normally live at 37°C)
  – For unknown reasons, they then take the DNA into the cell

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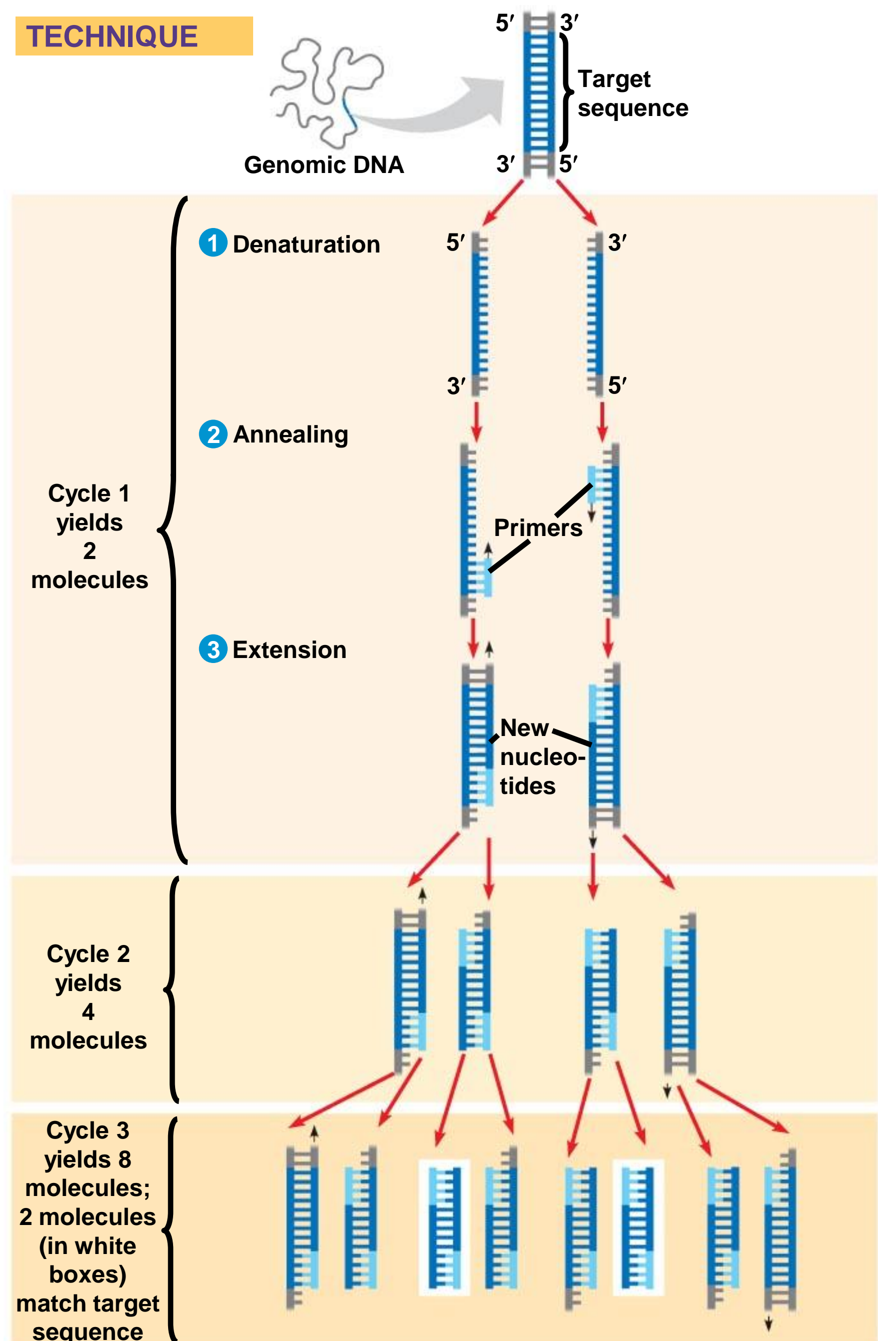
Amplifying DNA *in Vitro*: The Polymerase Chain Reaction (PCR)

- The **polymerase chain reaction, PCR**, can produce many copies of a specific target segment of DNA
- A three-step cycle—heating, cooling, and replication—brings about a chain reaction that produces an exponentially growing population of identical DNA molecules



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DNA technology allows us to study the sequence, expression, and function of a gene

- DNA cloning allows researchers to
  - Compare genes and alleles between individuals
  - Locate gene expression in a body
  - Determine the role of a gene in an organism
- Several techniques are used to analyze the DNA of genes
  - Gel electrophoresis
  - Southern Blotting
  - Restriction Fragment Analysis
  - DNA sequencing

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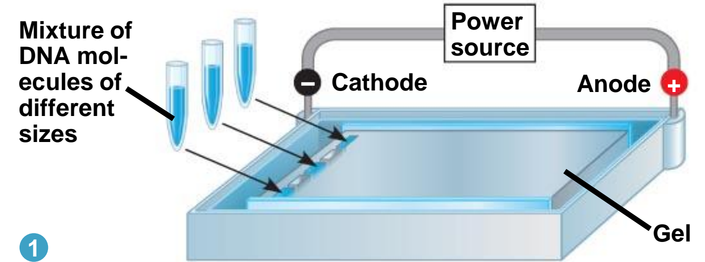
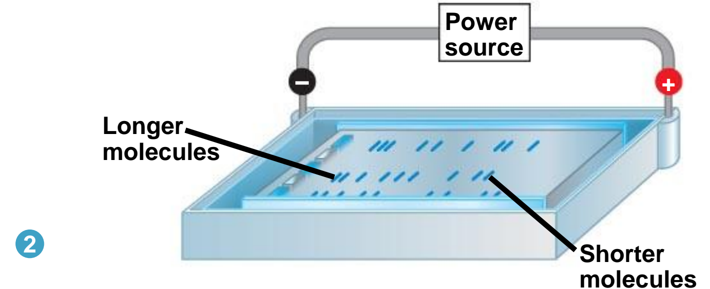
Gel Electrophoresis

- One indirect method of rapidly analyzing and comparing genomes is **gel electrophoresis**
- This technique uses a gel as a molecular sieve to separate nucleic acids or proteins by size
- A current is applied that causes charged molecules to move through the gel - due to their negative charge
- Molecules are sorted into “bands” by their size





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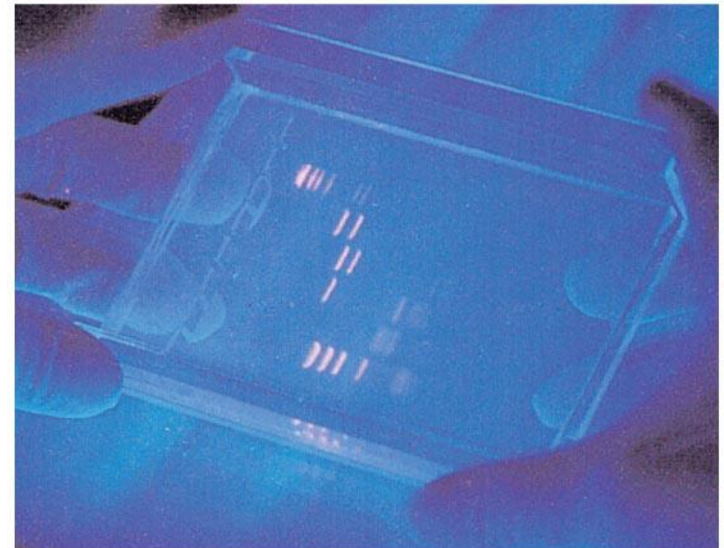

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Southern Blotting is another application of extension of Gel Electrophoresis

- A technique called **Southern blotting** combines gel electrophoresis of DNA fragments with nucleic acid hybridization
- Specific DNA fragments can be identified by Southern blotting, using labeled probes that hybridize to the DNA immobilized on a "blot" of gel

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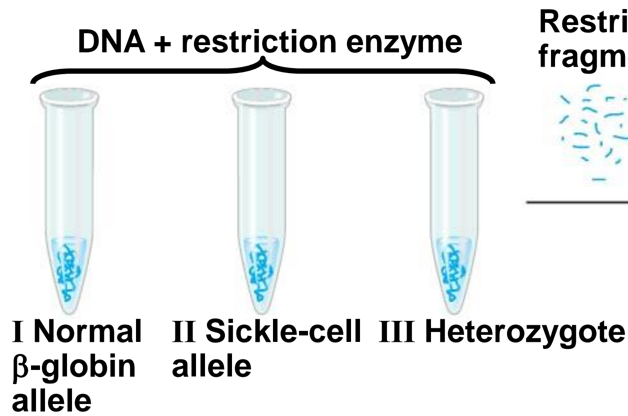
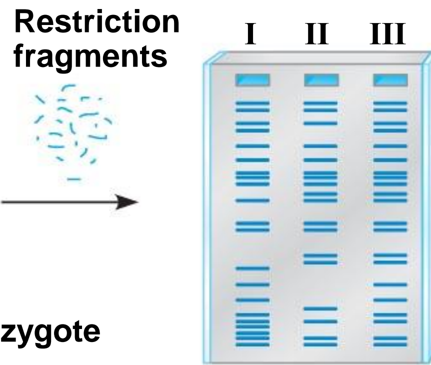
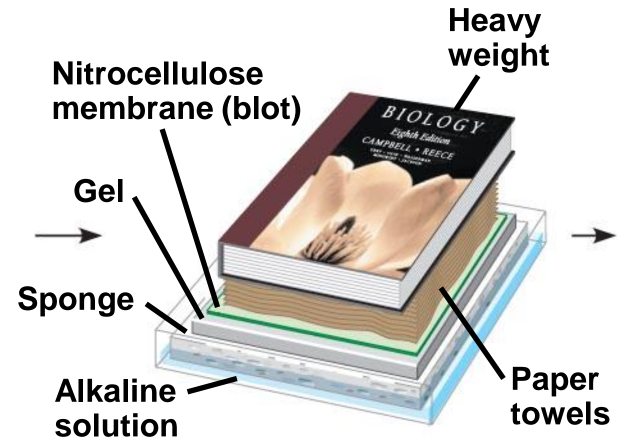
Southern blotting of DNA fragments

TECHNIQUE



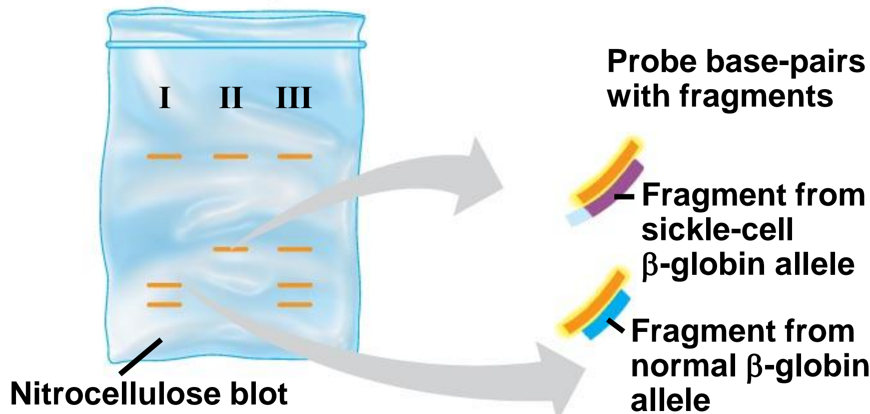


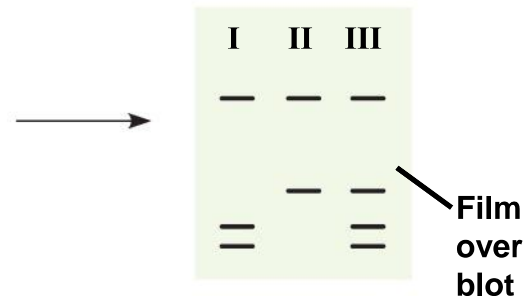


1 Preparation of restriction fragments

2 Gel electrophoresis

3 DNA transfer (blotting)





4 Hybridization with radioactive probe

5 Probe detection

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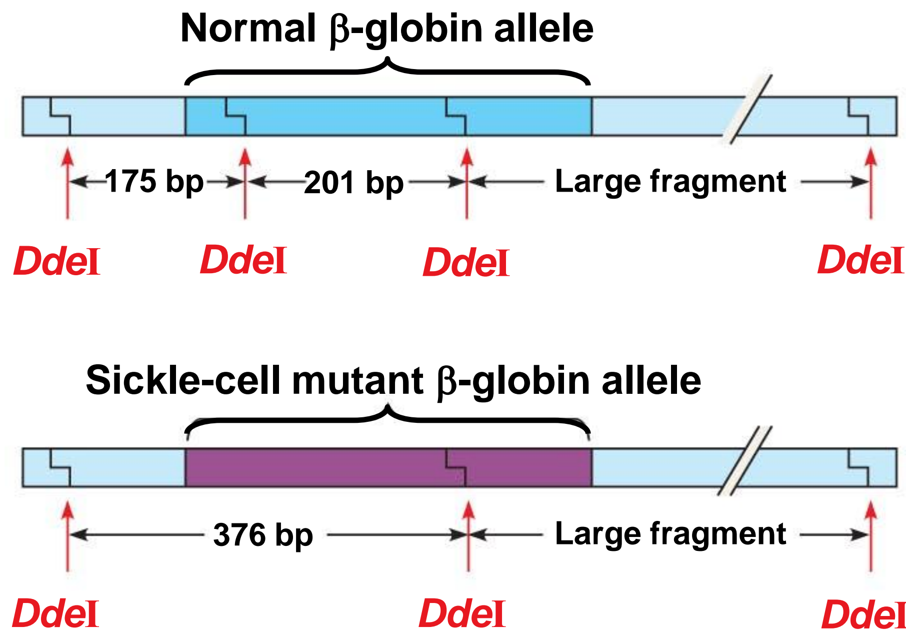
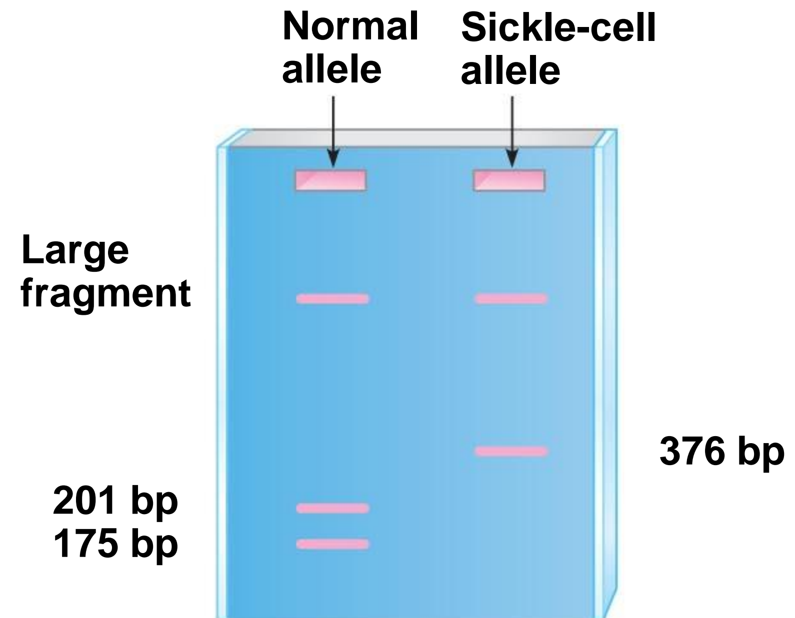
Restriction Fragment Analysis

- In *restriction fragment analysis*, DNA fragments produced by restriction enzyme digestion of a DNA molecule are sorted by gel electrophoresis
- Restriction fragment analysis is useful for comparing two different DNA molecules, such as two alleles for a gene
- The procedure is also used to prepare pure samples of individual fragments



(a) *Dde*I restriction sites in normal and sickle-cell alleles of β-globin gene



(b) Electrophoresis of restriction fragments from normal and sickle-cell alleles

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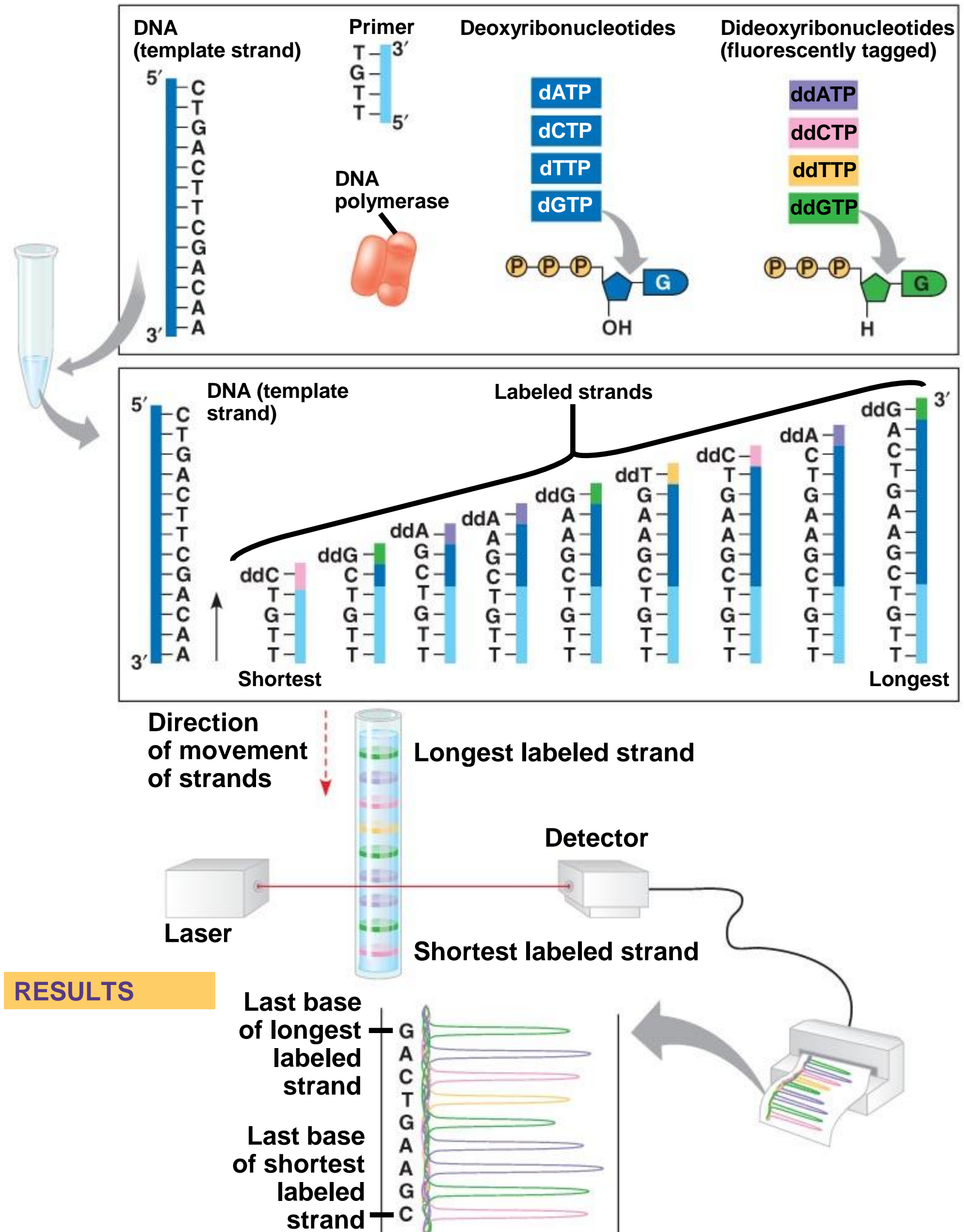
DNA Sequencing

- Relatively short DNA fragments can be sequenced by the *dideoxy chain termination method*
- Modified nucleotides called dideoxyribonucleotides (ddNTP) attach to synthesized DNA strands of different lengths
- Each type of ddNTP is tagged with a distinct fluorescent label that identifies the nucleotide at the end of each DNA fragment
- The DNA sequence can be read from the resulting spectrogram



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Analyzing Gene *Expression* of *one* gene

- Nucleic acid probes can hybridize with mRNAs transcribed from a gene and can be used to identify where or when a gene is transcribed in an organism

- Changes in the expression of a gene during embryonic development can be tested using

  - **Northern blotting** combines gel electrophoresis of mRNA followed by hybridization with a probe on a membrane (it is pretty much like Southern blotting, except it is used for mRNA detection)

  - **Reverse transcriptase-polymerase chain reaction (RT-PCR)**

  - ***In situ* hybridization**

- Both methods are used to compare mRNA from different developmental stages because identification of mRNA at a particular developmental stage suggests protein function at that stage

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RT-PCR analysis of expression of single genes

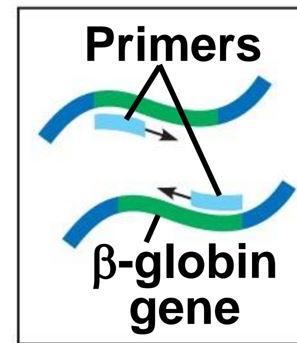
- Reverse transcriptase is added to mRNA to make cDNA, which serves as a template for PCR amplification of the gene of interest

- The products are run on a gel and the mRNA of interest identified

TECHNIQUE

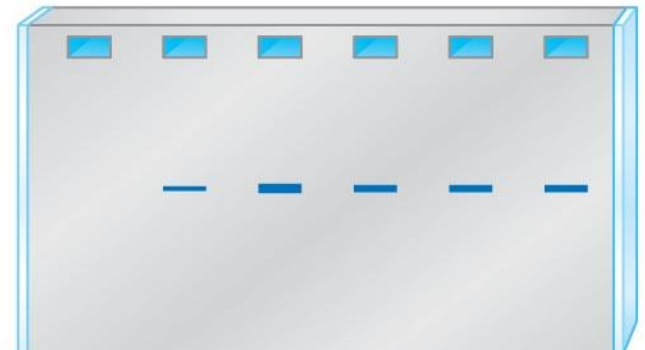
1 cDNA synthesis

2 PCR amplification



3 Gel electrophoresis

RESULTS



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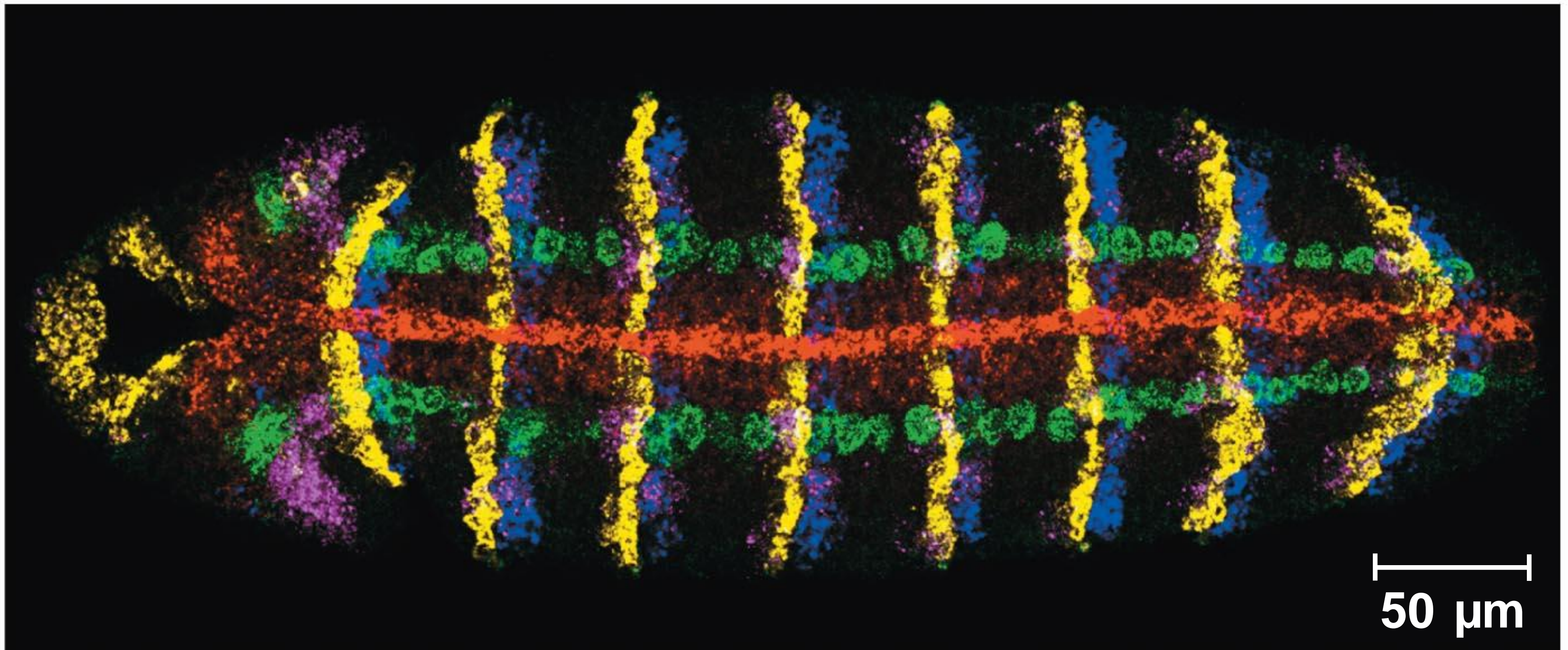
Determining where genes are expressed by *in situ* hybridization analysis

- ***In situ* hybridization** uses fluorescent dyes attached to probes to identify the location of specific mRNAs in place in the intact organism



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Studying the Expression of Interacting Groups of Genes

- Automation has allowed scientists to measure expression of thousands of genes at one time using DNA microarray assays
- **DNA microarray assays** compare patterns of gene expression in different tissues, at different times, or under different conditions

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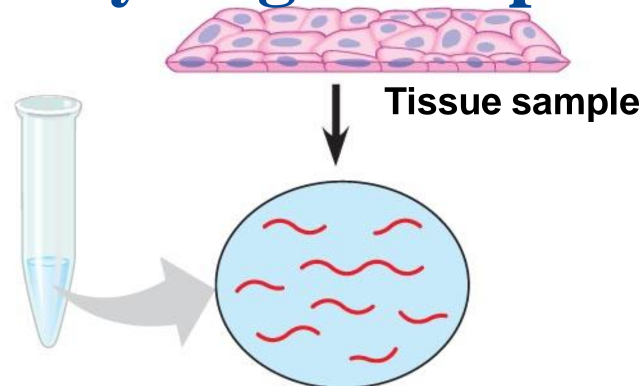
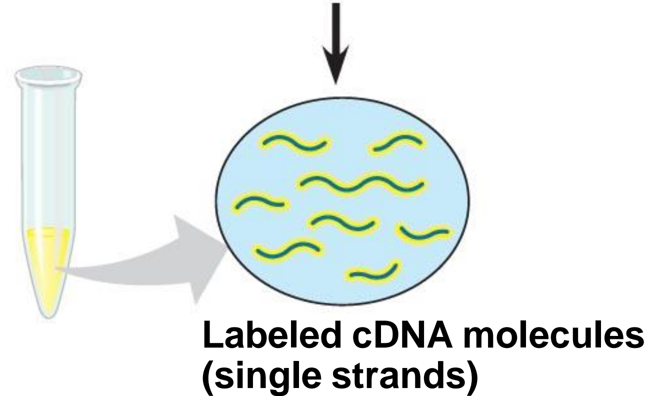
DNA microarray assay of gene expression levels

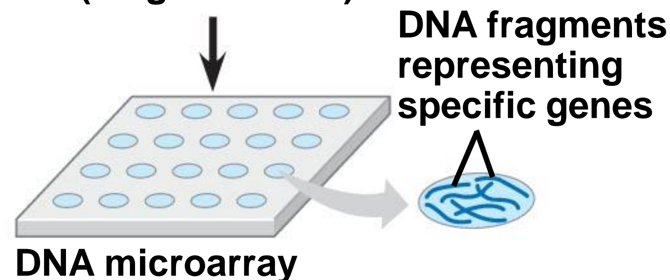
TECHNIQUE



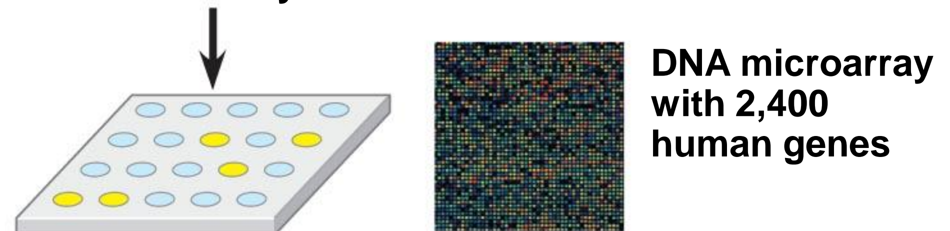
1 Isolate mRNA.



2 Make cDNA by reverse transcription, using fluorescently labeled nucleotides.



3 Apply the cDNA mixture to a microarray, a different gene in each spot. The cDNA hybridizes with any complementary DNA on the microarray.



4 Rinse off excess cDNA; scan microarray for fluorescence. Each fluorescent spot represents a gene expressed in the tissue sample.

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Determining Gene Function

- One way to determine function is to disable the gene and observe the consequences (This is a very important aspect of genetics!)
  - Using ***in vitro* mutagenesis**, mutations are introduced into a cloned gene, altering or destroying its function
    - When the mutated gene is returned to the cell, the normal gene's function might be determined by examining the mutant's phenotype
  - Gene expression can also be silenced using **RNA interference (RNAi)**
    - Synthetic double-stranded RNA molecules matching the sequence of a particular gene are used to break down or block the gene's mRNA

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Cloning organisms may lead to production of stem cells for research and other applications

- Organismal cloning produces one or more organisms genetically identical to the "parent" that donated the single cell
- One experimental approach for testing genomic equivalence is to see whether a differentiated cell can generate a whole organism
  - This approach can be utilized to clone plants, but has thus far been unsuccessful for animals
  - A **totipotent** cell (stem cell) is one that can generate a complete new organism
  - A **pluripotent** cell (stem cell) is a type that can generate a few cell types of an organism

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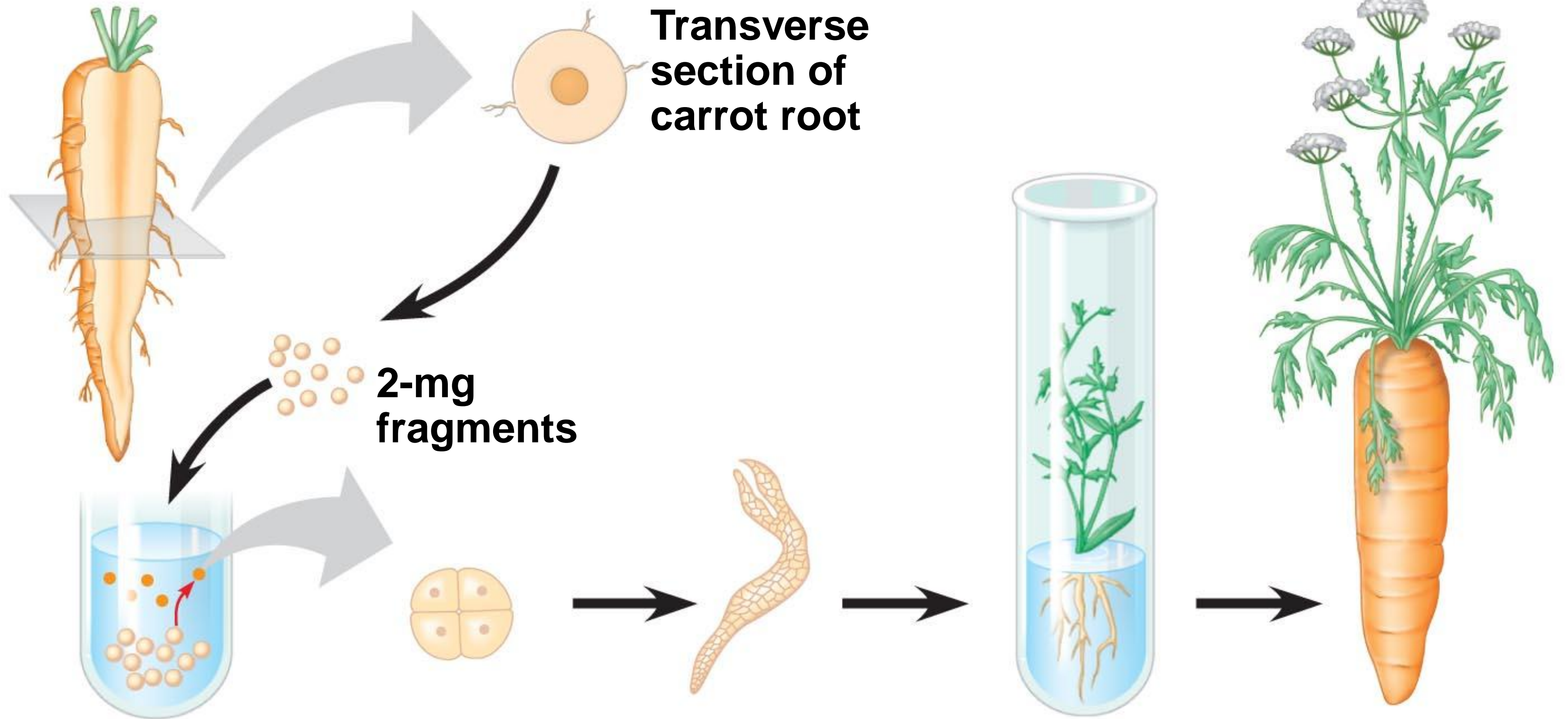
Can a differentiated plant cell develop into a whole plant?



Fragments were cultured in nu-trient medium; stirring caused single cells to shear off into the liquid.

Single cells free in suspension began to divide.

Embryonic plant developed from a cultured single cell.

Plantlet was cultured on agar medium. Later it was planted in soil.

A single somatic carrot cell developed into a mature carrot plant.

Cloning Animals: Nuclear Transplantation

- In **nuclear transplantation**, the nucleus of an unfertilized egg cell or zygote is replaced with the nucleus of a differentiated cell
- Experiments with frog embryos have shown that a transplanted nucleus can often support normal development of the egg
- However, the older the donor nucleus, the lower the percentage of normally developing tadpoles

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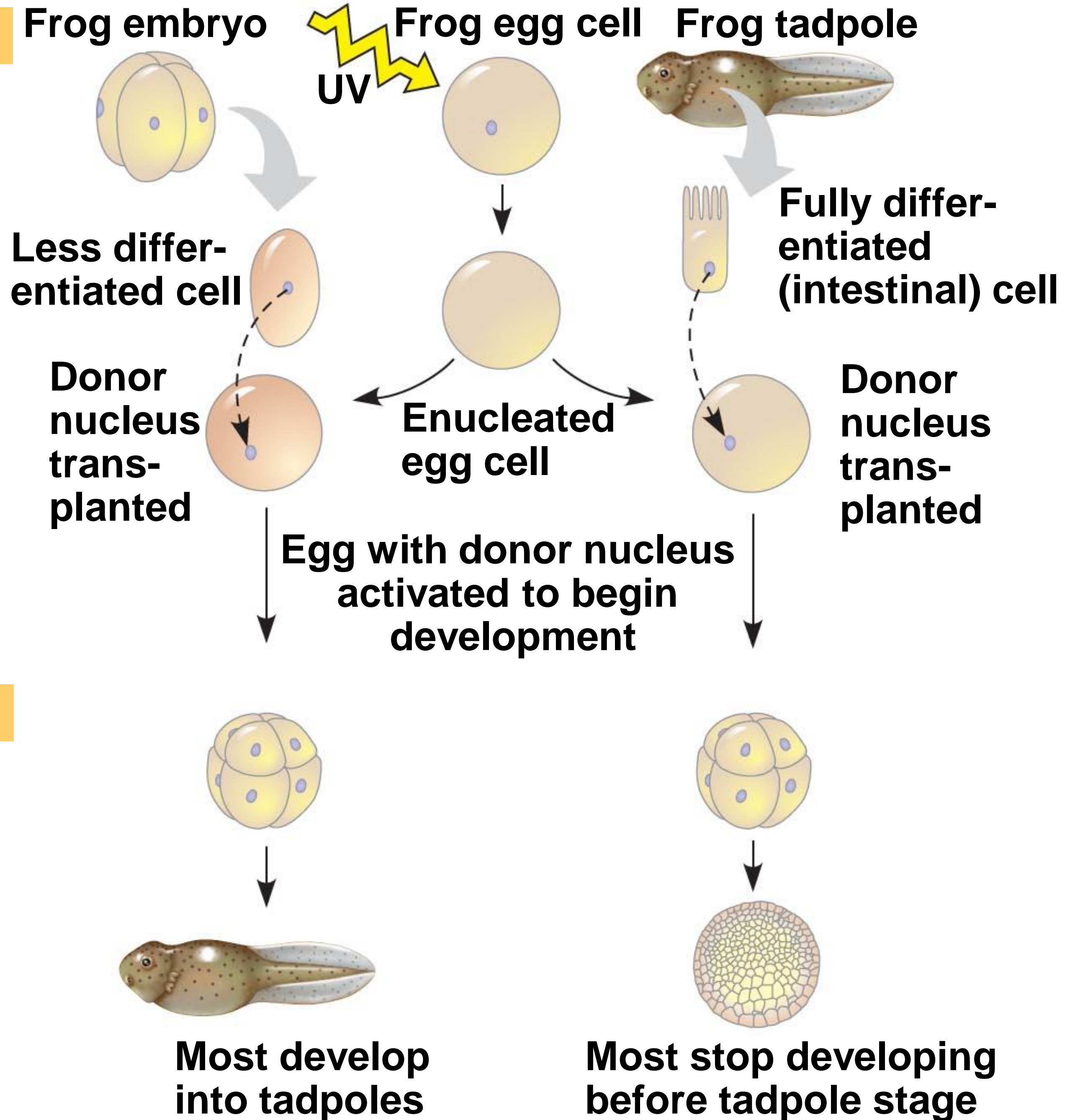
EXPERIMENT

Can the nucleus from a differentiated animal cell direct development of an organism?



RESULTS

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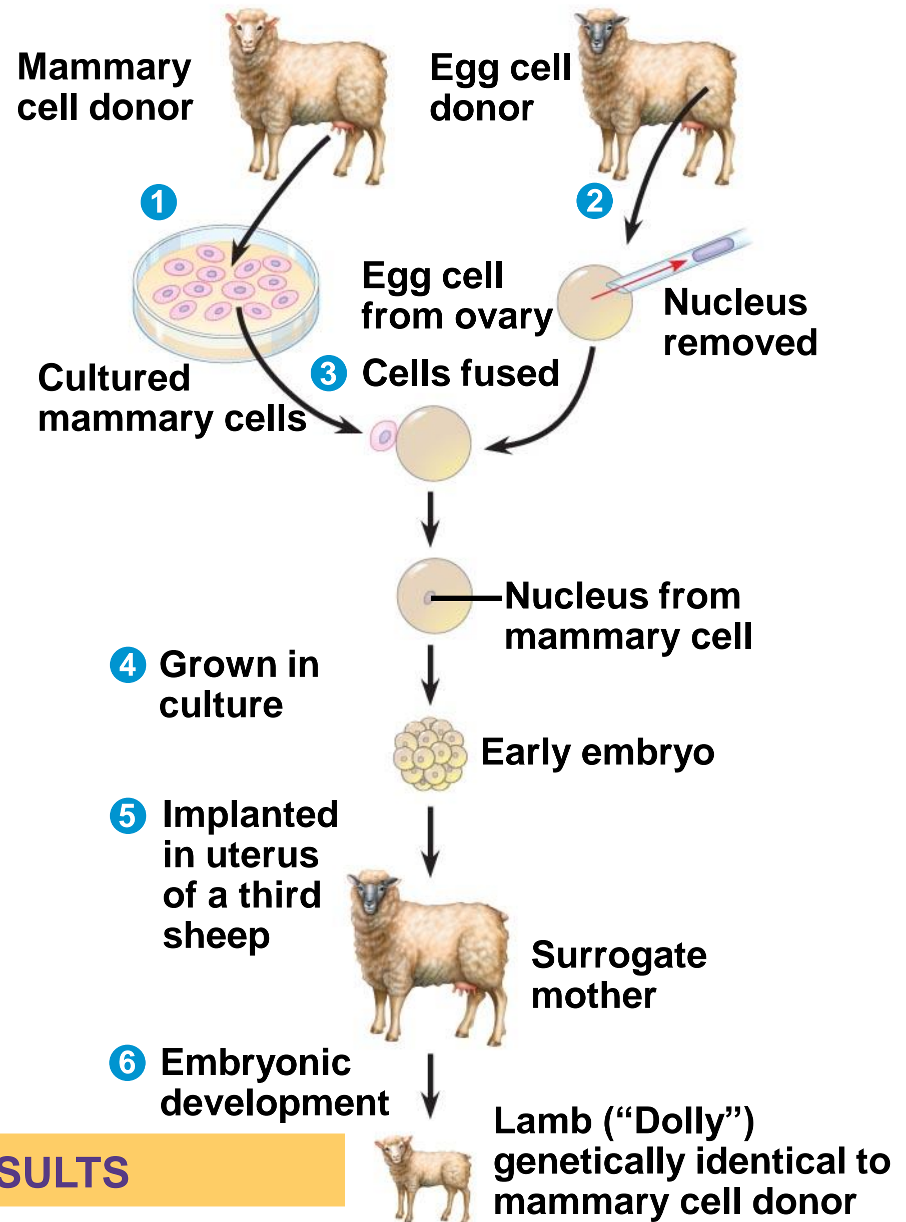
Reproductive Cloning of Mammals

- In 1997, Scottish researchers announced the birth of Dolly, a lamb cloned from an adult sheep by nuclear transplantation from a differentiated mammary cell
- Dolly's premature death in 2003, as well as her arthritis, led to speculation that her cells were not as healthy as those of a normal sheep, possibly reflecting incomplete reprogramming of the original transplanted nucleus



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Reproductive Cloning of Mammals

- Since 1997, cloning has been demonstrated in many mammals, including mice, cats, cows, horses, mules, pigs, and dogs - CC (for Carbon Copy) was the first cat cloned; however, CC differed somewhat from her female "parent"

- In most nuclear transplantation studies, only a small percentage of cloned embryos have developed normally to birth

- Many epigenetic changes, such as acetylation of histones or methylation of DNA, must be reversed in the nucleus from a donor animal in order for genes to be expressed or repressed appropriately for early stages of development

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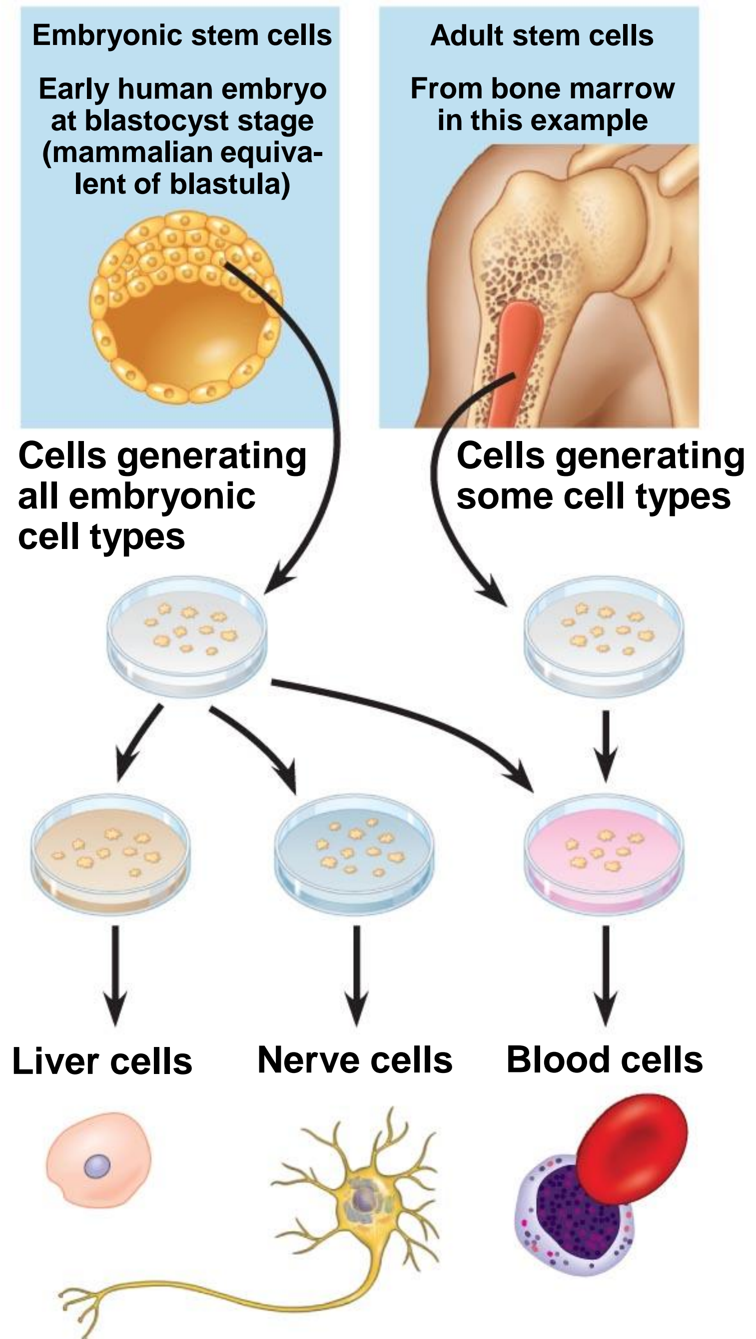
Stem Cells of Animals

- A **stem cell** is a relatively unspecialized cell that can reproduce itself indefinitely and differentiate into specialized cells of one or more types
- Stem cells isolated from early embryos at the blastocyst stage are called *embryonic stem cells*; these are able to differentiate into all cell types (Totipotent)
- The adult body also has stem cells, called *adult stem cells*, which replace nonreproducing specialized cells and can only form a few types of cells (Pluripotent)



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The practical applications of DNA technology affect our lives in many ways

- Many fields benefit from DNA technology and genetic engineering
- One benefit of DNA technology is identification of human genes in which mutation plays a role in genetic diseases
  - Scientists can diagnose many human genetic disorders by using PCR and primers corresponding to cloned disease genes, then sequencing the amplified product to look for the disease-causing mutation
  - Genetic disorders can also be tested for using genetic markers that are linked to the disease-causing allele
  - **Single nucleotide polymorphisms (SNPs),** which are signle base-pair sites that differ in a population, are useful genetic markers
  - When a restriction enzyme is added, SNPs result in DNA fragments with different lengths, or **restriction fragment length polymorphism (RFLP)**

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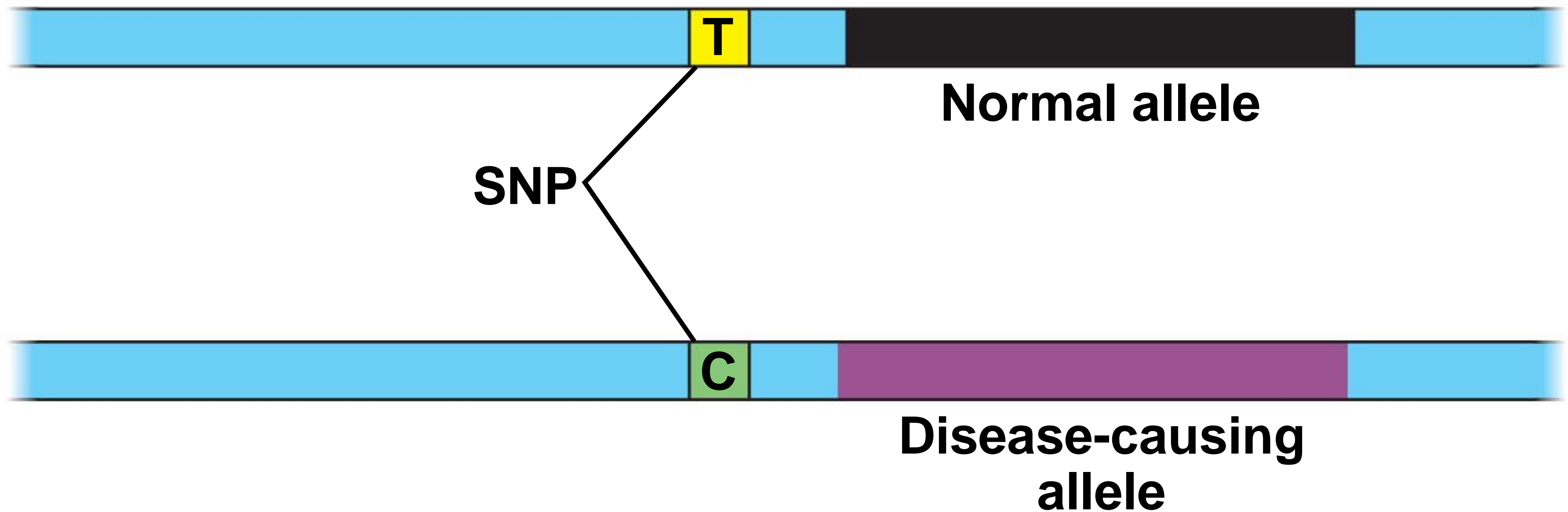
Single nucleotide polymorphisms (SNPs) as genetic markers for disease-causing alleles



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Human Gene Therapy

- **Gene therapy** is the alteration of an afflicted individual's genes
  - Gene therapy holds great potential for treating disorders traceable to a single defective gene
  - Vectors are used for delivery of genes into specific types of cells, for example bone marrow
  - Gene therapy raises ethical questions, such as whether human germ-line cells should be treated to correct the defect in future generations

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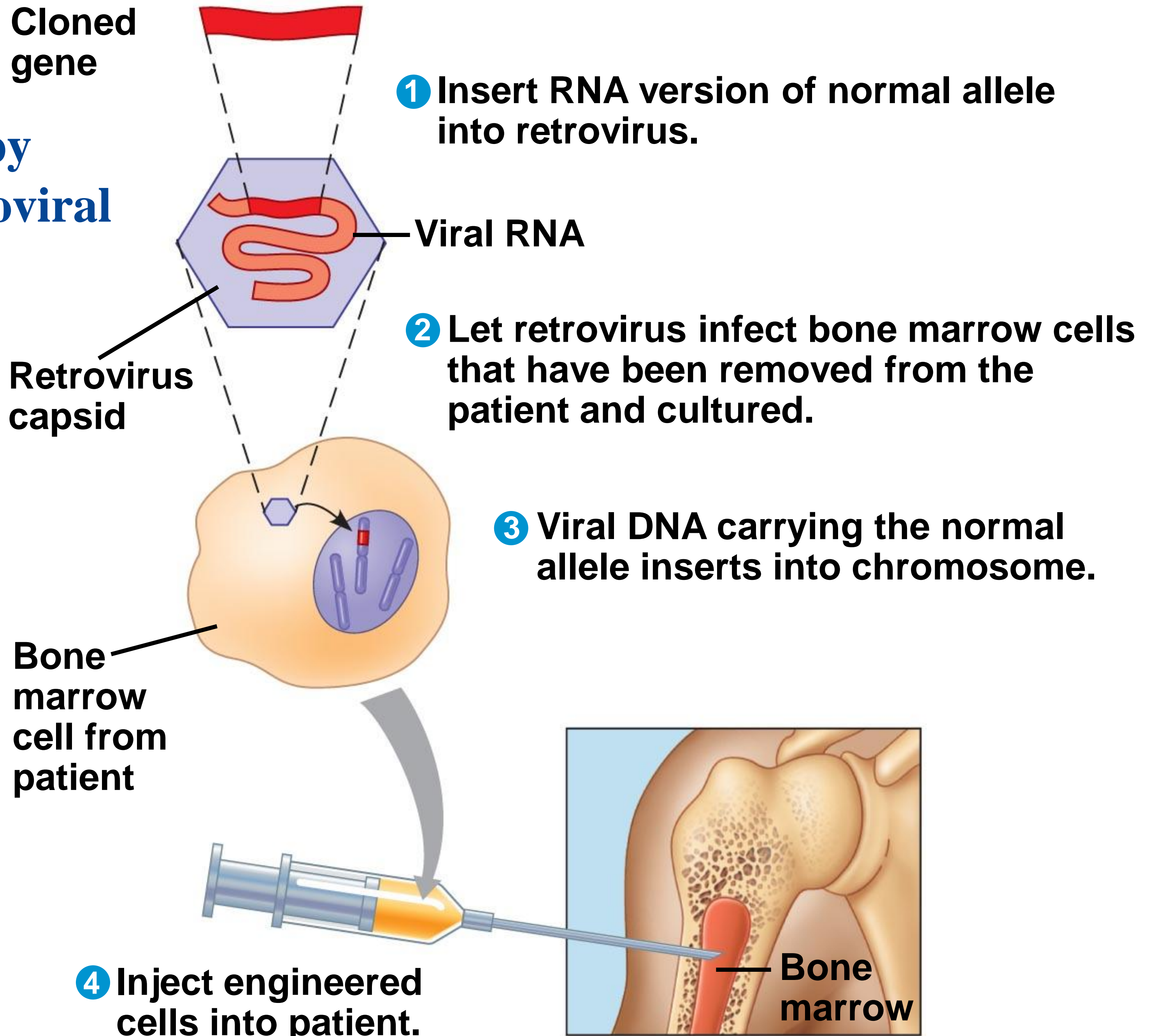
Gene therapy using a retroviral vector



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Pharmaceutical Products

- Advances in DNA technology and genetic research are important to the development of new drugs to treat diseases
- **Synthesis of Small Molecules for Use as Drugs -** The drug imatinib is a small molecule that inhibits overexpression of a specific leukemia-causing receptor
  - Pharmaceutical products that are proteins can be synthesized on a large scale
- **Protein Production in Cell Cultures -** cells in culture can be engineered to secrete a protein as it is made
  - This is useful for the production of insulin, human growth hormones, and vaccines
- **Protein Production by "Pharm" Animals and PlantsTransgenic** animals are made by introducing genes from one species into the genome of another animal
  - Transgenic animals are pharmaceutical "factories," producers of large amounts of otherwise rare substances for medical use
  - "Pharm" plants are also being developed to make human proteins for medical use

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Goats as “pharm” animals

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Forensic Evidence and Genetic Profiles

- An individual's unique DNA sequence, or **genetic profile**, can be obtained by analysis of tissue or body fluids
  - Genetic profiles can be used to provide evidence in criminal and paternity cases and to identify human remains
- Genetic profiles can be analyzed using RFLP analysis by Southern blotting
- Even more sensitive is the use of genetic markers called **short tandem repeats (STRs)**, which are variations in the number of repeats of specific DNA sequences
  - PCR and gel electrophoresis are used to amplify and then identify STRs of different lengths
  - The probability that two people who are not identical twins have the same STR markers is exceptionally small

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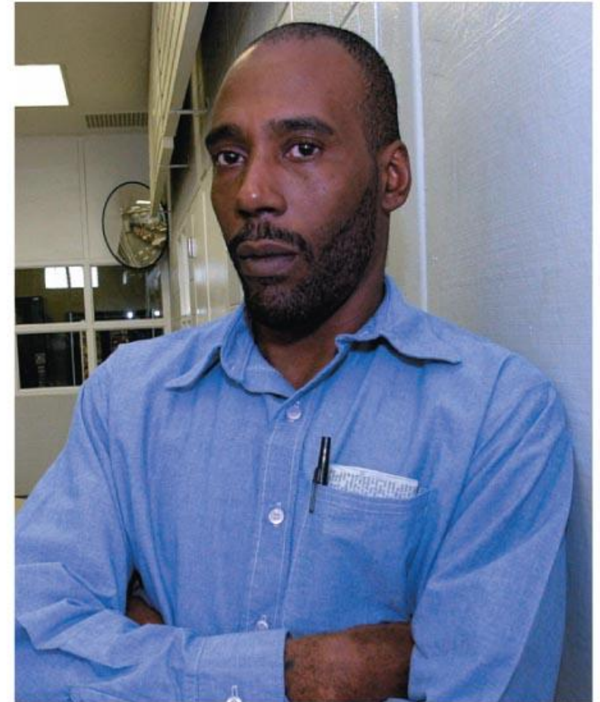
STR analysis used to release an innocent man from prison

(a) This photo shows Earl Washington just before his release in 2001, after 17 years in prison.



Source of sample	STR marker 1	STR marker 2	STR marker 3
Semen on victim	17, 19	13, 16	12, 12
Earl Washington	16, 18	14, 15	11, 12
Kenneth Tinsley	17, 19	13, 16	12, 12

(b) These and other STR data exonerated Washington and led Tinsley to plead guilty to the murder.

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Other uses of genetic engineering

- Genetic engineering can be used to modify the metabolism of microorganisms
  - Some modified microorganisms can be used to extract minerals from the environment or degrade potentially toxic waste materials
  - Biofuels make use of crops such as corn, soybeans, and cassava to replace fossil fuels
- DNA technology is being used to improve agricultural productivity and food quality - Beneficial genes can be transferred between varieties or species
- Genetic engineering in plants has been used to transfer many useful genes including those for herbicide resistance, increased resistance to pests, increased resistance to salinity, and improved nutritional value of crops
- Genetic engineering of transgenic animals speeds up the selective breeding process

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Safety and Ethical Questions Raised by DNA Technology

- Potential benefits of genetic engineering must be weighed against potential hazards of creating harmful products or procedures
- Guidelines are in place in the United States and other countries to ensure safe practices for recombinant DNA technology
- Most public concern about possible hazards centers on **genetically modified (GM) organisms** used as food
  - Some are concerned about the creation of “super weeds” from the transfer of genes from GM crops to their wild relatives
- As biotechnology continues to change, so does its use in agriculture, industry, and medicine
  - National agencies and international organizations strive to set guidelines for safe and ethical practices in the use of biotechnology

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