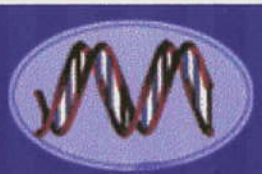
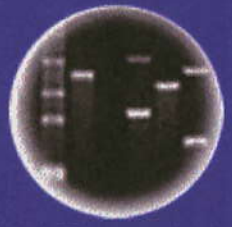


DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

Plants of Tomorrow

HC70A Winter 2008
Genetic Engineering in Medicine, Agriculture, and Law

Professor Bob Goldberg

Lecture 4
The Nuts & Bolts of Genetic Engineering: The Factor VIII Story - From Gene To Drug

Had Rosalind Franklin Lived, She Deserved to Share the Nobel Prize with Watson, Crick, and Wilkins.

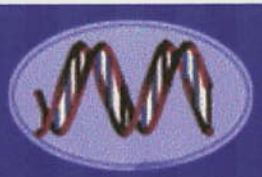
a. Yes
b. No

iClicker

Wilkins Deserved to Share the Nobel Prize With Watson And Crick in 1962:

a. Yes
b. No

iClicker



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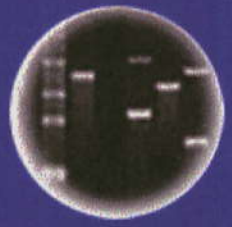


DNA Fingerprinting

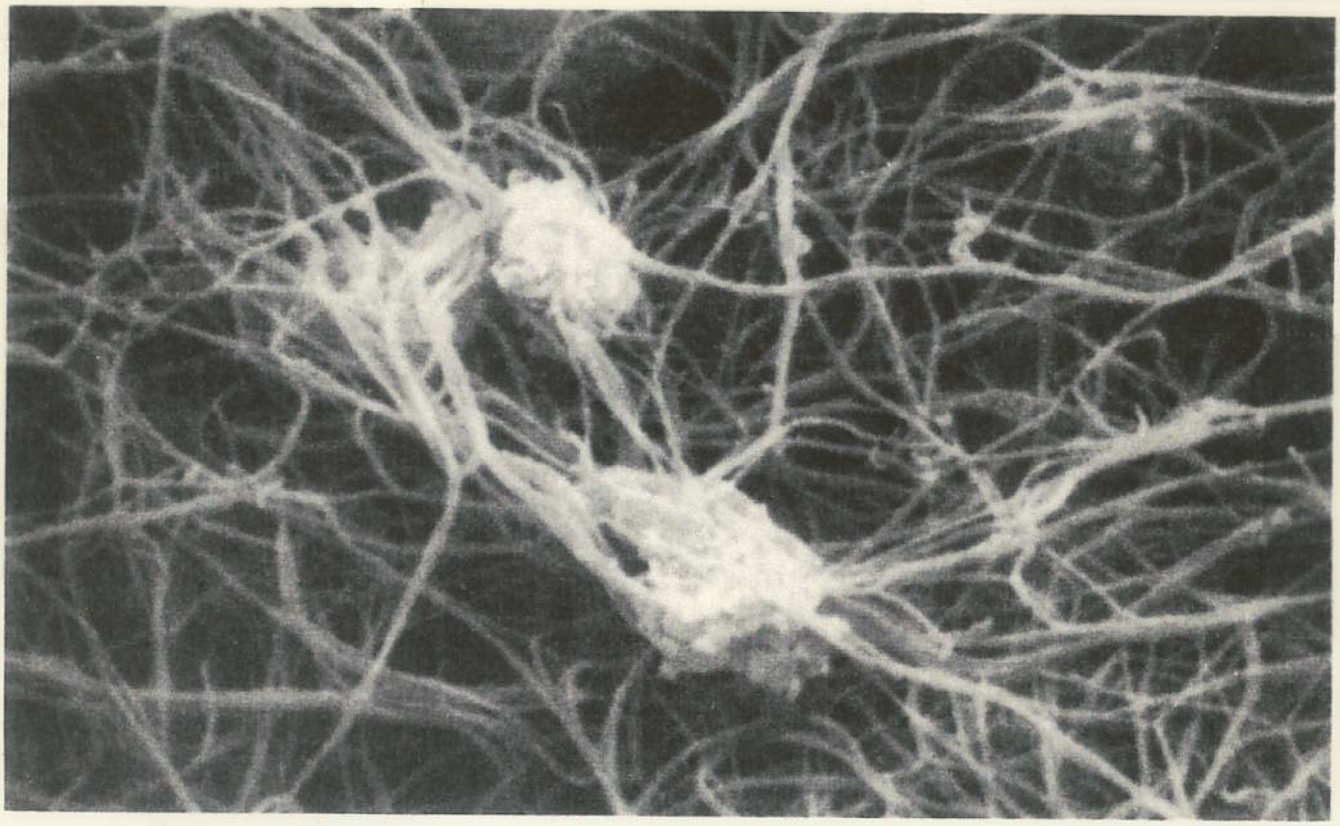
Cloning: Ethical Issues
and Future Consequences

Plants of Tomorrow

THEMES

1. What is Hemophilia?
2. How is Hemophilia Inherited?
3. What is the Pedigree Pattern of a Sex-Linked Gene?
4. How Find a Disease Gene When It is Not Known Where the Gene is Expressed?
5. What Vectors Can Be Used For Cloning DNA?
6. What Are the Advantages of Using a Virus Vector For Constructing Genome Libraries?
7. How Make a Library of the Human Genome?
8. How Find a Gene With Only a Knowledge of the Protein Sequence?
9. What is Chromosome Walking & What Role Did it Play in Cloning the Factor VIII Gene?
10. How Use DNA Testing to Detect Factor VIII Disease Alleles?
11. How Isolate a Factor VIII cDNA Clone?
12. How Produce Factor VIII Protein For Use as a Drug?

The Molecular Genetics of Hemophilia



FIBRIN STRANDS stabilize a blood clot at the site of a wound by trapping the platelets that form the bulk of the clot. The electron micrograph, which was made by Jon C. Lewis of Wake Forest University, shows a clot formed in a suspension of platelets and fibrin.

A clot in the bloodstream is the result of a complex cascade of enzymatic reactions culminating in the conversion of fibrinogen, a soluble protein, into insoluble fibrin strands. In hemophiliacs a crucial protein in the blood-clotting cascade is either missing or defective.

A CASE STUDY OF CLONING GENES AND MRNAS

HEMOPHILIA HAS BEEN KNOWN AS AN INHERITED DISEASE FOR > 2500 Years!

HUMAN GENETICS SIDELIGHT

Hemophilia: Successful Treatment of a Once Deadly Disorder

A small defect in an important gene can cause a fatal human disease. In the past, hemophilia, excess bleeding caused by a defect in blood clotting, was such a disease—often fatal early in life. Before the 1960s, when scientist-physicians developed the first effective treatment, the life expectancy of individuals with hemophilia was about 20 years. Today, hemophiliacs in most of the world have a nearly normal life expectancy. An understanding of the molecular basis of the disease resulted in the development of an effective treatment.

There are two major types of hemophilia. About 80 percent of the individuals with this disease have hemophilia A (classical hemophilia), and about 20 percent have hemophilia B (also called Christmas disease because it was first detected in a patient named Stephen Christmas). Both types of hemophilia are caused by defective genes on the X chromosome, the human chromosome that is present in two copies in females and one copy in males (Chapter 6). Most hemophiliacs are males, because they only need one copy of the defective gene to have the disease. Hemophilia is rare in females, because they need two copies of the defective gene, one on each X chromosome, to have the disorder.

Hemophilia A is sometimes called "royal hemophilia" because of its prevalence in the royal families of Europe. England's Queen Victoria (Figure 1) did not have hemophilia, although she carried the defective gene that causes hemophilia A on one of her X chromosomes. However, she passed the defective gene to two of her daughters—Alice, who transmitted the gene to the imperial families of Russia (see Figure 6.9) and Germany, and Beatrice, who passed the gene to the royal family of Spain—and to her son Prince Leopold, who died at age 31 from hemorrhages after a fall. Several of the queen's grandsons and great-grandsons died early in life because of excess bleeding or hemorrhages after surgery or accidents.

Bible

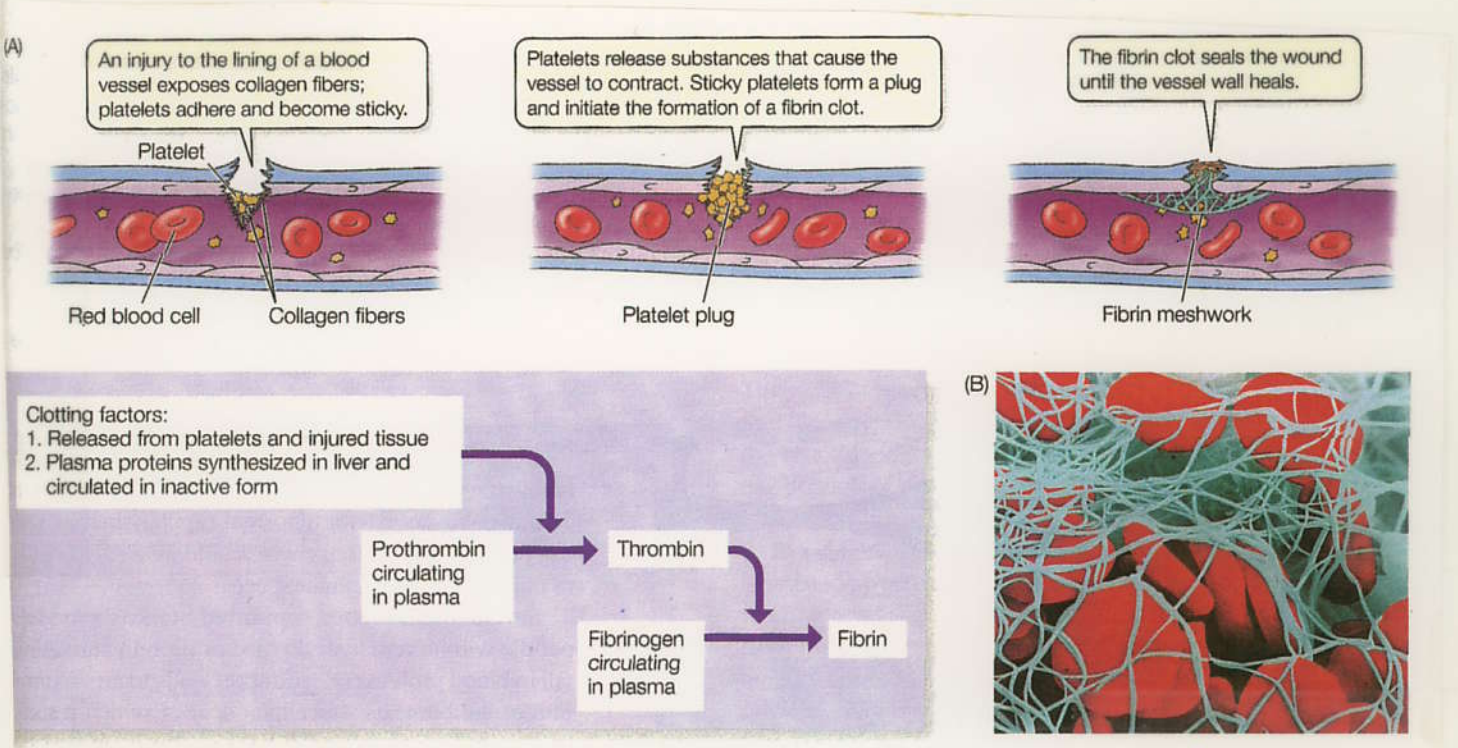
The mode of transmission of hemophilia was probably recognized in ancient civilizations. The Jewish Talmud, which dates to about 400 B.C. and was compiled into a single document in the 4th and 5th centuries A.D., decreed that boys whose older brothers or male cousins had died from excessive bleeding after circumcision were exempt from this procedure.

Hemophilia A and hemophilia B both result from defects in blood coagulation—the cascade of reactions that causes blood to clot at the site of a wound. A simplified version of part of this pathway is shown in Figure 2. Individuals with hemophilia A are deficient in a gene product called factor VIII; those with hemophilia B are lacking factor IX. In the absence of either of these blood-clotting factors, an individual can bleed to death after suffering a small cut or can die from internal hemorrhages after an otherwise minor bruise.

When scientists discovered that hemophilia was caused by the absence of specific blood-clotting factors, they realized that the disease could be treated with transfusions of concentrates of the missing factor. Initially, beginning in the 1960s, the proteins were purified from blood obtained from large numbers of donors. This process was expensive, and the concentrates were either unavailable or were too expensive for use by hemophiliacs in many countries. Fortunately, the advent of genetic engineering brought positive changes. The genes that encode factor VIII and factor IX were both isolated, and each gene was introduced into mammalian cells growing in culture. By this procedure, cell culture lines were produced that synthesize large quantities of either factor VIII or factor IX. The clotting factors are now purified from these cells and used to prepare concentrates for use in transfusions. As a result, both clotting factors are now available in essentially unlimited quantity to treat people suffering from hemophilia.

Figure 1 A portrait of Great Britain's Queen Victoria, her husband Prince Albert, and five of their nine children. Queen Victoria passed the defective gene that is responsible for hemophilia to at least three of her children. They, in turn, passed the gene to the royal families of Germany, Russia, and Spain (see Figure 6.9). The present British royal family is free of hemophilia. They are descendants of Victoria's son King Edward VII, who did not inherit the hemophilia gene from his mother.

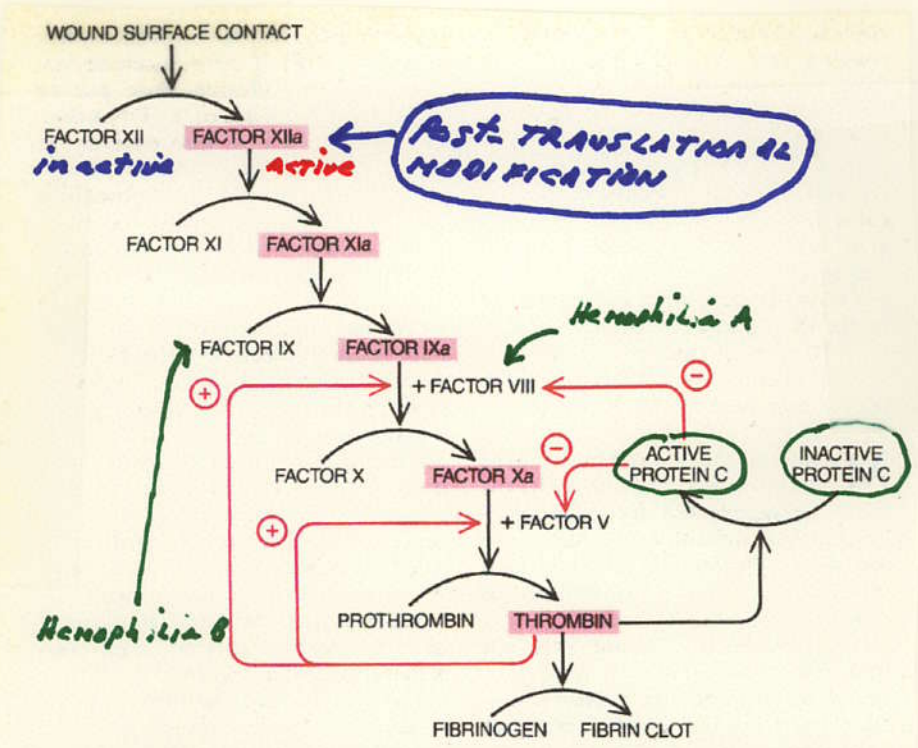
A CASCADE OF EVENTS AFTER WOUNDING LEADS TO A FIBRIN CLOT



.10 Blood Clotting (A) Damage to a blood vessel initiates a cas-de of events that produces a fibrin meshwork. (B) As the meshwork ms, red blood cells are enmeshed in the fibrin threads, forming a t, as shown in this color-enhanced electron micrograph.

CLOTTING FACTORS SUCH AS FACTOR VIII PLAY A CRITICAL ROLE IN THIS PROCESS

HOW DOES BLOOD CLOT AFTER WOUNDING?



CLOTTING CASCADE begins when cell damage at a wound somehow activates the enzyme factor XII; it ends with the conversion of fibrinogen into fibrin by thrombin. At each step an inactive protein is converted into a protease, or protein-cutting enzyme (*color*), which activates the next protein. Some steps require cofactors such as factors VIII and V. The cascade includes positive- and negative-feedback loops (*colored arrows*). Thrombin activates factors VIII and V; it also deactivates them (by activating protein *C*), which helps to halt clotting. Some 85 percent of hemophiliacs lack factor VIII. The rest lack factor IX.

Eight Proteins/Genes Required

1. Factor VII
2. Factor XI
3. Factor IX
4. Factor VIII
5. Factor X
6. Protein C
7. Prothrombin
8. Fibrinogen

What happens if any of these proteins or genes are Mutated?

↓

NO BLOOD CLOT!

Anti-Thrombin ??

CASCADE

↳ Anti-Thrombin Deficiency (AT-III) → genetic disease

(4)

Hemophiliacs Have Mutations in Either FACTOR VIII or FACTOR IX Genes

Table 13.2 Some Important Genetic Disorders

Disorder	Symptom	Defect	Dominant/ Recessive	Frequency among Human Births
Cystic fibrosis	Mucus clogs lungs, liver, and pancreas	Failure of chloride ion transport mechanism	Recessive	1/2500 (Caucasians)
Sickle cell anemia	Poor blood circulation	Abnormal hemoglobin molecules	Recessive	1/625 (African Americans)
Tay-Sachs disease	Deterioration of central nervous system in infancy	Defective enzyme (hexosaminidase A)	Recessive	1/3500 (Ashkenazi Jews)
Phenylketonuria	Brain fails to develop in infancy	Defective enzyme (phenylalanine hydroxylase)	Recessive	1/12,000
Hemophilia	Blood fails to clot	Defective blood clotting factor VIII	Sex-linked recessive	1/10,000 (Caucasian males)
Huntington's disease	Brain tissue gradually deteriorates in middle age	Production of an inhibitor of brain cell metabolism	Dominant	1/24,000
Muscular dystrophy (Duchenne)	Muscles waste away	Degradation of myelin coating of nerves stimulating muscles	Sex-linked recessive	1/3700 (males)
Hypercholesterolemia	Excessive cholesterol levels in blood, leading to heart disease	Abnormal form of cholesterol cell surface receptor	Dominant	1/500

Hemophilia A — Defective FACTOR VIII Gene — 1/10,000 Males

Hemophilia B — Defective FACTOR IX Gene — 1/30,000 Males

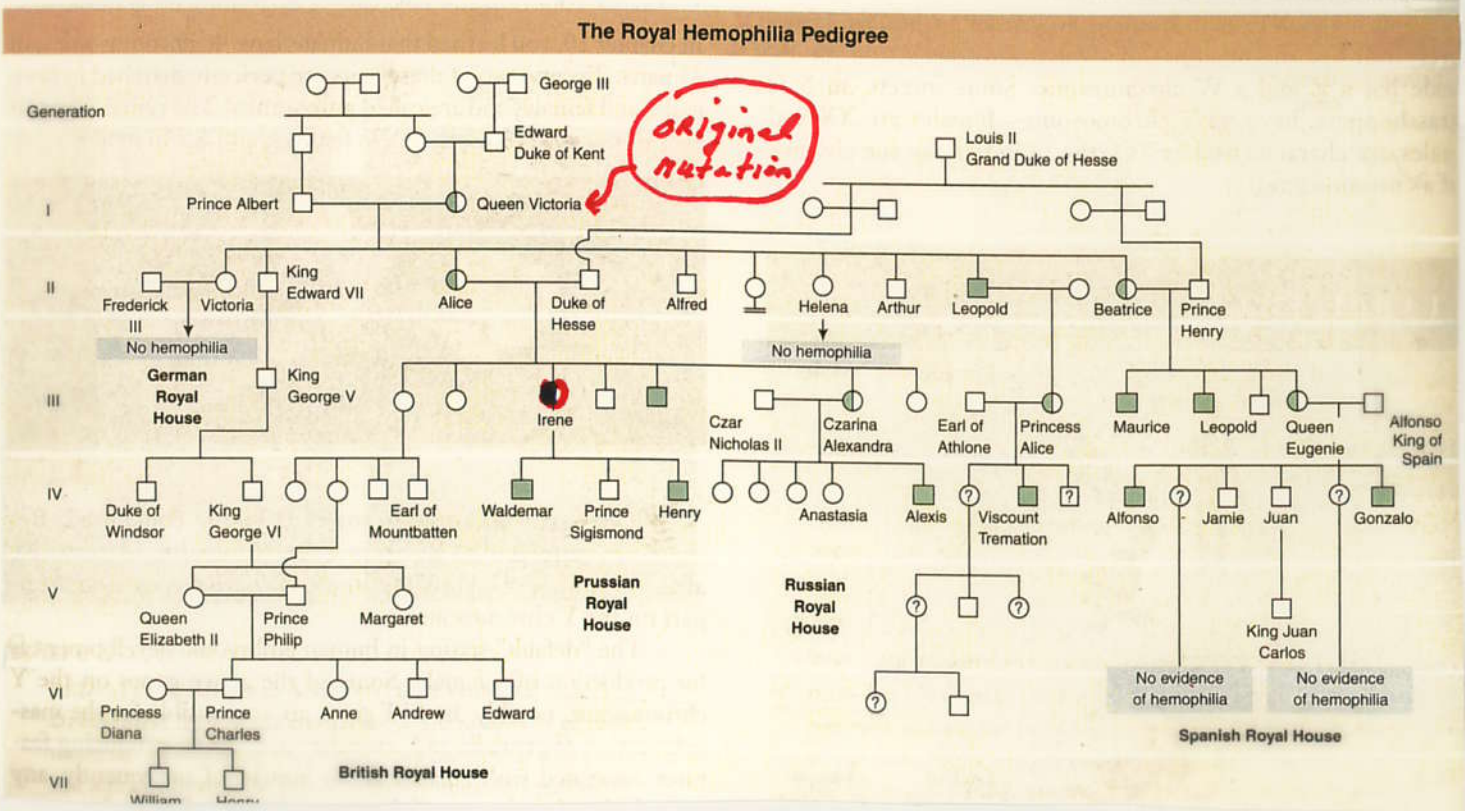
Hypothesis For High Frequency?

Both Genes on X-Chromosome
♀ → ♂'s

HEMOPHILIA A and B GENES (TRAITS) ARE SEX-LINKED

figure 13.3

THE ROYAL HEMOPHILIA PEDIGREE. Queen Victoria, shown at the bottom center of the photo, was a carrier for hemophilia. Two of Victoria's four daughters, Alice and Beatrice, inherited the hemophilia allele from Victoria. Two of Alice's daughters are standing behind Victoria (wearing feathered boas): Princess Irene of Prussia (*right*) and Alexandra (*left*), who would soon become czarina of Russia. Both Irene and Alexandra were also carriers of hemophilia. From the pedigree, it is clear that Alice introduced hemophilia into the Russian and Prussian royal houses, and Victoria's daughter Beatrice introduced it into the Spanish royal house. Victoria's son Leopold, himself a victim, also transmitted the disorder in a third line of descent. Half-shaded symbols represent carriers with one normal allele and one defective allele; fully shaded symbols represent affected individuals.

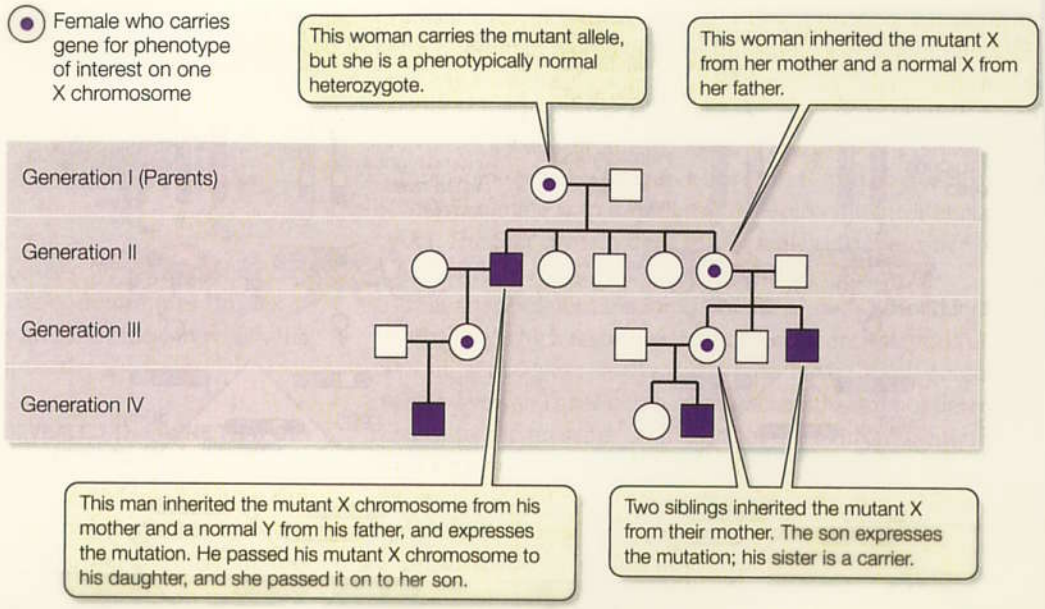


NOTE! ① Males obtain Defective Gene FROM Mothers

② 50% of Sons of a Maternal CARRIER Have the Defective Gene

SEX-LINKED INHERITANCE PATTERN FOLLOWS X-CHROMOSOME DISTRIBUTION TO GAMETES

10.24 Red-Green Color Blindness Is a Sex-Linked Trait in Humans The mutant allele for red-green color blindness is inherited as an X-linked recessive.



HUMAN DIPLOID KARYOTYPE A MALE XY

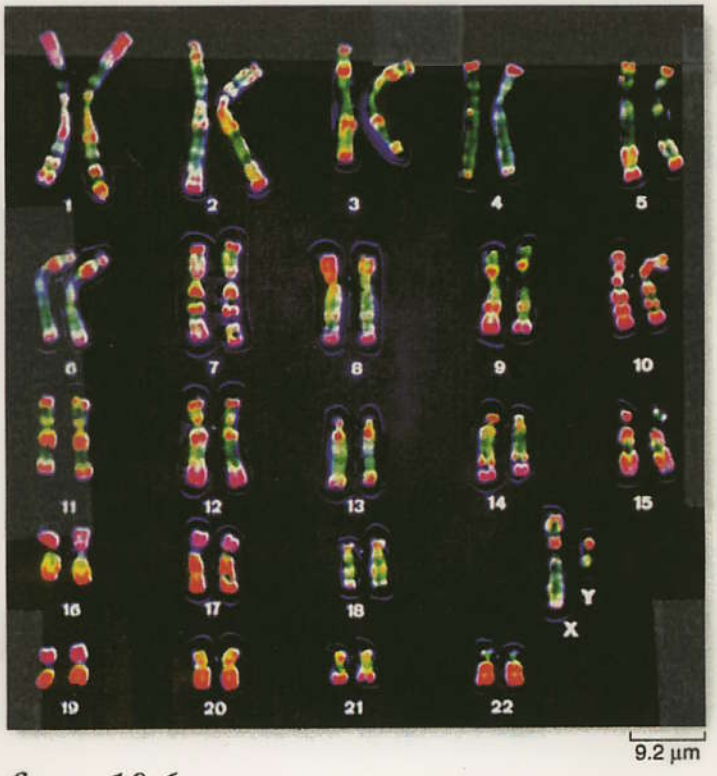


figure 10.6

A HUMAN KARYOTYPE. The individual chromosomes that make up the 23 pairs differ widely in size and in centromere position. In this preparation, the chromosomes have been specifically stained to indicate differences in their composition and to distinguish them clearly from one another. Notice that members of a chromosome pair are very similar but not identical.

Hemophilia A Disease Alleles Can Arise Because of:

a. A Change in a Base-Pair Sequence
b. An Addition of One or More Base Pairs
c. A Deletion of One or More Base Pairs
d. All of the Above

iClicker

The Hemophilia A Disease Allele Resides at the Same X-Chromosome Locus as the Normal (Wild Type) Hemophilia A Gene:

a. Yes
b. No

iClicker

An XY Individual is Always a Male:

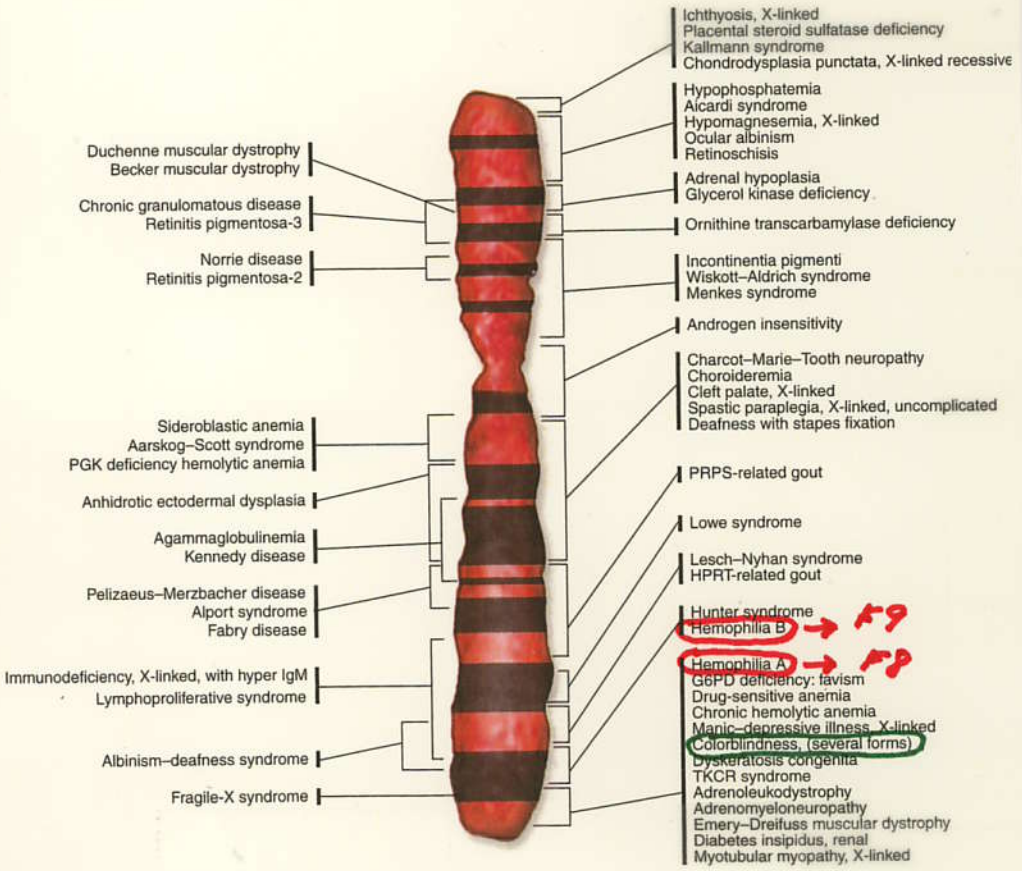
a. Yes
b. No

iClicker

FACTOR VIII AND FACTOR IX GENES ARE CLOSELY LINKED ON THE X CHROMOSOME

figure 13.10

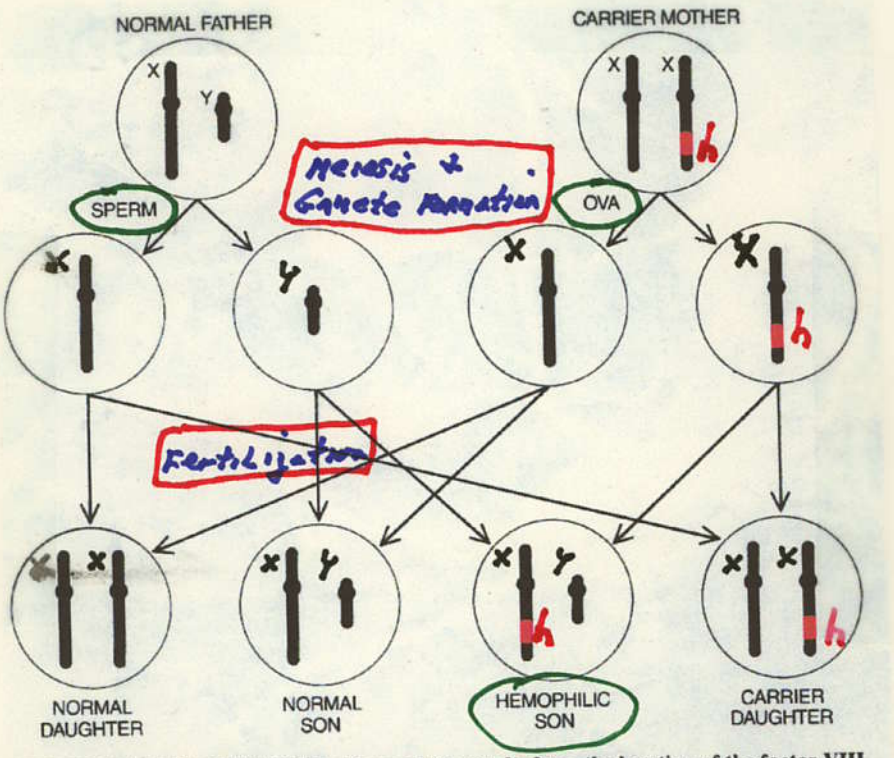
THE HUMAN X CHROMOSOME GENE MAP. A partial map for the human X chromosome, a more detailed map would require a much larger figure. The black bands represent staining patterns that can be seen in the microscope, and the constriction represents the centromere. Analysis of the sequence of the X chromosome indicates 1098 genes on the X chromosome. Many of these may have mutant alleles that can affect disease states. The 59 diseases shown have been traced to specific segments of the X chromosome, indicated by brackets, by analyzing inheritance patterns of affected and unaffected individuals.



Note: Factor VIII gene is Closely Linked to Color-blindness Gene.

The X chromosome has ~ 1500 Genes (2008) and 150,000,000 bp (150 Mb).

HEMOPHILIA A and B Inheritance



SEX-LINKED INHERITANCE of hemophilia results from the location of the factor VIII gene on the X chromosome. A male carrying a mutant factor VIII gene lacks normal factor VIII and is hemophilic. A female carrier is protected by the normal gene on her second X chromosome, but half of her daughters will be carriers and half of her sons will be hemophilic. In the case of a hemophilic father (not shown), his sons will not be hemophilic, because they receive his Y (not his X) chromosome, but his daughters will be carriers.

PARENTS 2n
↓ MEIOSIS
GAMETES n
↓ Fertilization
CHILDREN 2n

SEX-LINKED INHERITANCE

♀ CARRIERS → ½ SONS & NO DAUGHTERS!

ONLY ONE X-CHROMOSOME in ♂

FROM DISEASE TO GENE - USING PROTEIN TO IDENTIFY FACTOR EIGHT GENE

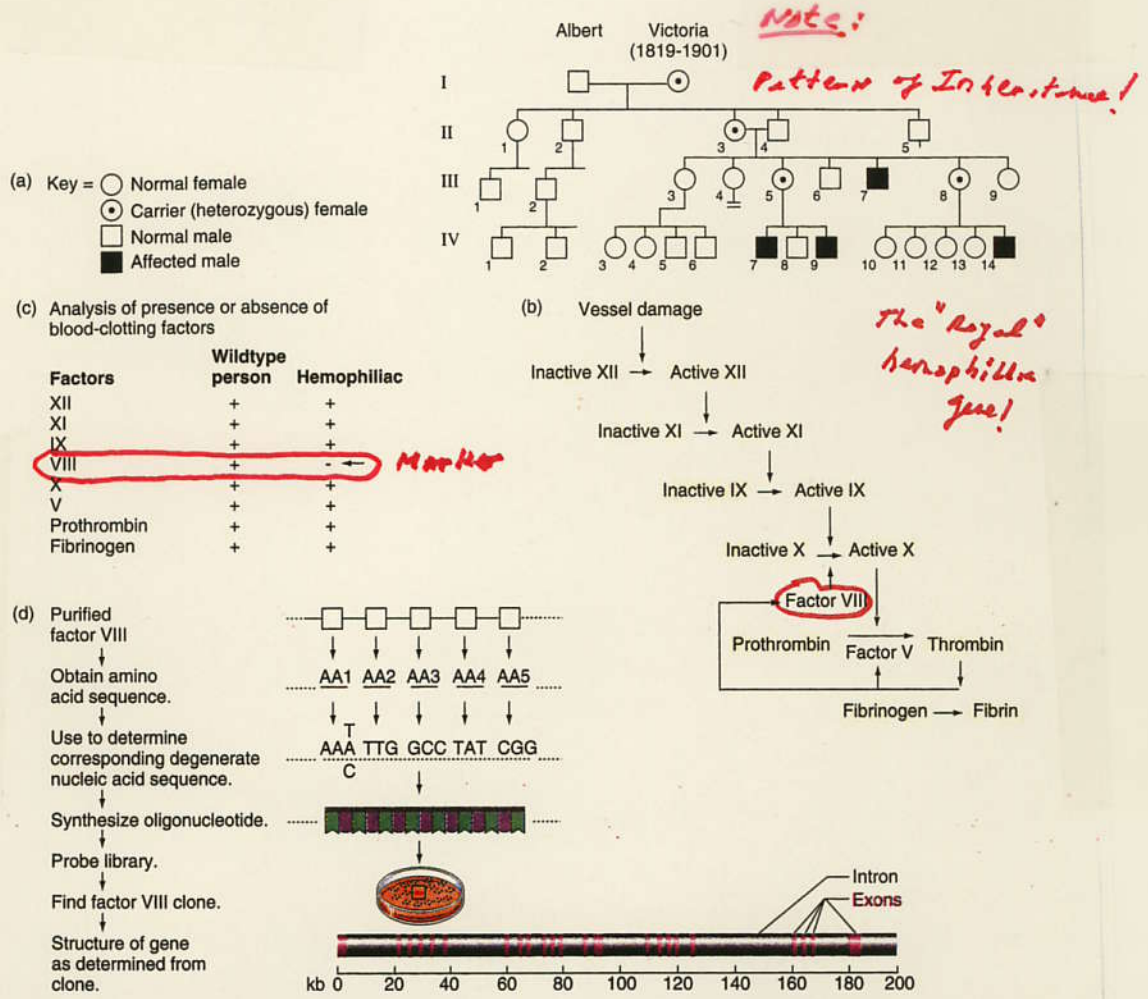


Figure 10.1 How geneticists identified the hemophilia A gene. (a) A pedigree of the royal family descended from Queen Victoria. This family tree uses the standard pedigree symbols. Black boxes represent males with hemophilia. (b) The blood clotting cascade. Vessel damage induces a cascade of enzymatic events that convert inactive factors to active factors. The cascade results in the transformation of fibrinogen to fibrin and the formation of a clot. (c) Many hemophiliac patients do not have an active form of Factor VIII. Blood tests can determine the presence or absence of the active form of each factor involved in the clotting cascade. The results of such analyses show that hemophiliacs, such as those found in Queen Victoria's pedigree, lack an active Factor VIII in their blood. (d) Starting with purified Factor VIII, scientists isolated DNA clones containing the *Factor VIII* gene. Researchers determined the amino-acid sequence of purified protein. Knowledge of this sequence enabled them to synthesize a degenerate oligonucleotide. They then used the oligonucleotide as a probe to screen a genomic library for clones containing all or parts of the gene. Finally, they sequenced the positive clones (that is, the clones with which the probe hybridizes) to determine the structure and coding sequence of the *Factor VIII* gene.

Key Concept ⇒ HOW CLONE A GENE WHEN YOU DON'T KNOW WHERE IT IS EXPRESSED!

What was known about Factor VIII before gene cloned?

1. Blood Protein (but perhaps synthesized elsewhere!)
2. Could be purified in small amounts from 25,000 liters of cow's blood! & pig's blood
3. Short stretch of both proteins sequenced & sequence known
4. Hemophilia A could be treated by blood transfusions from normal individuals ∴ clotting factor in blood.

∴ How to go from protein to gene?

KNOWLEDGE OF THE PROTEIN SEQUENCE AND THE GENETIC CODE MAKES IT POSSIBLE TO IDENTIFY A GENE

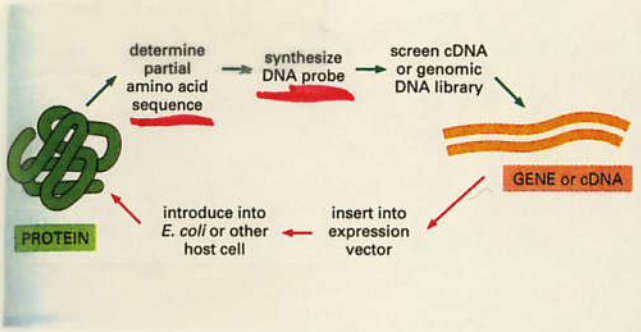


Figure 8–44 Knowledge of the molecular biology of cells makes it possible to experimentally move from gene to protein and from protein to gene. A small quantity of a purified protein is used to obtain a partial amino acid sequence. This provides sequence information that enables the corresponding gene to be cloned from a DNA library. Once the gene has been cloned, its protein-coding sequence can be inserted into an expression vector and used to produce large quantities of the protein from genetically engineered cells.

∴ (1) PROTEIN → GENE → DRUG

OR

(2) GENE → PROTEIN USING SEQUENCING and Genetic Code

Genomics

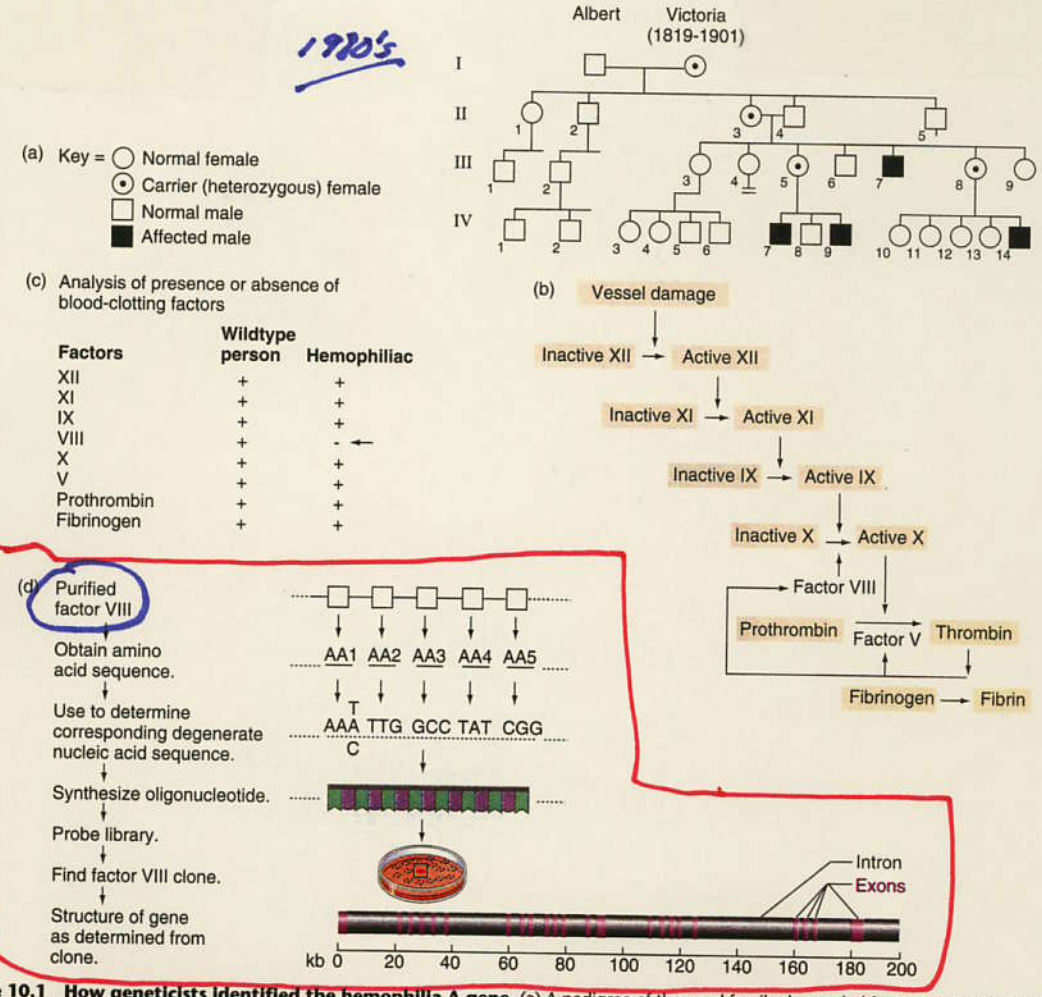
GENBANK

2008 → JUST SEQUENCE Everything + Identify Protein – GENBANK HUGE

THE PROBLEM

FOR FACTOR VIII – NOT KNOWN WHERE GENE IS EXPRESSED ∴ MUST USE GENOME LIBRARY

1980's



Key protein sequence known

Figure 10.1 How geneticists identified the hemophilia A gene. (a) A pedigree of the royal family descended from Queen Victoria. This family tree uses the standard pedigree symbols. Black boxes represent males with hemophilia. (b) The blood clotting cascade. Vessel damage induces a cascade of enzymatic events that convert inactive factors to active factors. The cascade results in the transformation of fibrinogen to fibrin and the formation of a clot. (c) Many hemophiliac patients do not have an active form of Factor VIII. Blood tests can determine the presence or absence of the active form of each factor involved in the clotting cascade. The results of such analyses show that hemophiliacs, such as those found in Queen Victoria's pedigree, lack an active Factor VIII in their blood. (d) Starting with purified Factor VIII, scientists isolated DNA clones containing the *Factor VIII* gene. Researchers determined the amino-acid sequence of purified protein. Knowledge of this sequence enabled them to synthesize a degenerate oligonucleotide. They then used the oligonucleotide as a probe to screen a genomic library for clones containing all or parts of the gene. Finally, they sequenced the positive clones (that is, the clones with which the probe hybridizes) to determine the structure and coding sequence of the *Factor VIII* gene.

HOW find Gene & cDNA?

protein → Gene → mRNA → DNA!

FACTOR VIII PROTEIN → GENE USING GENOME LIBRARY

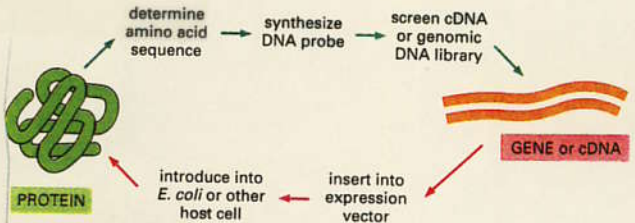


Figure 10–28 Knowledge of the molecula biology of cells makes it possible to experimentally move from gene to protein and from protein to gene. A small quantity of a purified protein is used to obtain a partial amino acid sequence. This provides sequence information that enables the corresponding gene to be cloned from a DNA library (see Figure 10–18). Once the gene has been cloned, its protein-coding sequence can be used to design a DNA that can then be used to produce large quantities of the protein from genetically engineered cells (see Figure 10–27).

FACTOR VIII

FROM PROTEIN TO GENE	FROM GENE TO PROTEIN
Isolate protein on the basis of its molecular function (e.g., enzymatic or hormonal activity)	Isolate genomic clone corresponding to an altered trait in mutants (e.g., nutritional auxotrophy, inherited disease, developmental defect)
Determine partial amino acid sequence of the protein	Use genomic DNA to isolate a cDNA for the mRNA encoded by the gene
Synthesize oligonucleotides that correspond to portions of the amino acid sequence	Sequence the cDNA to deduce amino acid sequence of the encoded protein
Use oligonucleotides as probes to select cDNA or genomic clone encoding the protein from library	Compare deduced amino acid sequence with that of known proteins to gain insight into function of the protein
Sequence isolated gene	Use expression vector to produce the encoded protein

* STOP

pure protein → gene from library

Gene Clone → cDNA → protein in expression vector

Gradually fill GenBank to identify by direct sequencing

SCREening Library

Genome

1. Sequence → Data base
2. Probe from cDNA/Switch
3. Probe from pure mRNA
4. Synthetic Probe from translated DNA Sequence ← ↓ Genetic Code

cDNA

1. Sequence → Data base
2. pure mRNA probe
3. Synthetic probe from translated protein sequence / genetic code
4. clone probe
5. Antibody probe using expression vector

CAN'T USE Antibody - don't know where gene active ∴ can't make cDNA library

STEPS REQUIRED TO CLONE FACTOR VIII GENE AND cDNA

① Make Genome Library Because Factor VIII GENE in genome!

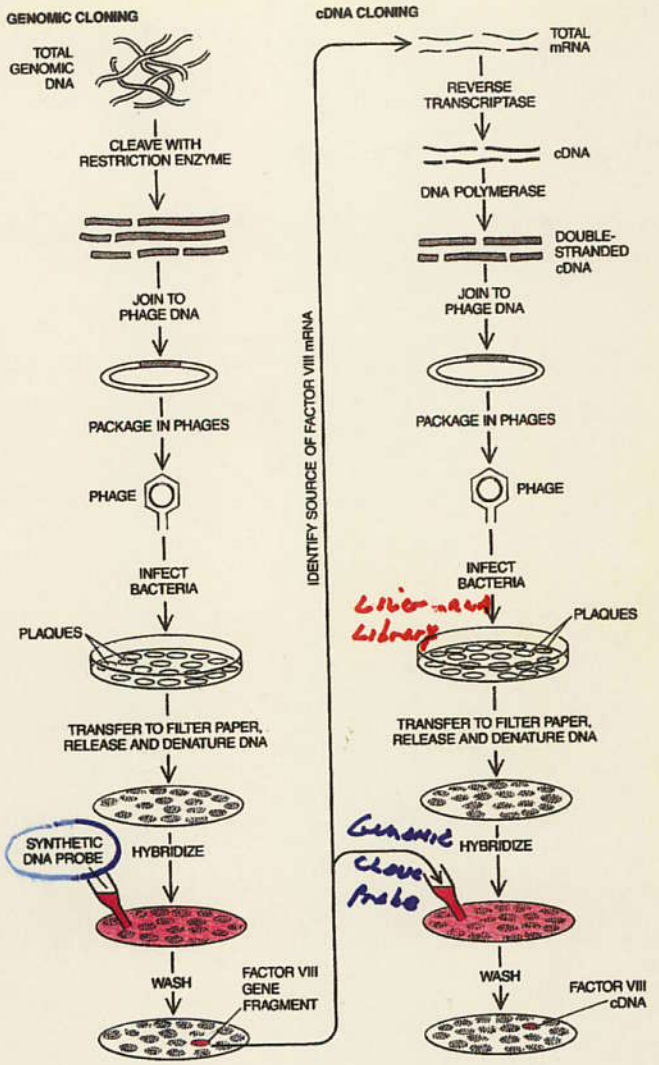
② Purify protein from Blood – That's where it works (wasn't know where made)

③ Reverse Translate using the genetic code a portion of the protein sequence

④ Synthesize a DNA probe → complementary to Factor VIII gene corresponding to protein sequence

⑤ Screen Genome Library

Entire Gene or re Clone?



GENE CLONING involves finding a specific gene among thousands in a human cell. The standard method, if one knows which cells make the desired protein, is to screen a copy DNA (cDNA) library derived by reverse transcription from the messenger RNA (mRNA) of those cells (*right*). In looking for the factor VIII gene, however, the authors did not know where the protein is produced. Hence they screened the entire human genome (*left*). Chromosomal DNA fragments were joined to the DNA of the bacterial virus phage lambda. Each phage contained one human DNA fragment; each phage multiplied and formed a plaque in a distinct region of a bacterial culture. To identify the plaque containing the factor VIII gene, the phages were blotted onto filter paper and broken open to release their DNA. The DNA was exposed to a radioactive probe: a small piece of synthetic DNA encoding part of factor VIII. The probe hybridized with part of the factor VIII gene, thereby labeling it. To produce factor VIII in cultured cells, it was still necessary to make factor VIII cDNA, which lacks the introns (noncoding sequences) that complicate the full gene. Now fragments of the cloned gene could serve as reliable probes, first for identifying cells that make factor VIII mRNA and then for finding factor VIII cDNA in the cDNA library.

① Use Gene probe to screen cDNA library for Factor VIII cDNA clone

② How know what mRNA to use to make cDNA library?

③ Use gene probe to probe RNA Blots containing mRNA from all major organs (liver, kidney, blood etc.) –

④ Find Factor VIII mRNA in liver – Made, Liver – Secreted into Blood

Why need cDNA?

STORY continued

WANT cDNA to MANUFACTURE FACTOR VIII AS A DRUG TO TEAT Hemophilia A!

HOW TO CONSTRUCT A HUMAN GENOME LIBRARY TO FIND THE FACTOR VIII GENE?

If it is not known where Gene is Active CAN "Look" to genome instead of mRNA to find & clone Gene!

VECTORS USED IN GENETIC ENGINEERING HAVE SIMILAR CONCEPTUAL PROPERTIES BUT ARE USED IN DIFFERENT SITUATIONS

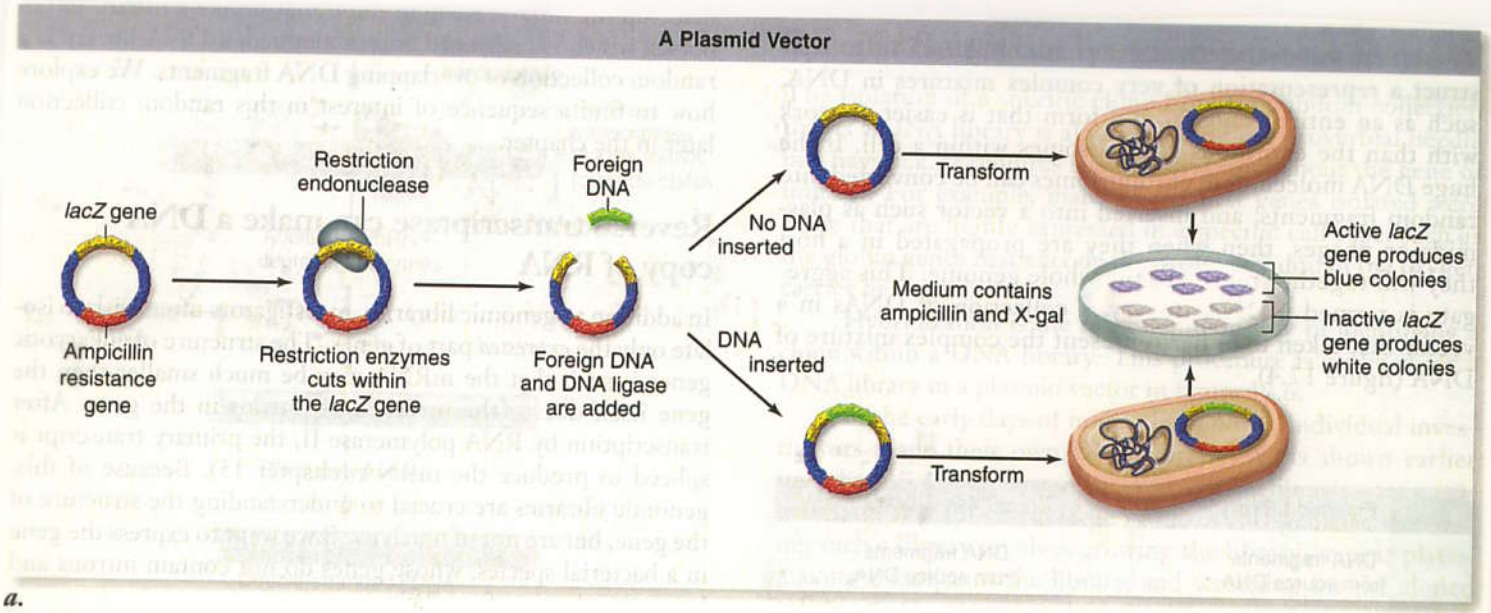
TABLE 3.2 A COMPARISON OF DNA VECTORS AND THEIR APPLICATIONS

Vector Type	Maximum Insert Size (kb)	Applications	Limitations
Bacterial plasmid vectors (circular)	~6–12	DNA cloning, protein expression, subcloning, direct sequencing of insert DNA	Restricted insert size; limited expression of proteins; copy number problems; replication restricted to bacteria
Bacteriophage vectors (linear)	~25	cDNA, genomic and expression libraries	Packaging limits DNA insert size; host replication problems
Cosmid (circular)	~35	cDNA and genomic libraries, cloning large DNA fragments	Phage packaging restrictions; not ideal for protein expression; cannot be replicated in mammalian cells
Bacterial artificial chromosome (circular)	~300	Genomic libraries, cloning large DNA fragments	Replication restricted to bacteria; cannot be used for protein expression
Yeast artificial chromosome (circular)	200–1,000 (1 megabase)	Genomic libraries, cloning large DNA fragments	Must be grown in yeast; cannot be used in bacteria
Ti vector (circular)	Varies depending on type of Ti vector used	Gene transfer in plants	Limited to use in plant cells only; number of restriction sites randomly distributed; large size of vector not easily manipulated.

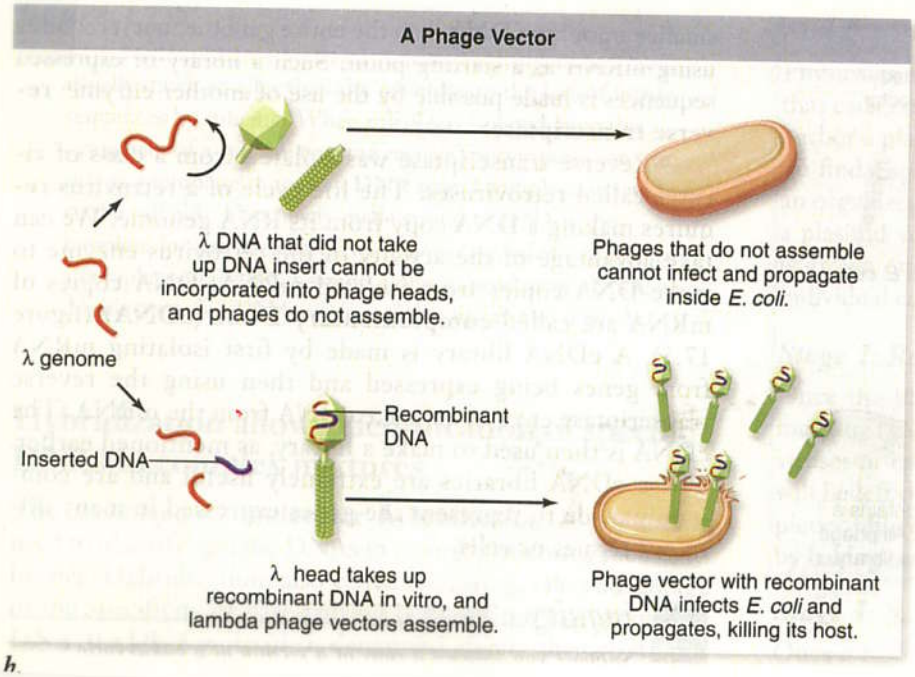
Plasmids vs. Bacteriophage Vectors

1. Replicate
2. Selectable
3. Can be used to insert foreign Genes / Restriction Sites
4. Easily Isolated & Transformed back to cells

PLASMID VS. BACTERIOPHAGE VECTORS FOR CLONING DNA FRAGMENTS



a.

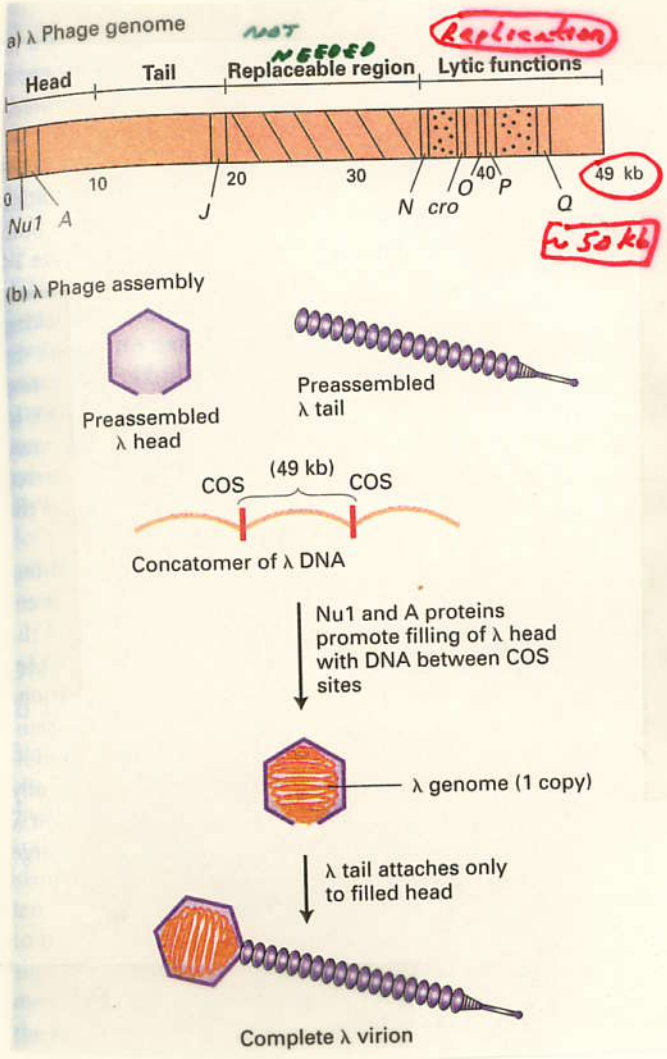


b.

figure 17.3

USING PLASMID AND PHAGE VECTORS. ***a.*** Plasmids are cut within the β-galactosidase gene (*lacZ*), and foreign DNA and DNA ligase are added. Foreign DNA inserted into *lacZ* interrupts the coding sequence inactivating the gene. Plating cells on medium containing the antibiotic ampicillin selects for plasmid-containing cells. The medium also contains X-gal and when *lacZ* is intact (*top*), the expressed enzyme cleaves the X-gal producing blue colonies. When *lacZ* is inactivated (*bottom*), X-gal is not cleaved and colonies remain white. ***b.*** Phage vectors are selected for the presence of recombinant DNA by the ability of the phage to assemble in vitro, infect a host, and propagate inside its host. The phage has been engineered such that only phage genomes with inserted DNA are long enough for the packaging machinery to produce mature phage that can infect cells.

STRUCTURE OF THE λ PHAGE AND ITS GENOME



▲ FIGURE 9-14 The bacteriophage λ genome and packaging of bacteriophage λ DNA. (a) Simplified map of the λ phage genome. There are about 60 genes in the λ genome, only a few of which are shown in this diagram. Genes encoding proteins required for assembly of the head and tail are located at the left end; those encoding additional proteins required for the lytic cycle, at the right end. Some regions of the genome can be replaced by exogenous DNA (diagonal lines) or deleted (dotted) without affecting the ability of λ phage to infect host cells and assemble new virions. Up to ≈25 kb of exogenous DNA can be stably inserted between the *J* and *N* genes. (b) In vivo assembly of λ virions. Heads and tails are formed from multiple copies of several different λ proteins. During the late stage of λ infection, long DNA molecules called *concatomers* are formed; these multimeric molecules consist of multiple copies of the 49-kb λ genome linked end to end and separated by COS sites (red), protein-binding nucleotide sequences that occur once in each copy of the λ genome. Binding of λ head proteins Nu1 and A to COS sites promotes insertion of the DNA segment between two adjacent COS sites into an empty head. After the heads are filled with DNA, assembled λ tails are attached, producing complete λ virions capable of infecting *E. coli* cells.

ONE OF FIRST GENOME SEQUENCES

λ Phage infects E. coli & destroys (lyses) cells

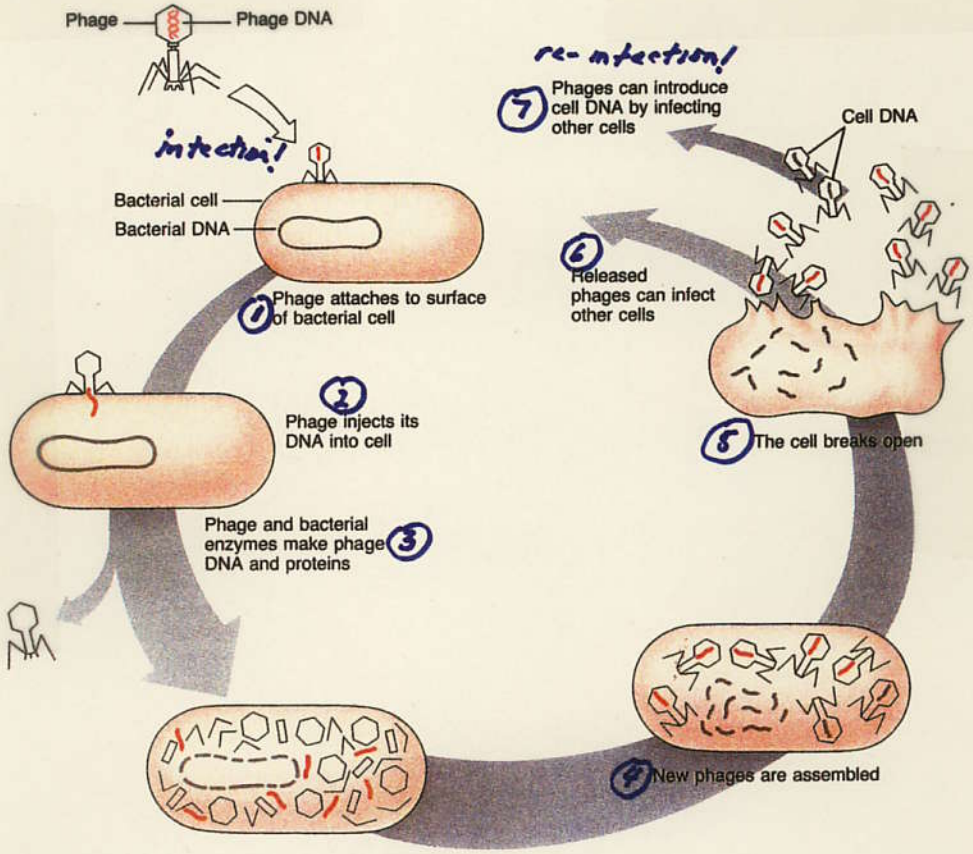
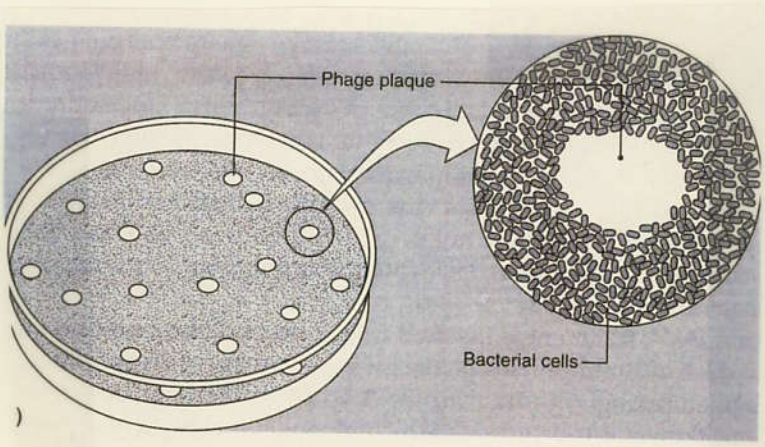
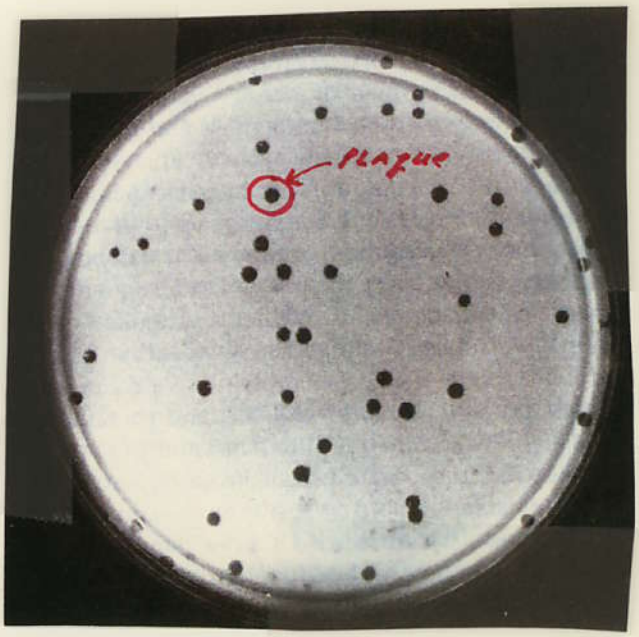


Figure 4.4 Events that occur when a phage infects a bacterial cell.

LYSED CELLS CAN BE SEEN AS CLEAR PLAQUES ON AGAR PLATES





EACH PLAQUE IS A VIRUS CLONE REPRESENTING ONE VIRAL infection!

SELECTABLE MARKER IS BACTERIAL CELL DESTRUCTION & PLAQUE FORMATION

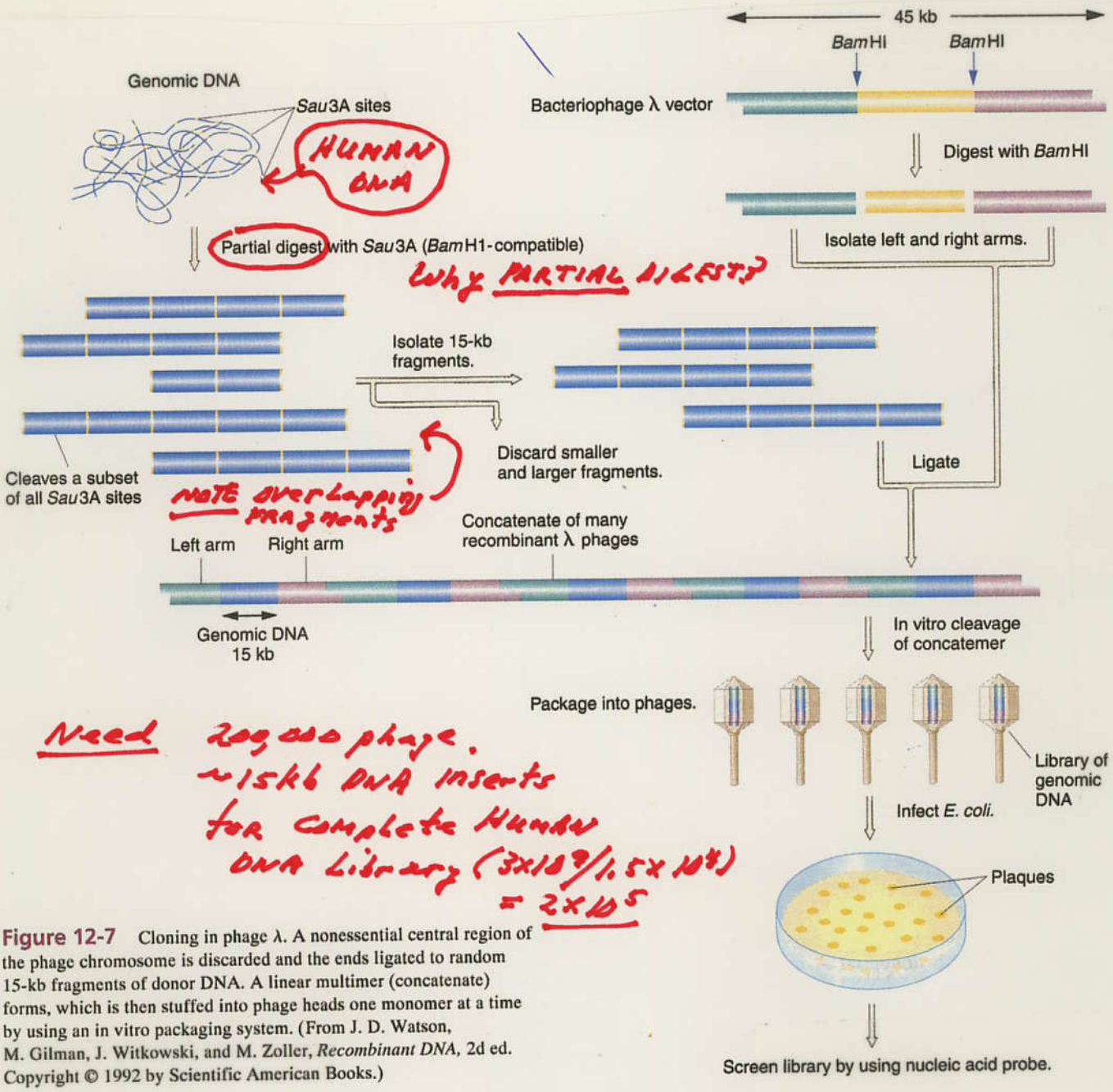
ADVANTAGES OF A VIRUS AS A VECTOR FOR CLONING DNA

(1) Long DNA segments can be cloned (~20kb)
Need fewer clones for whole genome!

(2) Can clone DNA segments in viral genome & self-assemble with viral proteins into virus in a test tube!
Make recombinant viruses in the lab!

(3) Use "natural" infection process to generate large number of clones for a eukaryotic genome library.
Much higher efficiency for getting recombinant DNA → bacterial cells compared with DNA transformation.
∴ Get more clones per amount of recombinant DNA!

CLONING THE HUMAN GENOME AND SCREENING FOR THE FACTOR VIII GENE



Need 2 or 3 x 10^5 phage. ~15kb DNA inserts for complete Human DNA Library ($3 \times 10^9 / 1.5 \times 10^4$) = 2×10^5

Figure 12-7 Cloning in phage λ. A nonessential central region of the phage chromosome is discarded and the ends ligated to random 15-kb fragments of donor DNA. A linear multimer (concatenate) forms, which is then stuffed into phage heads one monomer at a time by using an in vitro packaging system. (From J. D. Watson, M. Gilman, J. Witkowski, and M. Zoller, *Recombinant DNA*, 2d ed. Copyright © 1992 by Scientific American Books.)

Why Partial Digestion? An Important Concept!

What is Complete & Partial Digestion?

An EcoRI Restriction Enzyme Site is Found Only Once in the Human Genome:

a. Yes
b. No

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What is the Purpose of Partial Digestion of HUMAN DNA?

Sau 3A = 4bp = 5' GATC 3' ∴ 1 site every 280bp it digest to completion ≈ 1×10^7 DNA fragments

EcoRI = 6bp = 5' GAATTC 3' ∴ 1 site every 3100bp it digest to completion (cleave every site) ≈ 972,000 DNA fragments!

∴

1. Complete Digestion produces fragments that are too small to clone in λ virus (need 20kb)
2. Complete digestion would create huge genome libraries with large # clones to screen
3. Complete digestion would break up genes on different DNA fragments – particularly if human genes big – ∴ would have one gene on many different clones – parts separated!
4. Complete digestion provides no way to find neighbors of clones in genome – what's next to gene in chromosome!

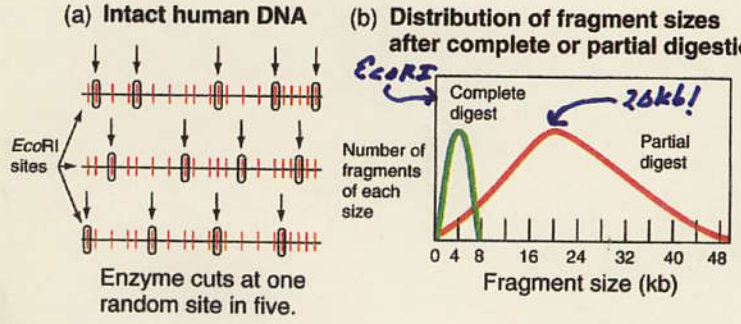
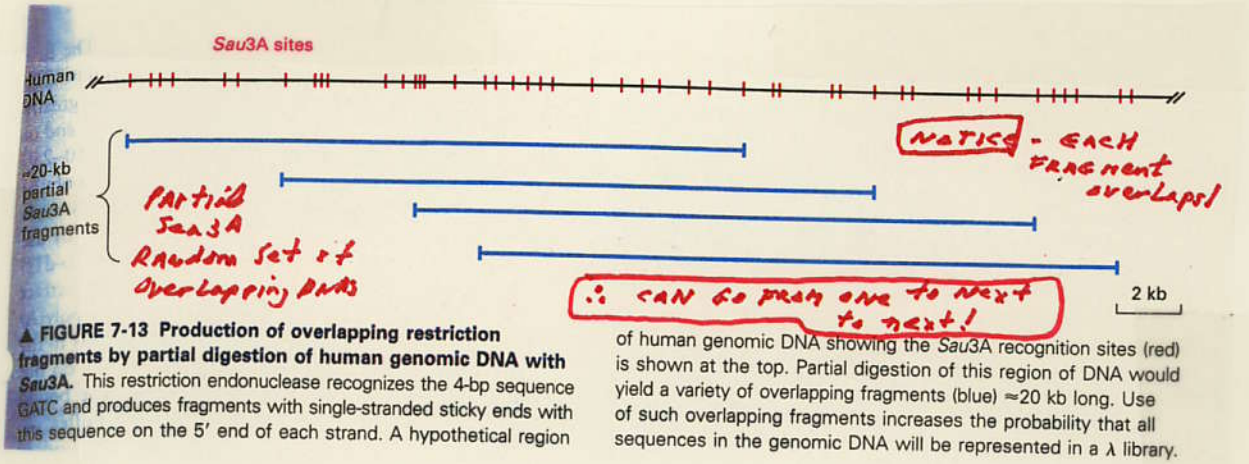


Figure 8.4 Comparison of results from partial and complete digests. (a) By reducing the time available for the reaction to occur, you can ensure that an enzyme actually cuts only a subset of the total recognition sites within a DNA sample. In this example, the chosen reaction time allowed only 1/5 of all *Eco*RI sites to be cut. The particular 20% of sites at which the cuts occur is totally random and different even on identical DNA molecules. (b) Most of the restriction fragments produced by partial digestion are larger than those produced by complete digestion with the same restriction enzyme.

PARTIAL DIGESTION PRODUCES A SERIES OF LARGE, OVERLAPPING DNA FRAGMENTS/CLONES!

CAN CONNECT ONE CLONE WITH ANOTHER?! BUILD UP CLONES OF EACH CHROMOSOME!!

CONSTRUCTING A HUMAN GENOME LIBRARY BY PARTIAL DIGESTION CREATES A SET OF OVERLAPPING DNA FRAGMENTS/CLONES



▲ **FIGURE 7-13 Production of overlapping restriction fragments by partial digestion of human genomic DNA with *Sau*3A.** This restriction endonuclease recognizes the 4-bp sequence GATC and produces fragments with single-stranded sticky ends with this sequence on the 5′ end of each strand. A hypothetical region of human genomic DNA showing the *Sau*3A recognition sites (red) is shown at the top. Partial digestion of this region of DNA would yield a variety of overlapping fragments (blue) ≈20 kb long. Use of such overlapping fragments increases the probability that all sequences in the genomic DNA will be represented in a λ library.

∴ WOULD AN overlapping set for each of the 24 chromosomes allowing clones to be ordered from beginning to end by restriction mapping because each chromosome contains one DNA molecule!

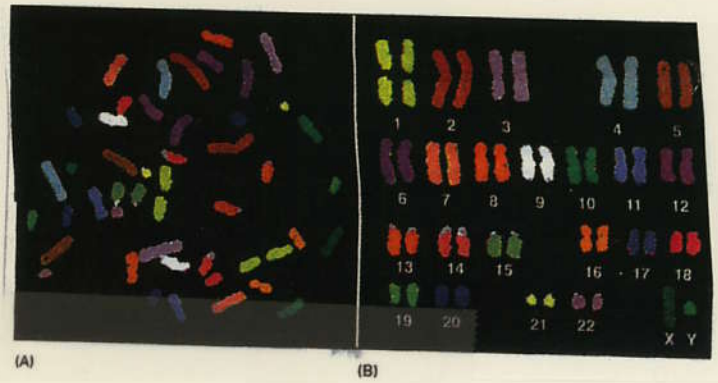
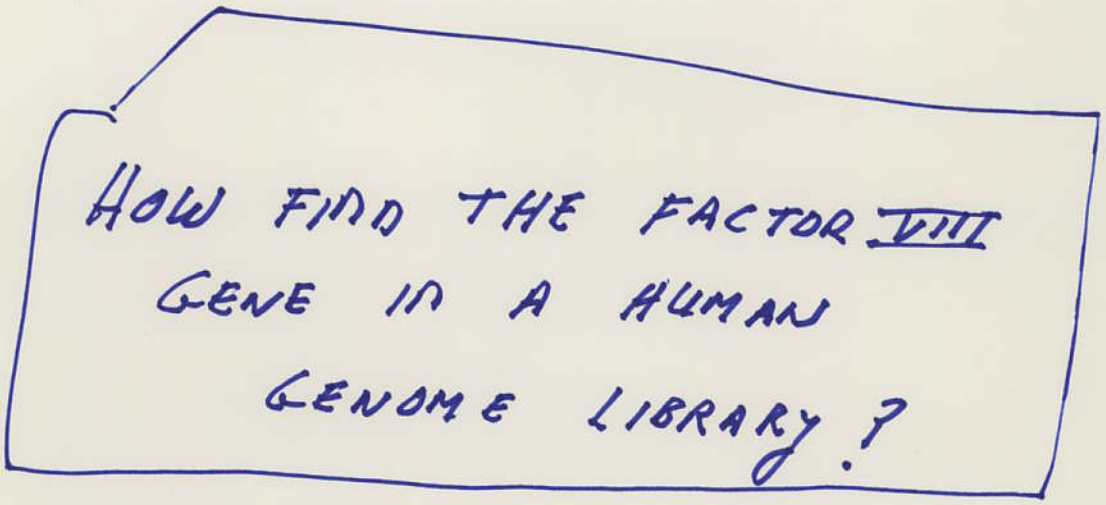


Figure 8–2 **Human chromosomes.** (A) The chromosomes as visualized as they originally spilled from the lysed cell. (B) The same chromosomes artificially lined up in order. This arrangement of the full chromosome set is called a karyotype. (From E. Schröck et al., *Science* 273:494–497, 1996.)


HOW FIND THE FACTOR VIII
GENE IN A HUMAN
GENOME LIBRARY ?

A Specific Gene Can Be Identified in a Genome Library if the Amino Acid Sequence of its Protein is Known Because of the :

a. Double Helical Structure of DNA
b. Antisense Strand DNA Sequence
c. Genetic Code
d. Mutant Gene Phenotype

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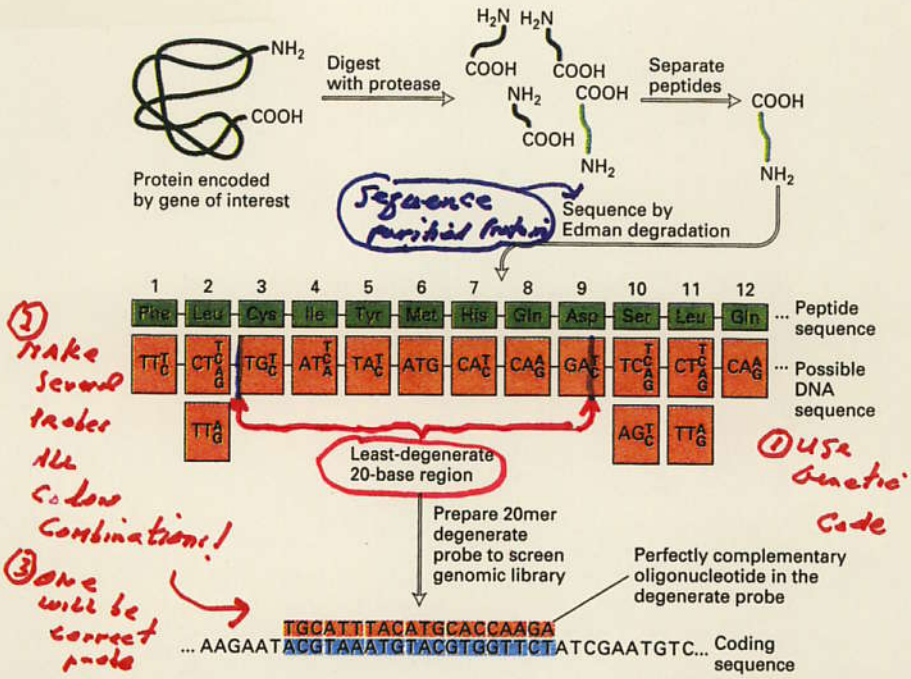
FACTOR VIII Protein → Gene

USING THE FACTOR VIII PROTEIN SEQUENCE AND GENETIC CODE AS A GUIDE TO SYNTHESIZE A FACTOR VIII Probe



▲ **FIGURE 7-19 Designing oligonucleotide probes based on protein sequence.** An isolated protein is digested with a selective protease such as trypsin, which specifically cleaves peptide bonds on the carboxy-terminal side of lysine and arginine residues. The resulting peptides are separated, and several are partially sequenced from their N-terminus by sequential Edman degradation. The determined sequences then are analyzed to identify the 6- or 7-aa region that can be encoded by the smallest number of possible DNA sequences. Because of the degeneracy of the genetic code, the 12-aa sequence (light green) shown here theoretically could be encoded by any of the DNA triplets below it, with the possible alternative bases at the same position indicated. For example, Phe-1 is encoded by TTT or TTC; Leu-2 is encoded by one of six possible triplets (CTT, CTC, CTA, CTG, TTA, or TTG). The region with the least degeneracy for a sequence of 20 bases (20-mer) is indicated by the red bracket. There are 48 possible DNA sequences in this 20-base region that could encode the peptide sequence 3–9. Since the actual sequence of the gene is unknown, a degenerate 20-mer probe consisting of a mixture of all the possible 20-base oligonucleotides is prepared. If a cDNA or genomic library is screened with this degenerate probe, the one oligonucleotide that is perfectly complementary to the actual coding sequence (blue) will hybridize to it.

How many combination of Probes?

2 × 3 × 2 × 2 × 2 = 48

Synthetic Probes!!

USING THE GENETIC CODE TO GO FROM PROTEIN SEQUENCE TO GENE SEQUENCE

(b) Synthesizing DNA probes based on reverse translation

Protein sequence	N	Glu	Asp	Met	Trp	Tyr	C
Degenerate coding sequences	5′	GAA GAG	GAT GAC	ATG	TGG	TAT TAC	3′
		(2)	(2)	(1)	(1)	(2)	

Sequences that must be present in the probe

= 8 codon combinations

GAAGATATGTGGTAT
GAGGATATGTGGTAT
GAAGACATGTGGTAT
GAGGACATGTGGTAT
GAAGATATGTGGTAC
GAGGATATGTGGTAC
GAAGACATGTGGTAC
GAGGACATGTGGTAC

(1) Need Amino Acid sequence of part of the protein

(2) Need DNA sequences representing ALL codon combinations

(3) Synthesize DNA sequence probes!

Probes Can Identify Genes in a Genome Library Because They Are: ?

a. Synthetic
b. Complementary to Specific DNA Sequences
c. Contain the Correct Amino Acid Sequence
d. Are Non-Radioactive

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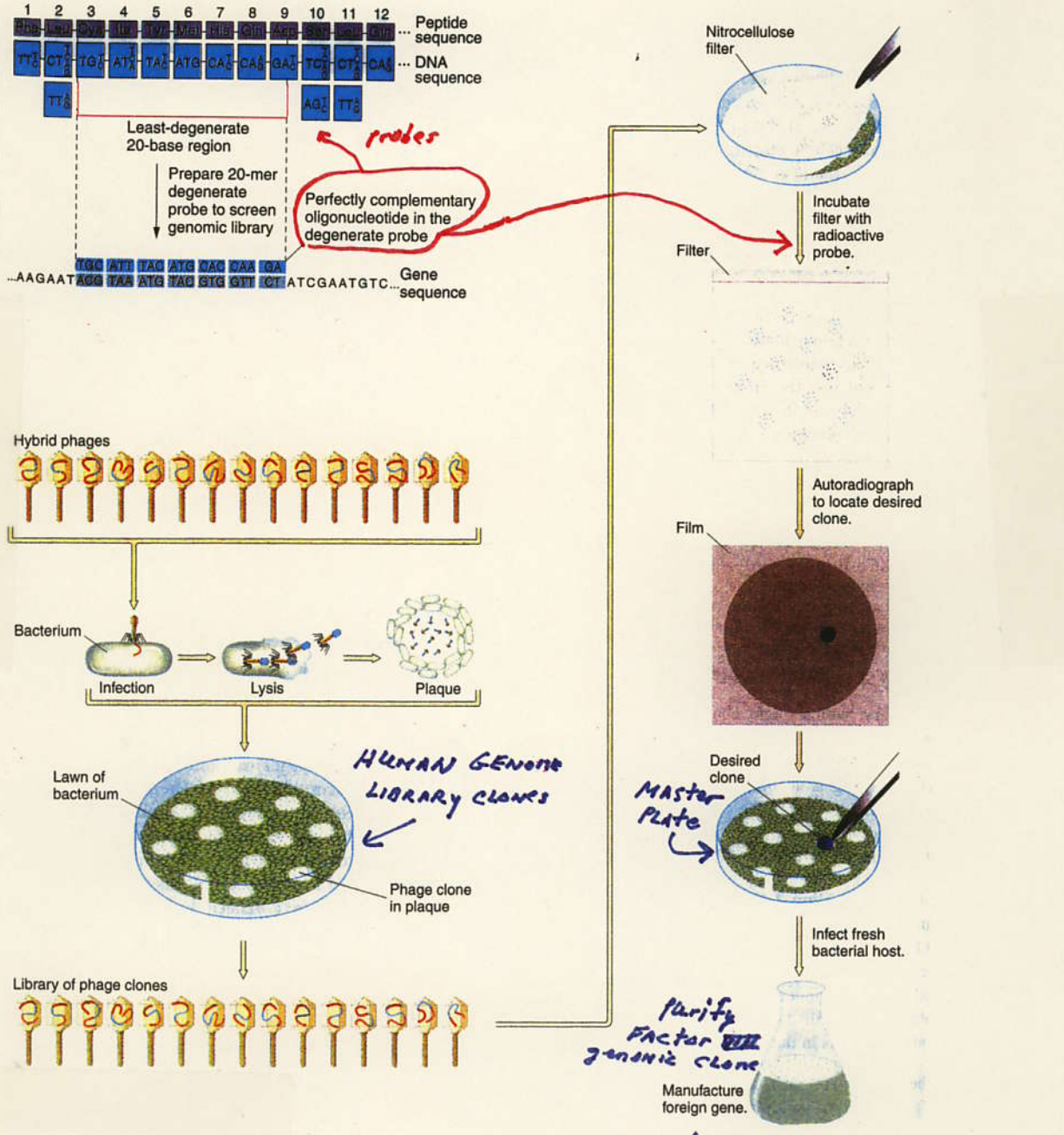
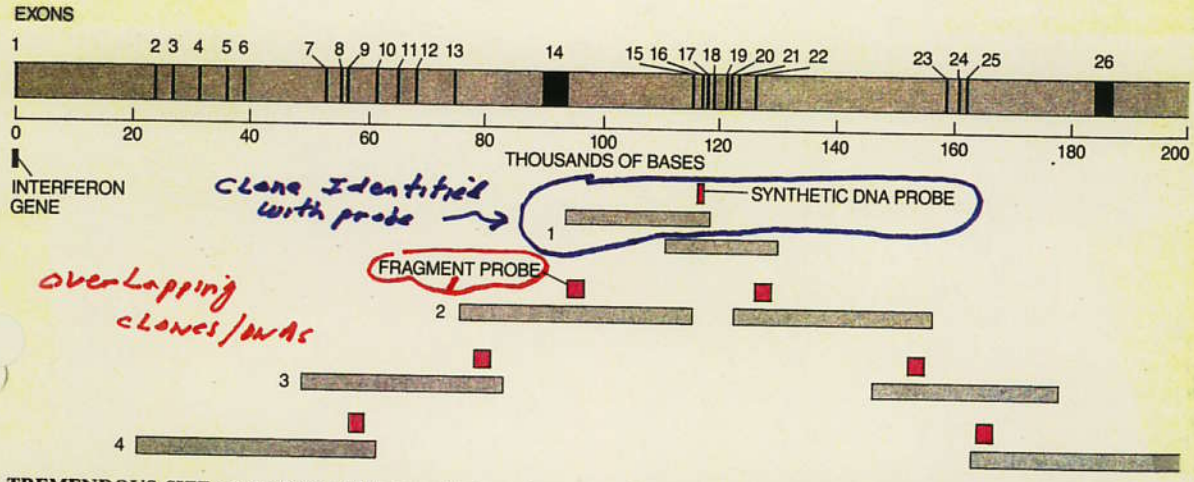
FINDING THE FACTOR VIII GENE
OR PART OF GENE!!

FACTOR VIII Protein Sequence
↳ Synthetic DNA Probe



THE RESULT - THE FACTOR VIII GENE IS HUGE - 186,000 bp - The Probe IDENTIFIED A CLONE CONTAINING ONLY One part of Gene!!!



TREMENDOUS SIZE of the factor VIII gene, the largest gene cloned to date, forced workers to apply a cloning technique called chromosome walking. The factor VIII gene is 186,000 bases long. In contrast the interferon gene, which was cloned in 1980, incorporates only about 600 bases. Because the factor VIII gene is too large to fit into a single phage, segments of it were found in different plaques in the genomic library. When the library was screened with a synthetic DNA probe, the probe hybridized with overlapping segments (*1*). Pieces of the segments then served as probes to rescreen the library and identify further segments (*2*). By repeating this procedure nearly all of the gene was identified (*3, 4*). (Its beginning was found once factor VIII cDNA was available as a probe.) Less than one-twentieth of the gene consists of exons, or coding sequences (*black bands*); the 26 exons are separated by 25 introns.

HOW FIND CLONES WITH Rest of Gene? Key Question!

Remember - the library contains overlapping DNA CLONES :: CAN use one part of First clone to Re-screen Library & "WALK" to other gene regions - using restriction maps & sequencing as guides!

SEQUENCE ~~~~~> GENBANK

FINDING THE ENTIRE FACTOR VIII GENE? WALKING + SEQUENCING

WALKING UP AND DOWN GENES AND CHROMOSOMES

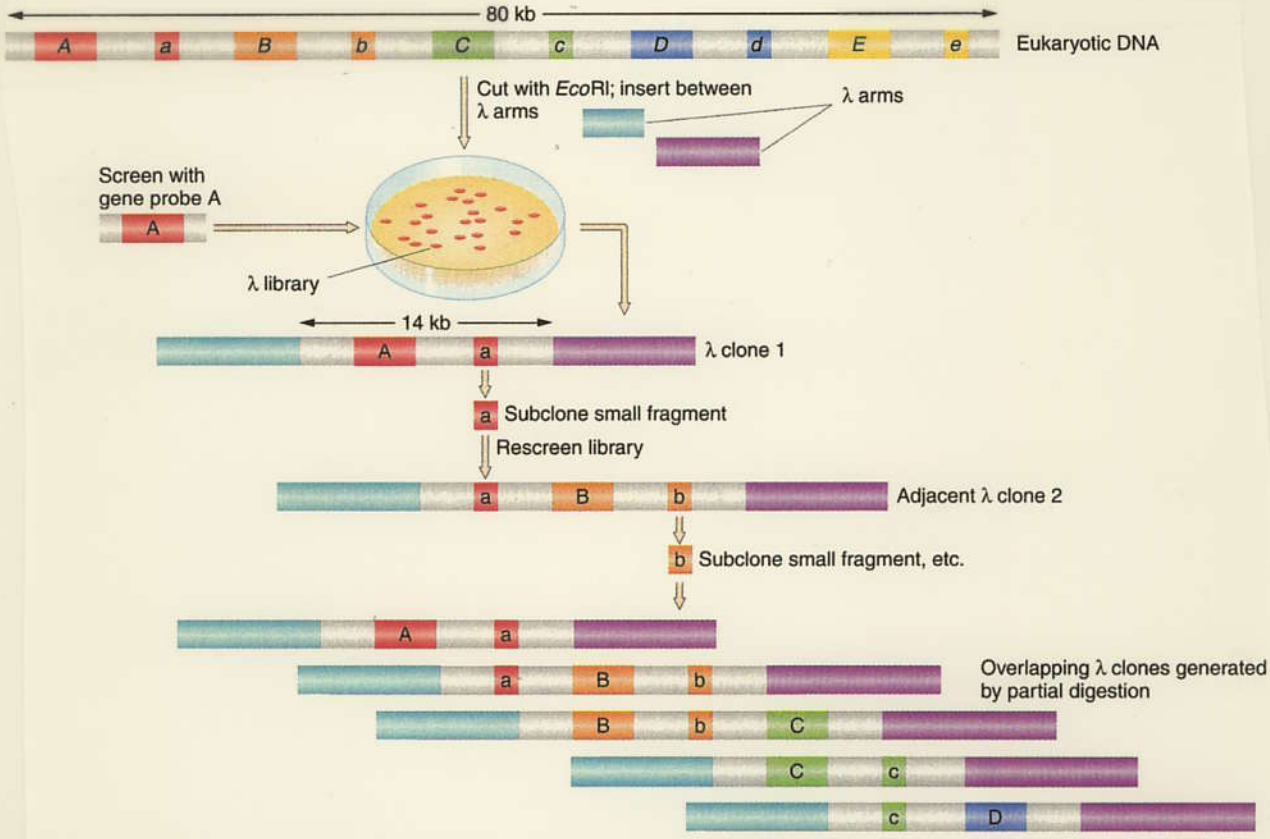


Figure 12-15 Chromosome walking. One recombinant phage obtained from a phage library made by the partial *Eco*RI digest of a eukaryotic genome can be used to isolate another recombinant phage containing a neighboring segment of eukaryotic DNA, as described in the text. (From J. D. Watson, J. Tooze, and D. T. Kurtz, *Recombinant DNA: A Short Course.* Copyright © 1983 by W. H. Freeman and Company.)

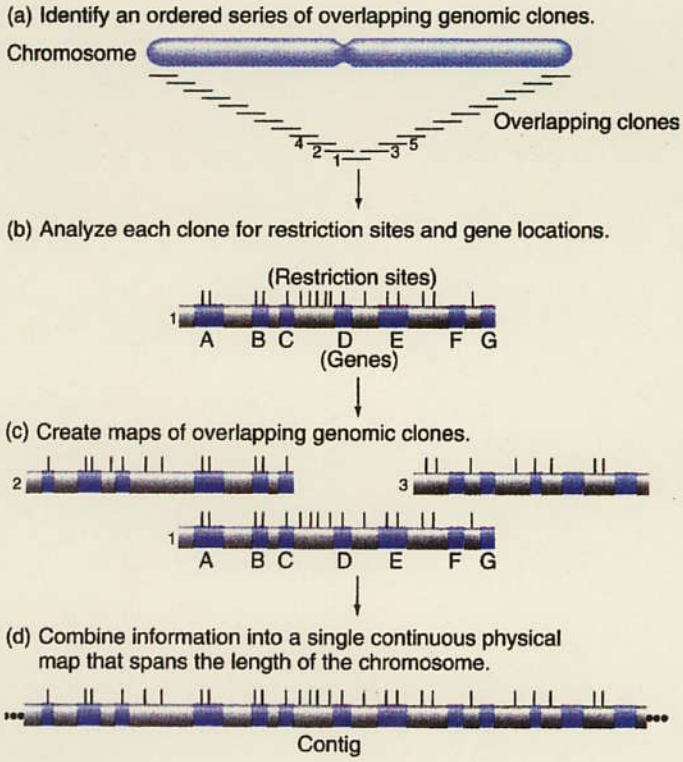
BASIS OF GENOME PROJECTS + WHOLE GENOME SEQUENCING

Key concept ⇒ HOW KNOW FIND COMPLETE FACTOR VIII GENE?

→ COMPARE PROTEIN + DNA SEQUENCES!

CAN WALK DOWN AN ENTIRE CHROMOSOME & OBTAIN AN ENTIRE SET OF OVERLAPPING CLONES CONTAINING EVERY GENE IN CHROMOSOME

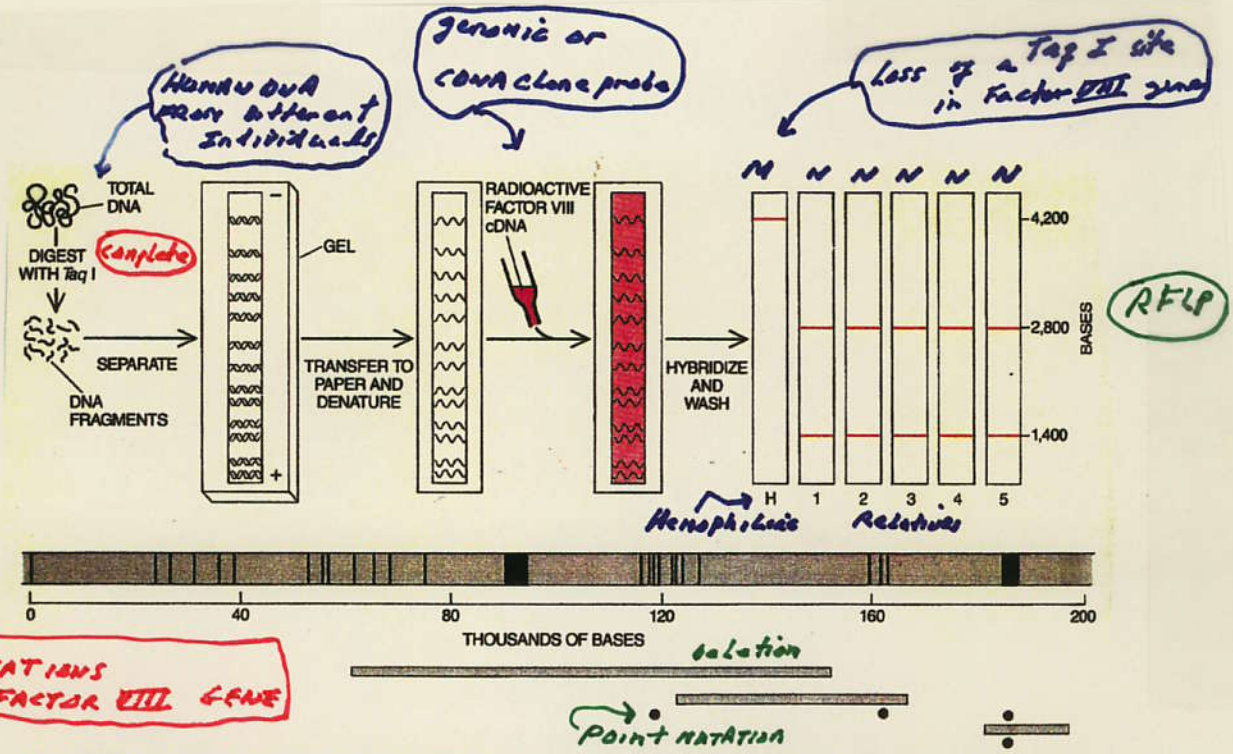
(1) Used to Sequence Human Genome
(2) Used to Map Genes to Chromosomes
(3) Used for MARKERS (RFLPs) to identify & follow Disease Genes



There are 24 sets of Clones for Human Genome
22 Autosomes + X chromosome + Y chromosome

Figure 10.5 Building a whole-chromosome physical map. (a) To produce a whole-chromosome physical map, you first order a set of overlapping genomic clones that extend from one end of the chromosome to the other. Subsequent figures describe various methods of obtaining this ordered set of clones. (b) You next map the restriction sites of each clone in the set through restriction analysis, and analyze individual restriction fragments in other ways, such as Northern blot analysis, to identify transcription units. (c) Computers overlay the different types of maps for each clone onto the overlapping clones to obtain a continuous map. (d) The result is a single continuous map extending the length of the chromosome.

FACTOR VIII GENE PROBES/SEQUENCE CAN BE USED TO CHARACTERIZE MUTANT GENES & DO DNA TESTING FOR CARRIERS



HEMOPHILIA-CAUSING MUTATIONS in the factor VIII gene can be detected by Southern blotting (*top*) if they happen to change the way the gene is fragmented by a restriction enzyme. DNA from blood cells is cut into millions of fragments, in this case with the enzyme *TaqI*. The fragments are separated according to size by electrophoresis, unraveled into single strands and blotted onto filter paper. The filter is bathed in a solution of radioactive factor VIII cDNA, which hybridizes only with fragments of the factor VIII gene. The size of the hybridizing fragments is revealed by exposing X-ray film to the filter. In the example shown here a point mutation in the factor VIII gene of a hemophiliac (*H*) has eliminated a *TaqI* cleavage site. The 2,800- and 1,400-base fragments on the blot patterns of his relatives (*1–5*) are replaced by a single, uncut 4,200-base fragment. So far seven different mutations have been located on hemophilic factor VIII genes (*bottom*). Four are point mutations, or changes of a single base (*dots*); three are extensive deletions (*bars*).

USE DNA Gel Blots & Factor VIII Probes to Investigate Presence of Mutant Alleles in FAMILIES (CARRIERS)

MUTATIONS ARISE INDEPENDENTLY in FAMILIES

ONCE gene = cDNA identified!

HOW IS A SPECIFIC GENE DETECTED IN GENOME?

DNA CAN BE TRANSFERRED "in situ" to PAPER & ANNEALED with RADIOACTIVE ANALOG

DNA BLOTS!

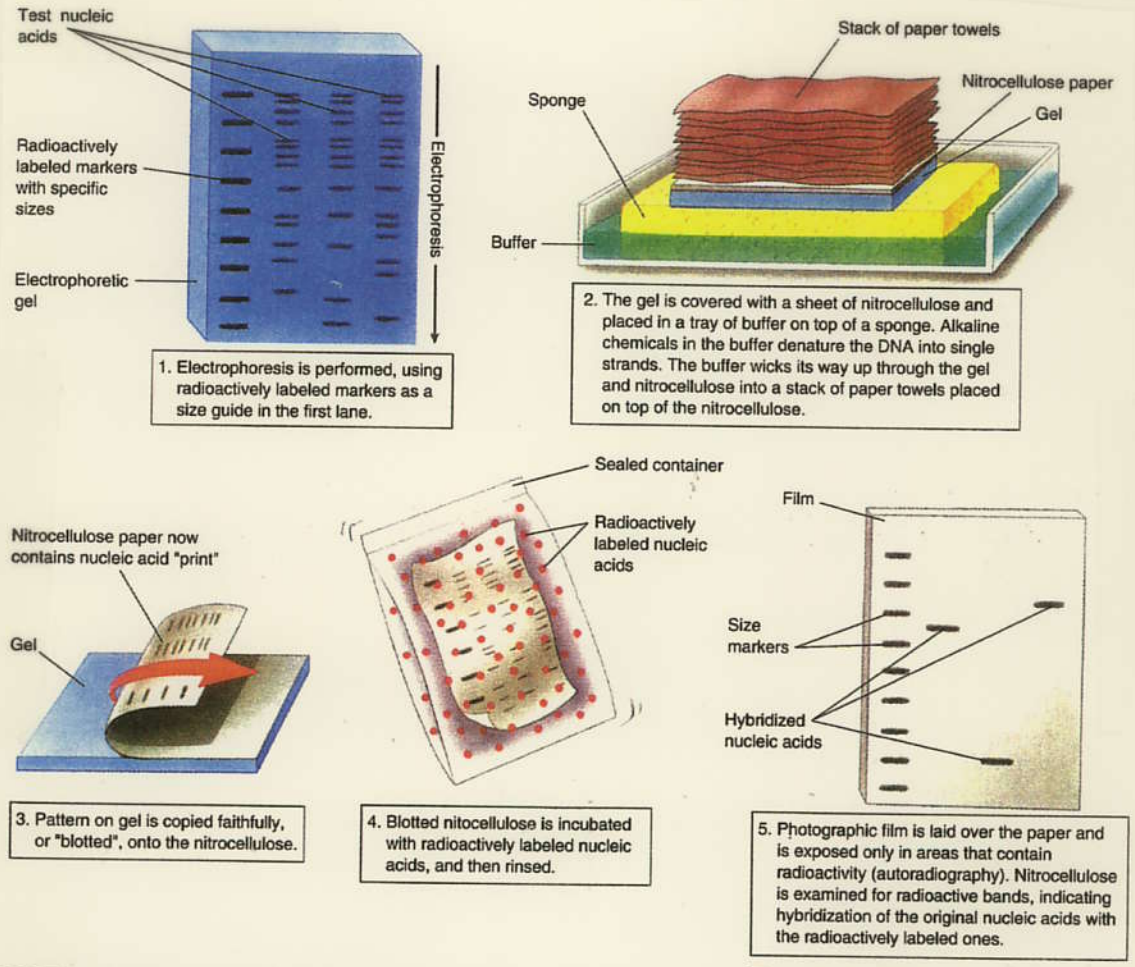


FIGURE 19.9
The Southern blot procedure. E. M. Southern developed this procedure in 1975 to enable DNA fragments of interest to be visualized in a complex sample containing many other fragments of similar size. The DNA is separated on a gel, then transferred ("blotted") onto a solid support medium such as nitrocellulose paper or a nylon membrane. It is then incubated with a radioactive single-strand copy of the gene of interest, which hybridizes to the blot at the location(s) where there is a fragment with a complementary sequence. The positions of radioactive bands on the blot identify the fragments of interest.

Probe Represents a CLONED FRAGMENT FROM GENOME with a unique sequence!

USING PCR and RFLPs To Detect the Hemophilia A Disease Allele/Gene

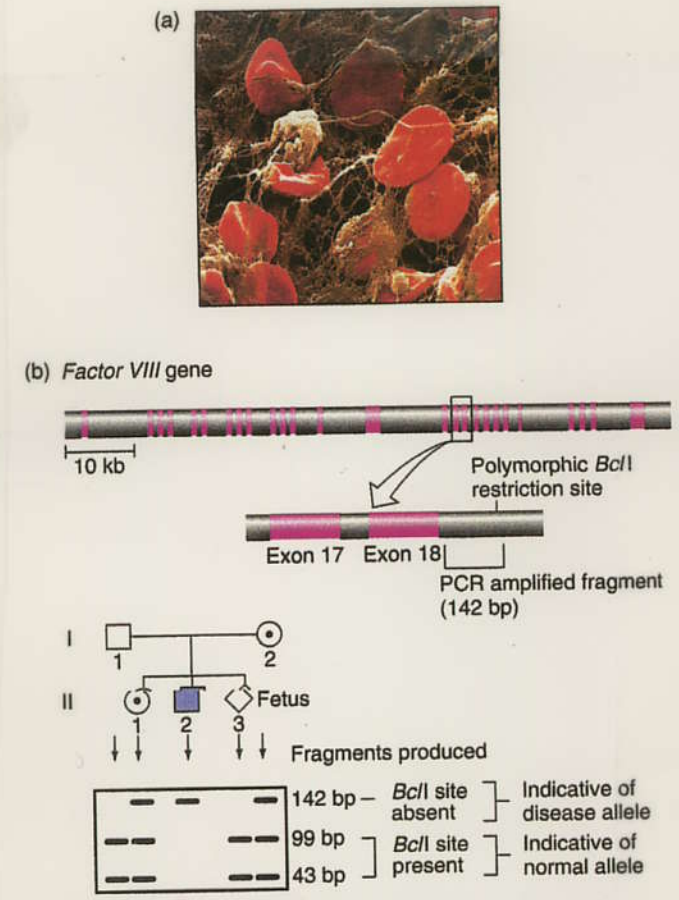


Figure 9.18 Diagnosis of hemophilia through the indirect detection of genotype at the factor VIII locus. The factor VIII protein participates in a cascade of reactions that result in formation of a blood clot. (a) A polymorphic *Bcl*I restriction site within intron 18 of the *factor VIII* gene has no effect on gene function but can provide a marker to follow the segregation of the gene from parents to children. (b) The family described by the pedigree has two healthy parents, but the mother is an obligate carrier of the disease mutation because she has passed this X-linked disease on to her son; her carrier status is signified by a circle with a dot in the middle. By comparing the RFLP pattern obtained from the mother's DNA with the pattern from her son's DNA, you can see that the disease allele is associated with the 142-bp *Bcl*I restriction fragment, and the wild-type allele in the mother's genome contains a *Bcl*I restriction site that causes this fragment to be cut into two pieces, one 43 bp and the other 99 bp in length. Using this information, you can determine that the firstborn sister is a carrier like her mother, while the male fetus will be disease free.

(1) Use PCR to Amplify a Specific Factor VIII Gene Region

(2) Use Restriction Enzyme (BclI) to Distinguish between Normal Allele (1 site) & Disease Allele (no sites)

= Normal Allele

= Disease Allele

The 21st CENTURY Approach!

ONLY CAN DO THIS WITH A KNOWLEDGE OF DNA SEQUENCE OF WILD-TYPE (NORMAL) AND Disease Genes (CAN VARY FAMILY to FAMILY.!)

Use Gene Probe to Test for Disease Gene Pre-Natally

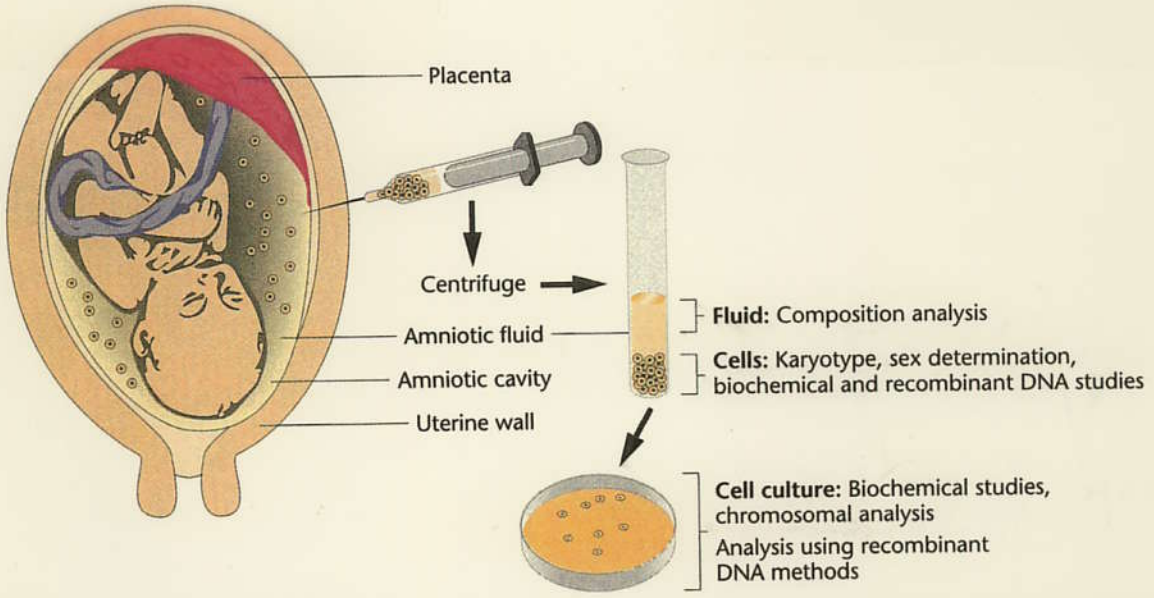
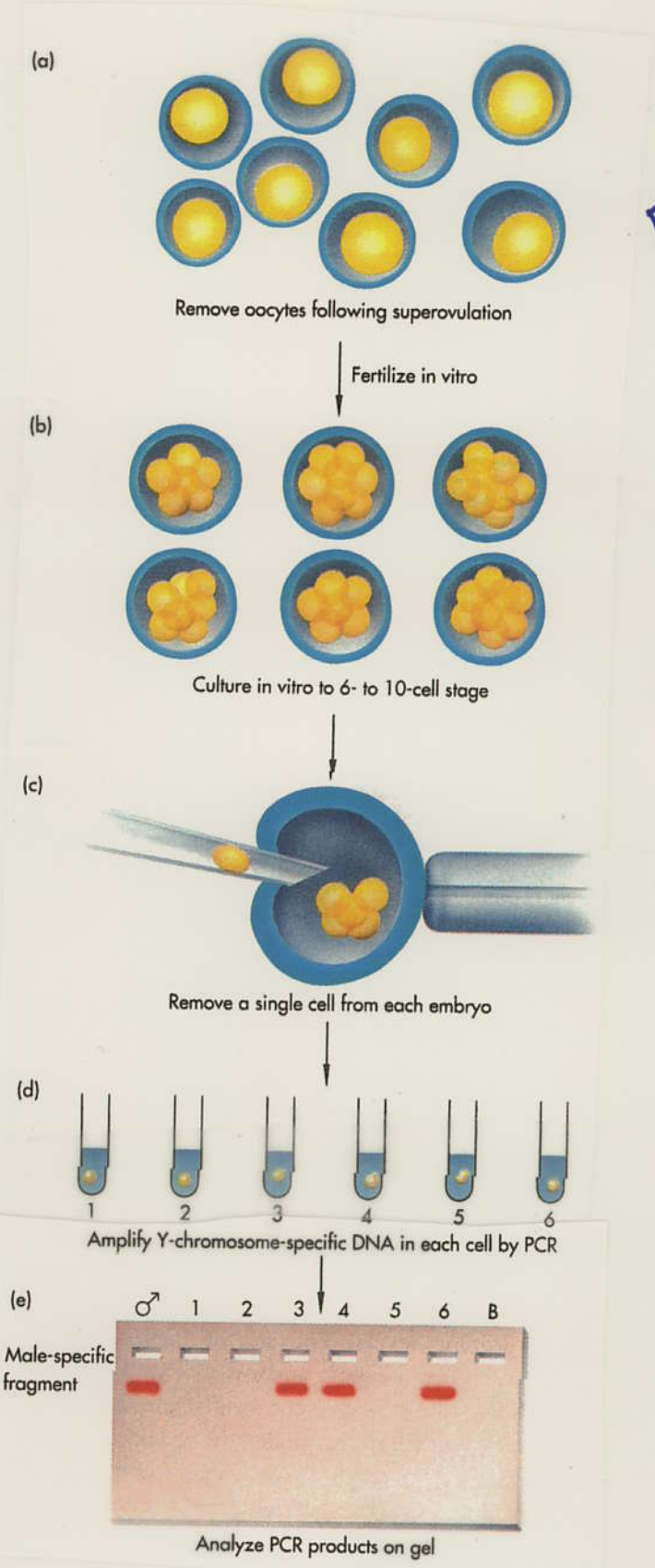


FIGURE 21.6 The technique of amniocentesis. The position of the fetus is first determined by ultrasound, and then a needle is inserted through the abdominal and uterine wall to recover fluid and fetal cells for cytogenetic and/or biochemical analysis.

USING PGD TO DETECT Hemophilia A Disease Alleles



Mother is a carrier X^HX^h

1. Test for male embryos
2. Test for presence of Hemophilia A disease alleles!

X^hY

HOW FIND FACTOR VIII mRNA to generate A cDNA FOR Protein Production in HOST CELLS?

RECALL: Eukaryotic Genes Provide Obstacles FOR Efficient Protein Production in Genetically Engineered Cells!

MAKING THE DRUG

NEED cDNA Not Gene

FACTOR VIII GENE CAN BE USED TO FIND OUT WHERE IT IS ACTIVE USING RNA BLOTS

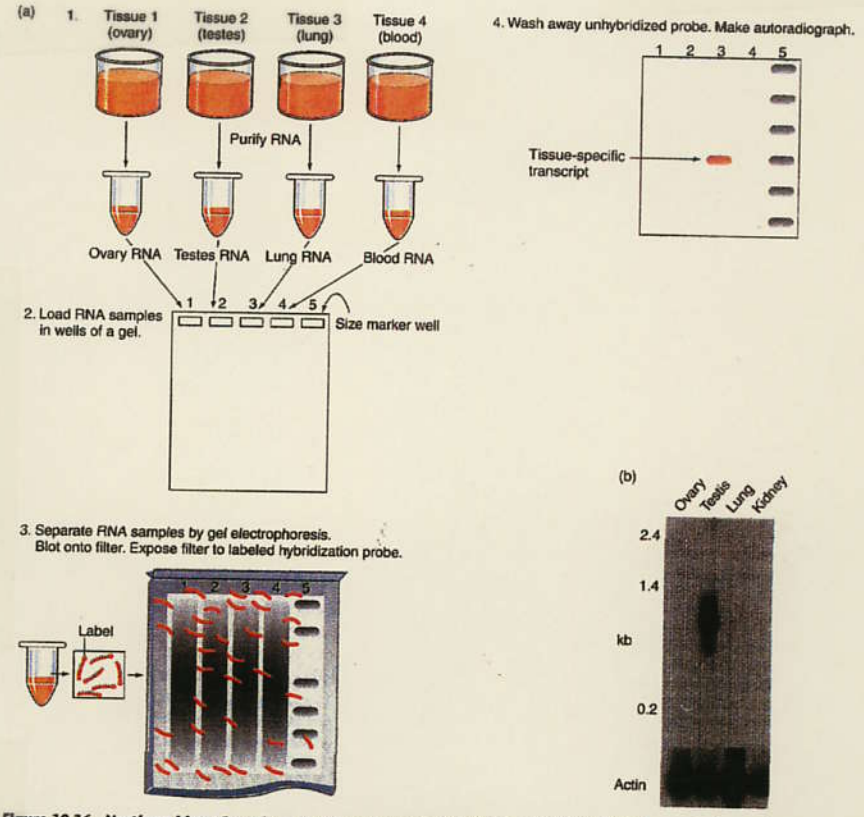
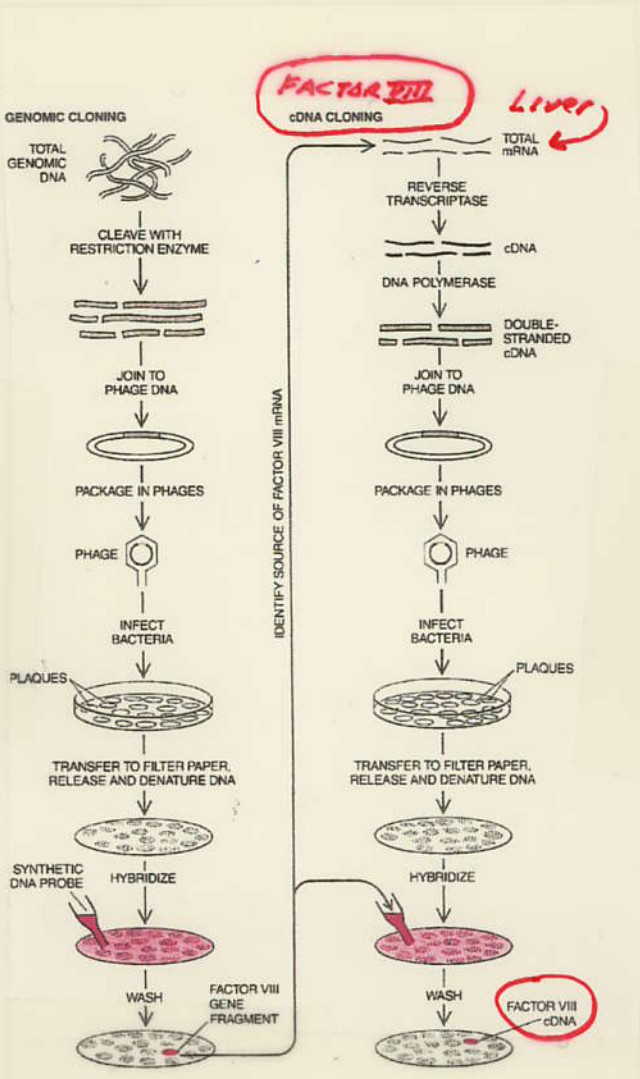


Figure 10.16 Northern blots: Snapshots of gene expression. (a) The protocol. (1) Purify RNA from each tissue to be examined for expression of the gene under investigation; here since you are looking at the *SRY* candidate for the testes-determining factor, the tissues to be examined are ovary, testes, lung, and blood. (2) Make an agarose gel and load each of the four RNA samples into a different well and load a fifth well with RNA size markers. Now subject the gel to an electric current that causes the RNA in each sample to migrate along a lane toward the bottom of the gel. The mobility of each RNA transcript in a sample depends on its size: smaller RNAs move faster, while larger RNAs migrate more slowly. When the smallest RNAs reach the bottom of the gel, turn off the current. Staining the RNAs in each lane would produce a smear reflecting the presence of so many RNAs of different sizes that they cannot be resolved from each other. (3) Blot the RNA within the gel and fix it to a filter so that each RNA molecule retains its position relative to all the other molecules. Expose the filter to labeled probe and allow the label to hybridize for several hours. (4) Wash away unhybridized probe. Place the filter on a film for autoradiography. Develop the film. You will see bands only in those lanes containing a tissue where the gene represented by the probe has been expressed. (b) Northern blot results obtained using the pY53.3 clone as a probe. This clone contains the *SRY* gene. The results show that *SRY* is expressed in the testes, but not the ovary, lung, or kidney. This result makes SRY a good candidate for the *TDF* locus. In a control experiment, researchers probed an identical blot with the same RNA samples using a clone containing the actin gene. As expected, a band of the same size appears in every lane. This control demonstrates the integrity of the RNA samples used in this study.

FACTOR VIII GENE is HIGHLY ACTIVE in LIVER!

USING FACTOR VIII GENE PROBE TO IDENTIFY FACTOR VIII cDNA CLONE



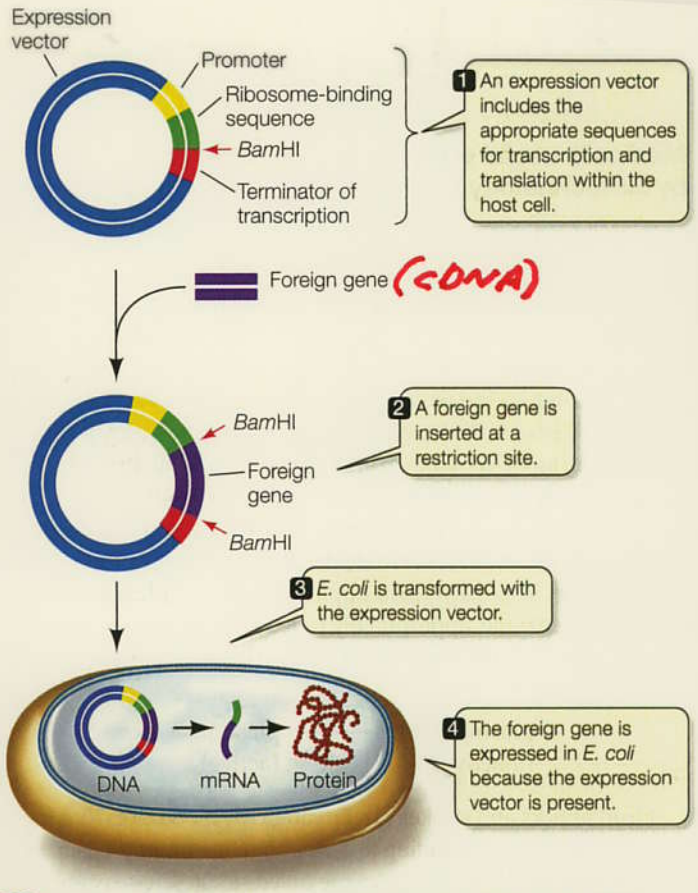
FACTOR VIII cDNA → USE TO MAKE drug!

An Expression Vector Must Have a Host Switch (Promoter) in Order to Direct the Synthesis of a Specific Protein?

a. Yes
b. No

iClicker

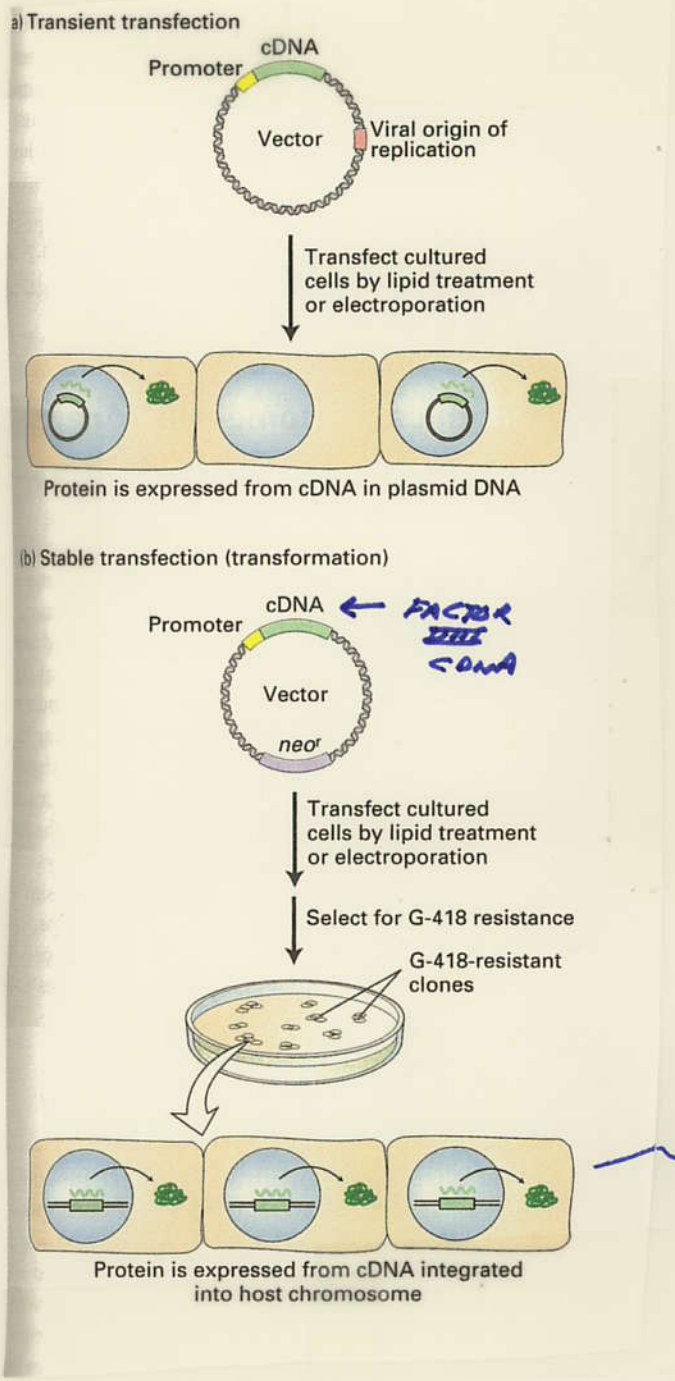
USE EXPRESSION VECTOR TO ALLOW cDNA TO Produce PROTEIN in Host Cell



16.16 An Expression Vector Allows a Transgene to Be Expressed in a Host Cell A transformed eukaryotic gene may not be expressed in *E. coli* if it lacks the necessary bacterial sequences for promotion, termination, and ribosome binding. Expression vectors contain these additional sequences, enabling the eukaryotic protein to be synthesized in the prokaryotic cell.

A FACTOR VIII DRUG/"CURE"

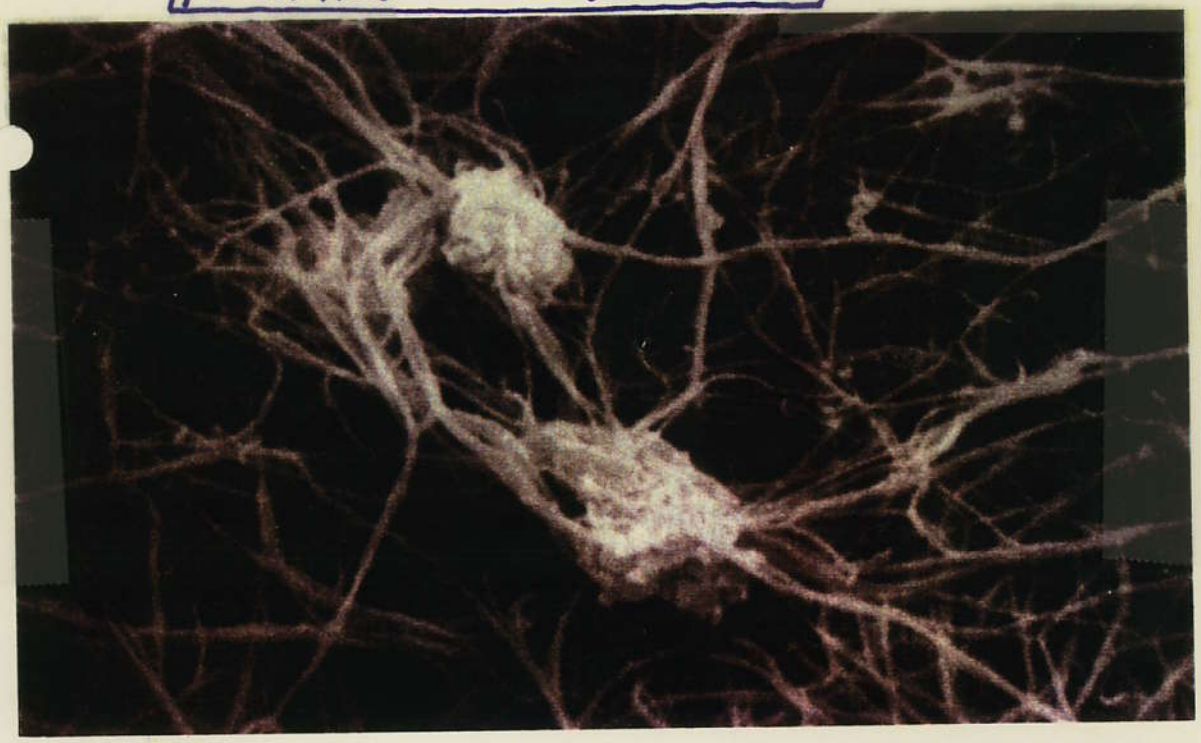
MAKING FACTOR VIII IN MAMMALIAN CELLS



PURIFY FACTOR VIII PROTEIN!

USING FACTOR VIII TO TREAT HEMOPHILIA

FORMATION OF A BLOOD CLOT



FIBRIN STRANDS stabilize a blood clot at the site of a wound by trapping the platelets that form the bulk of the clot. The electron ·icrograph, which was made by Jon C. Lewis of Wake Forest Uni- ·rsity, shows a clot formed in a suspension of platelets and fibrin.

A clot in the bloodstream is the result of a complex cascade of enzymatic reactions culminating in the conversion of fibrinogen, a soluble protein, into insoluble fibrin strands. In hemophiliacs a crucial protein in the blood-clotting cascade is either missing or defective.

A TRIUMPH of Genetic Engineering

RECOMBINANT FACTOR VIII

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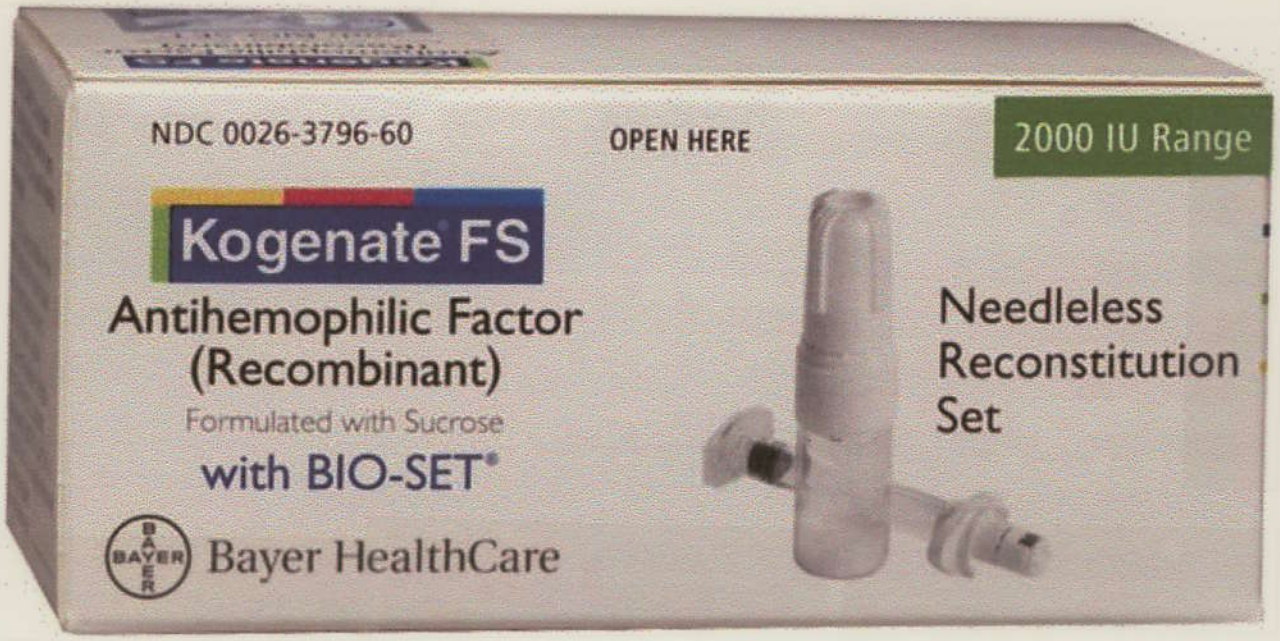
Recombinant factor VIII

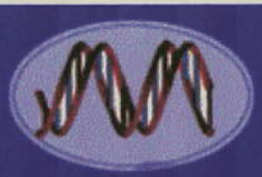
Recombinant factor VIII (rFVIII) is the antihaemophilic factor A, obtained using recombinant DNA technology. With this technology, pure protein is synthesized in the laboratory instead of being extracted from blood.

In the following pages, it will be explained in detail how the knowledge and analysis of DNA, using the new instruments of molecular genetics, have represented both the beginning and follow-up stages in the development of recombinant FVIII.



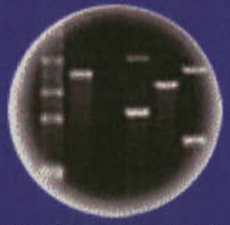
FACTOR VIII GENE CLONED IN 1983

FACTOR VIII (RECOMBINANT) APPROVED AS DRUG IN 1993! TEN YEARS FROM GENE → DRUG!



DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

Plants of Tomorrow

The Factor VIII Story -- A Summary

1. Purify Small Amounts of Factor VIII
2. Obtain Partial or Complete Amino Acid Sequence
3. Use the Genetic Code to Synthesize Degenerate DNA Probes
4. Isolate Factor VIII DNA Clones Complementary to Probe in Genome Library
5. Determine if Factor VIII Clones Contain the Complete Gene By Sequencing and Comparing With Protein Sequence
6. If Not, "Walk" to Obtain Overlapping DNA Clones That Collectively Contain the Factor VIII Gene
7. Sequence Clones To Determine Where the Factor VIII Gene Starts and Stops
8. Use Factor VIII Genome Probe to Find Out What Body Organ/Tissue Expresses the Factor VIII Gene
9. Make a cDNA Library From the Target Organ/Tissue and Isolate a Factor VIII cDNA Clone
10. Sequence the Factor VIII cDNA Clone and Compare With Factor VIII Gene Sequence to Map its Anatomy (I.e., introns, exons, swtiches) and Ensure That it Contains the Complete Protein Coding Sequence
11. Use Factor VIII cDNA and/or Genome Fragments as a Probe to Find RFLP Markers For Disease Alleles -- Or Sequence Disease Alleles to Find Relevant RFLP Markers By Comparison With Wild-Type Sequence
12. Insert Factor VIII cDNA Into an Expression Vector and Synthesize Factor VIII Protein in Host Cells (e.g., Mammalian Cells)

An Individual Should Be Allowed to Patent the Factor VIII DNA Sequence:

a. Yes
b. No

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Recombinant Factor VIII Should Have Been Released Immediately For Treatment of Individuals With Hemophilia Without Ten Years of Clinical Trials, Approval by the FDA, and a Cost of $200,000,000:

a. Yes
b. No

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