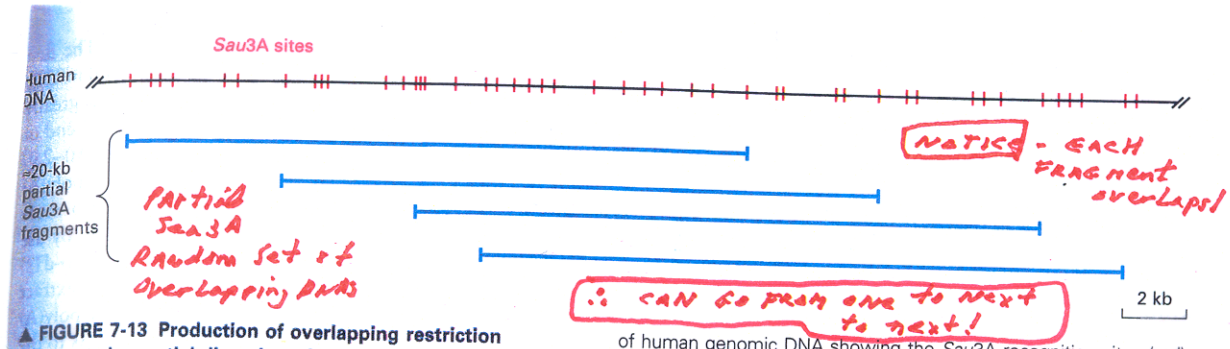
CONSTRUCTING A HUMAN GENOME LIBRARY BY PARTIAL DIGESTION CREATES A SET OF OVERLAPPING DNA FRAGMENTS/CLONES



▲ **FIGURE 7-13 Production of overlapping restriction fragments by partial digestion of human genomic DNA with *Sau3A*.** 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 *Sau3A* 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!

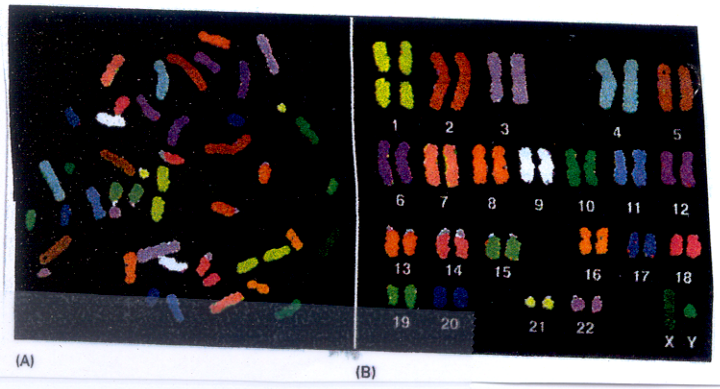
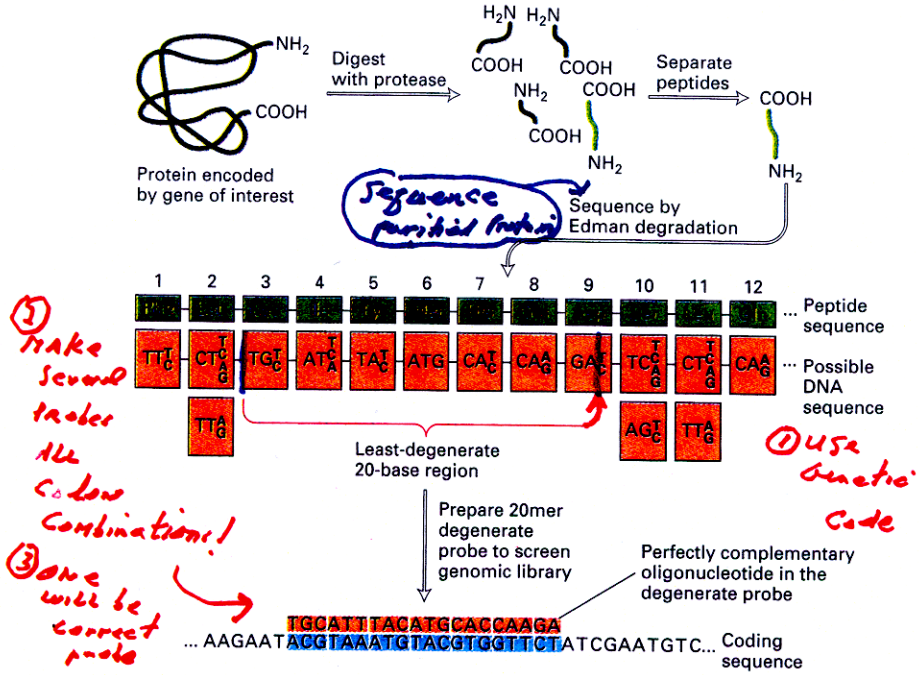


Figure 8–2 Human chromosomes. (A) The chromosomes as visualized as they originally spilled from the lysed cell. (B) The same chromosomes artificially lined up in order. This arrangement of the full chromosome set is called a karyotype. (From E. Schröck et al., *Science* 273:494–497, 1996.)

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.

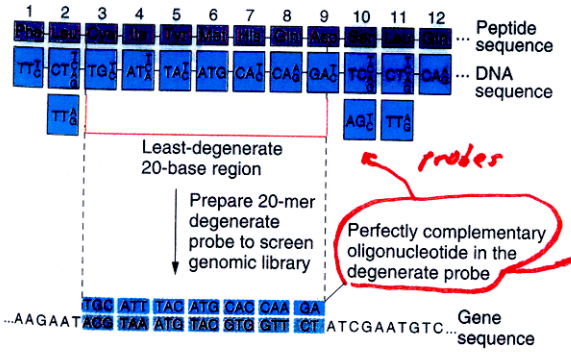
How many combination of Probes?

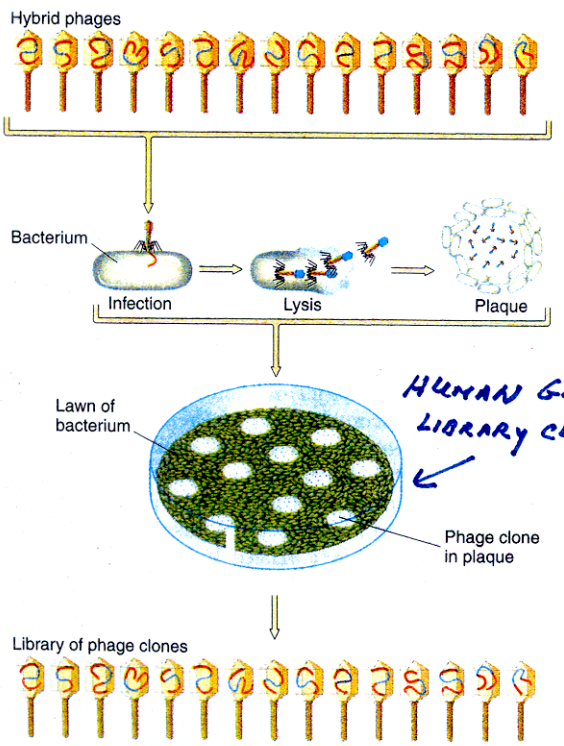
2 × 3 × 2 × 2 × 2 = 48

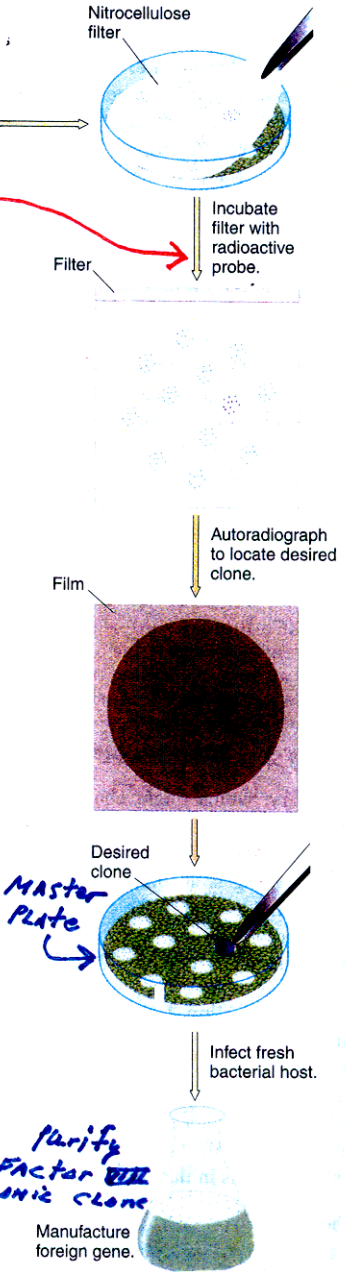
Synthetic Probes!!

FINDING THE FACTOR VIII GENE
OR PART OF GENE!!

FACTOR VIII Protein Sequence
↳ Synthetic DNA Probe

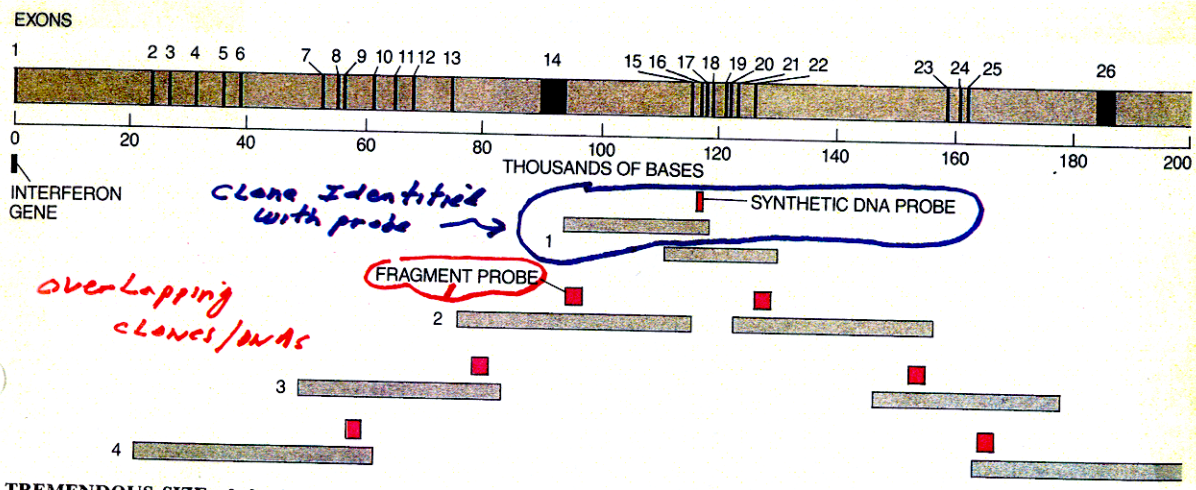






SEQUENCE TO SEE IF IT MATCHES PROBE/PROTEIN

THE RESULT – THE FACTOR VIII GENE IS HUGE – 186,000 bp – The Probe IDENTIFIED A CLONE containing only one part of Gene!!!



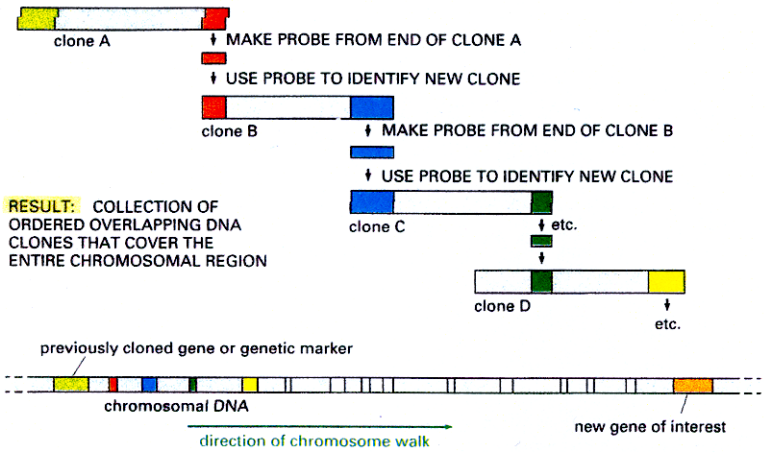
TREMENDOUS SIZE of the factor VIII gene, the largest gene cloned to date, forced workers to apply a cloning technique called chromosome walking. The factor VIII gene is 186,000 bases long. In contrast the interferon gene, which was cloned in 1980, incorporates only about 600 bases. Because the factor 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

CHROMOSOME WALKING CAN BE USED TO FIND ALL PARTS OF FACTOR VIII Gene



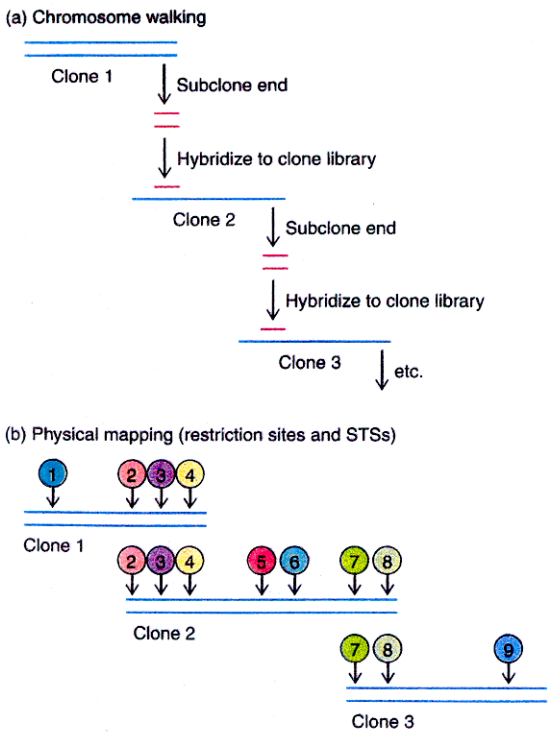


Figure 24.18 Mapping by chromosome walking. **(a)** Chromosome walking. To start the walk, choose a cloned piece of DNA (clone 1) and subclone one end of it. Then use this small end piece (red) as a probe to identify an overlapping clone (clone 2) in a library. Repeating the process, subclone the far end of clone 2 to generate a probe to identify yet another overlapping clone (clone 3). Repeat this cycle as many times as needed to build a set of overlapping clones spanning large stretches of DNA. **(b)** Physical mapping of restriction sites or STSs in each clone allows one to align the overlapping DNAs and build a map of the whole contig.

Align using Restriction Maps/Sequence of Each Clone

WALKING UP AND DOWN GENES AND CHROMOSOMES

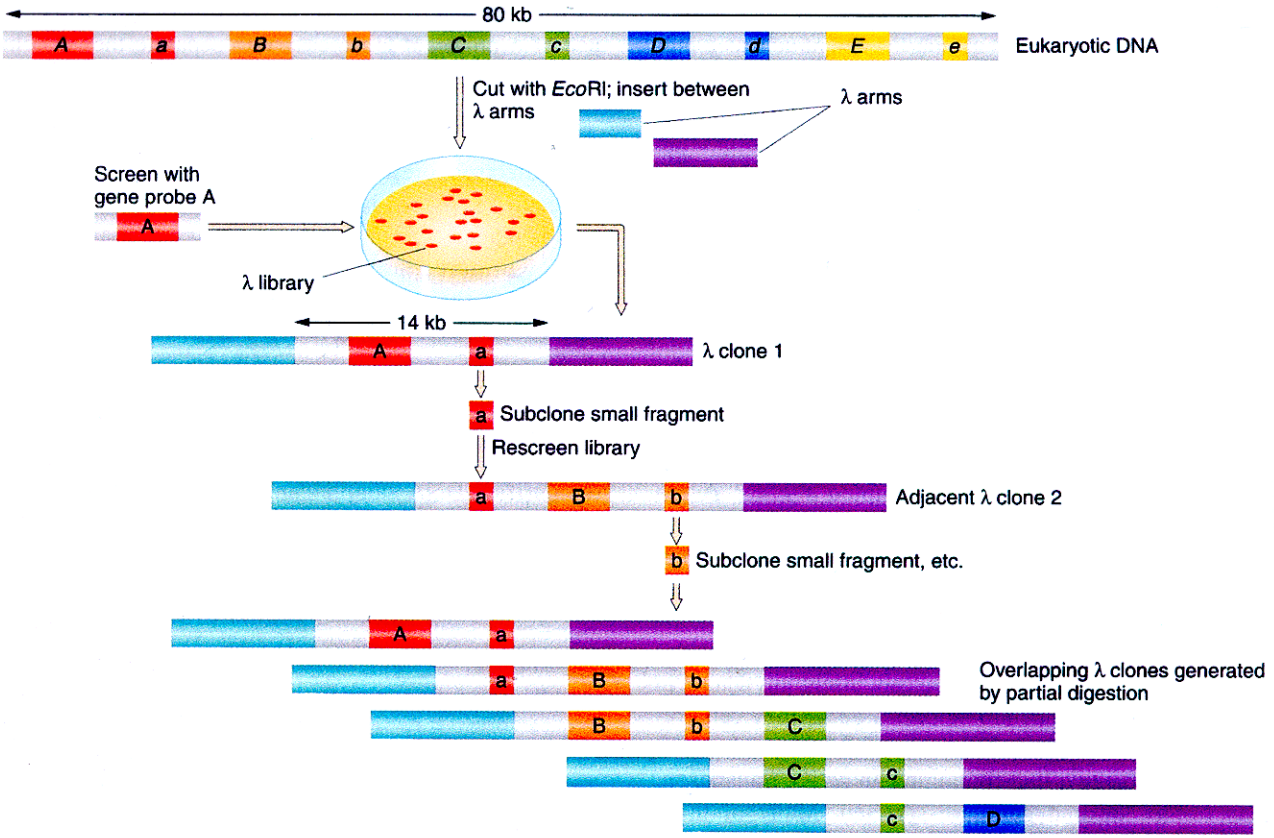


Figure 12-15 Chromosome walking. One recombinant phage obtained from a phage library made by the partial *Eco*RI digest of a eukaryotic genome can be used to isolate another recombinant phage containing a neighboring segment of eukaryotic DNA, as described in the text. (From J. D. Watson, J. Tooze, and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

BASIS OF GENOME PROJECTS + WHOLE GENOME SEQUENCING

→ HOW KNOW FIND COMPLETE FACTOR ~~VIII~~ GENE?

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

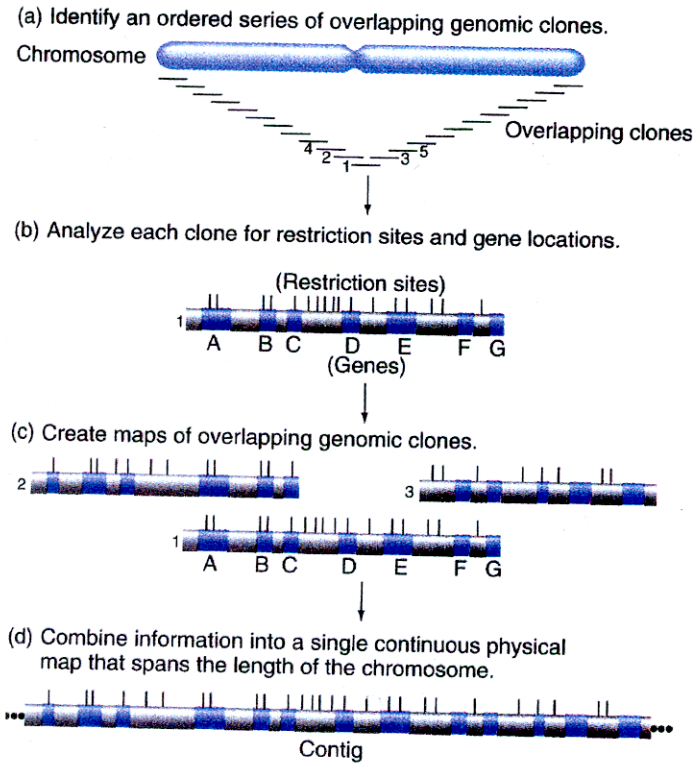
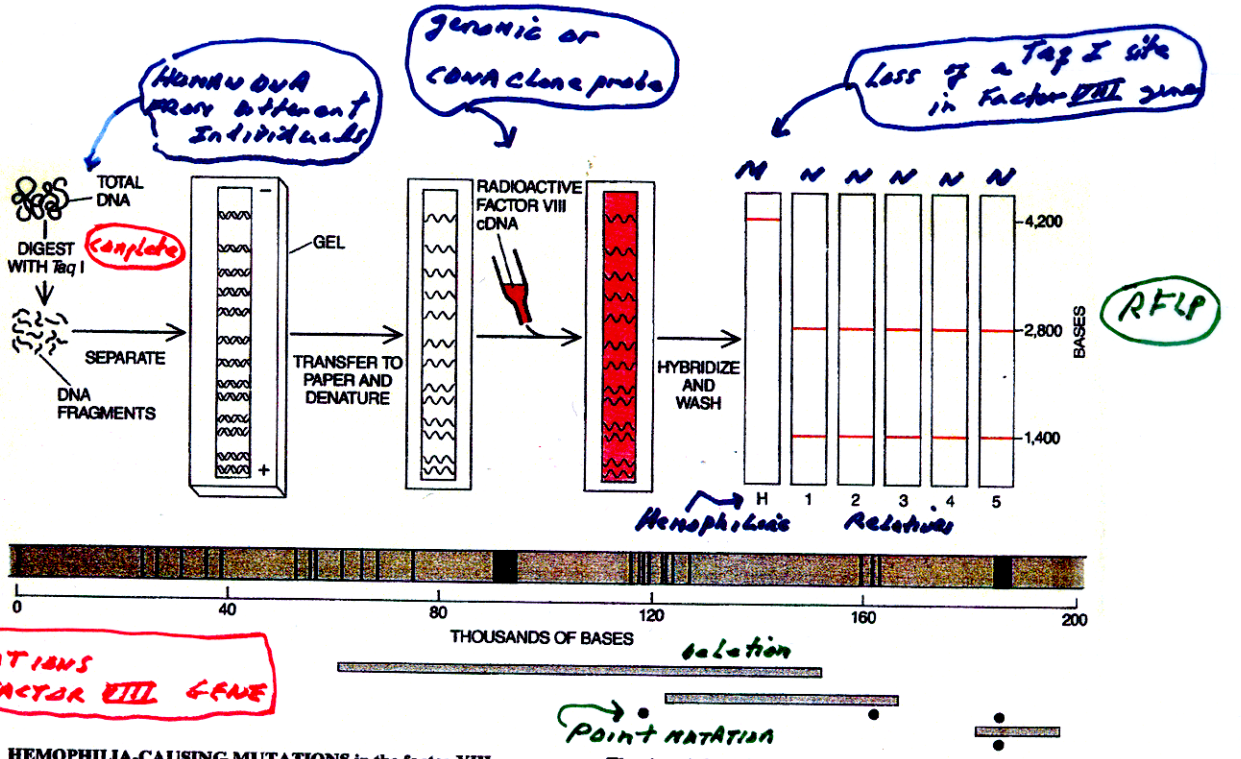


Figure 10.5 Building a whole-chromosome physical map. (a) To produce a whole-chromosome physical map, you first order a set of overlapping genomic clones that extend from one end of the chromosome to the other. Subsequent figures describe various methods of obtaining this ordered set of clones. (b) You 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.

There are 24 sets of Clones for HUMAN Genome

22 Autosomes
+
X chromosome
+
Y chromosome

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



MUTATIONS IN FACTOR VIII GENE

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 *Taq*I. 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 *Taq*I 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)

ONCE gene & cDNA identified!

USE GENE PROBE TO TEST FOR CARRIERS

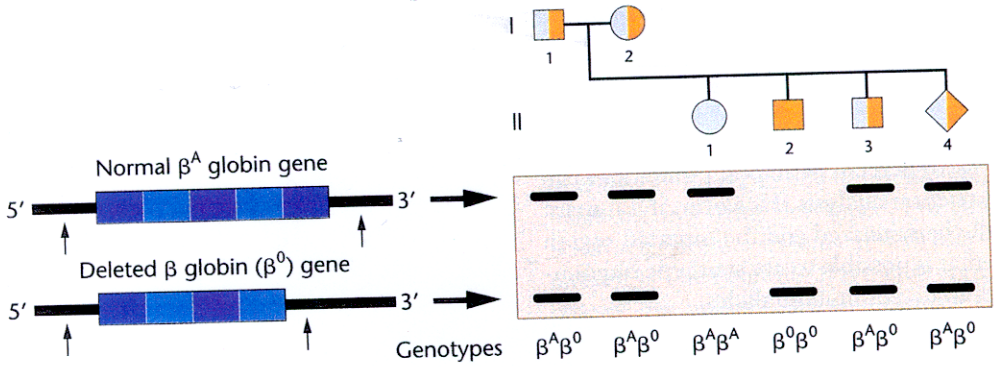


FIGURE 21.7 Diagnosis of β-thalassemia caused by a partial deletion of the β-globin gene. The family pedigree is shown positioned above each individual's genotype on a Southern blot. The normal β-globin gene (β^A) contains three exons and two introns. The deleted β-globin gene (β^0) has the third exon deleted. Arrows indicate the cutting sites for restriction enzymes used in this analysis. The normal gene produces a larger fragment (shown as the top row of fragments on the Southern blot); the smaller fragments produced by the deleted gene are represented at the bottom of the gel. The genotype of each individual in the pedigree can be determined from the pattern of bands on the blot, and these are shown below the blot.

RFLP = Restriction Fragment Length Polymorphism

Use Gene Probe to Test for Disease Gene Pre-Natally

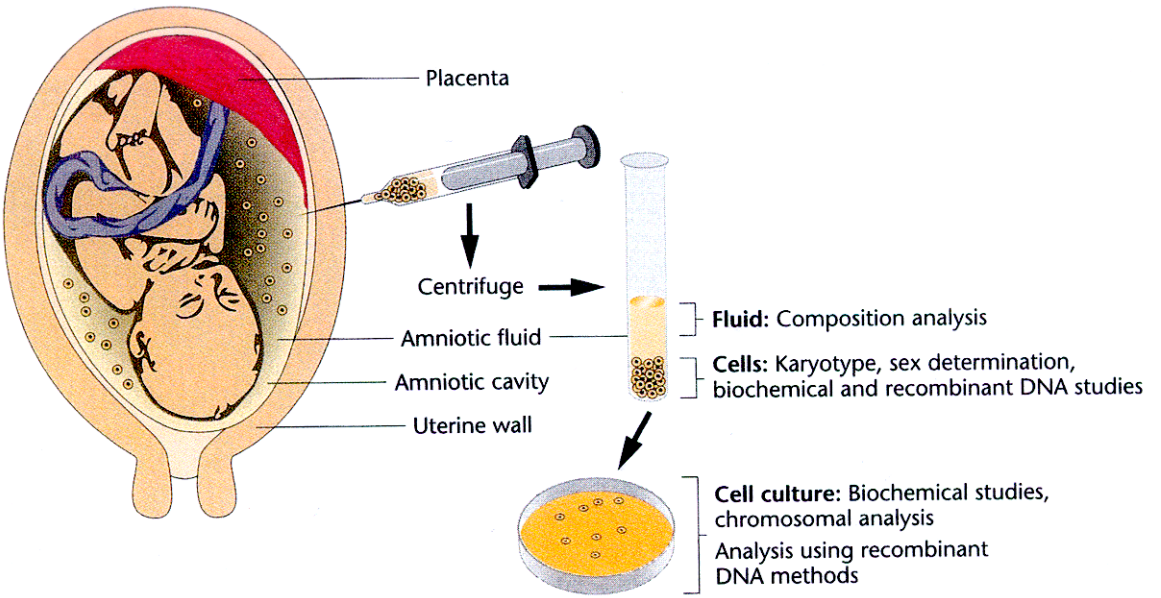


FIGURE 21.6 The technique of amniocentesis. The position of the fetus is first determined by ultrasound, and then a needle is inserted through the abdominal and uterine wall to recover fluid and fetal cells for cytogenetic and/or biochemical analysis.

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

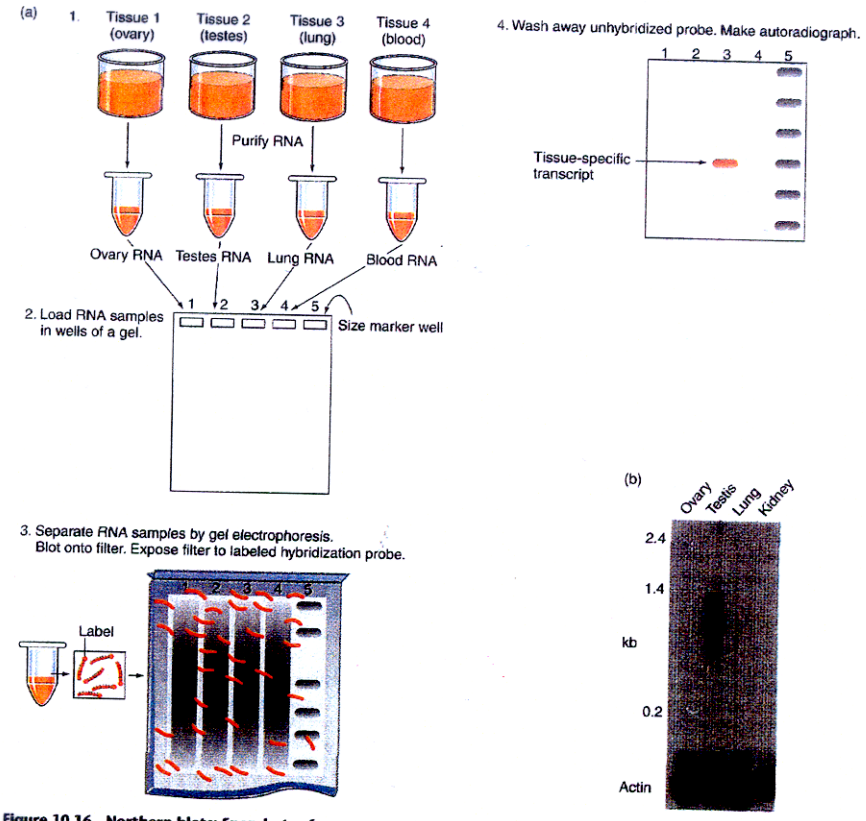


Figure 10.16 Northern blots: Snapshots of gene expression. (a) The protocol. (1) Purify RNA from each tissue to be examined for expression of the gene under investigation; here since you are looking at the *SRY* candidate for the testes-determining factor, the tissues to be examined are ovary, testes, 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!

FACTOR VIII GENE SEQUENCE CAN BE USED TO GUIDE PRIMER SYNTHESIS FOR USE IN PCR TO AMPLIFY FACTOR VIII GENE FROM GENOME OR mRNA FROM LIVER

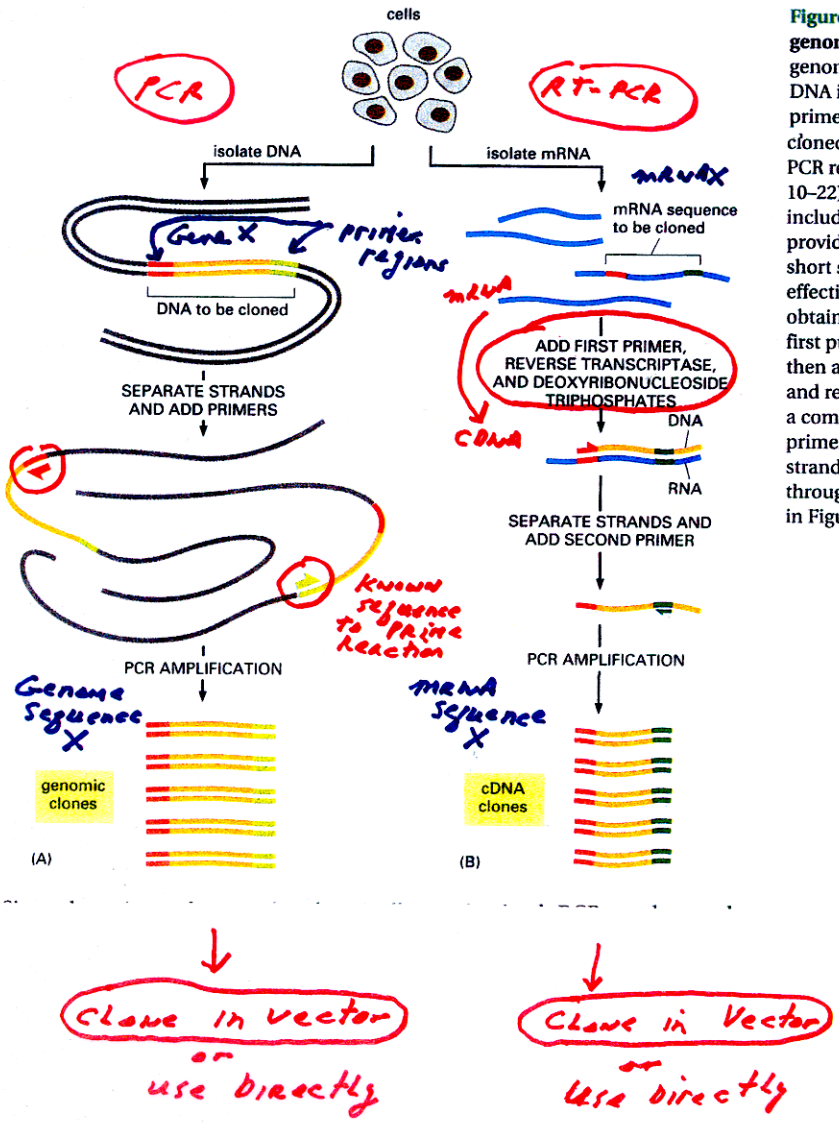


Figure 10–23 Use of PCR to obtain a genomic or cDNA clone. (A) To obtain a genomic clone using PCR, chromosomal DNA is first purified from cells. PCR primers that flank the stretch of DNA to be cloned are added, and many cycles of the PCR reaction are completed (see Figure 10–22). Since only the DNA between (and including) the primers is amplified, PCR provides a way to obtain selectively a short stretch of chromosomal DNA in an effectively pure form. (B) To use PCR to obtain a cDNA clone of a gene, mRNA is first purified from cells. The first primer is then added to the population of mRNAs, and reverse transcriptase is used to make a complementary DNA strand. The second primer is then added, and the single-stranded DNA molecule is amplified through many cycles of PCR, as shown in Figure 10–22.

FACTOR VIII GENE PROBE CAN BE USED TO VISUALIZE ITS LOCATION ON CHROMOSOMES

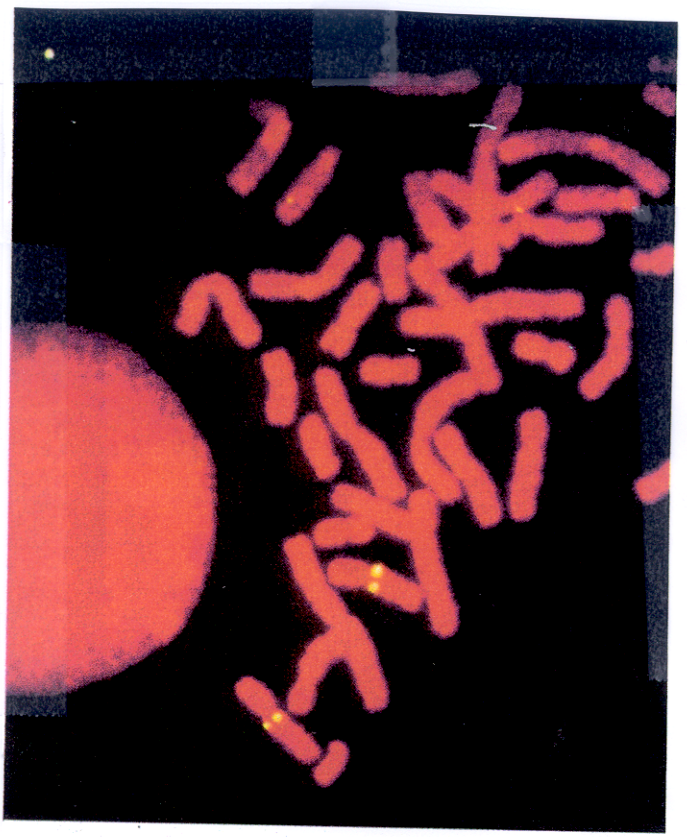
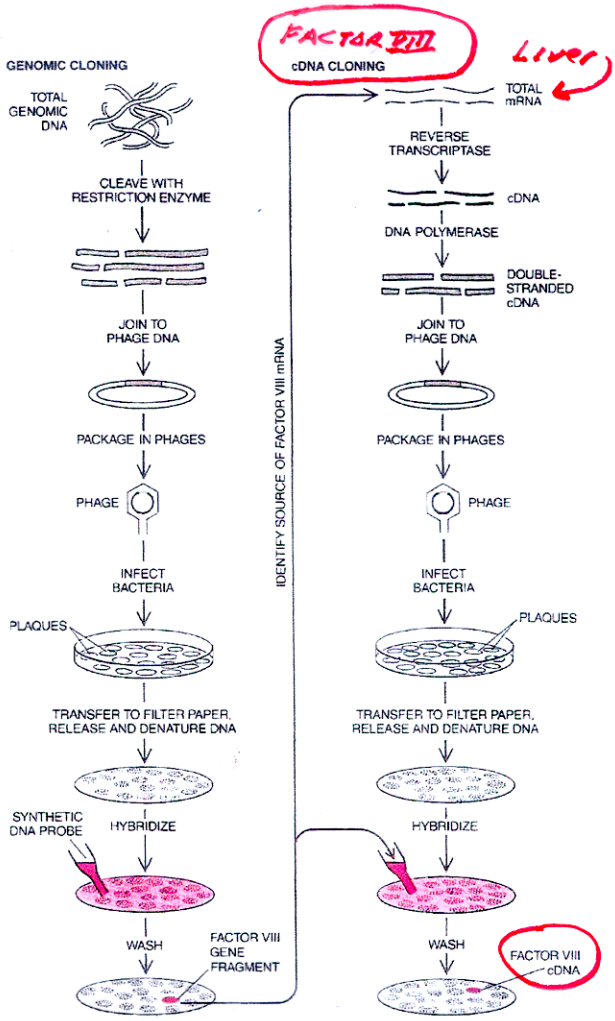


Figure 14-9 FISH analysis. Chromosomes probed in situ with a fluorescent probe specific for a gene present in a single copy in each chromosome set — in this case, a muscle protein. Only one locus shows a fluorescent spot corresponding to the probe bound to the muscle protein gene. (From P. Lichter et al., "High-Resolution Mapping of Human Chromosome 11 by in Situ Hybridization with Cosmid Clones," *Science* 247, 1990, 64.)

USING FACTOR VIII GENE PROBE TO IDENTIFY FACTOR VIII cDNA CLONE

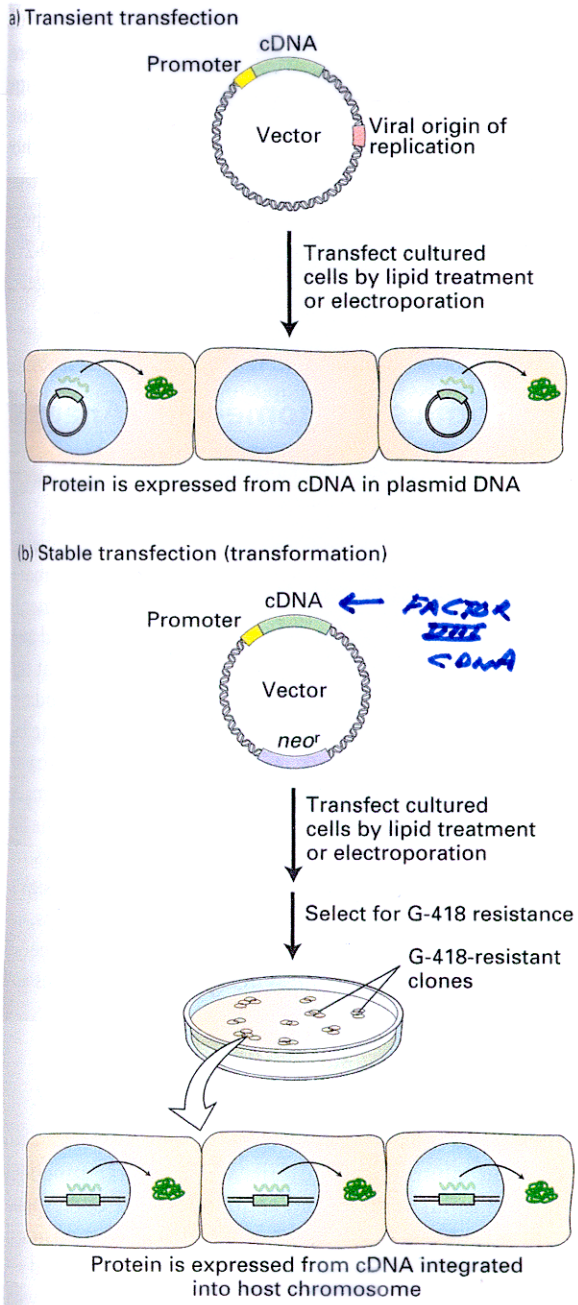


FACTOR VIII cDNA

→ USE TO MAKE DRUG!

A FACTOR VIII DRUG/"CURE"

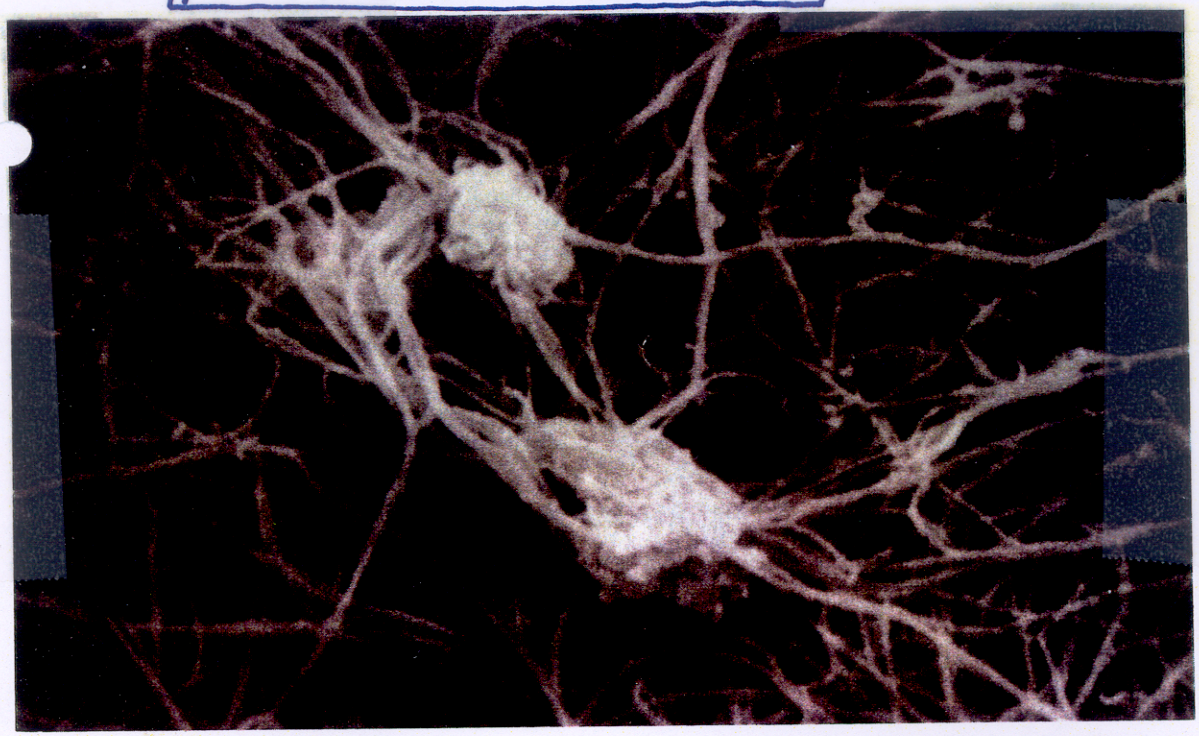
MAKING FACTOR VIII in MAMMALIAN CELLS



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 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 TRIUMPH of Genetic Engineering

RECOMBINANT FACTOR VIII

Factor VIII

Active Ingredients: Antihemophilic Factor (Human)
Pronunciation: an tee hee moe fil' ik fak tir
Representative Names: AHF (Human), AHG, Alphanate, Factor VIII, Hemofil M, Humate-P, Koate-HP, Monoclate-P, Profilate HP

Who is this for?

Your doctor has ordered antihemophilic factor (human), an antihemophilic factor, to help your blood to clot. The drug will be either injected directly into your vein or added to an intravenous fluid that will drip through a needle or catheter placed in your vein for approximately 5-10 minutes. It will be given as often as your doctor determines you need it, possibly as often as every other day. Antihemophilic factor (human), a substance naturally produced in your body, activates substances in your blood to form clots and decrease bleeding episodes. This medication is sometimes prescribed for other uses; ask your doctor or pharmacist for more information. Your health care provider (doctor, nurse, or pharmacist) may measure the effectiveness and side effects of your treatment using laboratory tests and physical examinations. It is important to keep all appointments with your doctor and the laboratory. The length of treatment depends on how your symptoms respond to the medication.