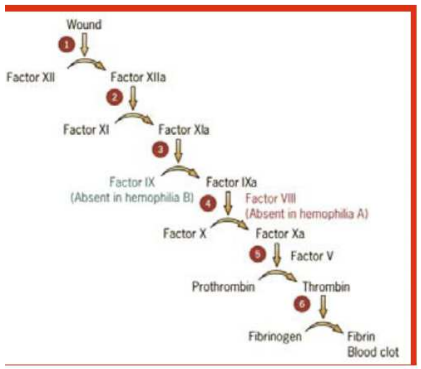
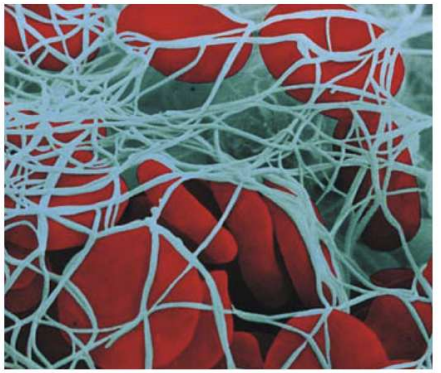
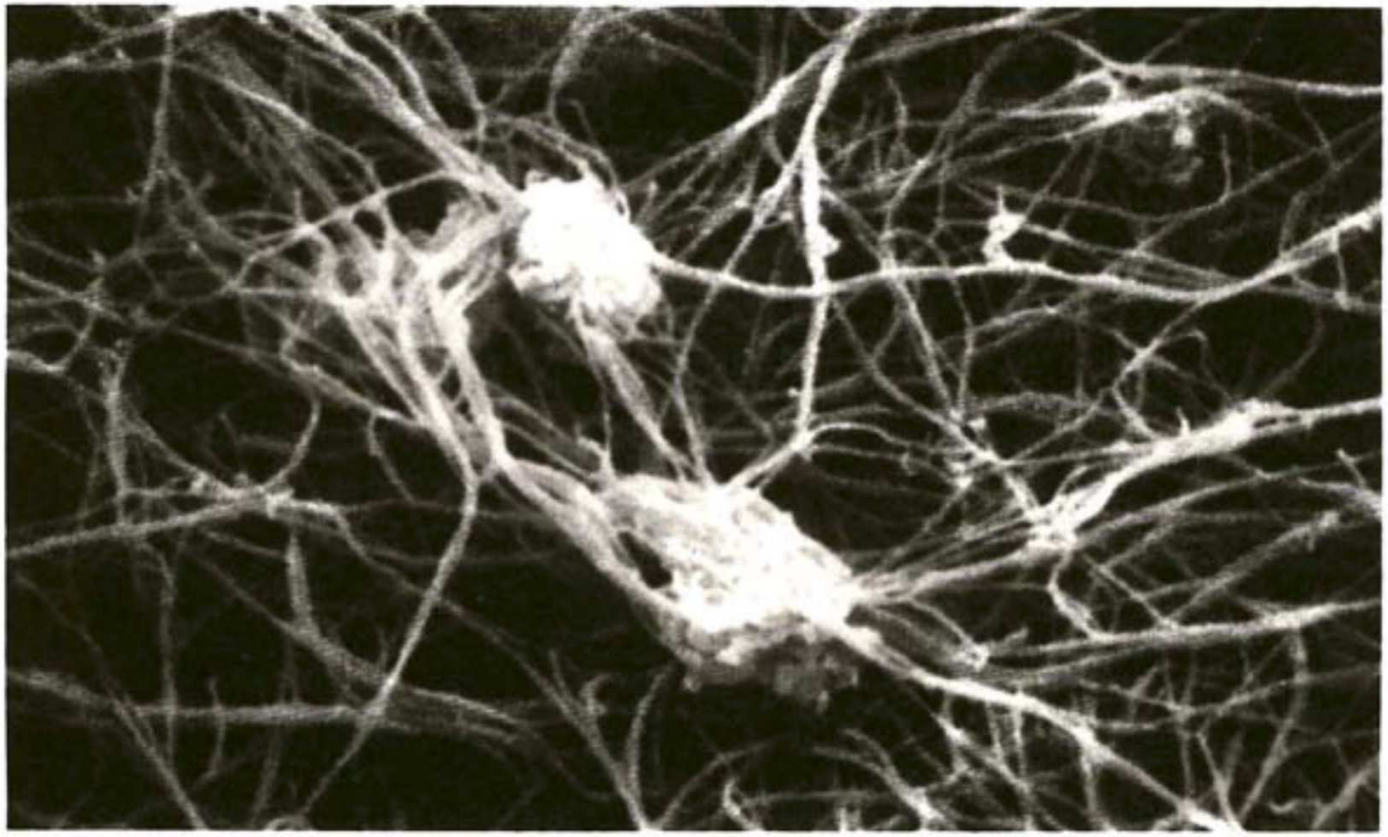
The Factor VIII Story: From Gene To Genetic Screening To Drug





How "genetic engineers" think and work
(used to work in eighties)

The Molecular Genetics of Hemophilia (Potentially Lethal Disease)



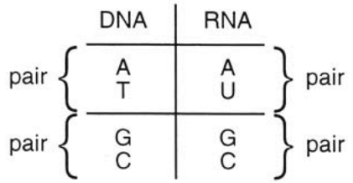
FIBRIN STRANDS stabilize a blood clot at the site of a wound by trapping the platelets that form the bulk of the clot. The electron micrograph, which was made by Jon C. Lewis of Wake Forest University, shows a clot formed in a suspension of platelets and fibrin.

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

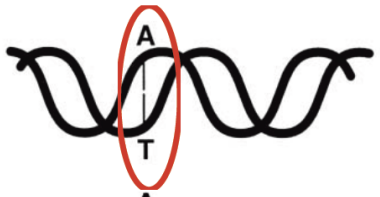
Reference: Lawn & Vehar, Sci. Amer., January, 1986

DNA Replication is Precise
But Mistakes or Mutations Can Occur!!



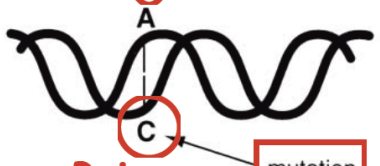
BASE PAIR RULES

Gene A



Rare Base Mismatch

Replication①



MUTATION DURING REPLICATION

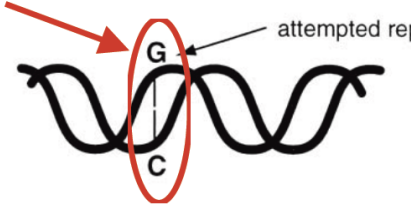
mutation

C mispairs with A

New Base Pair

See Mutation As Change in Phenotype

Replication②
Gene A'
Allelic Variant



RESULTING DEFECT

Change DNA Sequence From A = T to G ≡ C

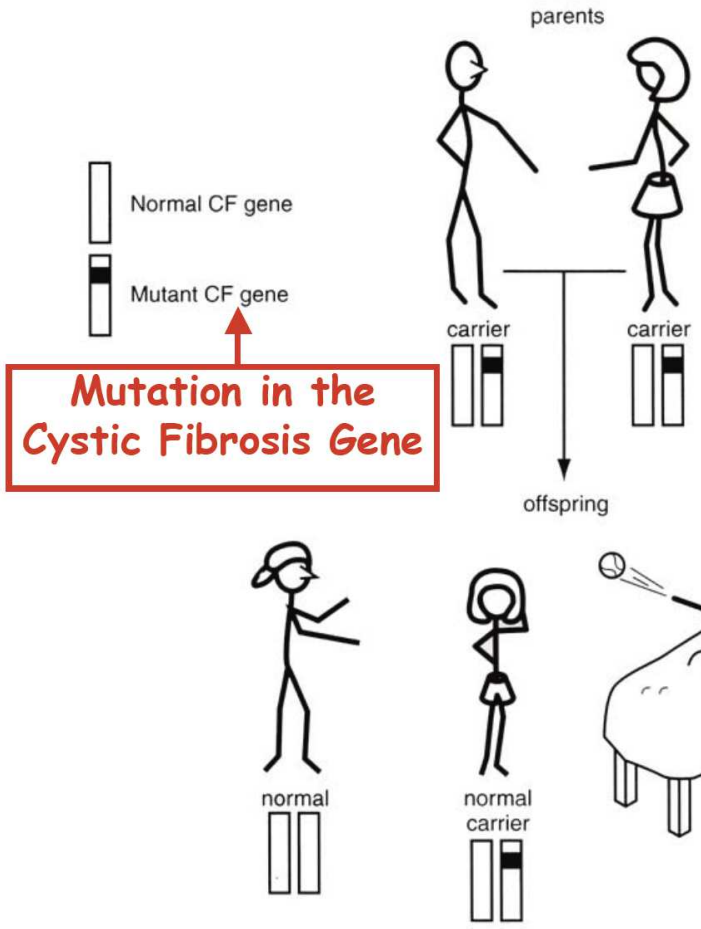
∴ Change Protein Amino Acid Sequence ⇨ Alter Function!

Mutation in Genes Are Rare But Are Inherited (1 out of 10^7 replications)

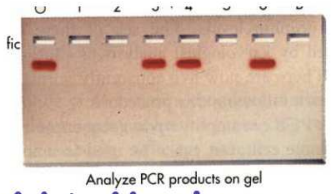
One Gene Per Gamete

♀ + ♂

Two Genes per Somatic Cells



CF - abnormal transport of chloride and sodium across an epithelium, leading to thick, viscous secretion – Lungs problem

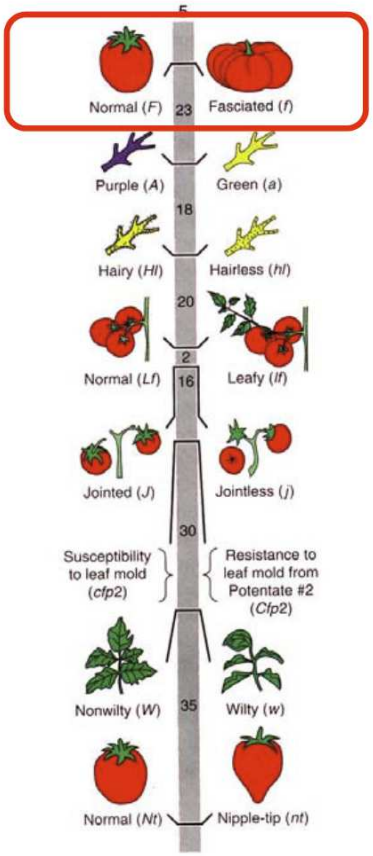


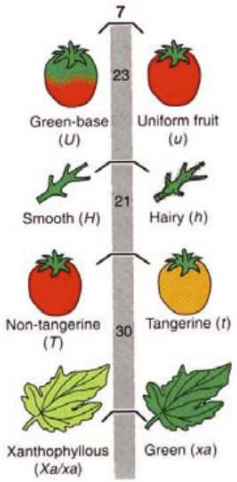
DNA Marker or Fingerprint!

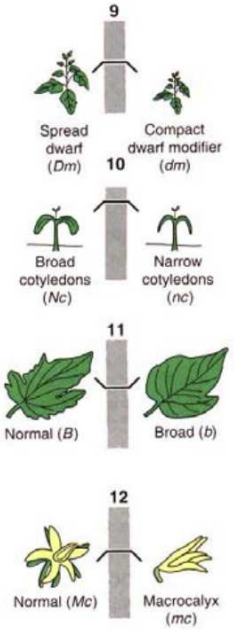
How Follow Inheritance?
What Allows Disease To Be Followed?

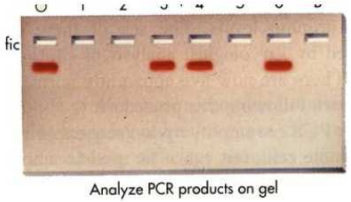
Alternative Forms of the Same Gene Lead to Genetic Diversity

Alleles







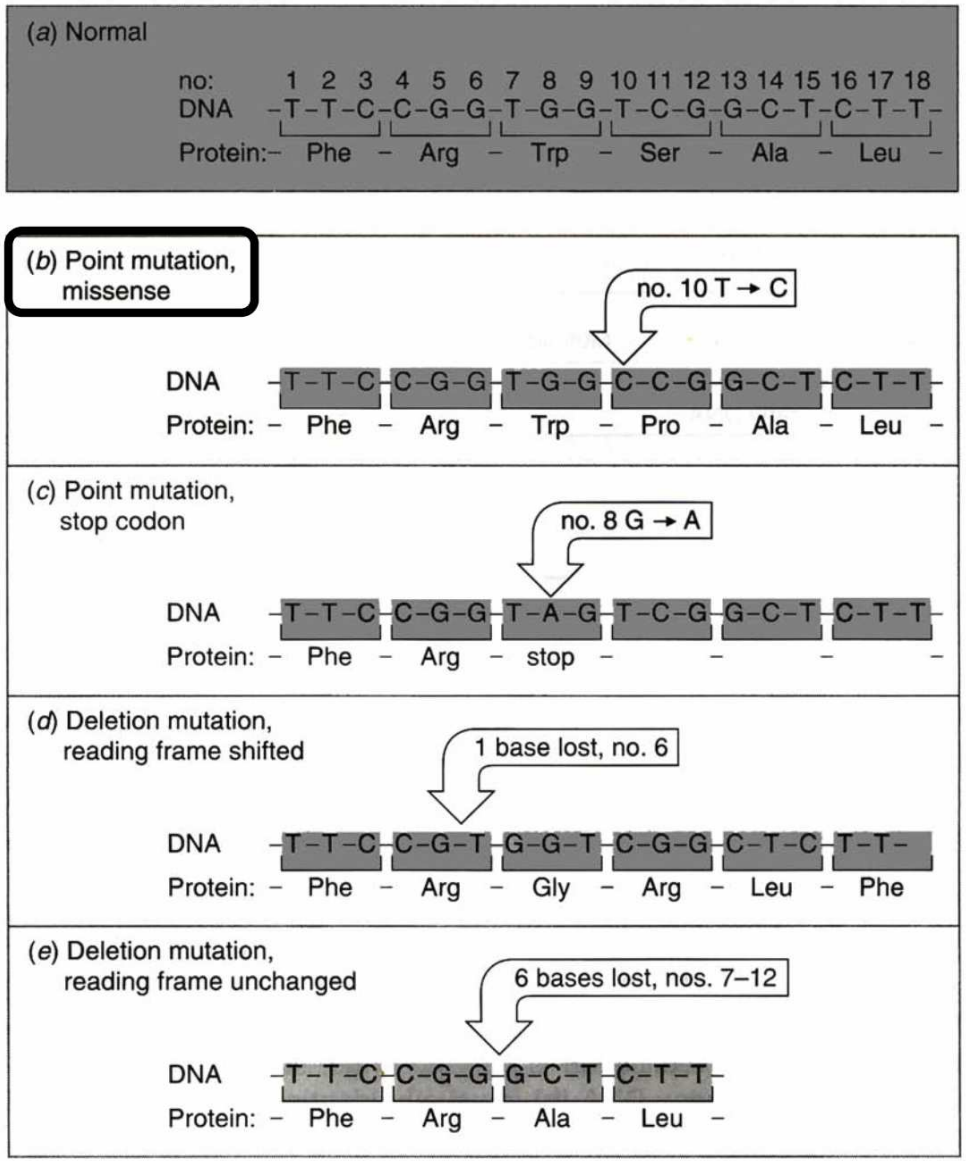


Can Follow These Traits With DNA Markers As Well

mutations result in genetic diversity!!!

Spontaneous Mutations Give Rise To Alleles, or Different Forms of the Same Gene, And result in Small DNA Sequence Changes (e.g., SNPs or Single Nucleotide Polymorphisms)

Mutations Can Occur Different Ways



1. Base-Pair Change
2. Insert or Delete Base (Indel)
3. Move Gene, or Part of Gene, to New Location (Switches Change)!

Function of Protein Lost and/or Changed

∴

Phenotype Changes

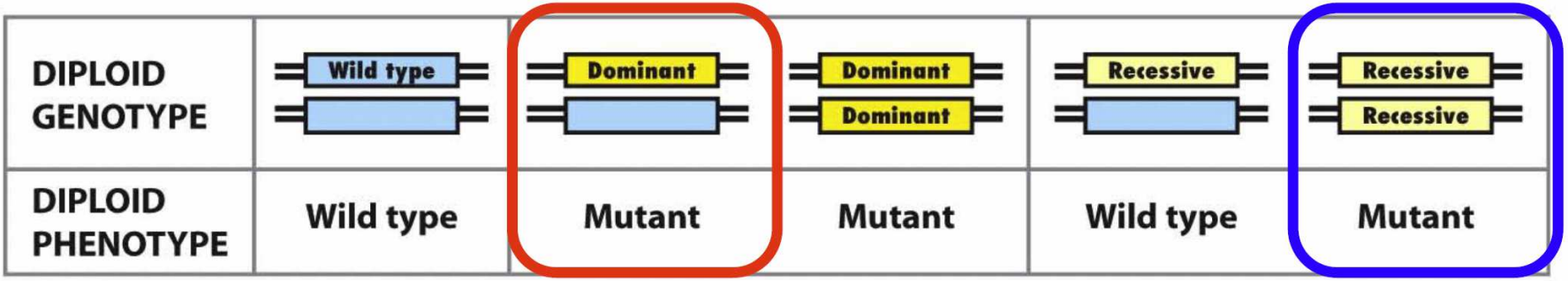
Human Genetic Disorders Occur As a Result of Mutations

TABLE 13.2 Some Important Genetic Disorders

Disorder	Symptom	Defect	Dominant/ Recessive	Frequency Among Human Births
Hemophilia	Blood fails to clot	Defective blood-clotting factor VIII	X-linked recessive	1/10,000 (Caucasian males)
Huntington 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	X-linked recessive	1/3700 (males)
Hypercholesterolemia	Excessive cholesterol levels in blood lead to heart disease	Abnormal form of cholesterol cell surface receptor	Dominant	1/500

Dominant

Recessive

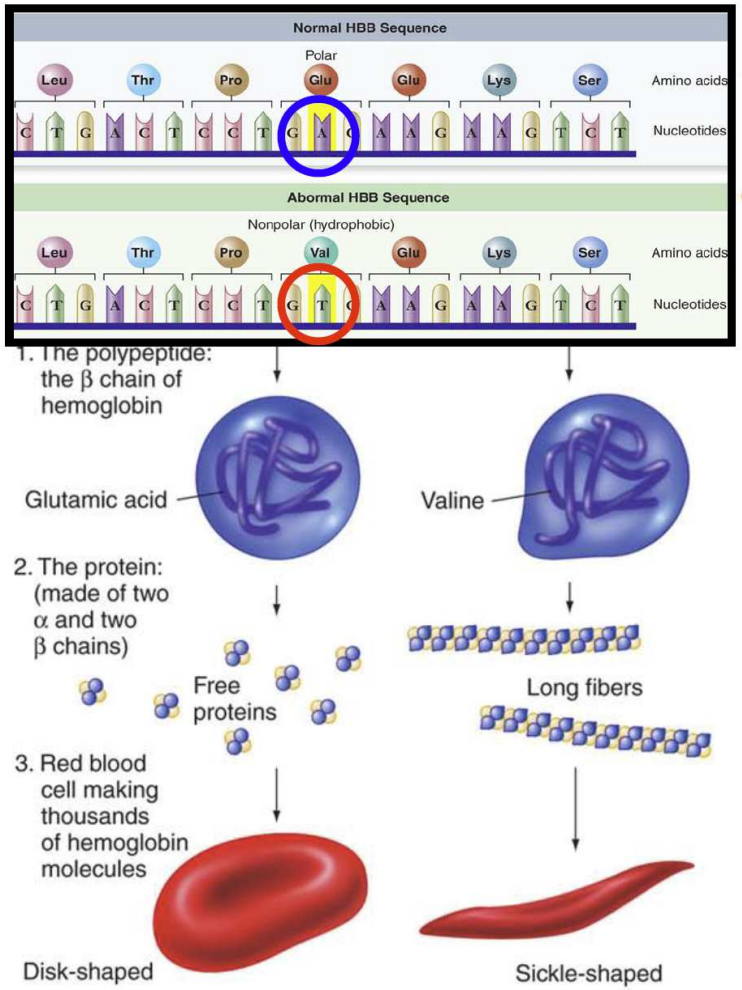


Need One Allele

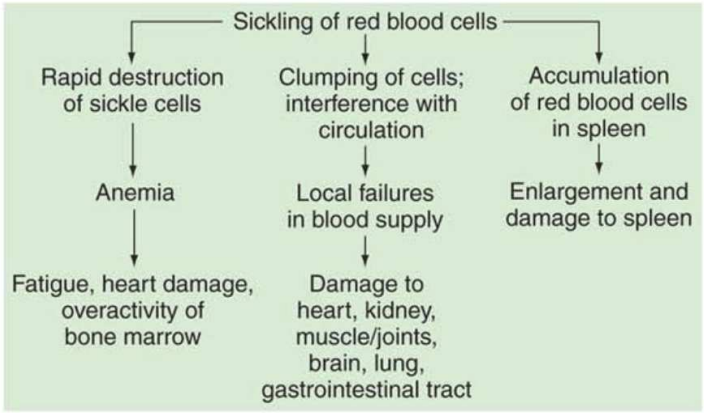
Need Two Alleles

Figure 5-2
Molecular Cell Biology, Sixth Edition

Human Genetic Disorders Occur As A Result of Mutations: *Change Code-Alter Protein*



(b) Sickle-cell anemia is pleiotrophic

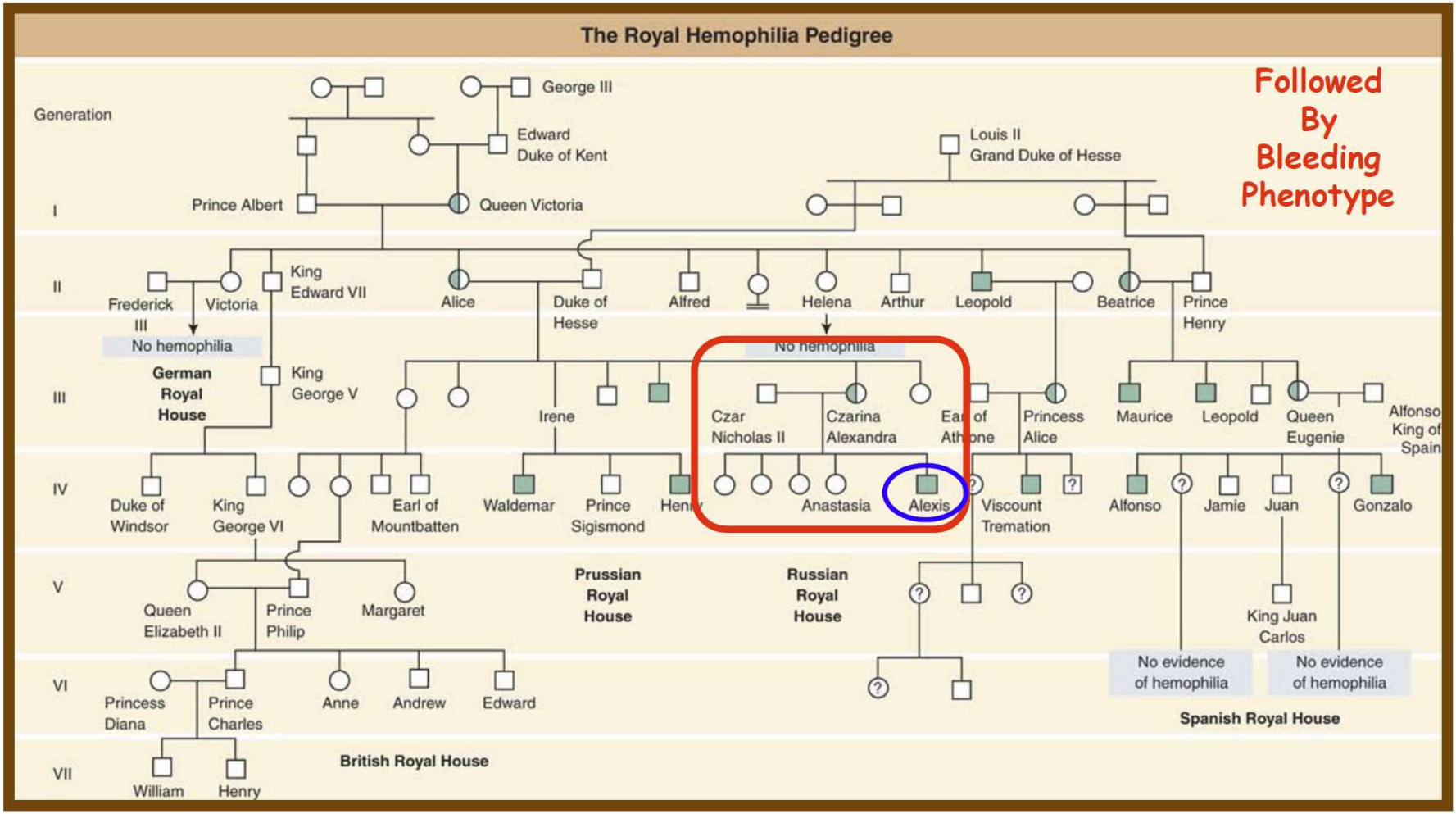


(c) β-chain substitutions/variants

	Amino-acid position									
	1	2	3 ···	6	7 ···	26 ···	63 ···	67 ···	125 ···	146
Normal (HbA)	Val	His	Leu	Glu	Glu	Glu	His	Val	Glu	His
HbS	Val	His	Leu	Val	Glu	Glu	His	Val	Glu	His
HbC	Val	His	Leu	Lys	Glu	Glu	His	Val	Glu	His
HbG San Jose	Val	His	Leu	Glu	Gly	Glu	His	Val	Glu	His
HbE	Val	His	Leu	Glu	Glu	Lys	His	Val	Glu	His
HbM Saskatoon	Val	His	Leu	Glu	Glu	Glu	Tyr	Val	Glu	His
Hb Zurich	Val	His	Leu	Glu	Glu	Glu	Arg	Val	Glu	His
HbM Milwaukee 1	Val	His	Leu	Glu	Glu	Glu	His	Glu	Glu	His
HbDβ Punjab	Val	His	Leu	Glu	Glu	Glu	His	Val	Gln	His

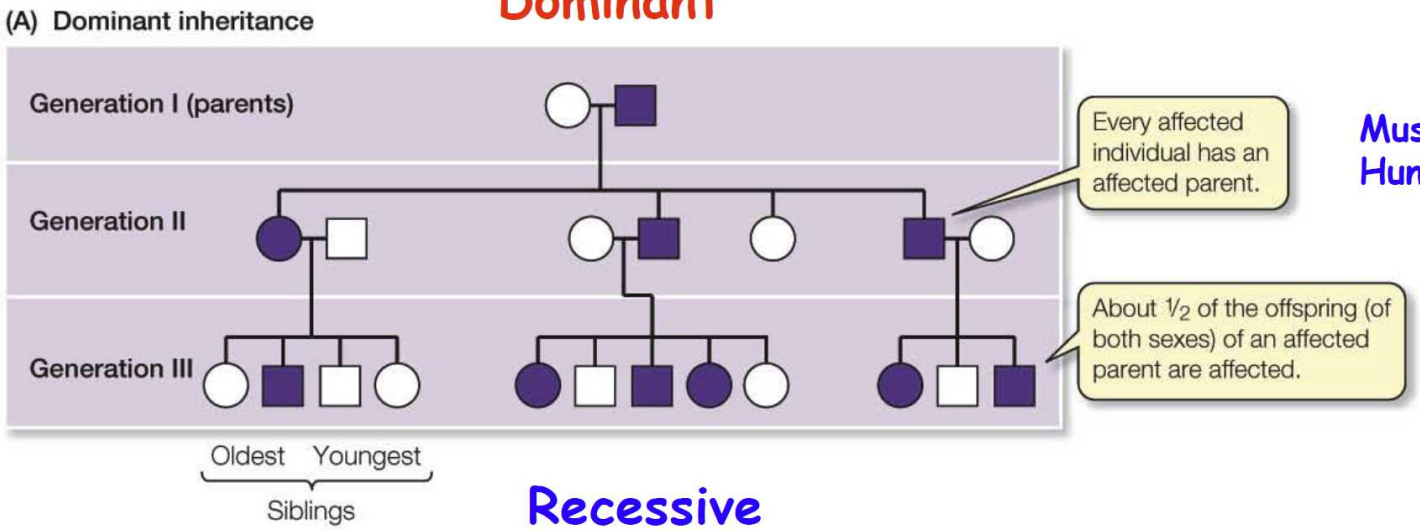
Sickle-Cell Anemia

Pedigrees Can Be Used To Follow Disease Genes in Human Families



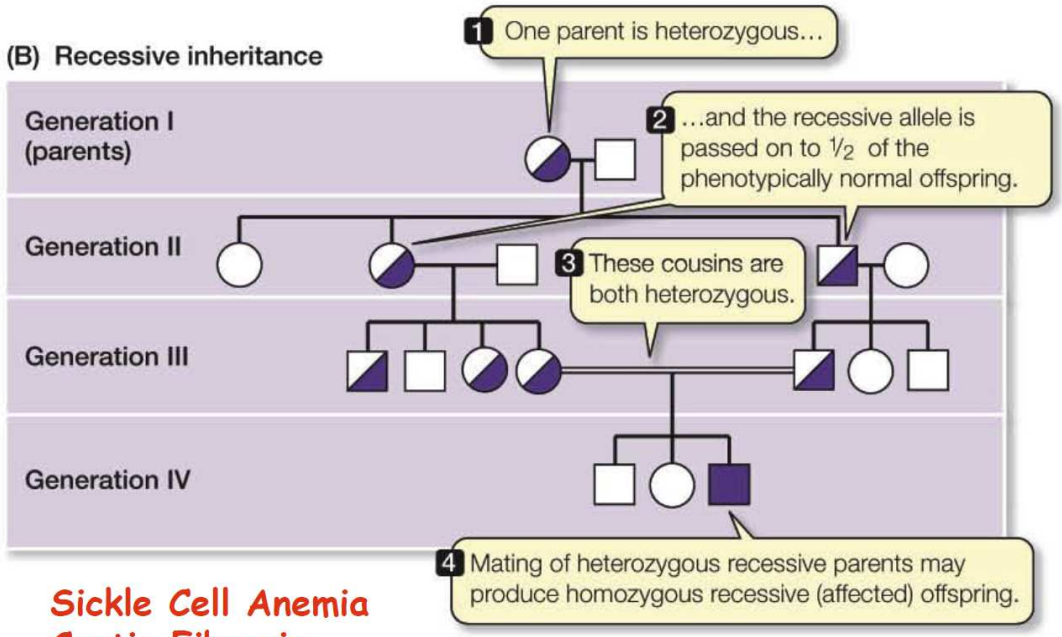
Recessive Sex Linked

Dominant

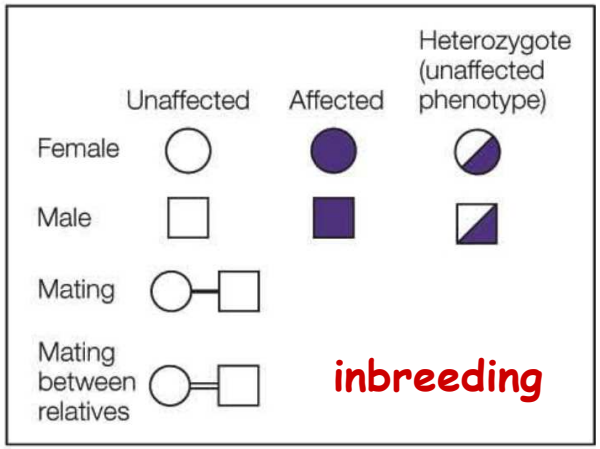


Muscular Dystrophy
Huntington Disease

Recessive



Sickle Cell Anemia
Cystic Fibrosis
Tay-Sachs Disease

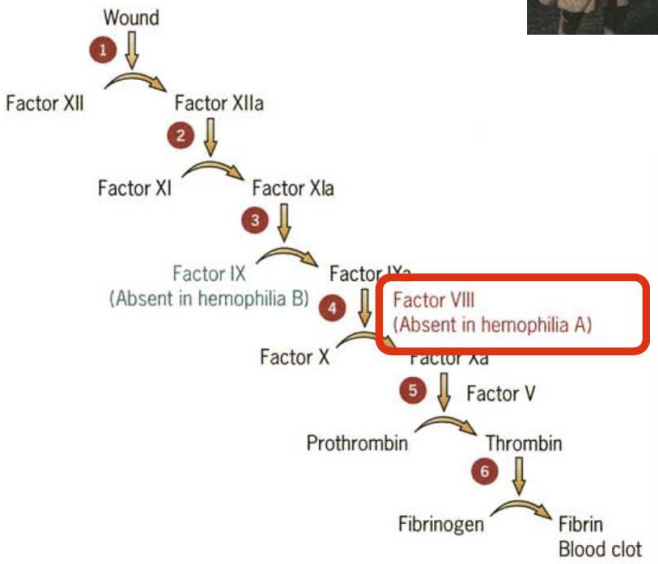


inbreeding

Hemophilia Has Been Known As An Inherited Disease For >2500 Years!

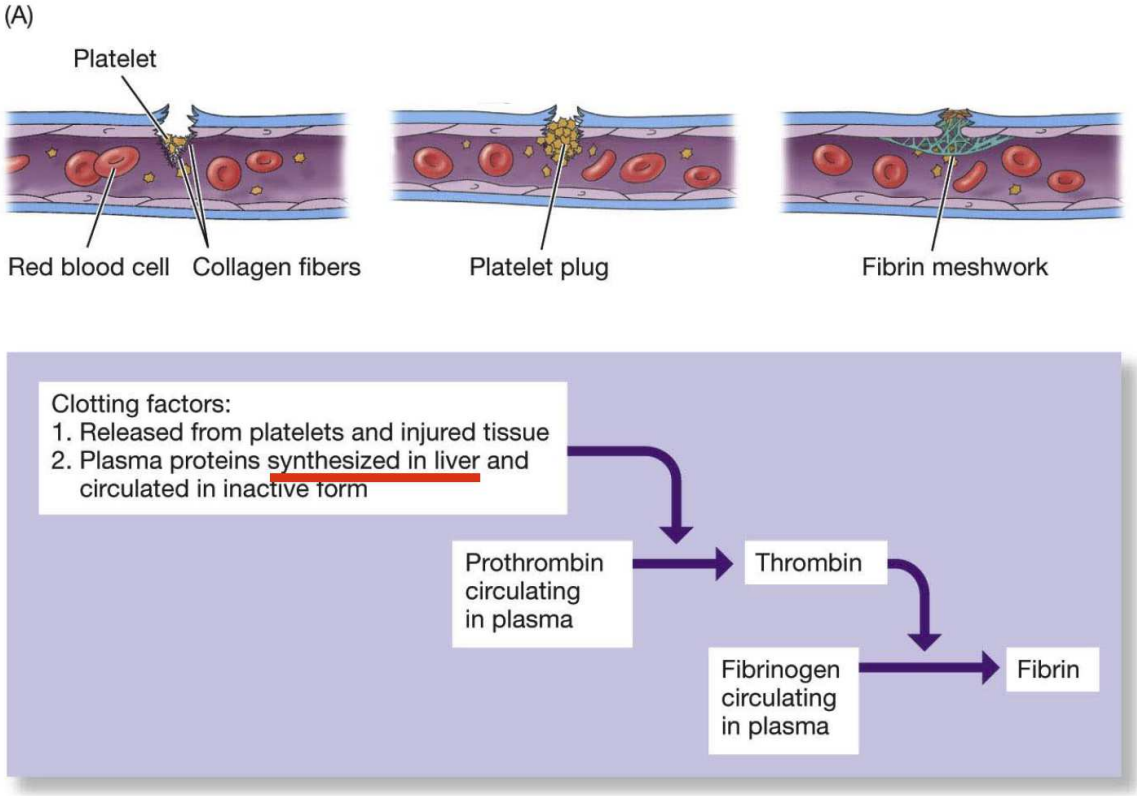
Talmud - Circumcisions

Royal Family-Europe

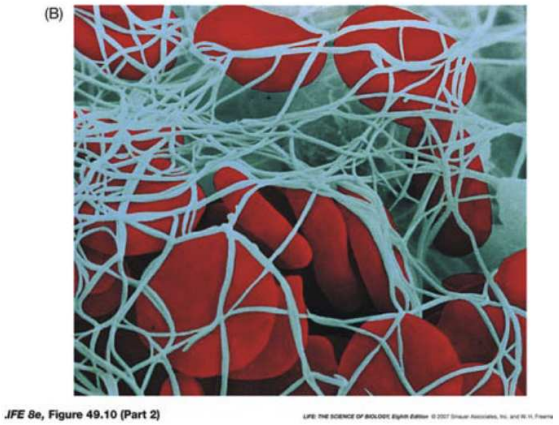


The Talmud also makes reference to families in whom children have died as a result of circumcision (Babylonian Talmud, Chapter *Yevamoth* p64b) [6]. Should a mother lose two children or should two sisters lose a child each after circumcision, subsequent children of the woman, the two sisters or of any other sisters of the same family should not be circumcised until they are older, or possibly not at all. This is thought to be the earliest reference to haemophilia; it was recognized in the Talmud that this condition was transmitted by the mother.

A Cascade Of Events After Wounding Leads to A Fibrin Clot



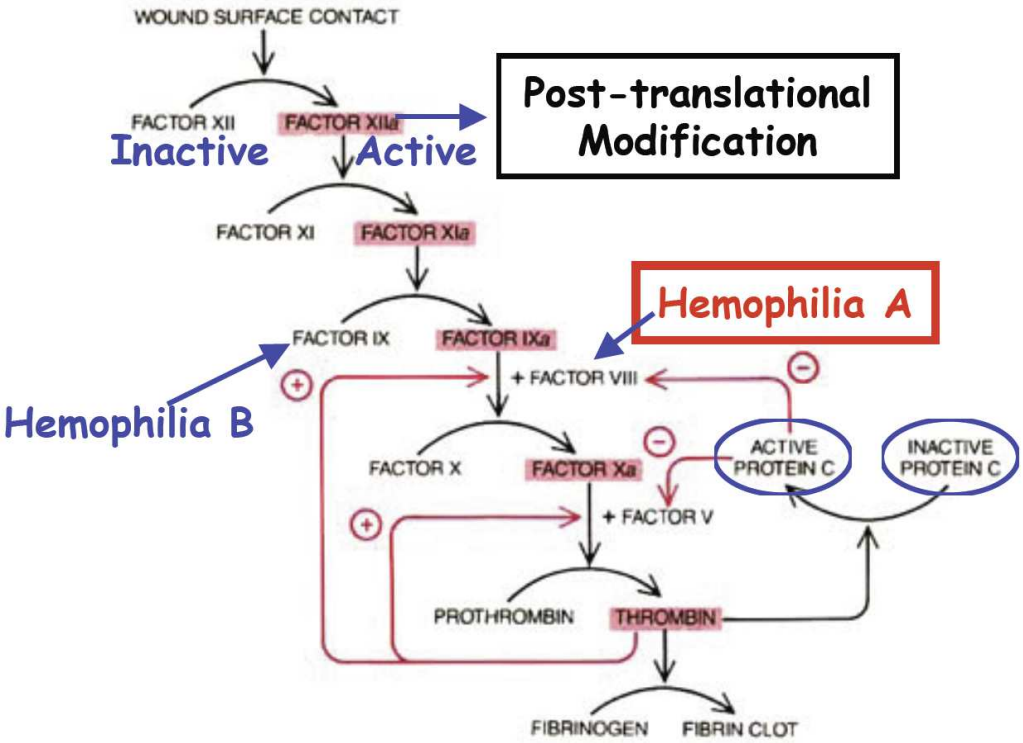
LIFE 8e, Figure 49.10 (Part 1)



LIFE 8e, Figure 49.10 (Part 2)

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! - bleeding

Hemophiliacs Have Mutations In Either Factor VIII or Factor IX Genes

TABLE 13.2 Some Important Genetic Disorders

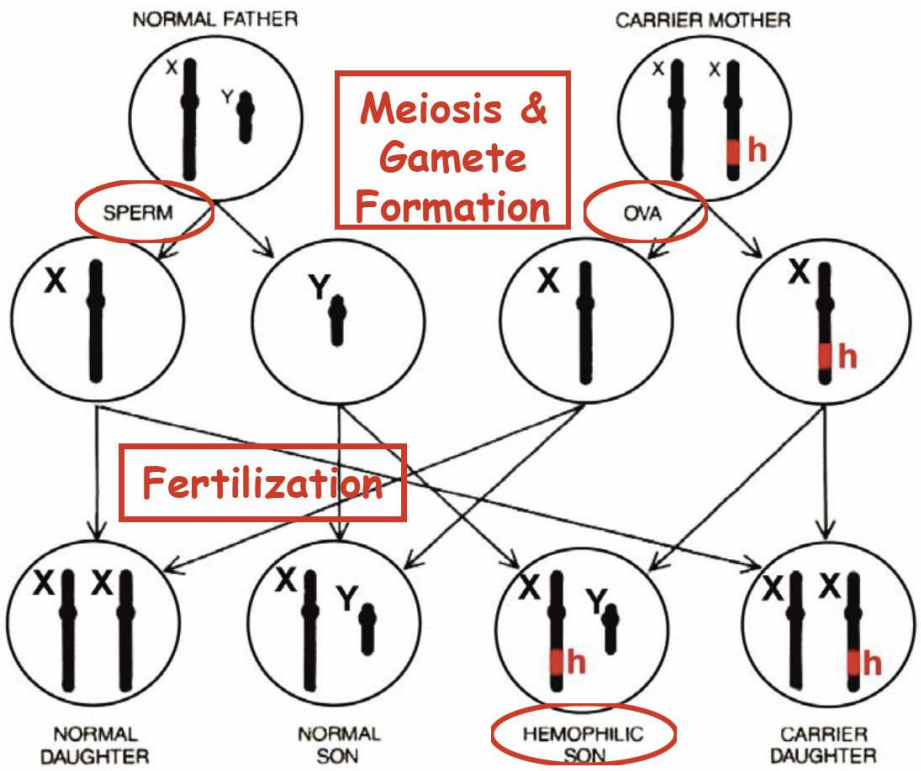
Disorder	Symptom	Defect	Dominant/ Recessive	Frequency Among Human Births
Cystic fibrosis	Mucus clogs lungs, liver, and pancreas	Failure of chloride ion transport mechanism	Recessive	1/2500 (Caucasians)
Sickle cell anemia	Blood circulation is poor	Abnormal hemoglobin molecules	Recessive	1/600 (African Americans)
Tay–Sachs disease	Central nervous system deteriorates 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	X-linked recessive	1/10,000 (Caucasian males)
Huntington 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	X-linked recessive	1/3700 (males)
Hypercholesterolemia	Excessive cholesterol levels in blood lead 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 in Males?

Both Genes On X-Chromosome ♀ → ♂'s

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

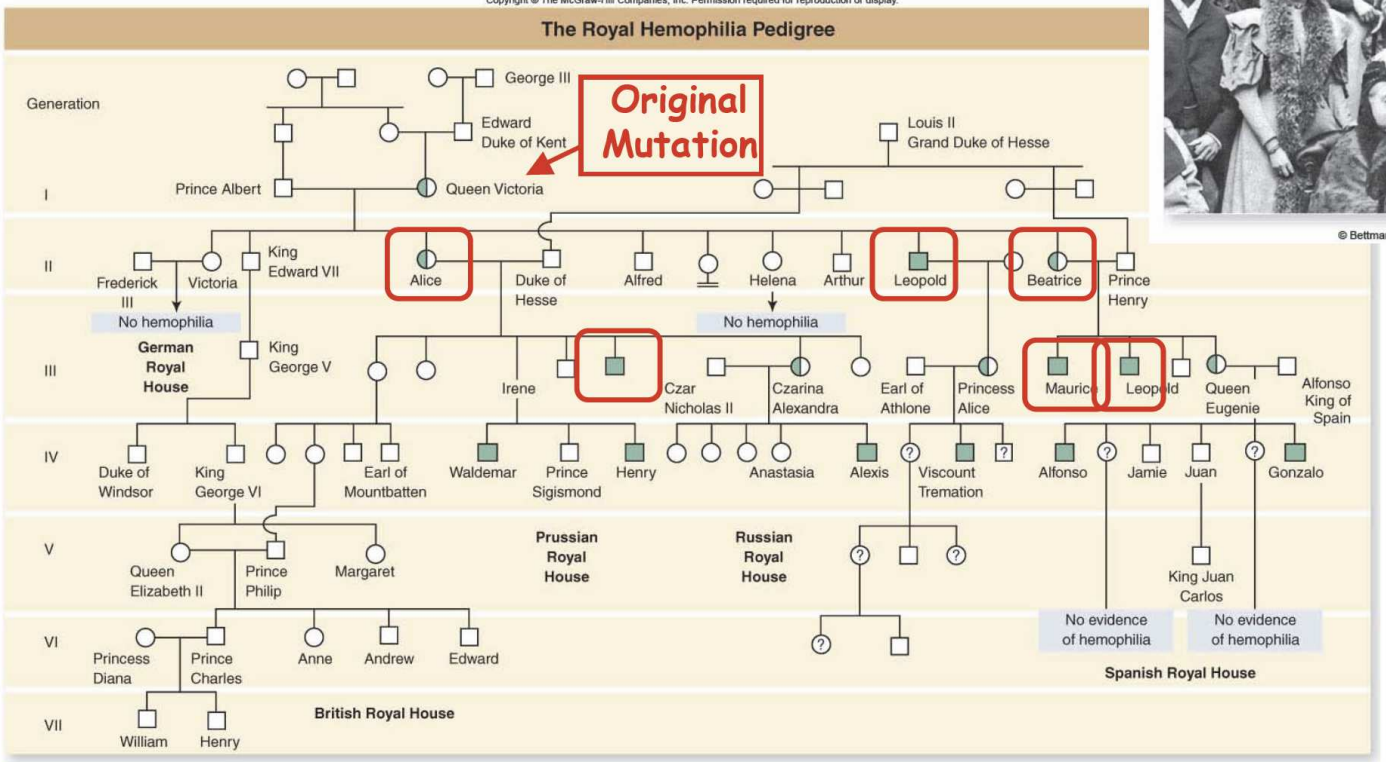
Children 2n

Sex-Linked Inheritance

♀ Carriers → 1/2 Sons + No Daughters!

Only One X-Chromosome is ♂

Hemophilia A and B Genes (Traits) Are Sex Linked



© Bettmann/Corbis

Note: 1. Males Obtain Detective Gene From Mothers

2. 50% of Sons Of A Maternal Carrier Have The Defective Gene

Search for the gene

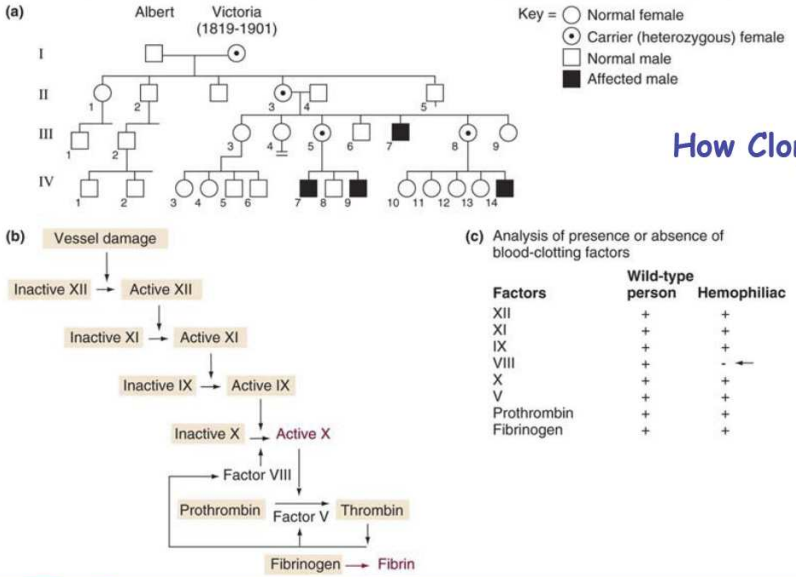
1. Blood Protein (But Perhaps Synthesized Elsewhere!)
2. Could be purified in small amounts from >20 Liters of human blood +cow blood + pig blood
3. Short Stretch of Proteins Sequenced = Known Protein Sequence!
4. Hemophilia A could be treated by blood transfusions from normal individuals, ∴ clotting factor in blood.

∴ How to go From Protein to Gene

The Problem

For Factor VIII- *Not Known Where Gene is Expressed* ∴ Must Use Genome Library

Early 1980's

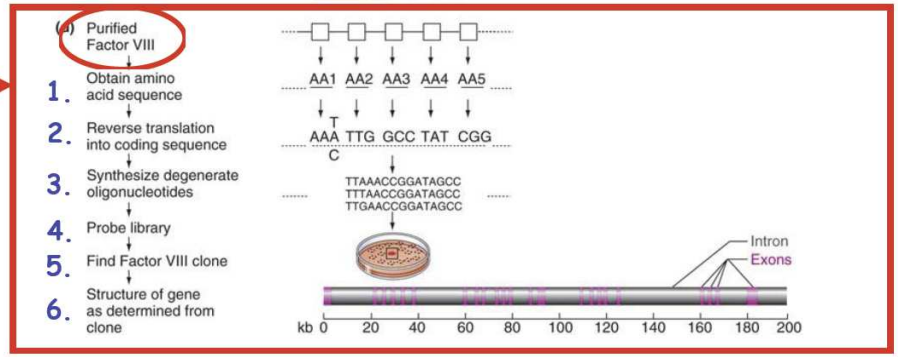


Key Concept

⬇

How Clone A Gene When You Don't Know Where it is Expressed !

Key:
Protein Sequence Known



How Find Gene & cDNA?

Protein → Gene → mRNA → Drug !

Knowledge of the Protein Sequence and the Genetic Code Makes it Possible to Identify a Gene

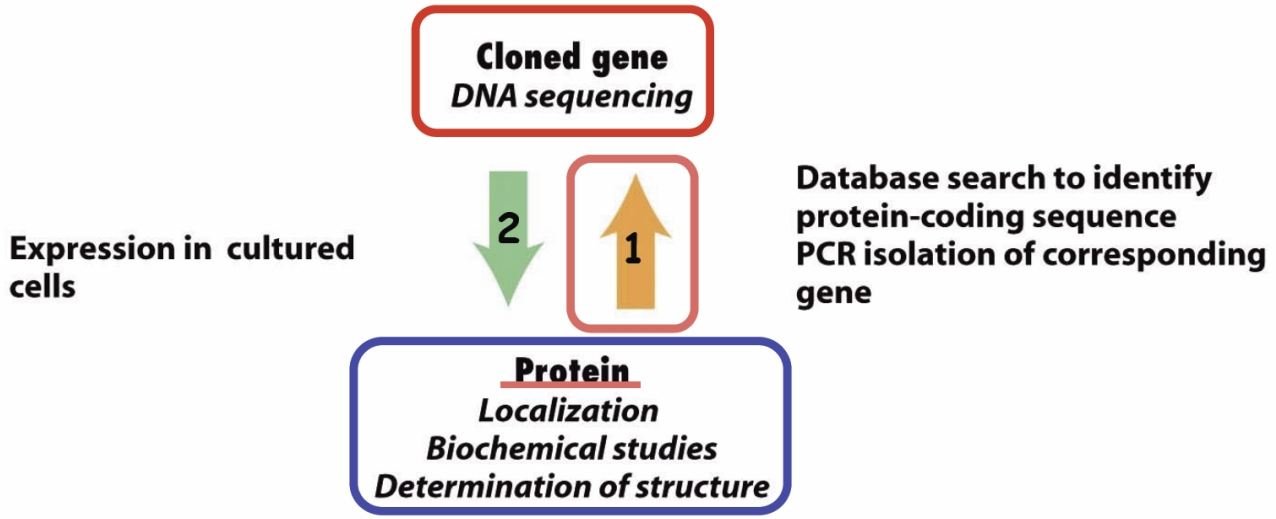


Figure 5-1
Molecular Cell Biology, Sixth Edition

∴ 1. Protein → Gene → Drug

Factor VIII Strategy (1985)

or

2011 →

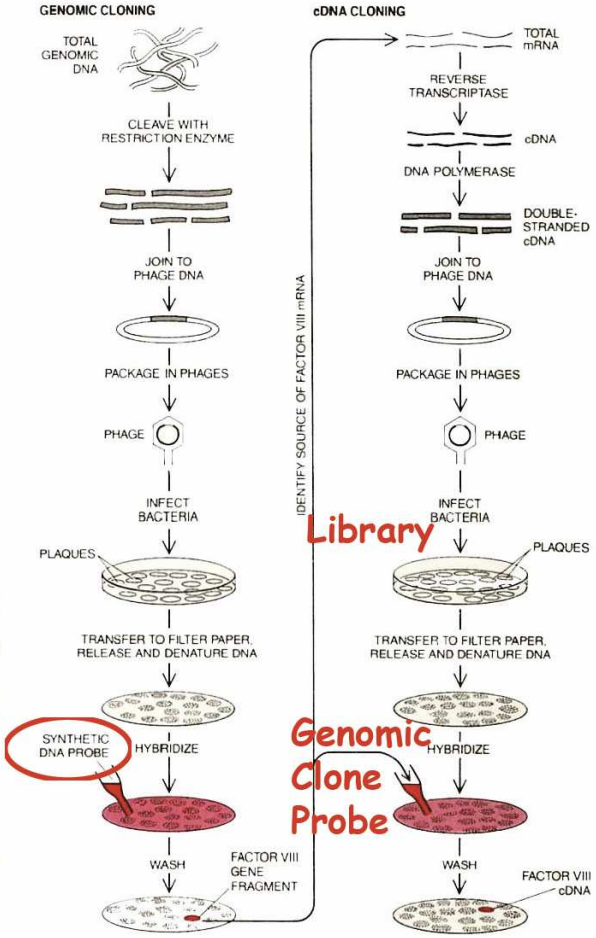
3. Just Sequence Everything + Identify Protein- GenBank Huge

Steps Required to Clone Factor VIII Gene and cDNA

Gene

1. Make Genome Library Because Factor VIII Gene in Genome!
2. Purify Protein from Blood- that's where it works (wasn't known 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 on The Clone?



cDNA

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- male, liver- secrete into blood

Why Need cDNA?

Story continued

Want cDNA to Manufacture Factor VIII as a Drug to Treat Hemophilia A!

Step One

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!

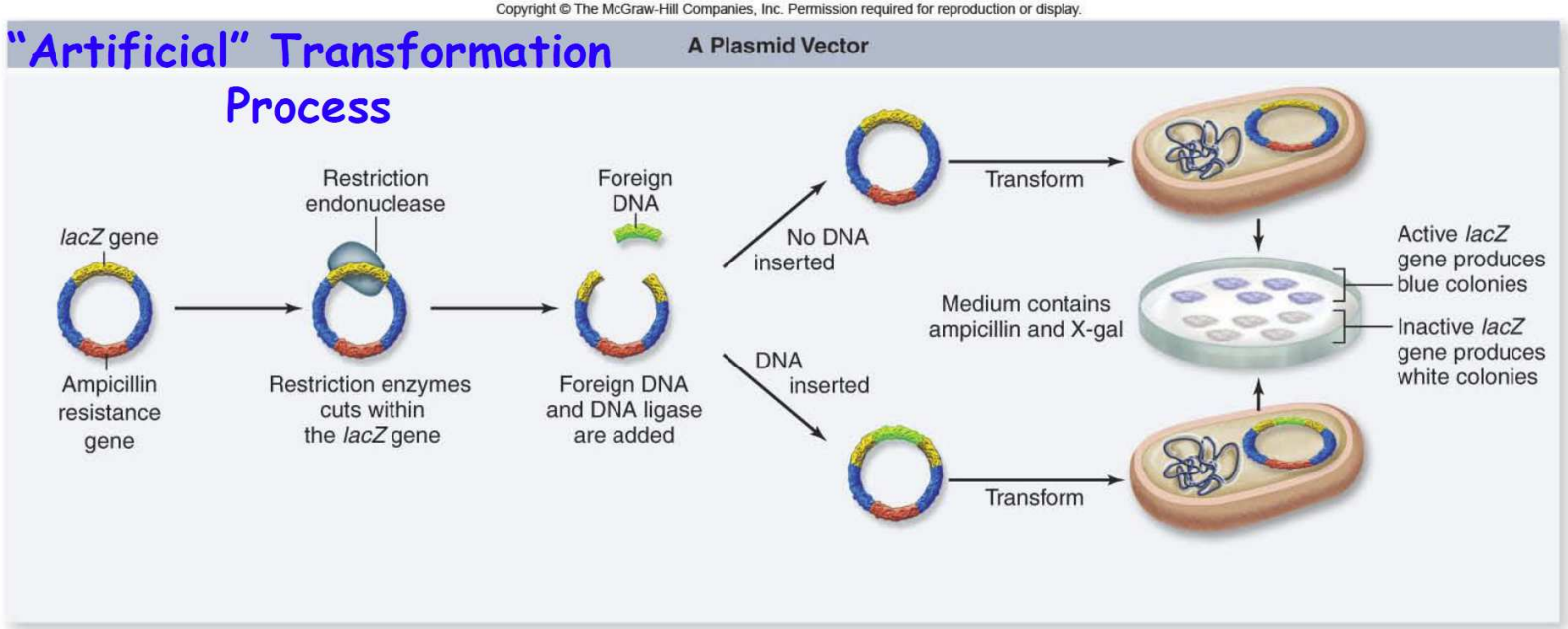
Vectors Used in Genetic Engineering Have Similar Conceptual Properties But are Used in Different Situations

Table 3.2 A COMPARISON OF DNA VECTORS AND THEIR APPLICATIONS

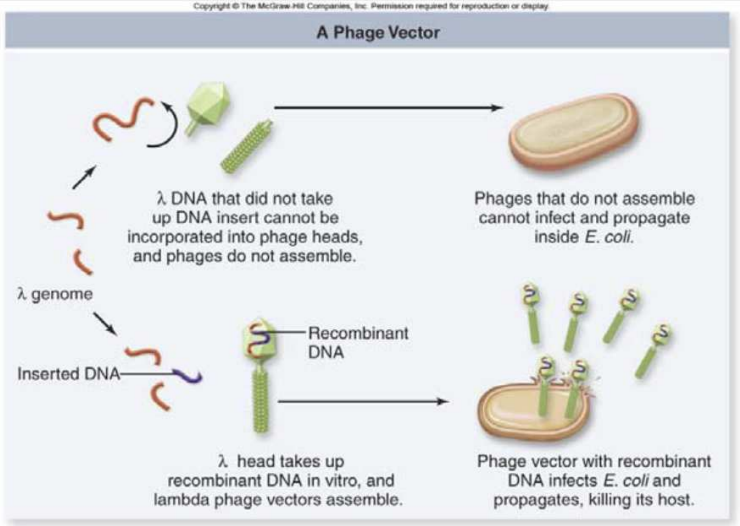
Vector Type	Maximum Insert Size (kb)	Applications	Limitations
Bacterial plasmid vectors (circular)	~6–12	DNA cloning, protein expression, subcloning, direct sequencing of insert	Restricted insert size; limited expression of proteins; copy number problems; replication restricted to bacteria
	DNA	DNA	
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 (BAC, circular)	~300	Genomic libraries, cloning large DNA fragments	Replication restricted to bacteria; cannot be used for protein expression
Yeast artificial chromosome (YAC, circular)	200–2,000	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

Plasmid vs. Bacteriophage Vectors for Cloning DNA Fragments

"Artificial" Transformation Process

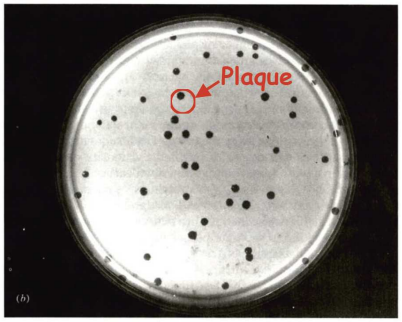


a.



b.

Natural Infection Process



Cloning the Human Genome and Screening for the Factor VIII Gene

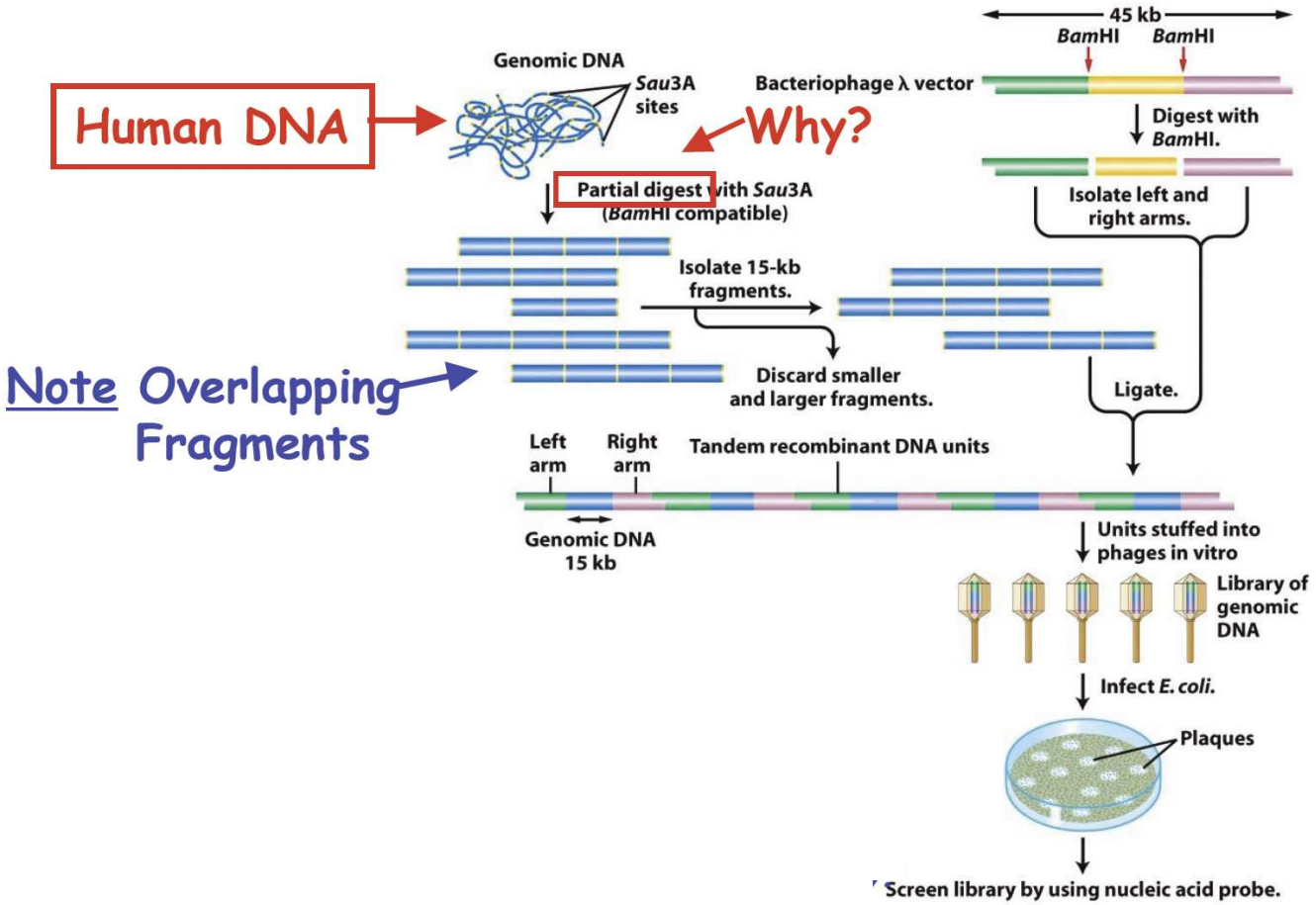
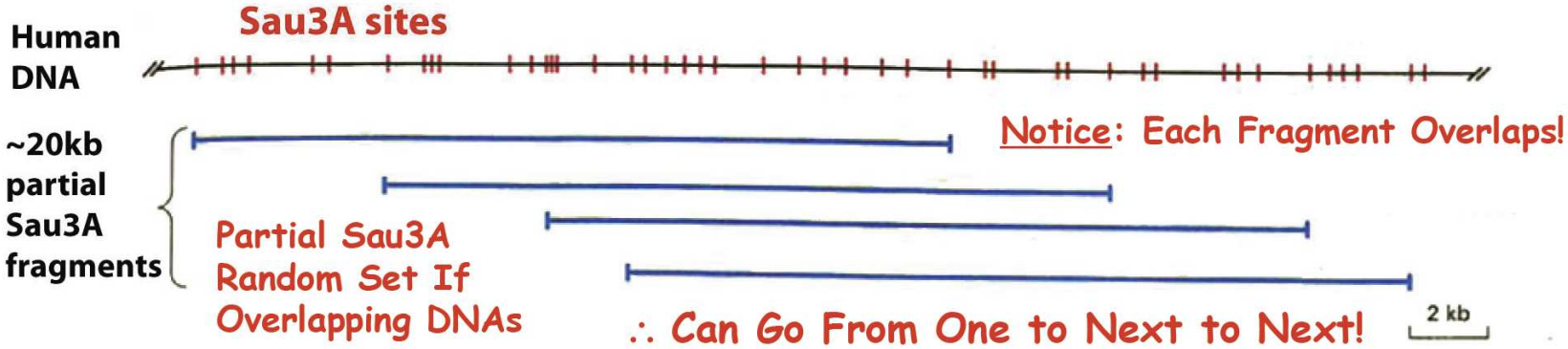


Figure 20-6
Introduction to Genetic Analysis, Ninth Edition

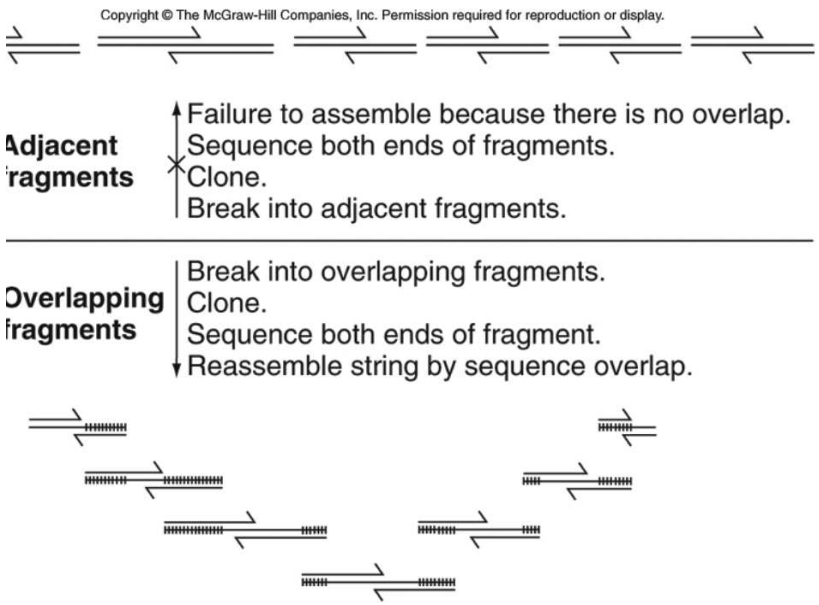
Why Partial Digestion? An Important Concept!

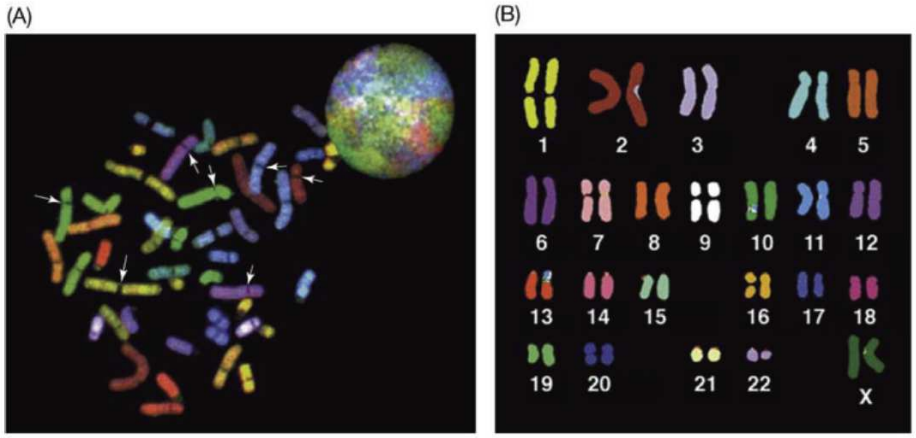
What is Complete & Partial Digestion?

Constructing a Human Genome Library by Partial Digestion Creates a Set of Overlapping DNA Fragments/ Clones



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



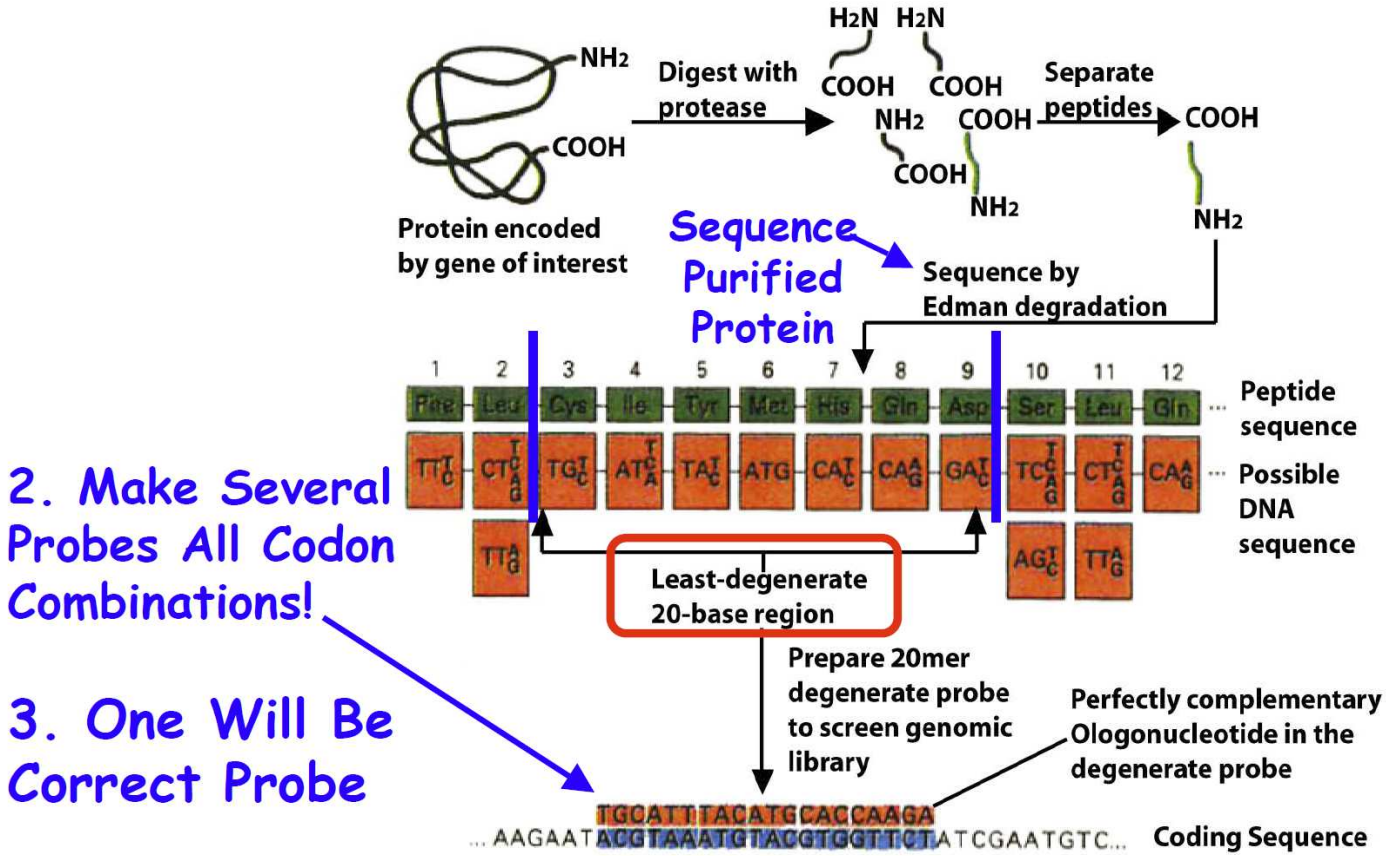


Step Two

How Find the Factor VIII Gene in a Human Genome Library?

Factor VIII Protein → Gene

Using the Factor VIII Protein Sequence and Genetic Code as Guide to Synthesize a Factor VIII Probe



2. Make Several Probes All Codon Combinations!

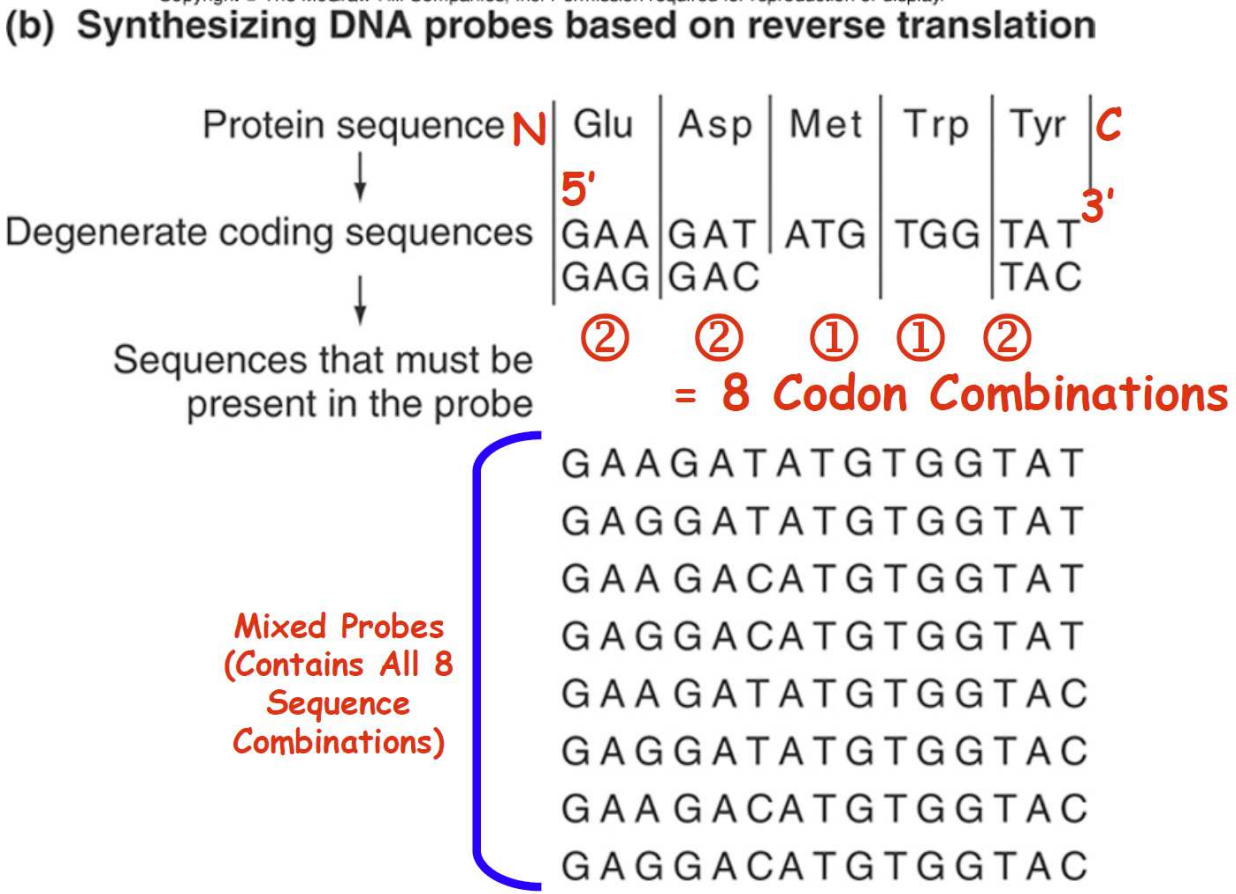
3. One Will Be Correct Probe

1. Use Genetic Code

How many Combinations of Synthetic Probes?

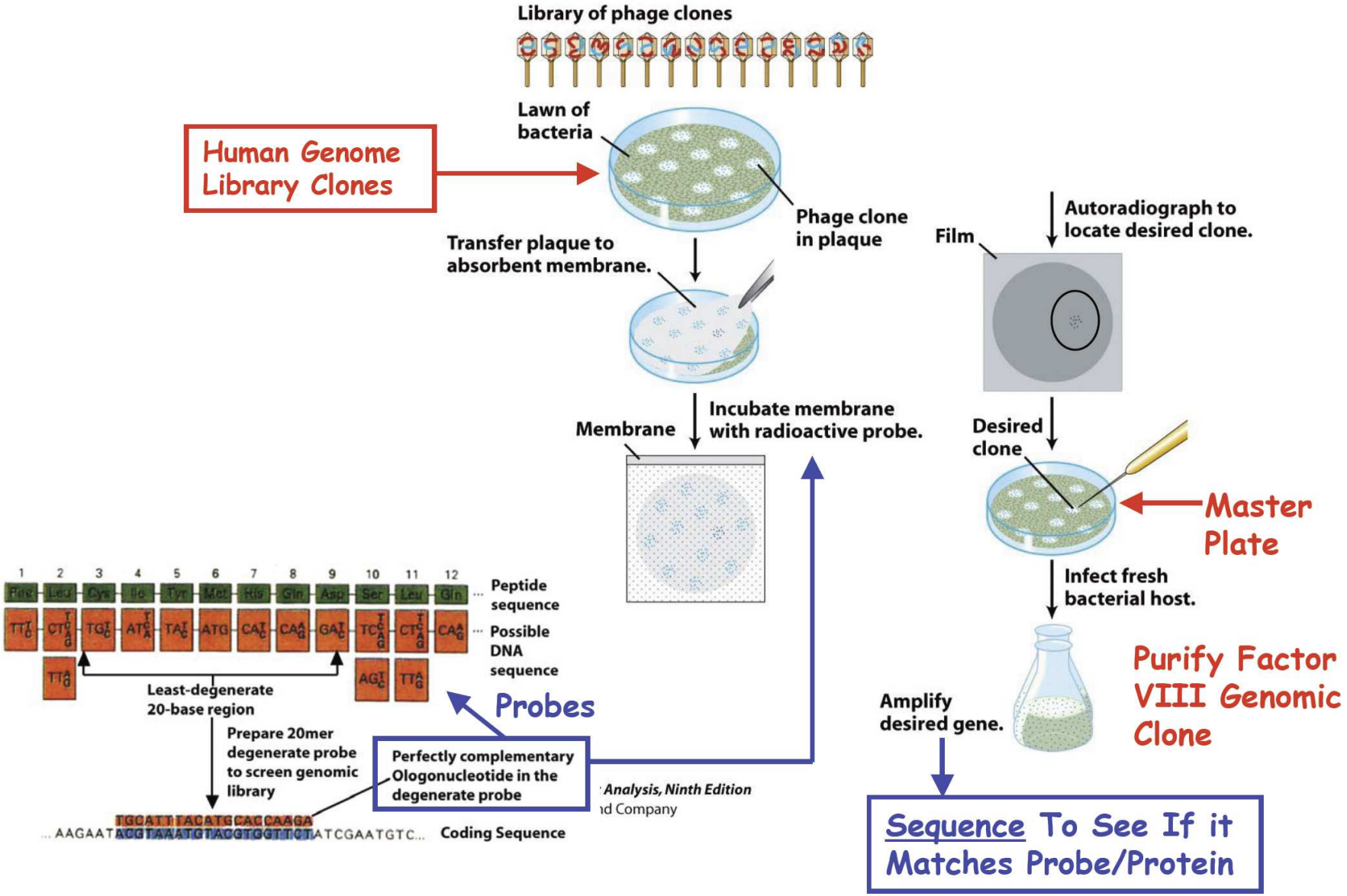
2x3x2x1x2x2x2=96

Using the Genetic Code to go From Protein Sequence to Gene Sequence

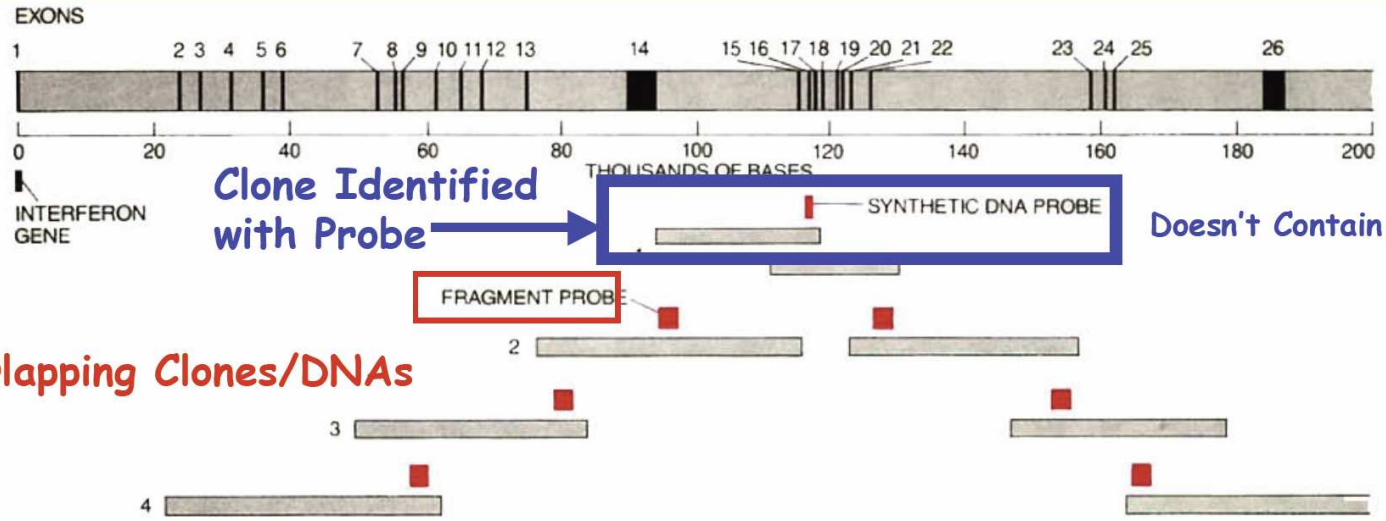


1. Need Amino Acid Sequence of Part of the Protein
2. Need DNA Sequences Representing all Codon Combinations
3. Synthesize DNA Sequence Probes!

Finding The Factor VIII Gene Or Part of Gene!!



The Result-The Factor VIII Gene is Huge- 186,000 bp- The Probe Identified a Clone Containing Only One Part of Gene !!! Why?



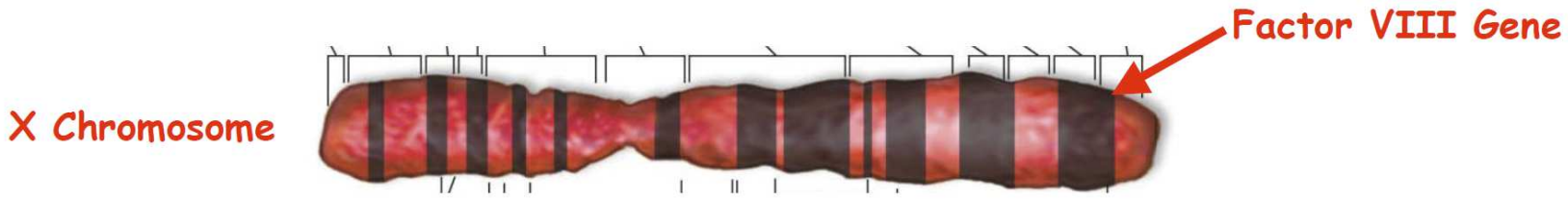
How Find Clones with Rest of Gene? Key Question !

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 (compare with protein sequence) as guides!

Sequence ----------> GenBank

The Factor VIII Gene Was Found To Be Very Large

- 186,000 Nucleotides in Length (Won't Fit in One Phage Clone)
- 25 Introns
- 9,000 Nucleotide Coding Sequence (cDNA)
- 2,351 Amino Acids in Protein

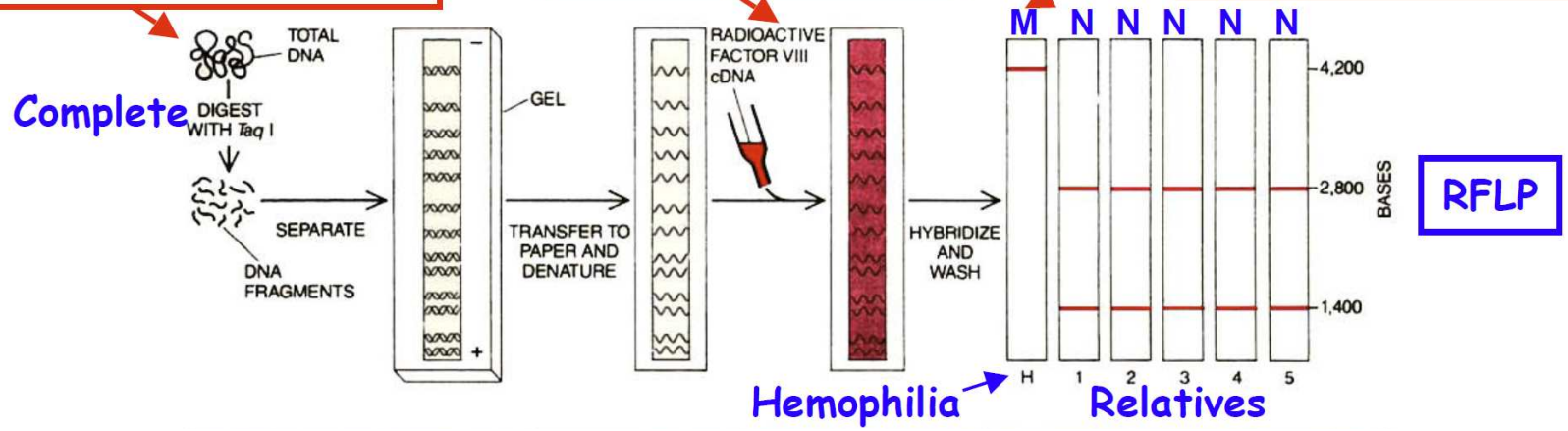


Factor VIII Gene Probes/ Sequence Can Be Used to Characterize Mutant Genes & Do DNA Testing for Carriers

Human DNA From Different Individuals

Genomic or cDNA Clone Probe

Loss of a TaqI site in Factor VIII Gene



RFLP

Once Gene & cDNA Identified!

Use DNA Gel Blots (or PCR) & Factor VIII Probes to Investigate Presence of Mutant Alleles in Families (carriers)

Mutations Arise Independently in Families

How is a Specific Gene Detected in Genome?

DNA can be Transferred "in situ" to paper
& annealed with radioactive probes

DNA Blots!

Probe Represents a Cloned Fragment from Genome with a Unique Sequence!

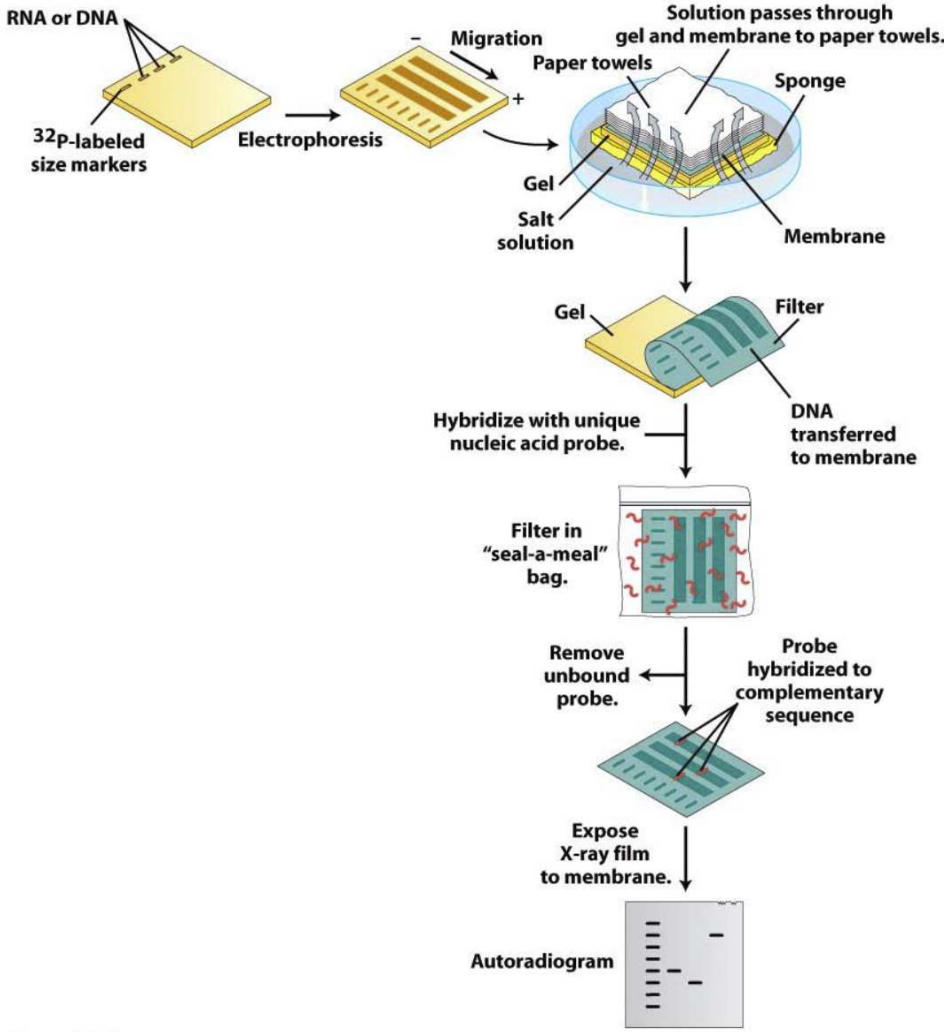


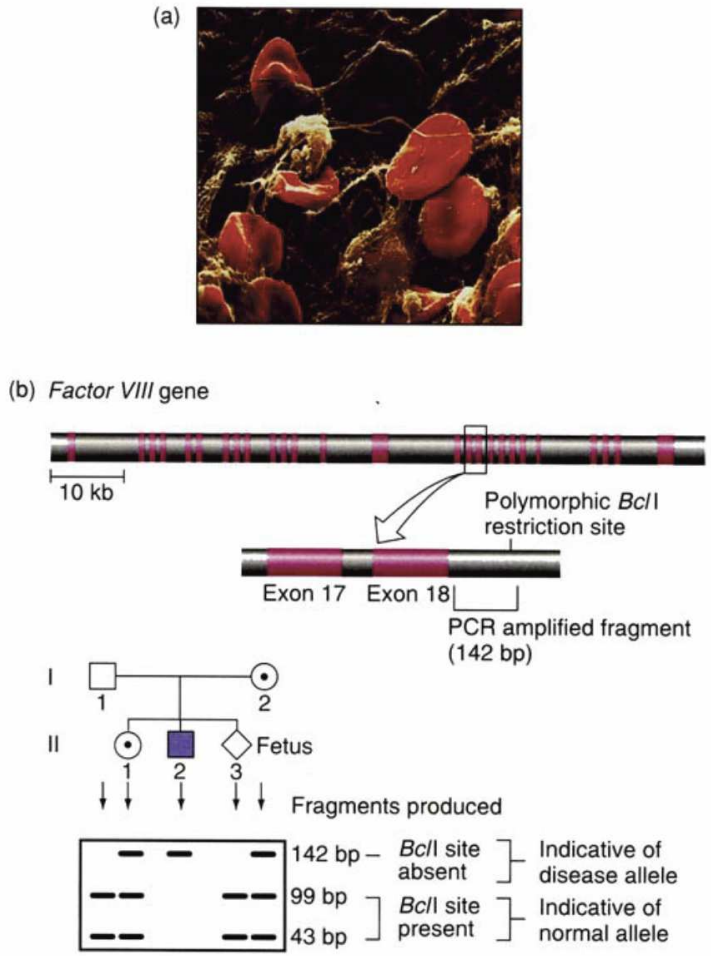
Figure 20-12
Introduction to Genetic Analysis, Ninth Edition

Using PCR and RFLPs (Markers) to Detect the Hemophilia A Disease Allele/ Gene

1. Use PCR to amplify a specific Factor VIII gene region
2. Use restriction enzyme (BcL I) to distinguish between normal allele (1 site) & disease allele (no site)

[=] = Normal allele

[–] = Disease allele



The 21st Century Approach!

1. Sequence the Entire Gene & Find Mutation
2. Then Synthesize Probes to Test Family Members Using PCR

Only Can Do This With a Knowledge of DNA Sequence of Wild-type (Normal) and Disease Genes (Can Vary family to Family)

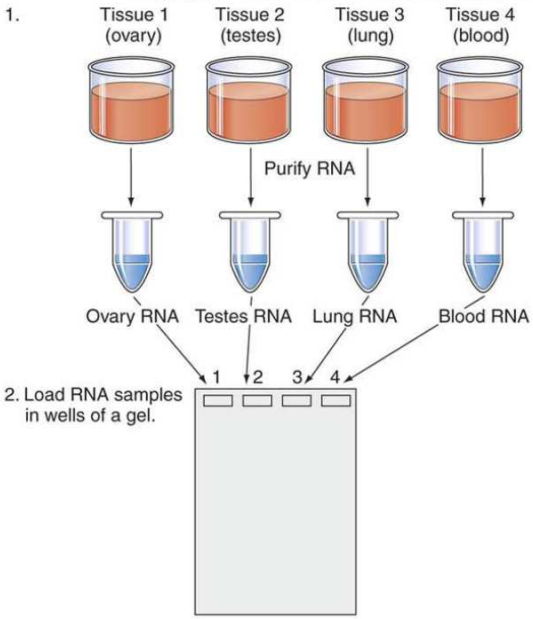
Step Four

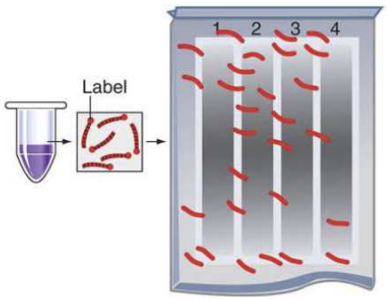
How Find Factor VIII mRNA to Generate a cDNA for Protein Production in Host Cells?

Making the Drug

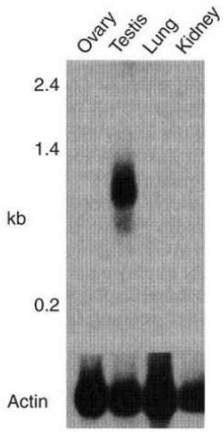
Need cDNA Not Gene

Factor VIII Gene Can Be Used to Find Out Where It is Active Using RNA Blots





4. Wash away unhybridized probe. Make autoradiograph.



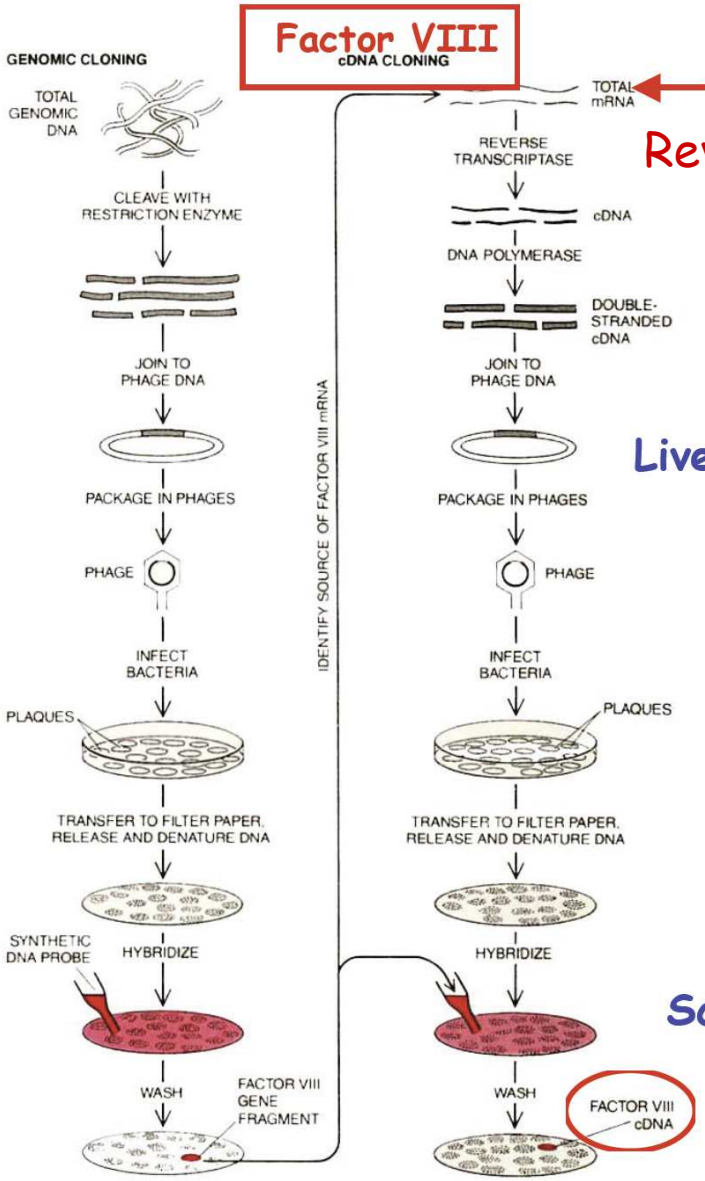
RNA Blot Is Like a DNA Blot Except That RNA is on Gel & Blotted

Factor VIII Gene Is Highly Active in Liver!

Could Also Use PCR (RT-PCR)

(4): Reprinted with permission from *Nature* 1990 Jul 19; 346(6281):216-7, Sinclair et al. © 1990 Macmillian Magazines Limited

Using Factor VIII Gene Probe to Identify Factor VIII cDNA clone



Liver mRNA

Reverse transctiptase!

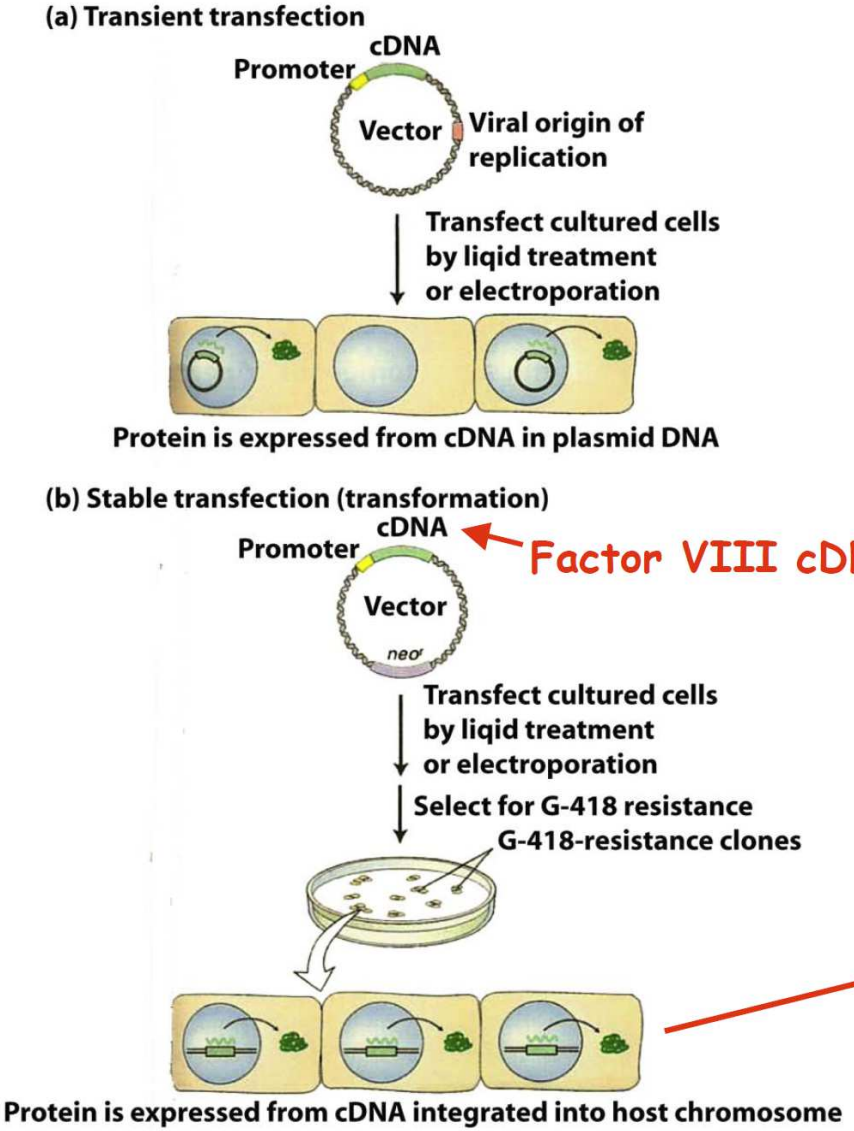
Liver cDNA Library

Factor VIII cDNA →

Used to Make Drug !

Screen Liver cDNA Library

A Factor VIII Drug/"Cure"
Making Factor VIII in Mammalian Cells



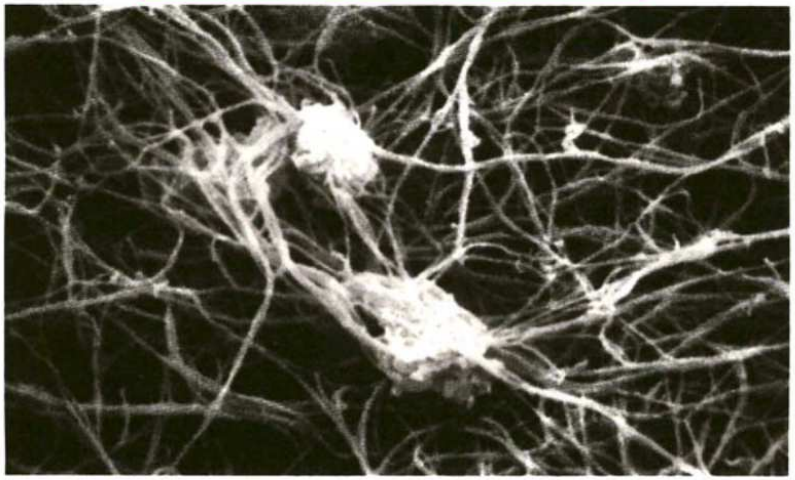
Why Mammalian Cells?

Factor VIII is glycoprotein

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.

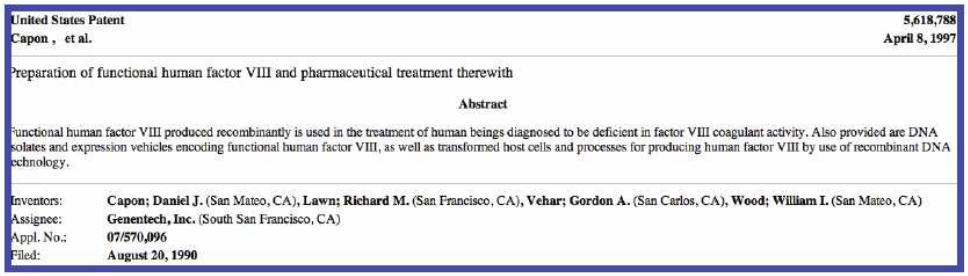
A Triumph of Genetic Engineering

Recombinant Factor VIII

Factor VIII gene cloned in 1983

Factor VIII (recombinant) approved as drug in 1993!
Ten years from gene → drug! (Off Patent in 2011)

A Patent on YOUR Factor VIII Gene!



United States Patent **5,618,788**
Capon , et al. **April 8, 1997**

Preparation of functional human factor VIII and pharmaceutical treatment therewith

Abstract

Functional human factor VIII produced recombinantly is used in the treatment of human beings diagnosed to be deficient in factor VIII coagulant activity. Also provided are DNA solates and expression vehicles encoding functional human factor VIII, as well as transformed host cells and processes for producing human factor VIII by use of recombinant DNA echnology.

Inventors: **Capon; Daniel J.** (San Mateo, CA), **Lawn; Richard M.** (San Francisco, CA), **Vehar; Gordon A.** (San Carlos, CA), **Wood; William I.** (San Mateo, CA)
Assignee: **Genentech, Inc.** (South San Francisco, CA)
Appl. No.: **07/570,096**
Filed: **August 20, 1990**

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)