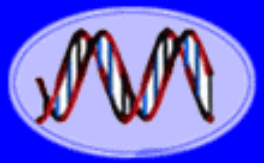
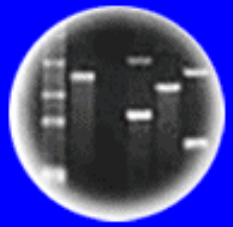


DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

Plants of Tomorrow

HC70A & SAS70A Winter 2012
Genetic Engineering in Medicine, Agriculture, and Law

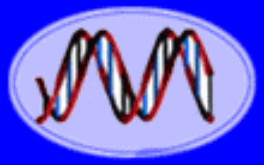
Professors Bob Goldberg & John Harada

Lecture 6

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

UCLA

UCDAVIS
UNIVERSITY OF CALIFORNIA



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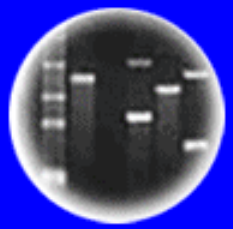

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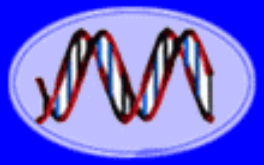
THEMES

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 Advantage 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?
13. Transgenic Protein Patent & Regulatory Concerns?
14. How About Gene Therapy?



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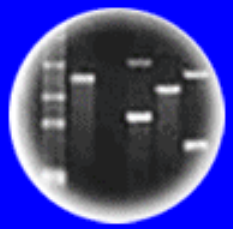

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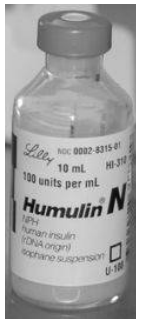
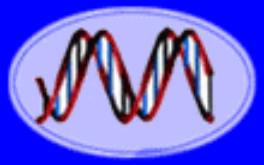
One of the Most Important Applications of Genetic Engineering Technology Has Been To Manufacture Drugs to Treat Human and Animal Diseases



Created a Multibillion Dollar Biotechnology Industry, Was Responsible For the Acceptance of Recombinant DNA Technology in the 1970s,& Lead to Pioneering Decisions in Patent Law



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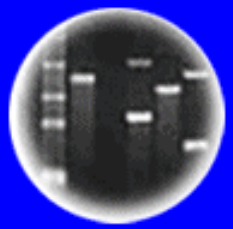

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Useful Proteins from Recombinant Bacteria

Sci. Amer., 1980

by Walter Gilbert and Lydia Villa-Komaroff

Edible Vaccines

by William H. R. Langridge

Sci. Amer., 2000

Transgenic Livestock as Drug Factories

Sci. Amer., 1997

Examples of Recombinant DNA Drugs

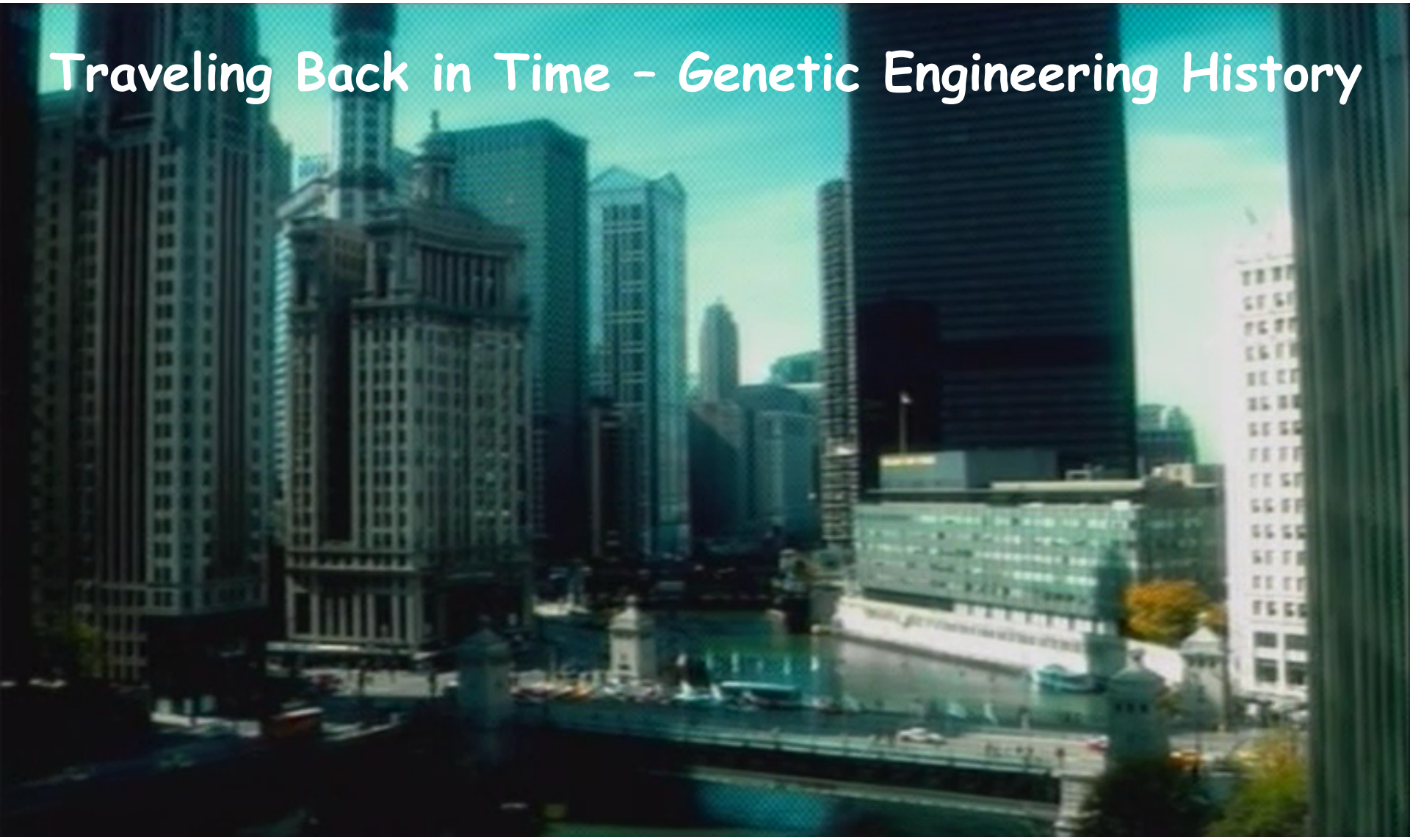
TABLE 1.2 EXAMPLES OF PROTEINS MANUFACTURED FROM CLONED GENES

Product	Application
Blood factor VIII (clotting factor)	Treat hemophilia
Epidermal growth factor	Stimulate antibody production in patients with immune system disorders
Growth hormone	Correct pituitary deficiencies and short stature in humans; other forms are used in cows to increase milk production
Insulin	Treat diabetes
Interferons	Treat cancer and viral infections
Interleukins	Treat cancer and stimulate antibody production
Monoclonal antibodies	Diagnose and treat a variety of diseases including arthritis and cancer
Tissue plasminogen activator	Treat heart attacks and stroke

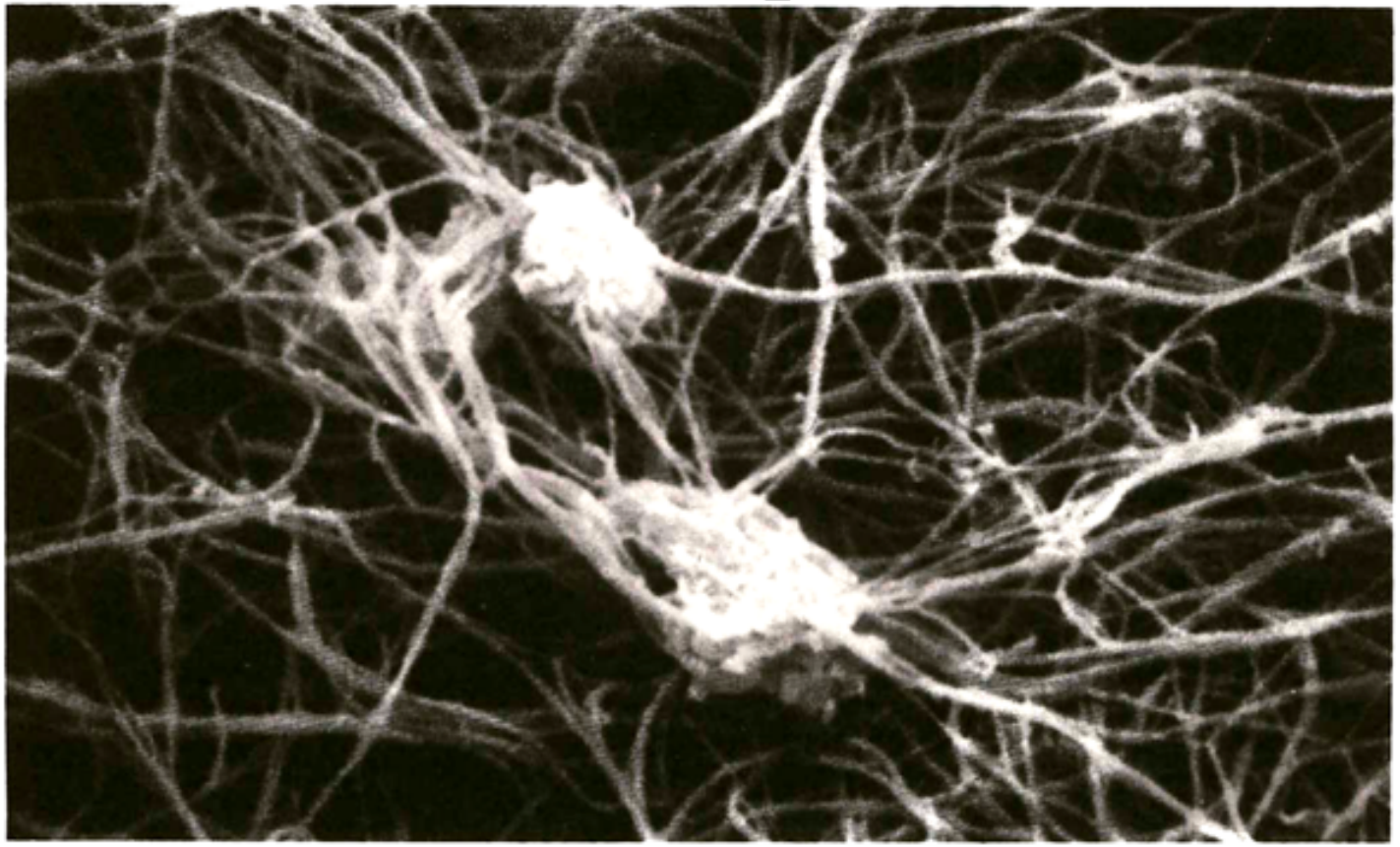
TABLE 1.1 TOP 10 BIOTECHNOLOGY DRUGS (WITH SALES OVER $1 BILLION) 2012

Drug	Developer	Function (Treatment of Human Disease Conditions)
Enbrel	Amgen & Wyeth	Rheumatoid arthritis
Remicade	Johnson & Johnson	Rheumatoid arthritis
Rituxan	Roche	Non-Hodgkin's lymphoma
Avastin	Roche	Colon cancer
Herceptin	Roche	Breast cancer
Humira	Abbott Labs	Rheumatoid arthritis
Levenox	sanofi-aventis	Blood clots
Lantus	sanofi-aventis	Diabetes
Aranesp	Amgen	Anemia

These Include: Hormones, Blood Factors, Anticoagulents, Growth Factors, Interferons, Vaccines, Monoclonal Antibodies, Bone Morphogenic Proteins, & Many Others


Traveling Back in Time – Genetic Engineering History

The Molecular Genetics of Hemophilia (Potentially Lethal Disease)



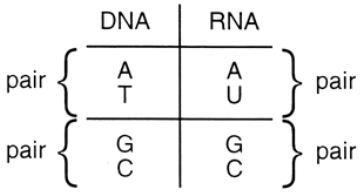
FIBRIN STRANDS stabilize a blood clot at the site of a wound by trapping the platelets that form the bulk of the clot. The electron micrograph, which was made by Jon C. Lewis of Wake Forest University, shows a clot formed in a suspension of platelets and fibrin.

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

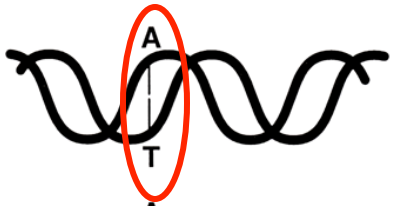
Reference: Lawn & Vehar, Sci. Amer., January, 1986

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



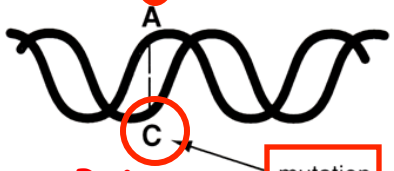
BASE PAIR RULES

Gene A



Rare Base Mismatch

Replication①



MUTATION DURING REPLICATION

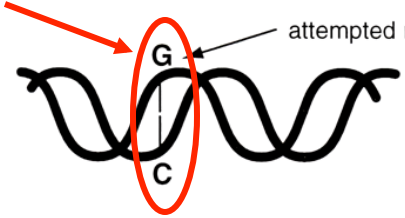
mutation

C mispairs with A

New Base Pair

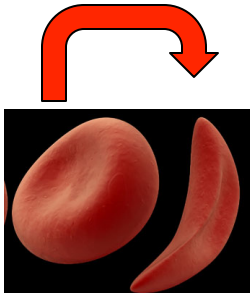
See Mutation As Change in Phenotype

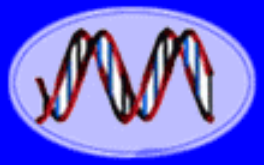
Replication②
Gene A'
Allelic Variant



Change DNA Sequence From A = T to G ≡ C

∴ Change Protein Amino Acid Sequence ⇨ Alter Function!





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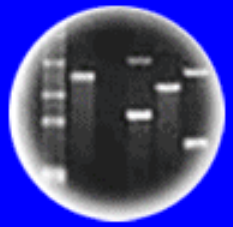

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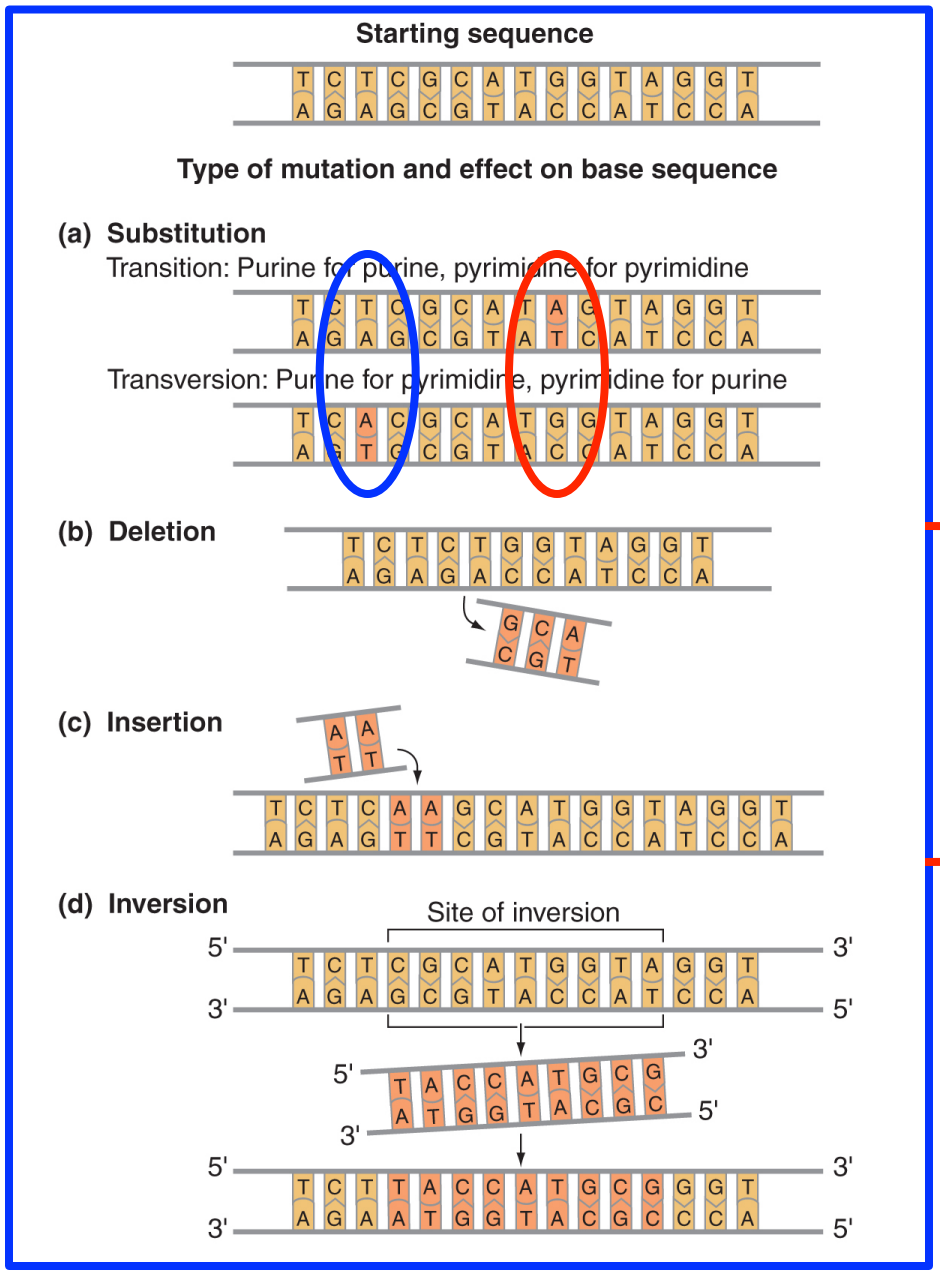
Different Events Cause Gene Mutations

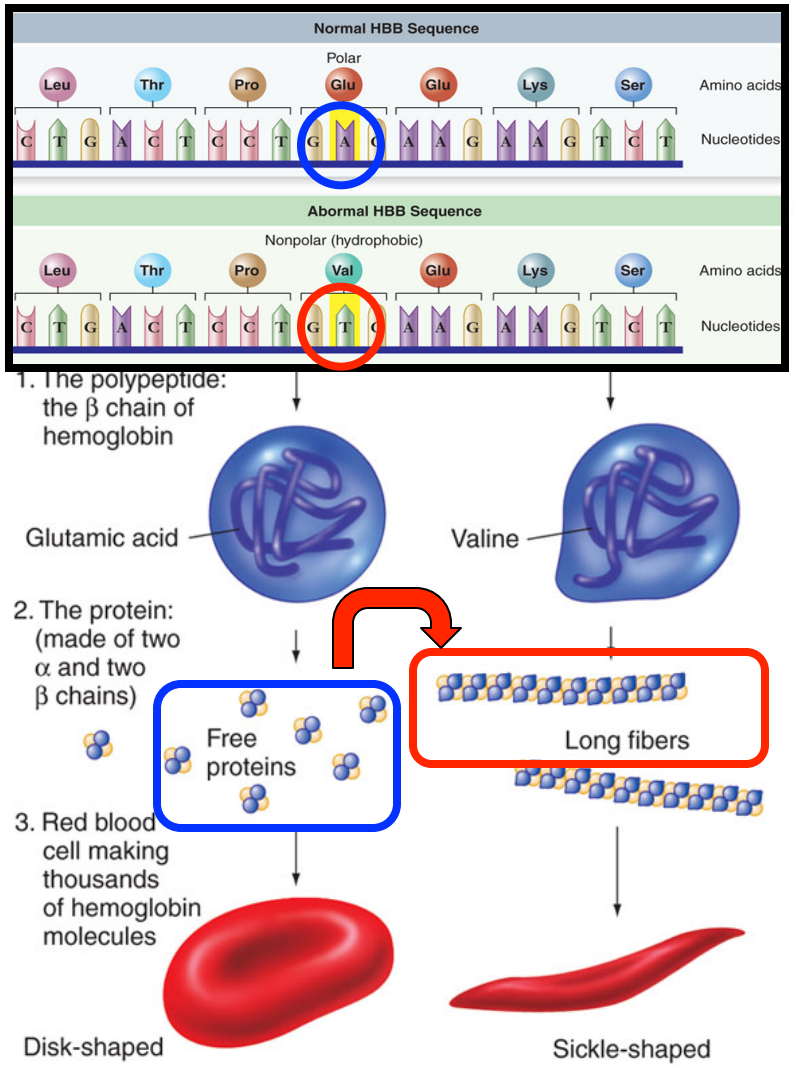


snps

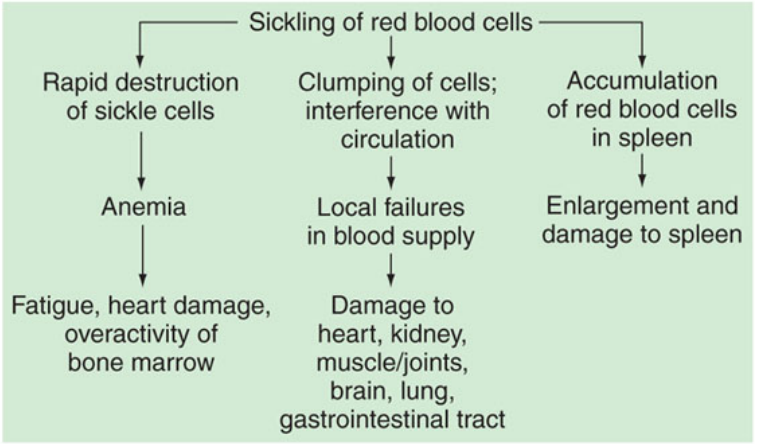
indels

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

Chromosome 11



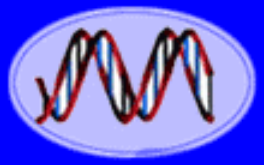
(b) Sickle-cell anemia is pleiotrophic



(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

Note Change in Protein Structure Leading to Sickle-Cell Anemia Phenotype!



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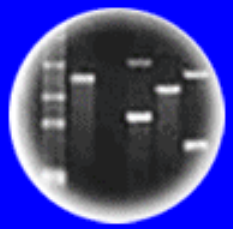

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ARTICLE

Nature, October 28, 2010

doi:10.1038/nature09534

A map of human genome variation from population-scale sequencing

The 1000 Genomes Project Consortium*

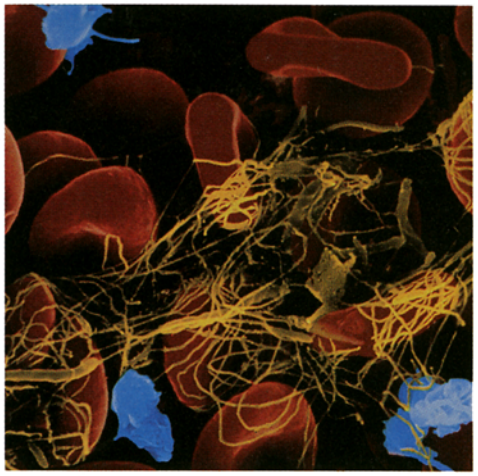
The 1000 Genomes Project aims to provide a deep characterization of human genome sequence variation as a foundation for investigating the relationship between genotype and phenotype. Here we present results of the pilot phase of the project, designed to develop and compare different strategies for genome-wide sequencing with high-throughput platforms. We undertook three projects: low-coverage whole-genome sequencing of 179 individuals from four populations; high-coverage sequencing of two mother–father–child trios; and exon-targeted sequencing of 697 individuals from seven populations. We describe the location, allele frequency and local haplotype structure of approximately 15 million single nucleotide polymorphisms, 1 million short insertions and deletions, and 20,000 structural variants, most of which were previously undescribed. We show that, because we have catalogued the vast majority of common variation, over 95% of the currently accessible variants found in any individual are present in this data set. On average, each person is found to carry approximately 250 to 300 loss-of-function variants in annotated genes and 50 to 100 variants previously implicated in inherited disorders. We demonstrate how these results can be used to inform association and functional studies. From the two trios, we directly estimate the rate of *de novo* germline base substitution mutations to be approximately 10^{-8} per base pair per generation. We explore the data with regard to signatures of natural selection, and identify a marked reduction of genetic variation in the neighbourhood of genes, due to selection at linked sites. These methods and public data will support the next phase of human genetic research.

- Sequenced Genomes of ~900 individuals
- From Seven Different Global Populations
- Found 250-300 Loss-Of-Function Mutations (KOs) Per Person
- 50-100 Variants in Disease Genes Per Person
- 10^{-8} Mutations Per bp Per Generation (~30 per Genome)
- 3,000,000 Unique SNPs Per Person
- 750,000 Unique Indels Per Person

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

Old Testament-Circumcisions

Royal Family-Europe



a = activated form

First Reference to Hemophilia is in the Old Testament

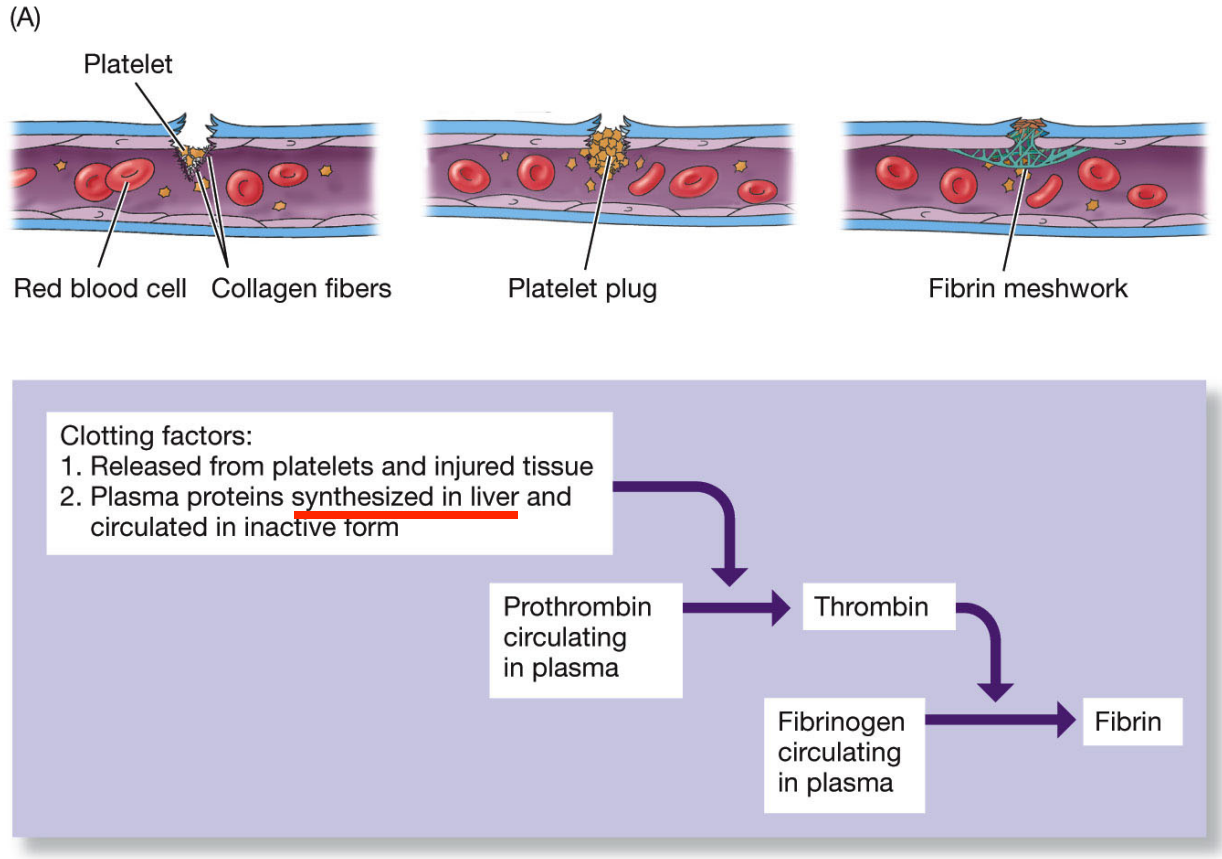
Genesis 17:10-14

'This is My covenant that you shall keep between Me and you and your descendants after you: every male among you shall be circumcised. You shall circumcise the flesh of the foreskin.....At the age of eight days every male among you shall be circumcised throughout your generations.....an uncircumcised male...that soul shall be cut off from its people, he has invalidated My covenant.'

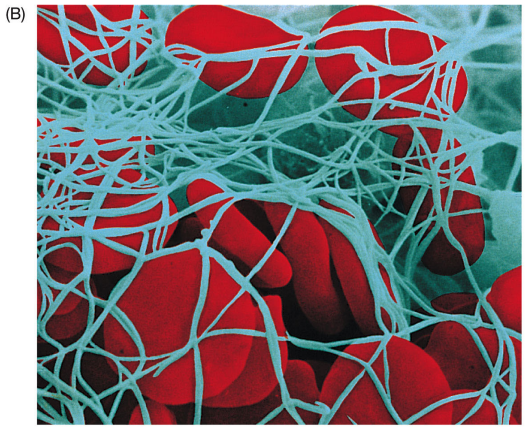
The Talmud also makes reference to families in whom children have died as a result of circumcision (Babylonian Talmud, Chapter *Yevamoth* p64b) [6]. Should a mother lose two children or should two sisters lose a child each after circumcision, subsequent children of the woman, the two sisters or of any 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.

Abraham was circumcised at 93 and gave birth to Isaac at 99. His wife - Sarah - was 90!

A Cascade Of Events After Wounding Leads to A Fibrin Clot



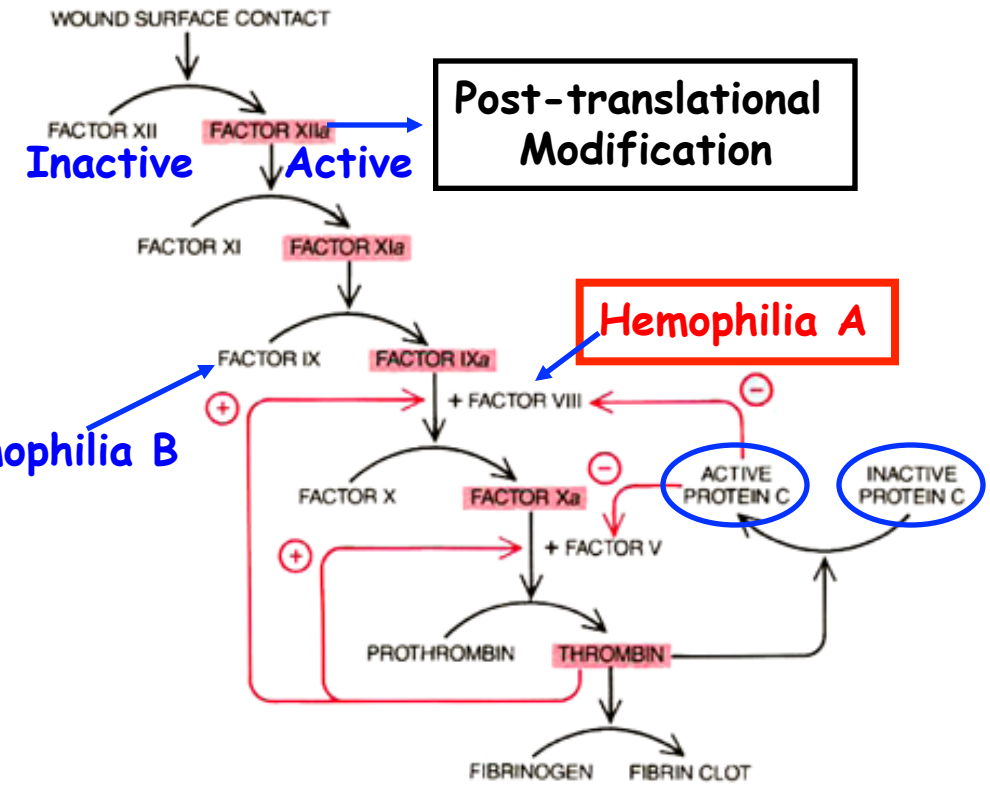
LIFE 8e, Figure 49.10 (Part 1)



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?



Post-translational Modification

Hemophilia A

Hemophilia B

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!

ATryn® 2009

Anti-Thrombin?? → Anti-Thrombin Deficiency (At-III) genetic disease

Cascade

Hemophiliacs Have Mutations in Factor VIII, Factor IX, or Factor XI Genes

TABLE 13.2 Some Important Genetic Disorders

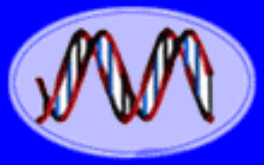
Disorder	Symptom	Defect	Dominant/ Recessive	Frequency Among Human Births
Cystic fibrosis	Mucus clogs lungs, liver, and pancreas	Failure of chloride ion transport mechanism	Recessive	1/2500 (Caucasians)
Sickle cell anemia	Blood circulation is poor	Abnormal hemoglobin molecules	Recessive	1/600 (African 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

18,000 People in US Have Hemophilia & 400 Babies/Year Are Born With Disorder
Prior to 1960s – Average Life Span Was 11 Years

Hemophilia A	Defective Factor VIII Gene	1/10,000 males	80%
Hemophilia B	Defective Factor IX Gene	1/30,000 males	20%
Henophilia C	Defective Factor XI Gene	Autosomal	<1%

Hypothesis For High Frequency in Males?

Both Factor VIII & IX Genes
on X-Chromosome (♀→♂'s)



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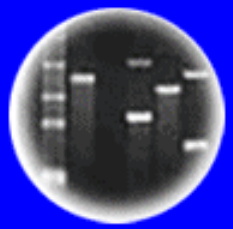

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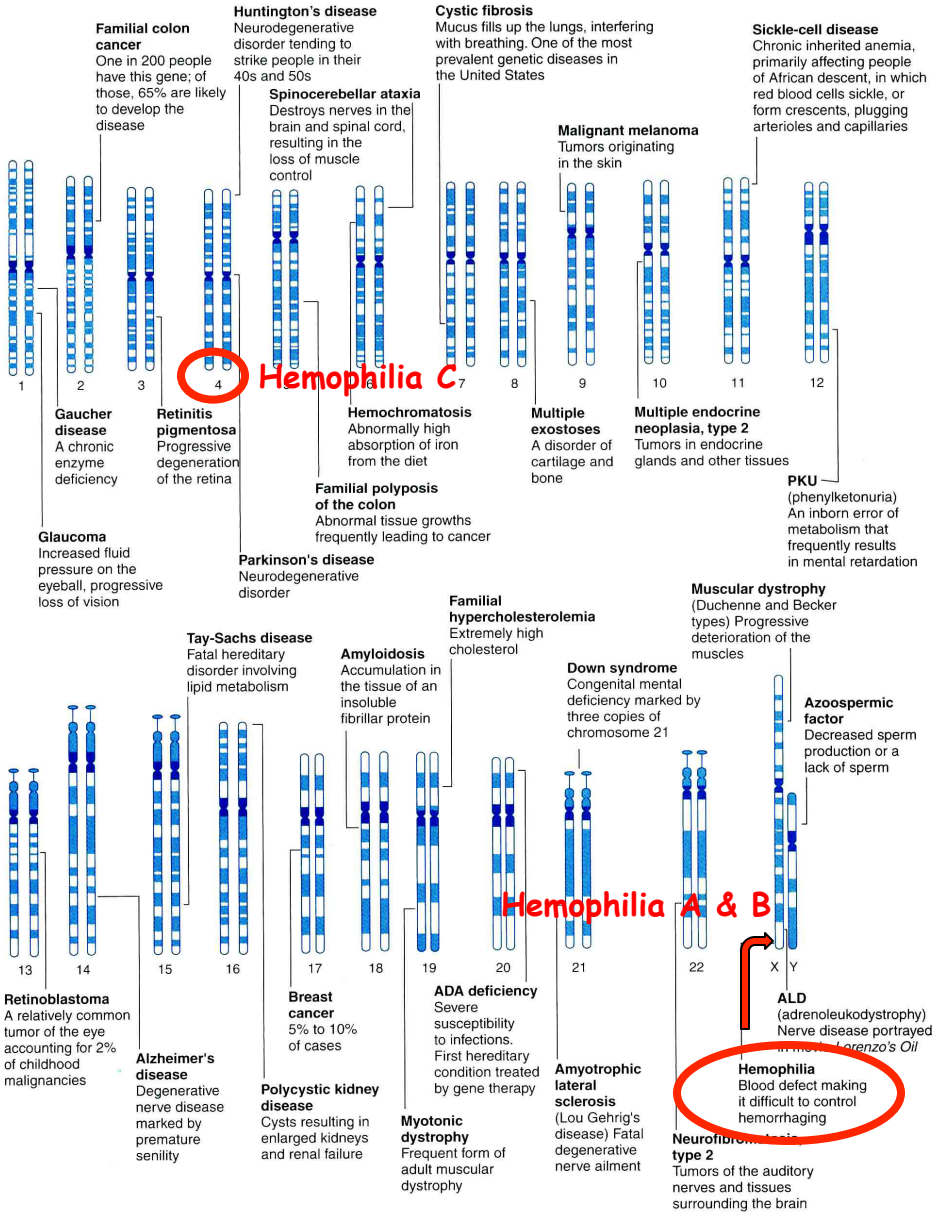
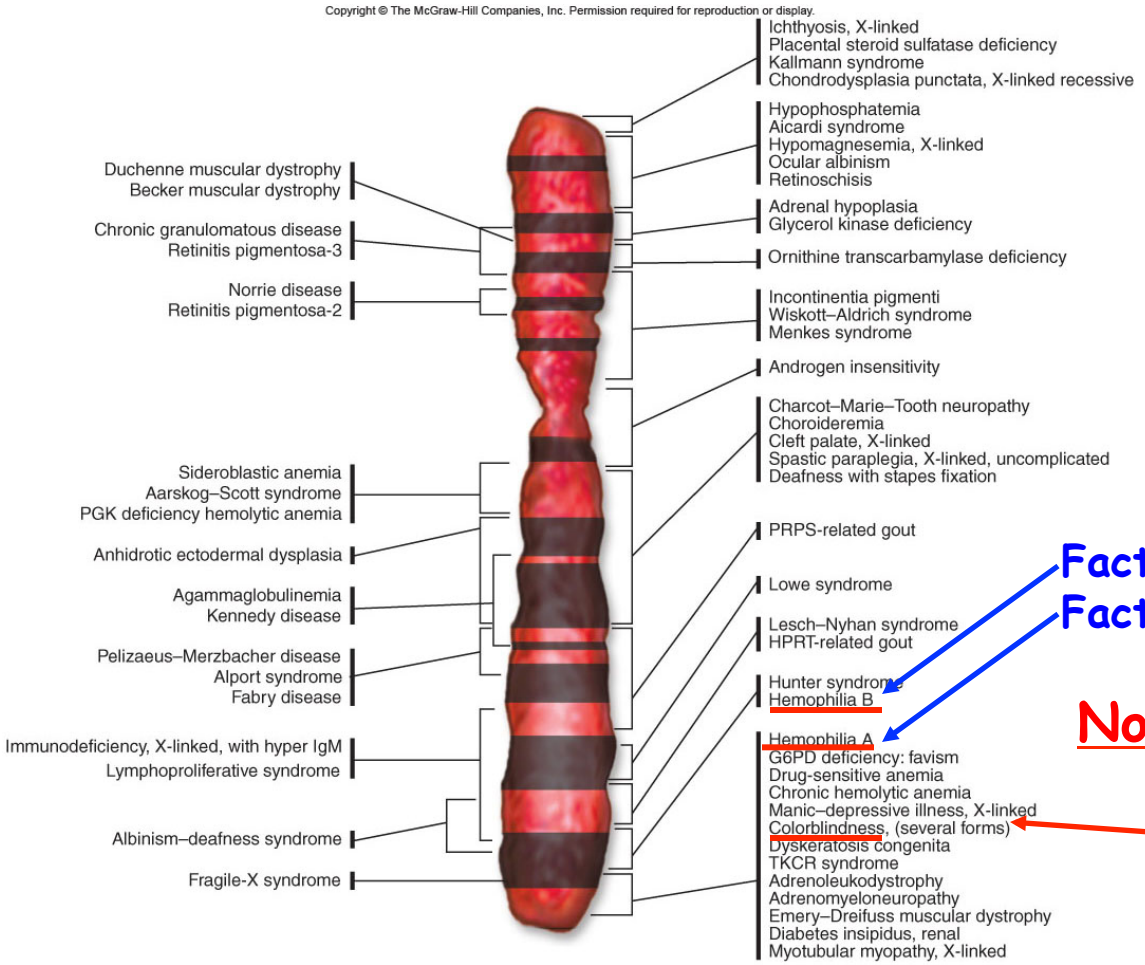
Human Disease Genes Have Been Mapped To Specific Chromosomal Locations

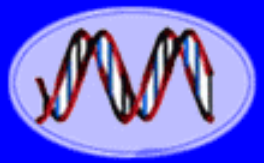


Factor VIII and Factor IX Genes are Closely Linked on the X Chromosome



Note: Factor VIII *gene* is closely *linked* to Colorblindness Gene

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



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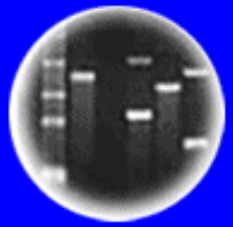

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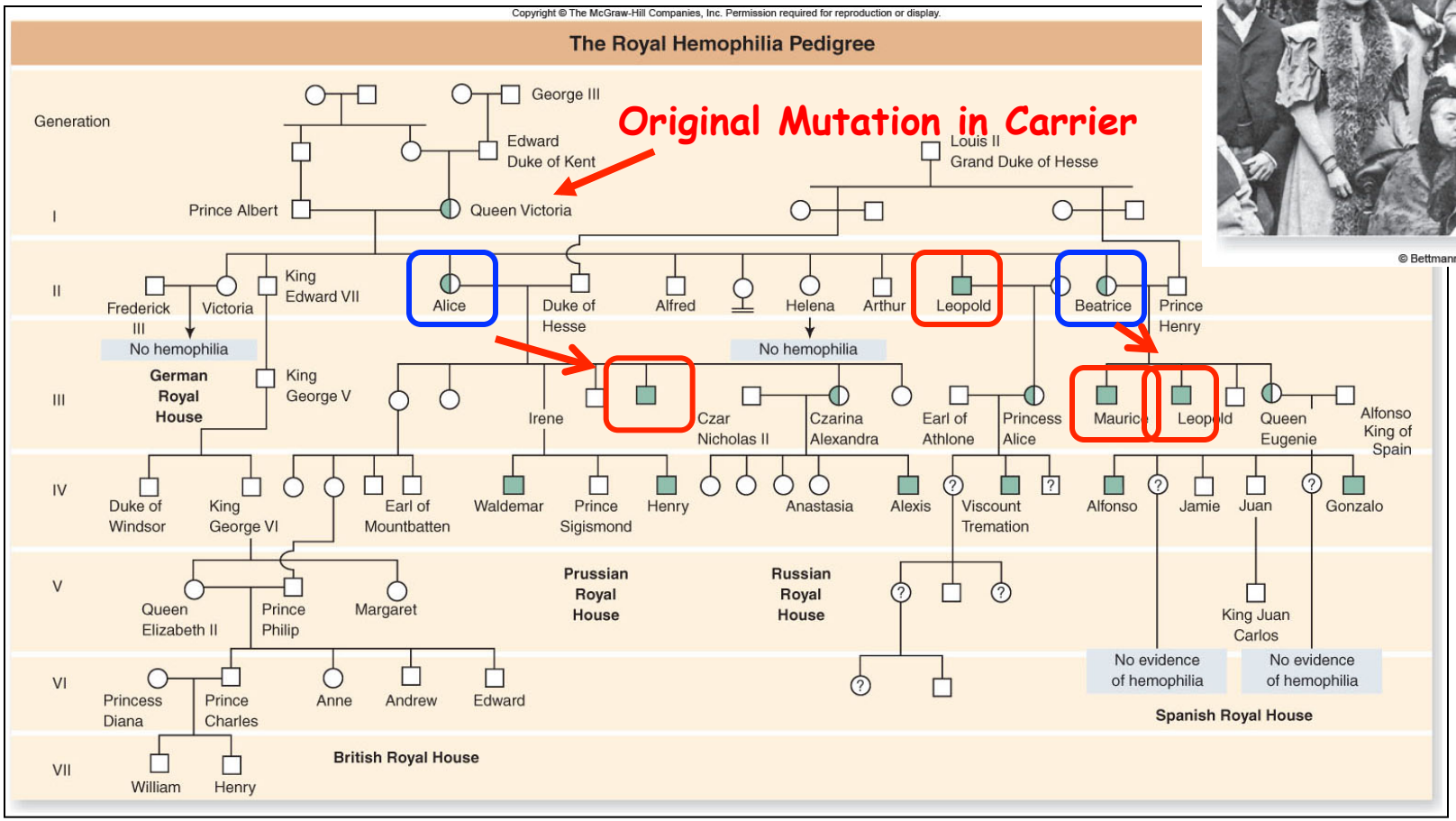
Pedigrees Can Be Used To Determine If a Trait is Dominant or Recessive

Each Type of Inheritance Predicts Specific Results in Each Generation

Hemophilia A and B Genes (Traits) Are Sex Linked

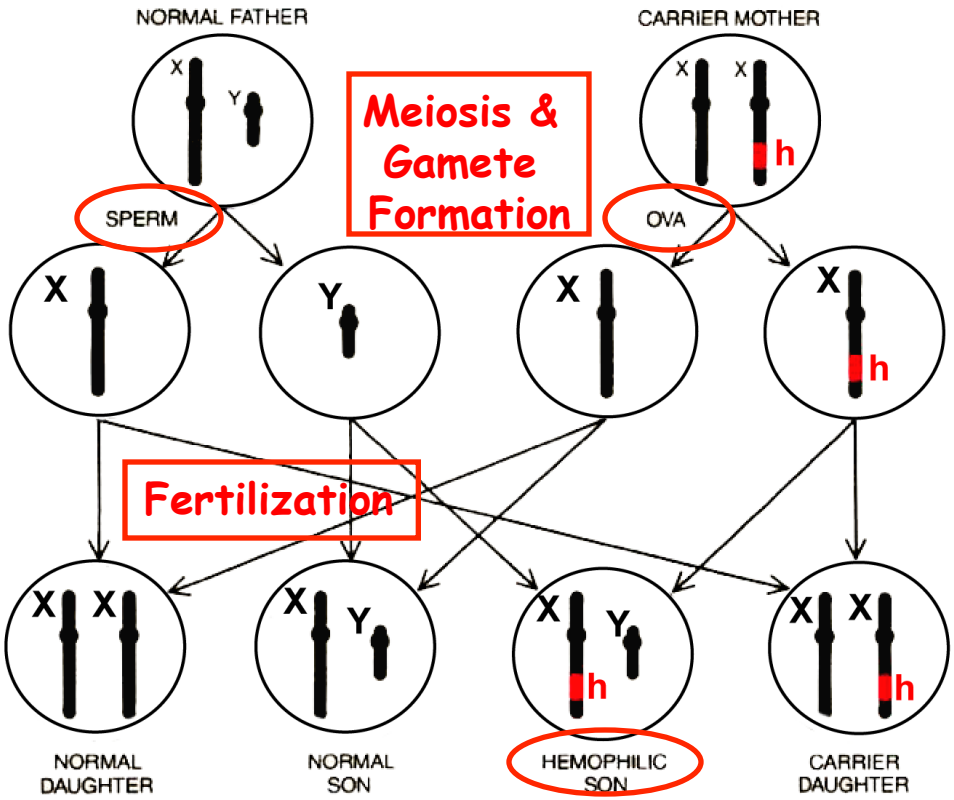
© Bettmann/Corbis



Note: 1. Males Obtain Detective Gene From Mothers

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

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

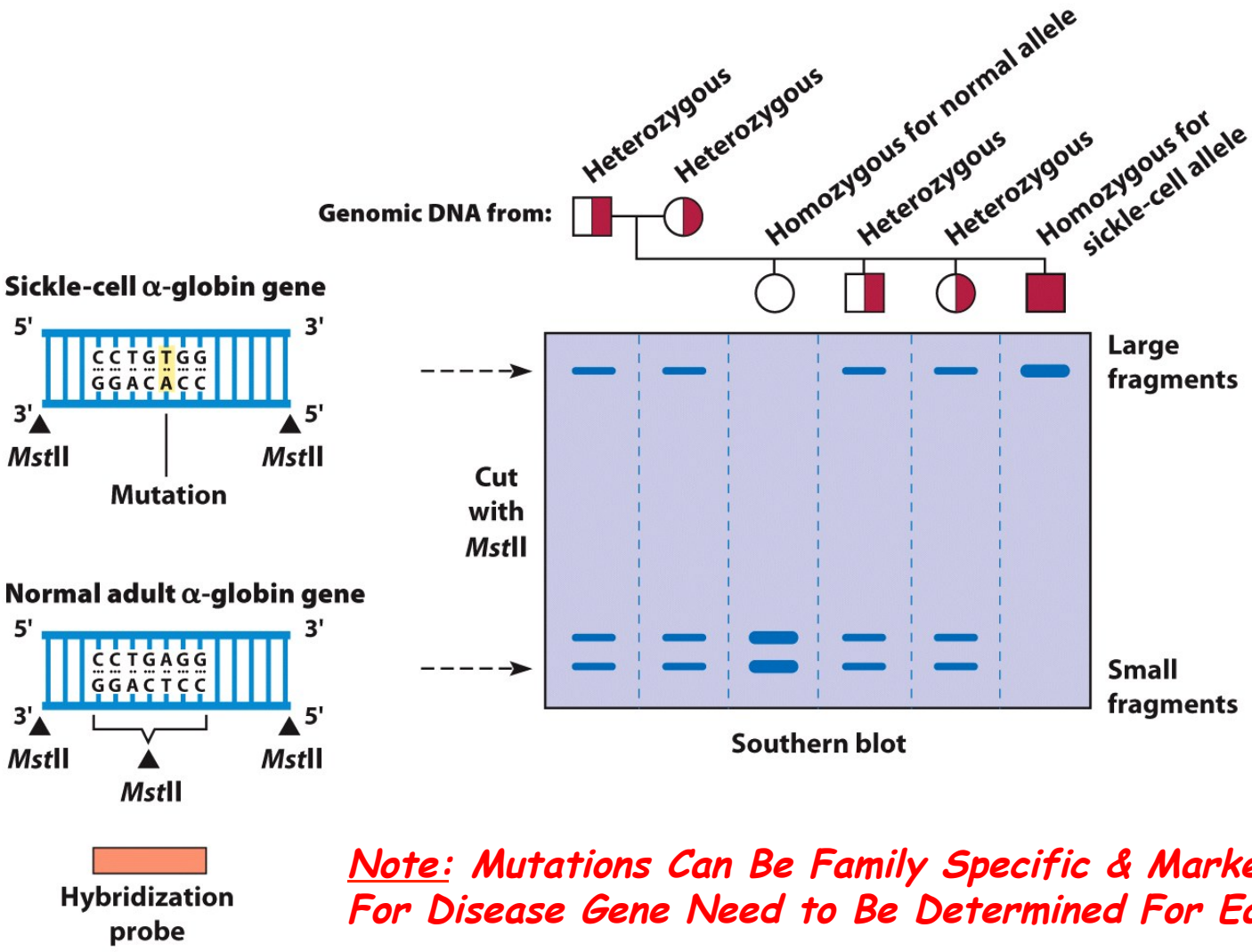
Children 2n

Sex-Linked Inheritance

♀ Carriers → 1/2 Sons + No Daughters!

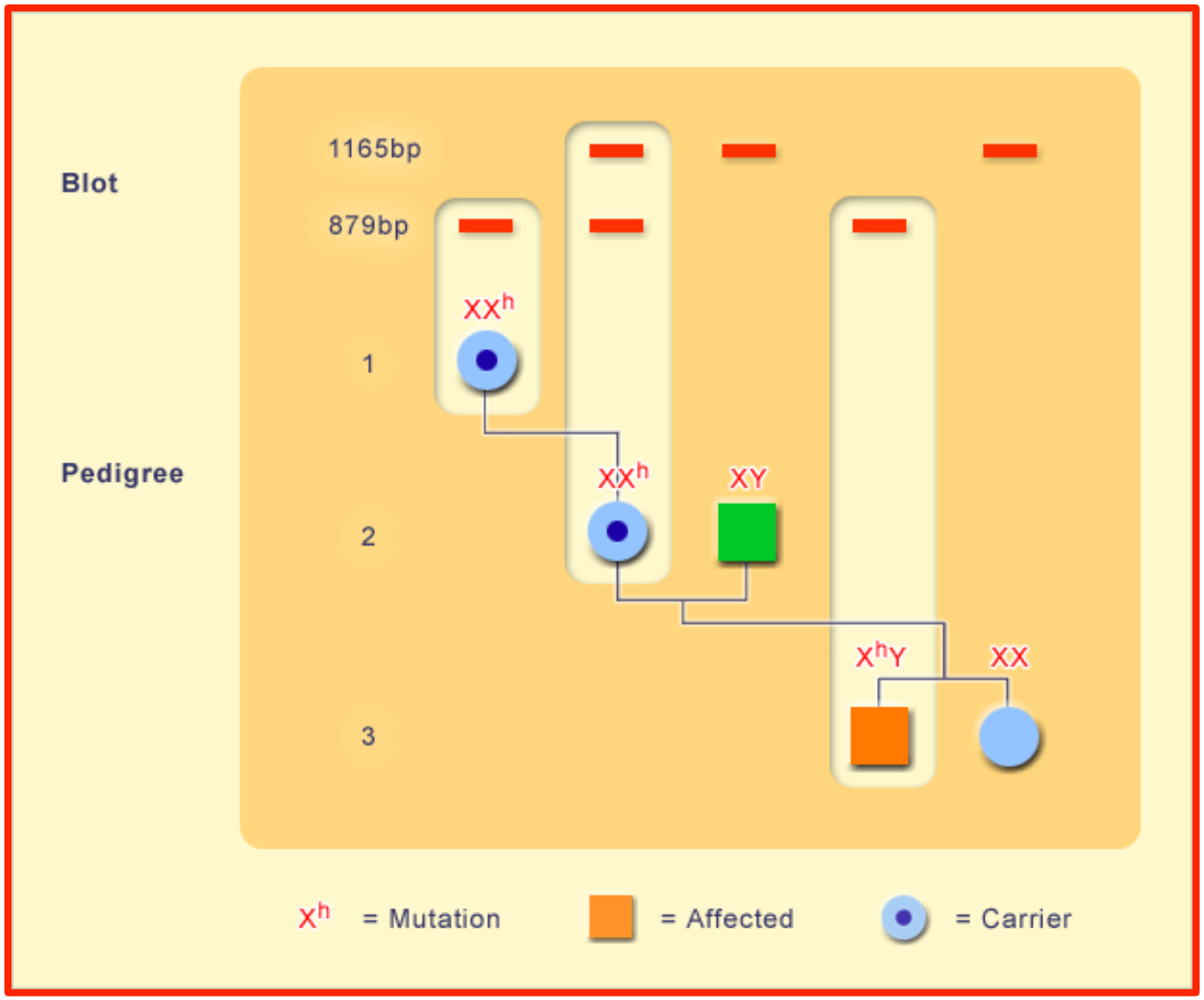
Only One X-Chromosome is ♂

DNA Testing Can Be Used To Detect The Presence of Disease Gene Alleles: *This is Now Done Using PCR*



***Note:* Mutations Can Be Family Specific & Markers For Disease Gene Need to Be Determined For Each Family**

DNA Testing For Hemophilia A



Do You See An Error in this Figure?

DNA Tests Can Now Be Used To Detects Hundreds of Genetic Disease Alleles

TABLE 11.1 GENETIC DISEASE TESTING

Genetic Disease Condition	Genetic Basis for Disease and Symptoms
Cancers (brain tumors; urinary bladder, prostate, ovarian, breast, brain, lung, and colorectal cancers)	A variety of different mutant genes can serve as markers for genetic testing.
Cystic fibrosis	Large number of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7. Causes lung infections and problems with pancreatic, digestive, and pulmonary functions.
Duchenne muscular dystrophy	Defective gene (dystrophin) on the X chromosome causes muscle weakness and muscle degeneration.
Familial hypercholesterolemia	Mutant gene on chromosome 19 causes extremely high levels of blood cholesterol.
Hemophilia	Defective gene on the X chromosome makes it difficult for blood to clot when there is bleeding.
Huntington disease	Mutation in gene on chromosome 4 causes neurodegenerative disease in adults.
Phenylketonuria (PKU)	Mutation in gene required for converting the amino acid phenylalanine into the amino acid tyrosine. Causes severe neurological damage, including mental retardation.
Severe combined immunodeficiency (SCID)	Immune system disorder caused by mutation of the adenosine deaminase gene.
Sickle cell disease	Mutation in ß-globin gene on chromosome 11 affects hemoglobin structure and shape of red blood cells, which disrupts oxygen transport in blood and causes joint pain.
Tay-Sachs disease	Rare mutation of a gene on chromosome 5 causes certain types of lipids to accumulate in the brain. Causes paralysis, blindness, retardation, and respiratory infections.

Kerry Mullis & PCR

What Was Known About Factor VIII *Before Gene Cloned?*

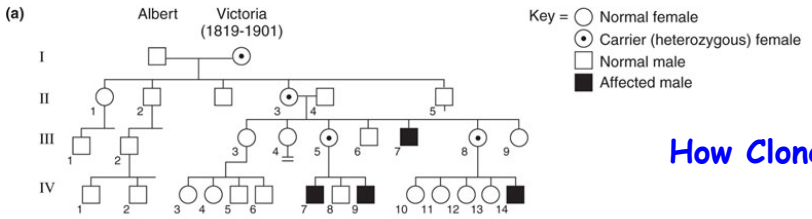
1. Blood Protein (But Perhaps Synthesized Elsewhere!)
2. Could be purified in small amounts from >20 Liters of human blood +cow blood + pig blood
3. Short Stretch of Protein Sequenced = Known Protein Sequence!
4. Hemophilia A could be treated by blood transfusions from normal individuals, ∴ clotting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

∴ How to go From Protein to Gene

The Problem

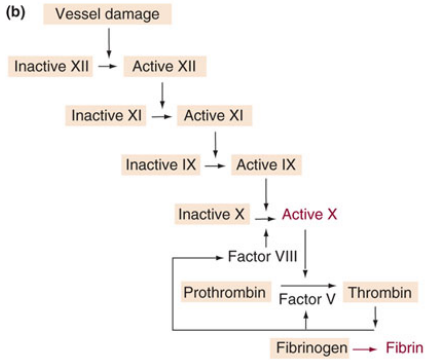
For Factor VIII- *Not Known Where Gene is Expressed* ∴ Must Use Genome Library

Early 1980's



Key Concept

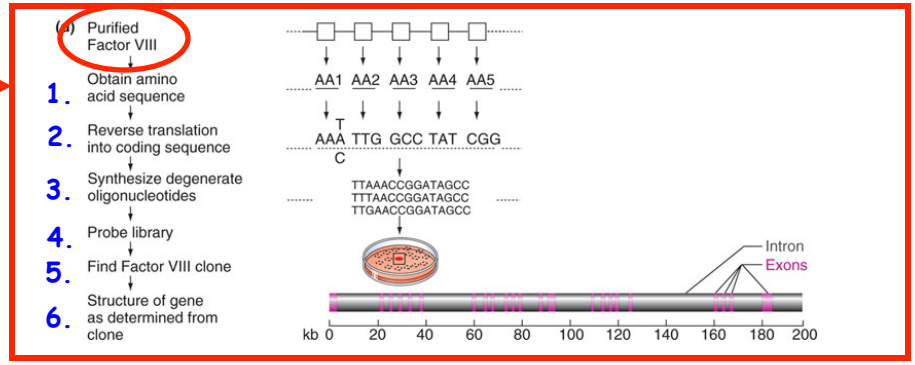
How Clone A Gene When You Don't Know Where it is Expressed !



(c) Analysis of presence or absence of blood-clotting factors

Factors	Wild-type person	Hemophiliac
XII	+	+
XI	+	+
IX	+	+
VIII	+	-
X	+	+
V	+	+
Prothrombin	+	+
Fibrinogen	+	+

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

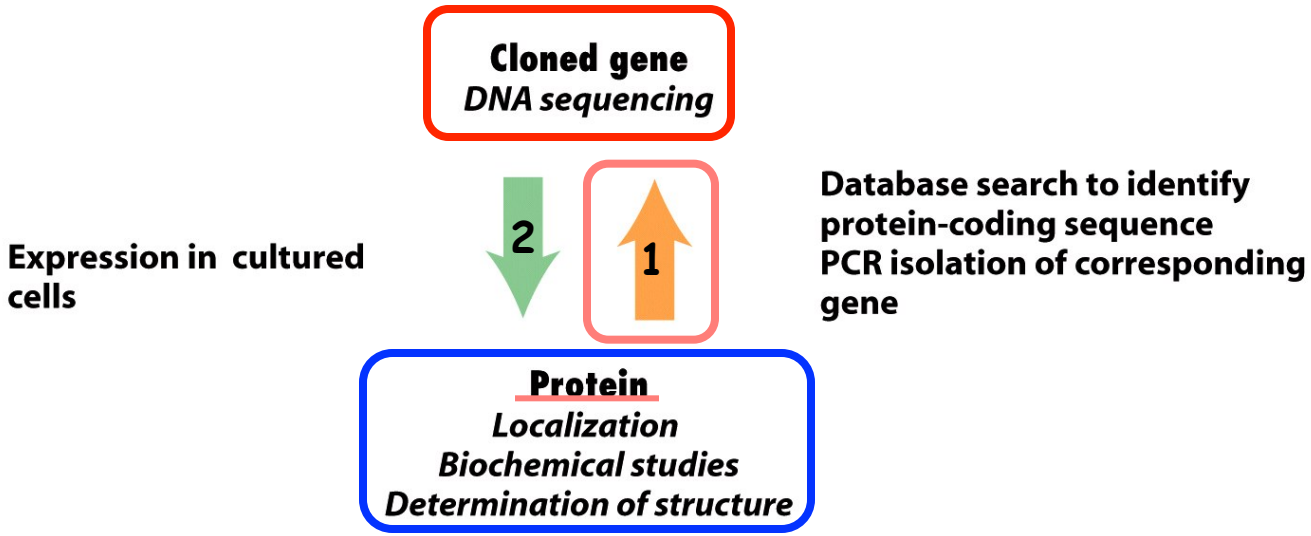


Figure 5-1
Molecular Cell Biology, Sixth Edition

∴ 1. Protein → Gene → Drug

Factor VIII Strategy (1985)

or

Genomics

2. Gene → Protein Using Sequencing and Genetic Code

GenBank

2012

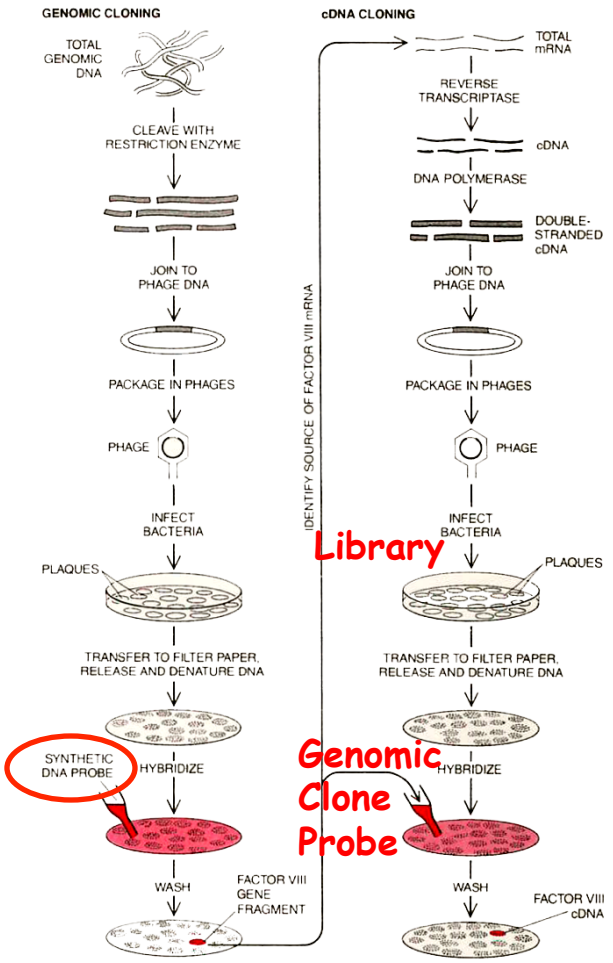
3. Just Sequence Everything + Identify Protein-GenBank Huge

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

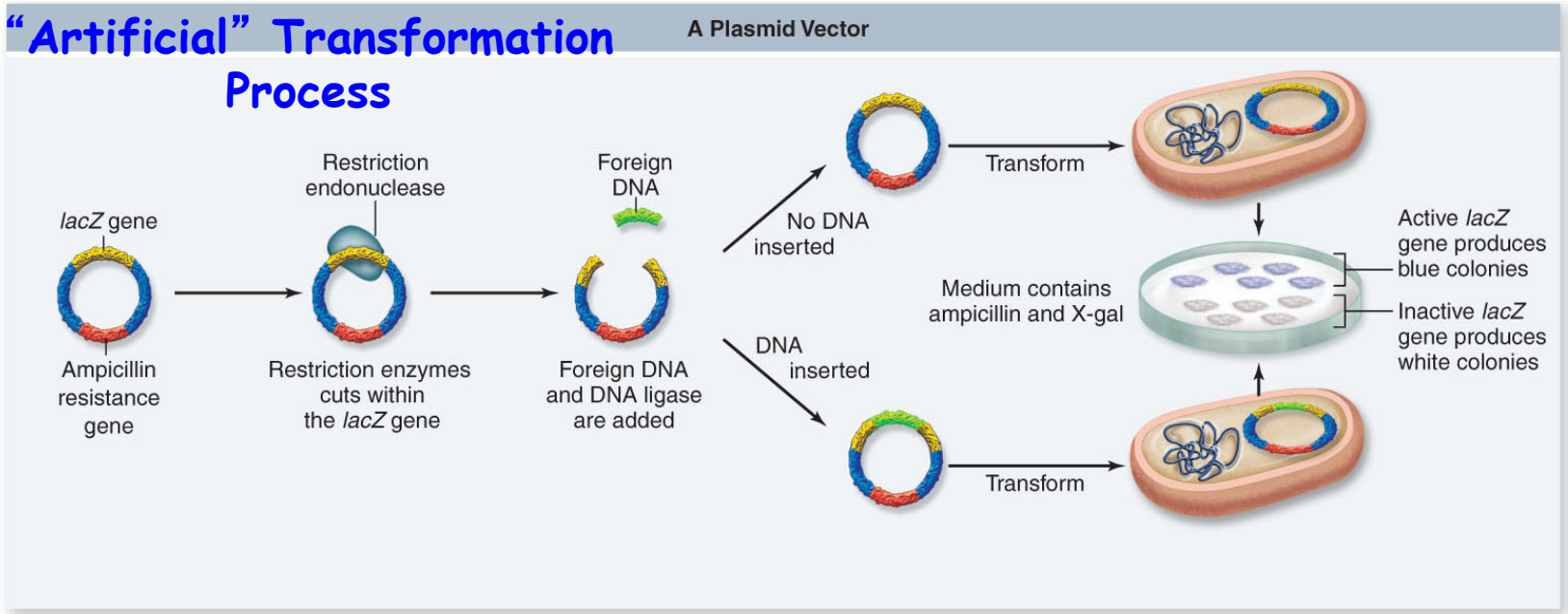
Vector Type	Maximum Insert Size (kb)	Applications	Limitations
Bacterial plasmid vectors (circular)	~6–12	DNA cloning, protein expression, subcloning, direct sequencing of insert	Restricted insert size; limited expression of proteins; copy number problems; replication restricted to bacteria
	DNA	DNA	
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

Plasmids vs. Bacteriophage Vectors

1. Replicate
2. Selectable
3. Can be used to insert foreign genes/restriction sites
4. Easily isolated + transferred back to cells

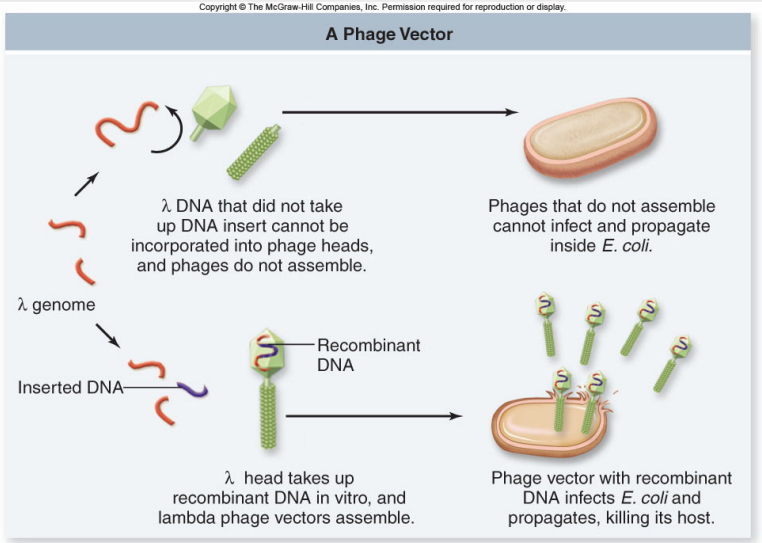
Plasmid vs. Bacteriophage Vectors for Cloning DNA Fragments

"Artificial" Transformation Process



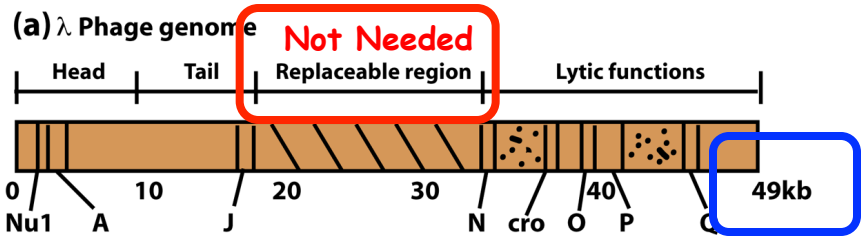
a.

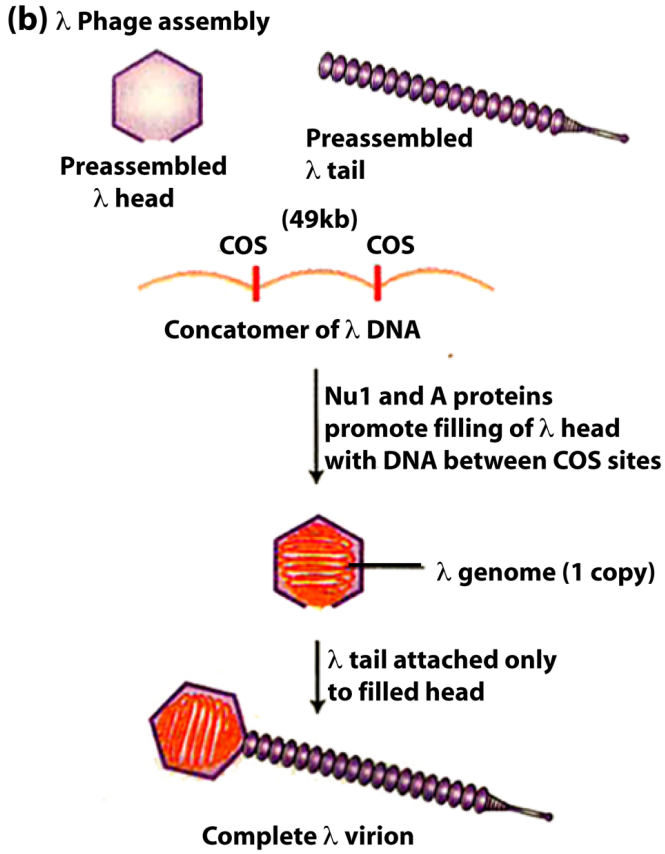
Natural Infection Process

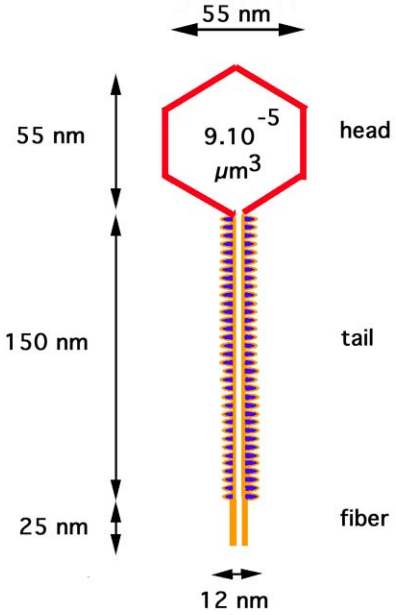


b.

Structure of the λ Phage and Its Genome







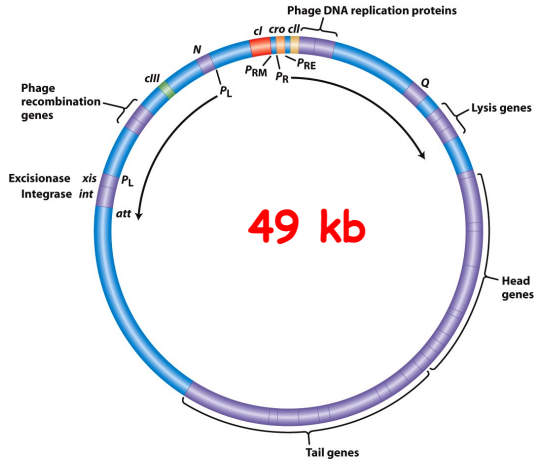
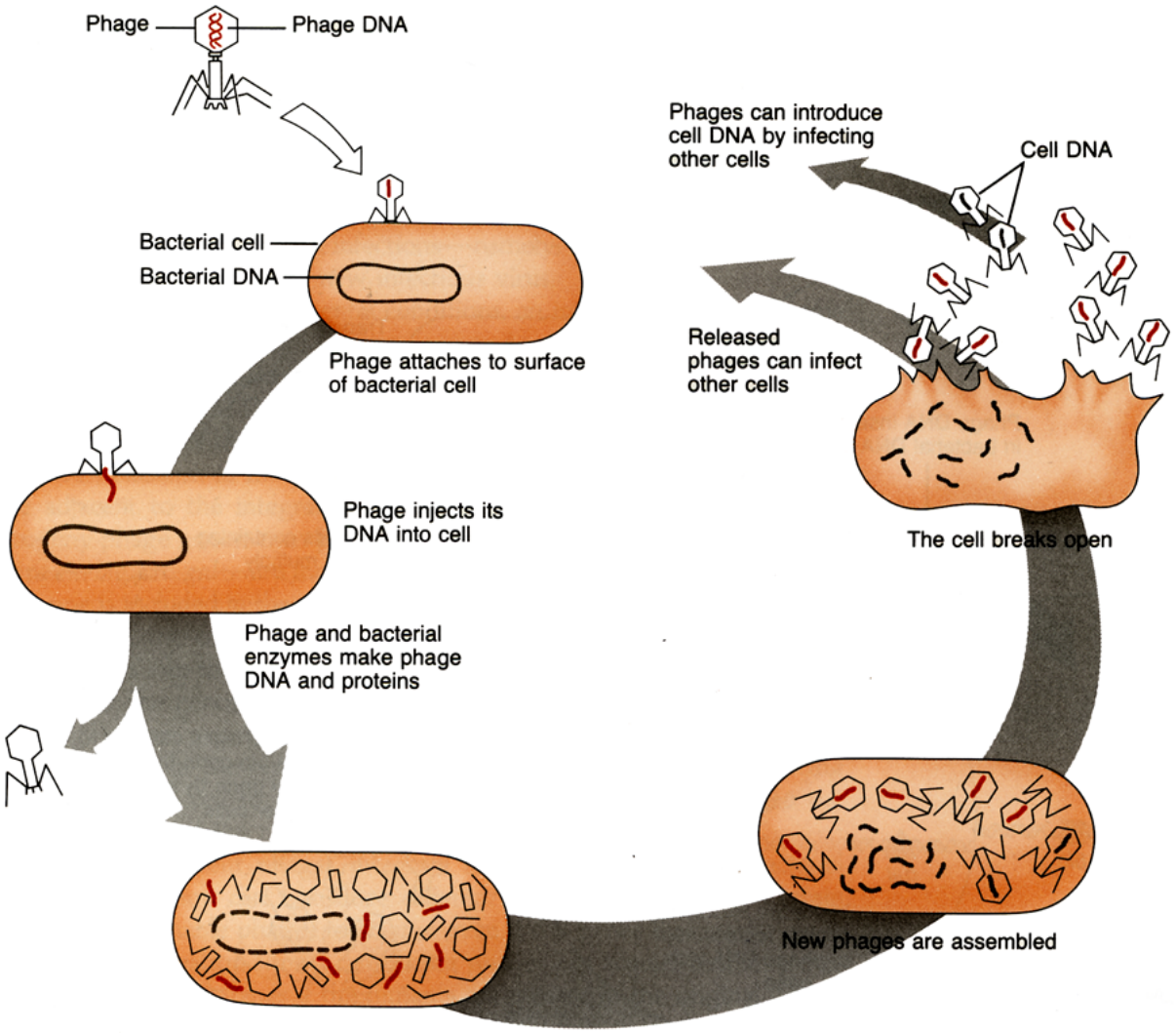


Figure 10-26

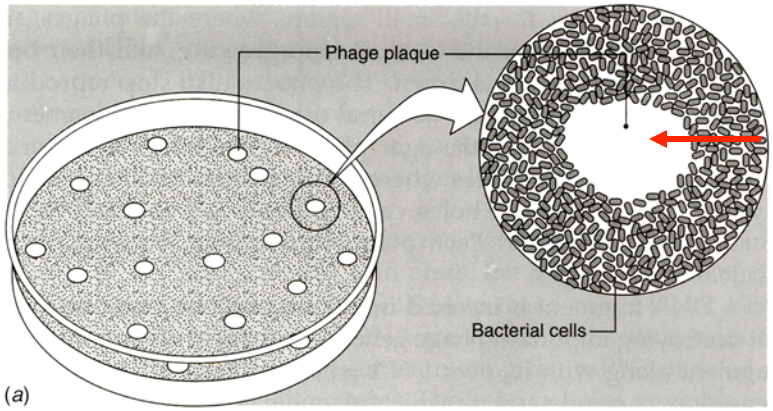
One of First Genome Sequences

How can the Genome "fit" into the Head?

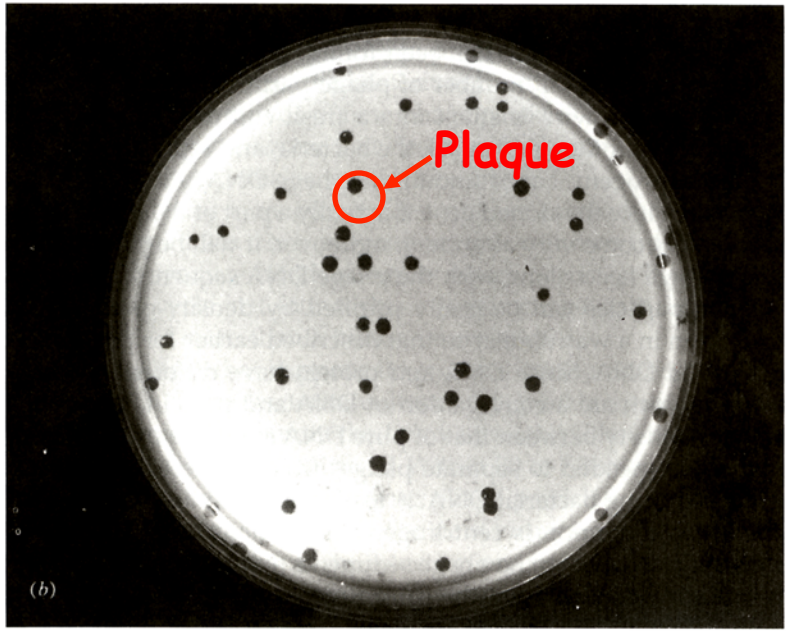
λ Phage Infects E.coli & Destroys (Lyses) cells



Lysed Cells Can Be Seen as Clear Plaques on Agar Plates



Plaque



1. Each Plaque is a Virus Clone Representing One Viral Infection!

2. Selectable Marker is Bacterial Cell Destruction & Plaque Formation

Advantages of λ 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!

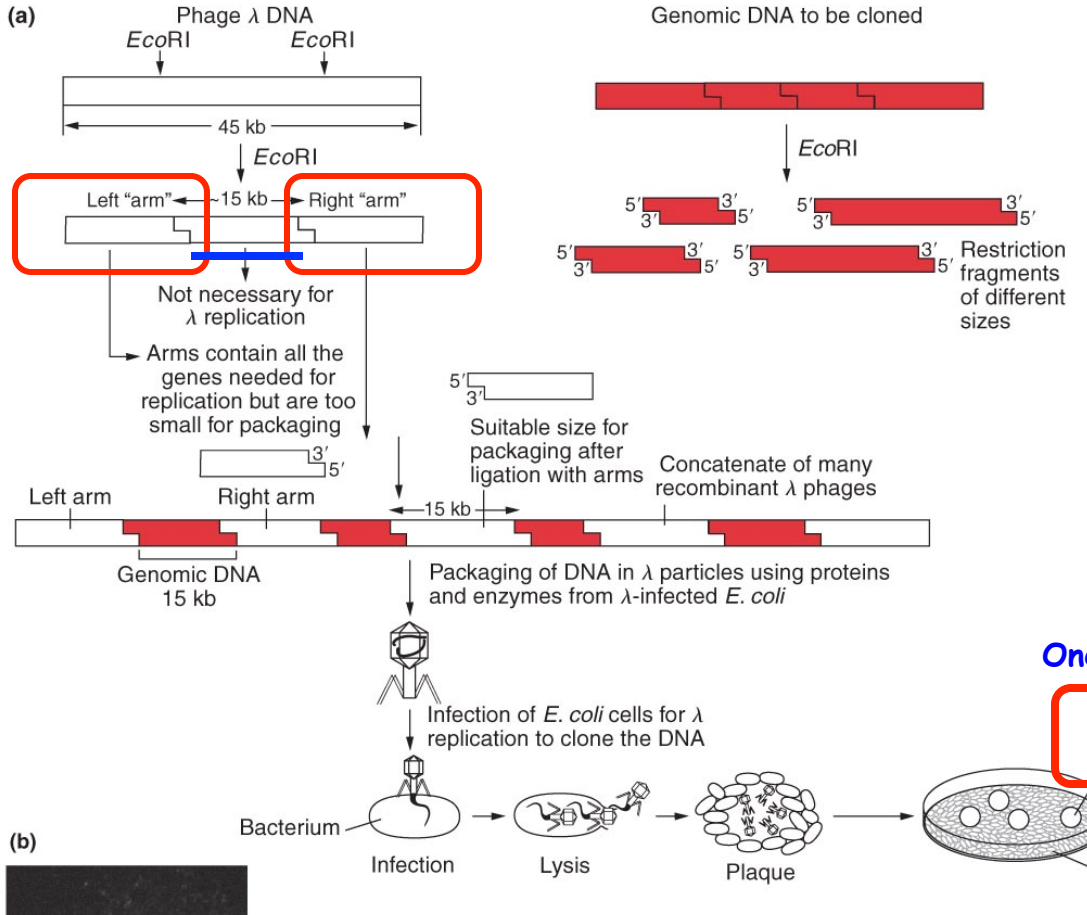
   ∴ Make Recombinant Viruses in the Lab!

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.

   ∴ set more clones per amount of recombinant DNA!

Using a Bacterial Virus To Clone the Human Genome

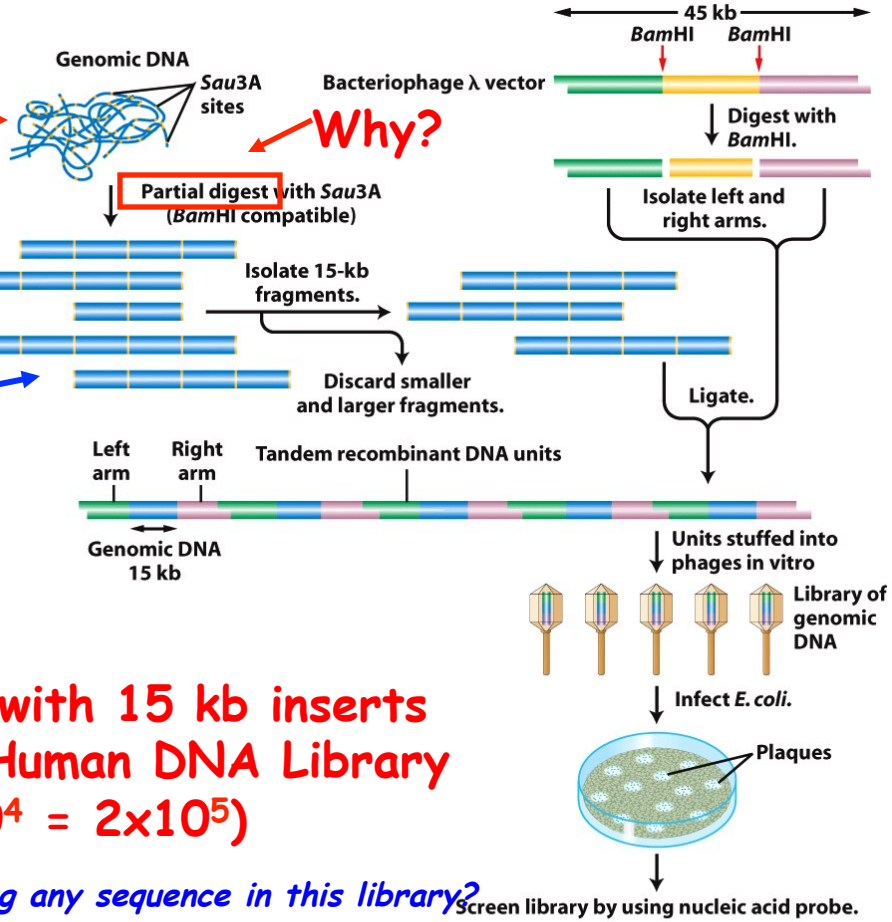


One Clone per Plaque

Mixture of Plaques = Library With All Human DNA Sequences Represented

Cloning the Human Genome and Screening for the Factor VIII Gene

Human DNA



Note Overlapping Fragments

Need 200,00 Phage with 15 kb inserts for Complete Human DNA Library ($3x10^9/1.5x10^4 = 2x10^5$)

What's the probability of having any sequence in this library?

Figure 20-6
Introduction to Genetic Analysis, Ninth Edition

Why Partial Digestion? An Important Concept!

What is Complete & Partial Digestion?

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

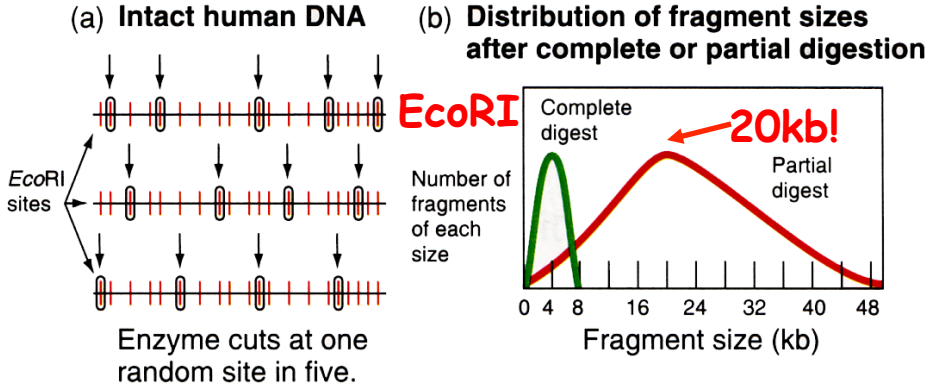
a. Yes
b. No

What is the Purpose of Partial Digestion of Human DNA?

Sau 3A= 4bp= $^{5'}GATC^{3'}$ ∴ 1 site every 280bp if digest to completion = $1x10^7$ DNA fragments

Eco RI= 6bp= $^{5'}GAATTC^{3'}$ ∴ 1 site every 3100 bp if digest to completion (cleaves 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 of 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!

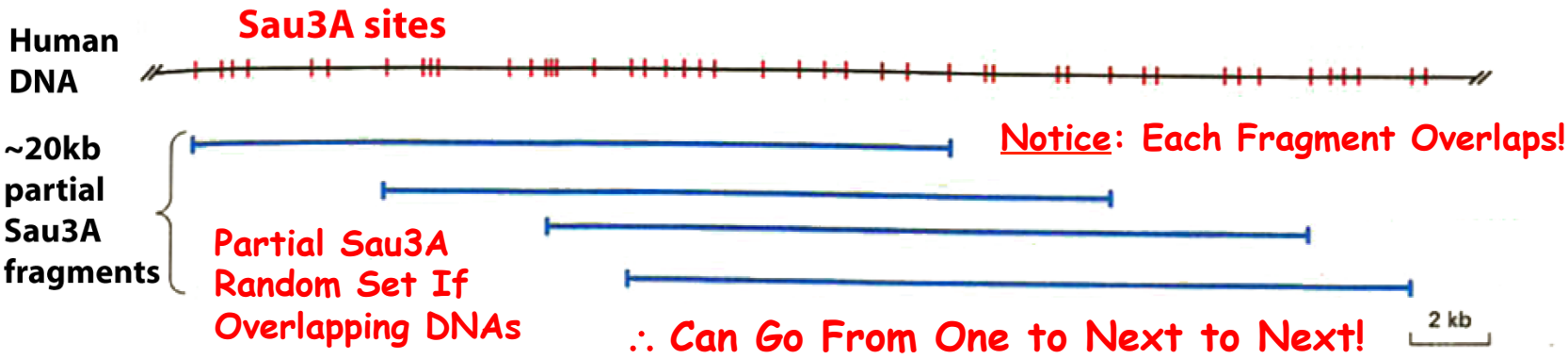


Principle of Genome Sequencing Too!!

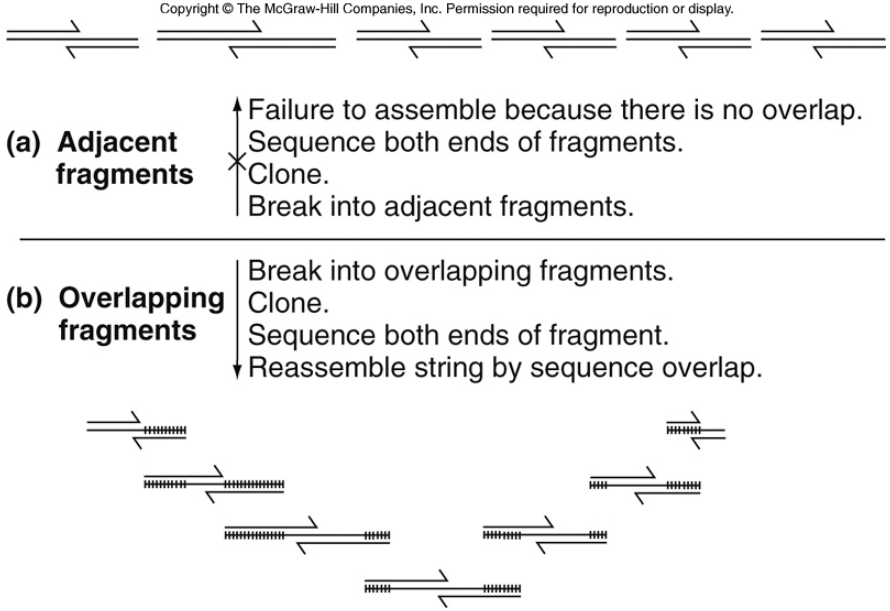
Partial Digestion Produces A series of Large, Overlapping DNA Fragments/ Clones

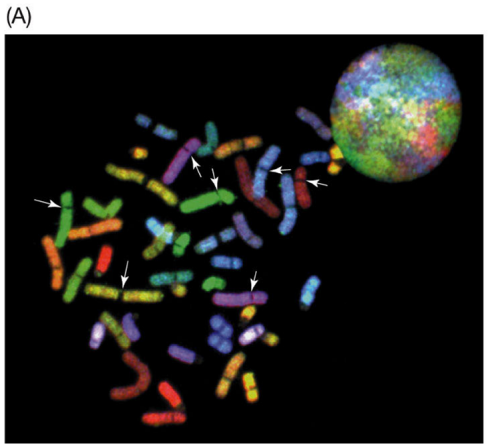
Can connect one clone with another!! Build up clones of each chromosome!!

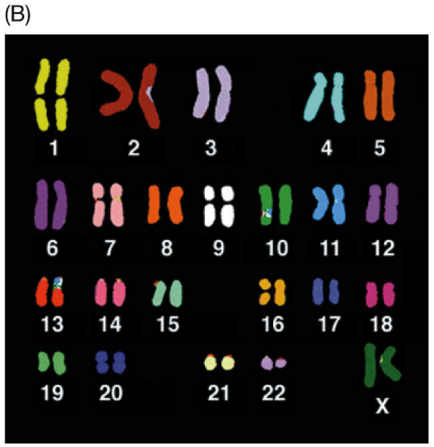
Constructing a Human Genome Library by Partial Digestion Creates a Set of Overlapping DNA Fragments/ Clones



∴ 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

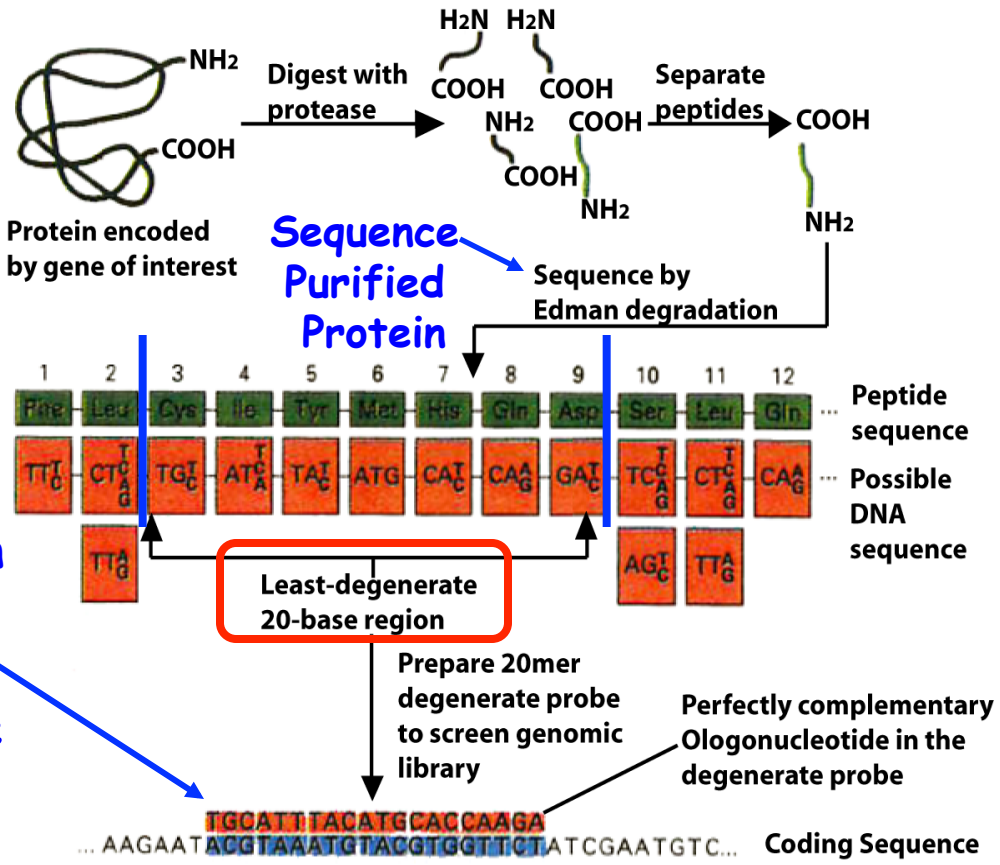
How Find the Factor VIII Gene in a Human Genome Library?

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

Factor VIII Protein → Gene

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



1. Use Genetic Code

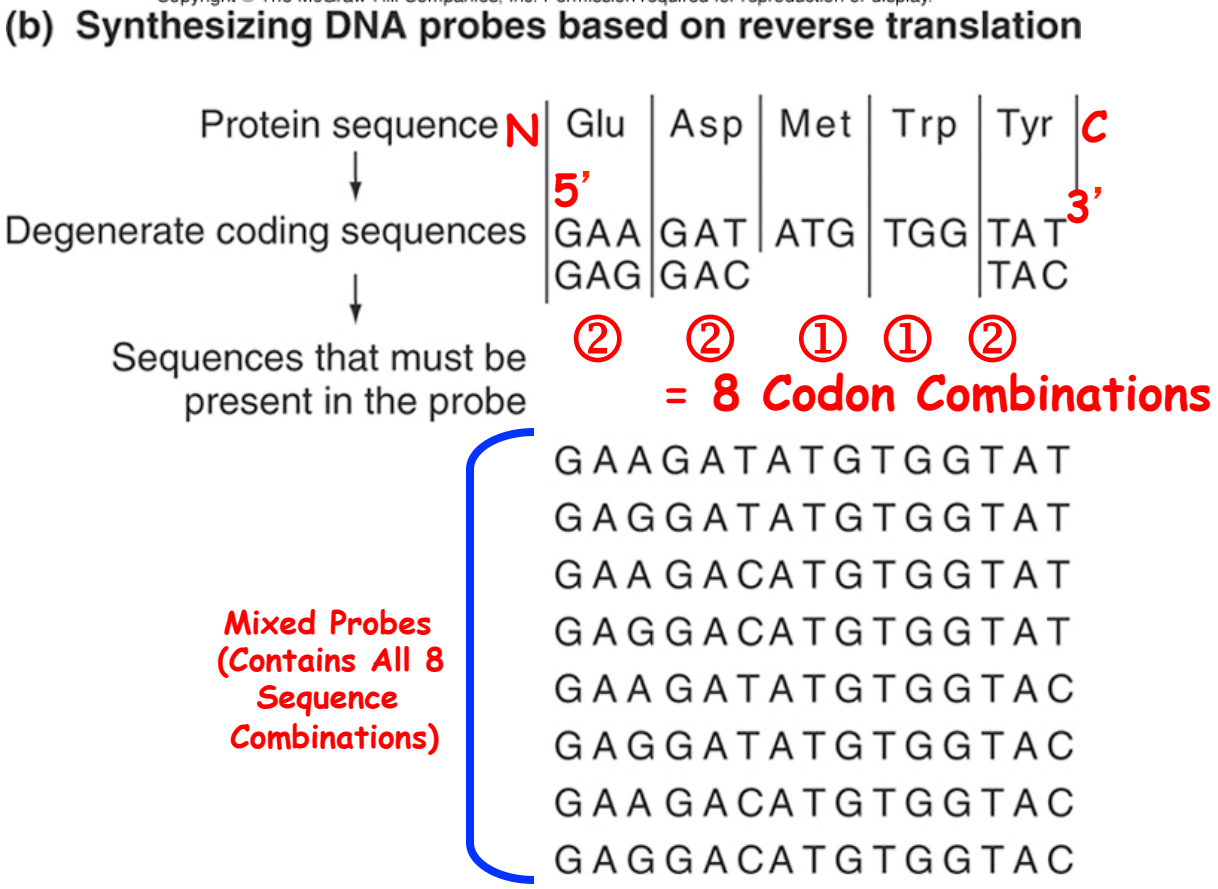
2. Make Several Probes All Codon Combinations!

3. One Will Be Correct Probe

How many Combinations of Synthetic Probes?

2x3x2x1x2x2x2=96

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

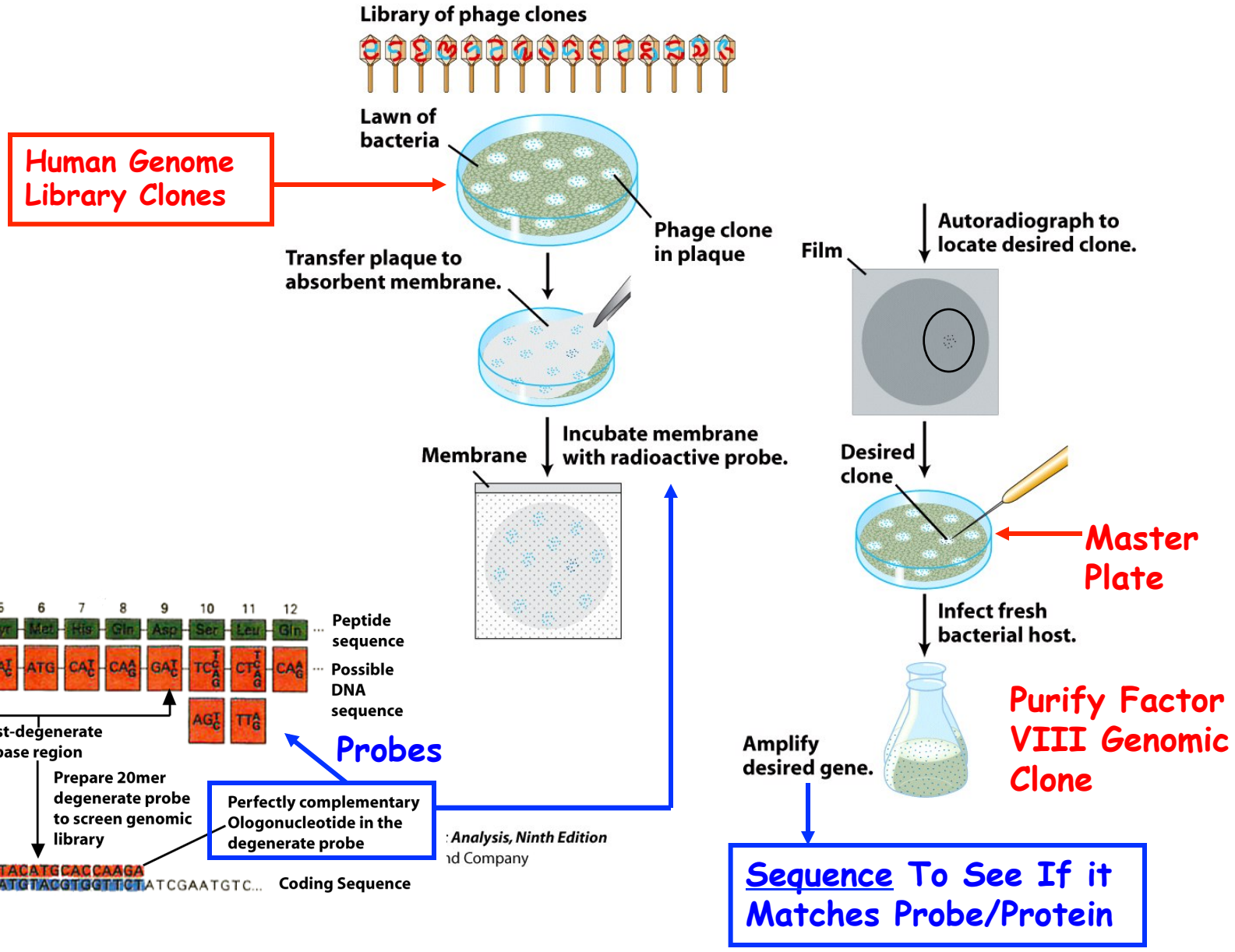


1. Need Amino Acid Sequence of Part of the Protein
2. Need DNA Sequences Representing all Codon Combinations
3. Synthesize DNA Sequence Probes!

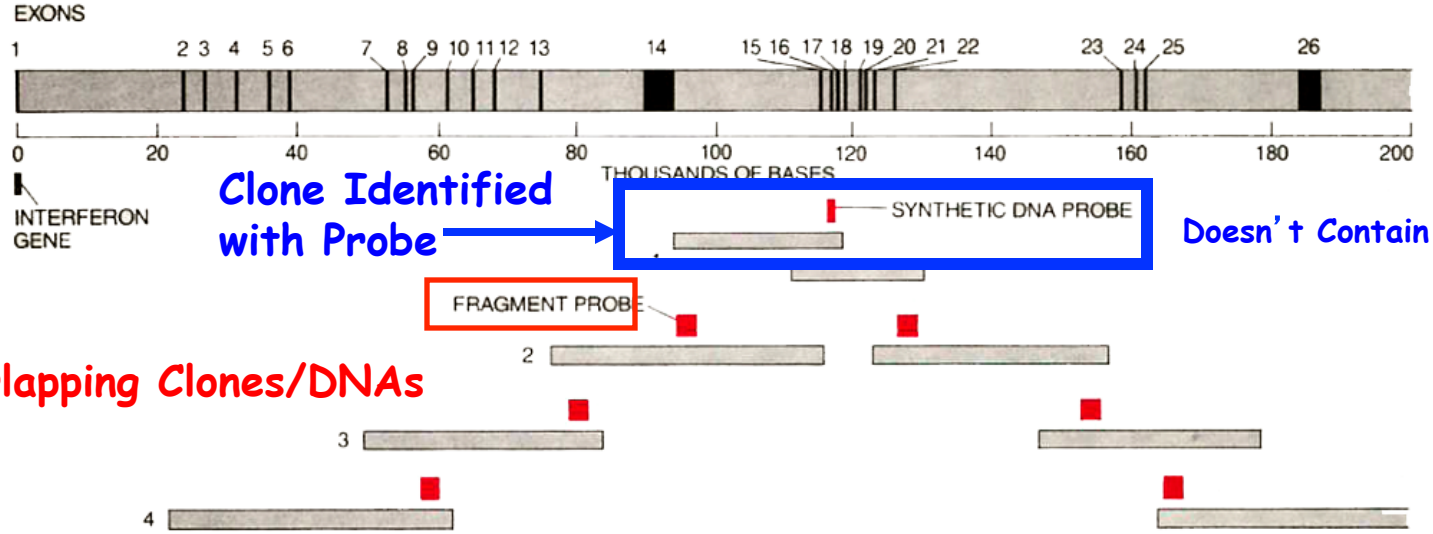
Probes Can Identify Genes in a Genome Library Because They Are: ?

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

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

Step Three

Finding the Entire Factor VIII Gene? Walking & Sequencing

Walking up and down Genes and Chromosomes

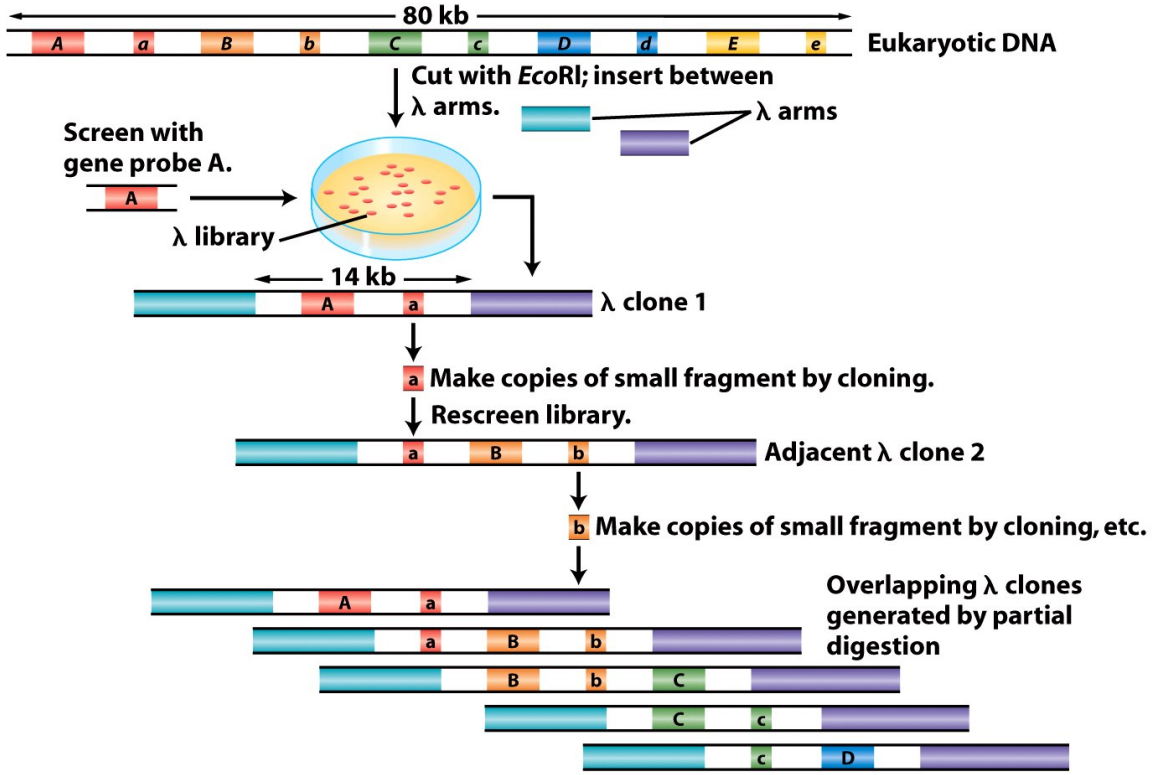


Figure 20-13
Introduction to Genetic Analysis, Ninth Edition

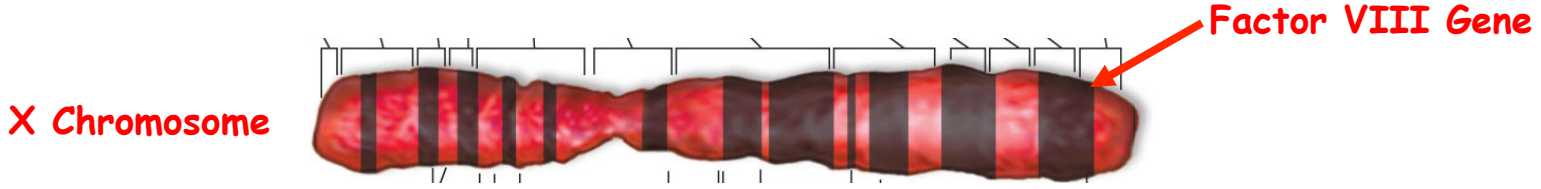
Basis of Genome Projects & Whole Genome Sequencing

Key Concepts → **How know Find Complete Factor VIII Gene?**

→ **Compare Protein & DNA Sequences**

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

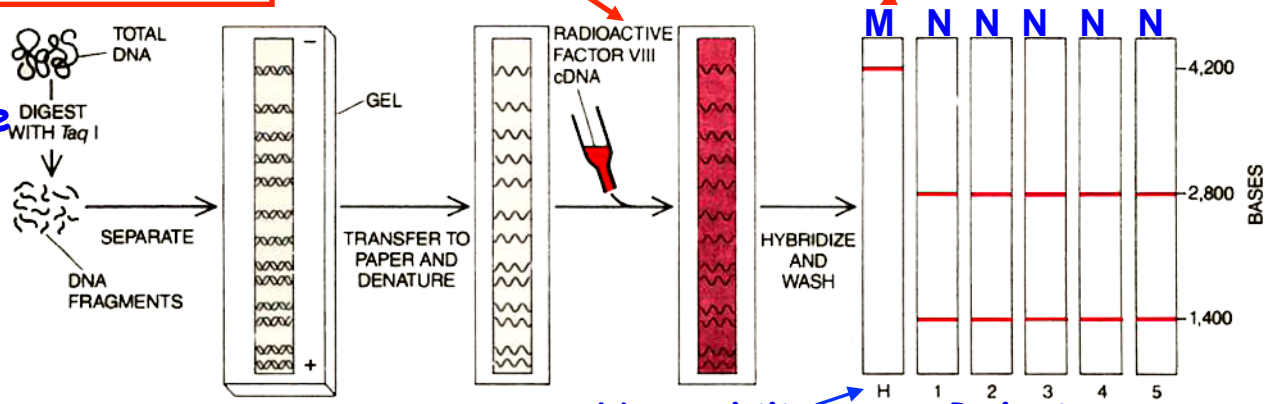


Factor VIII Gene Probes/ Sequence Can Be Used to Characterize Mutant Genes & Do DNA Testing for Carriers

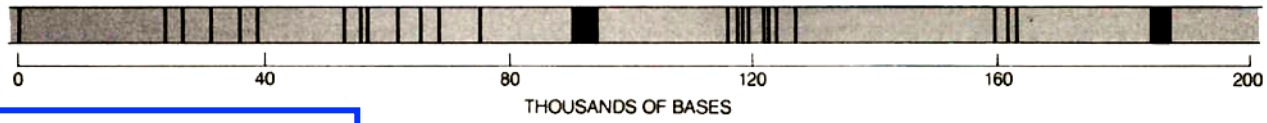
Human DNA From Different Individuals

Genomic or cDNA Clone Probe

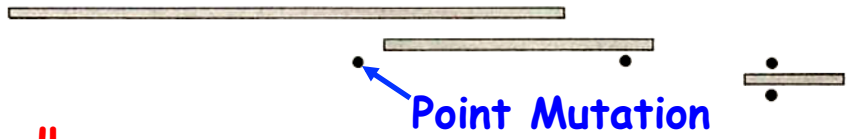
Loss of a TaqI site in Factor VIII Gene



RFLP



Mutations in Factor Gene



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

Factor VIII Mutations Occur Throughout the Gene

[*Haemophilia* 11, 481-491 (2005)]

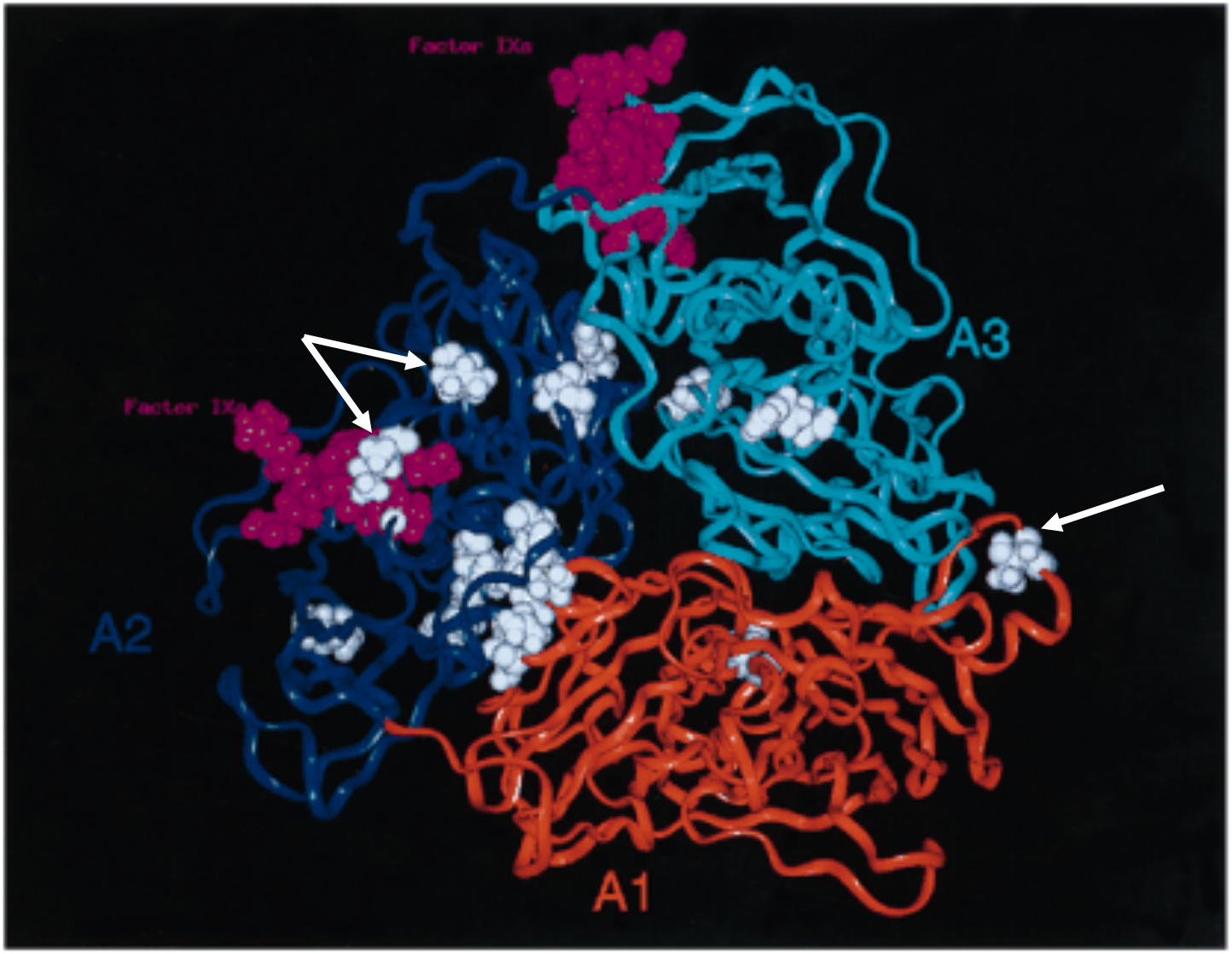
Factor VIII gene mutations in haemophilia A patients without intron 22 inversion.

VIII:C (%)	Family history	Consanguinity*	Inversion	Codon†	Mutation	Amino acid change	Exon	Conservation‡
1	Sporadic	NC	Normal	51	TTT → TCT§	Phe → Ser	2	FFFF, identical
1.20	Sporadic	NC	Normal	80	GTT → GAT	Val → Asp	3	VVVV, identical
1	Sporadic	NC	Normal	102	GGT → GTT§	Gly → Val	3	GGGG, identical
2	Sporadic	NC	Normal	104	TCC → CCC§	Ser → Pro	3	SSSS, identical
6	Sporadic	NC	Normal	143	GAG → AAG§	Glu → Lys	4	EEEE, identical
1	Sporadic	NC	Normal	233	delCA§	Thr → fs (TGA-264)	6	
2.70	Inherited	NC	Normal	321	GAA → AAA	Glu → Lys	8	EEEE, identical
0	Sporadic	NC	Normal	372	CGC → CAC	Arg → His	8	RRRR, identical
3	Inherited	NC	Normal	527	CGG → TGG	Arg → Trp	11	RRRR, identical
1	Sporadic	NC	Normal	528	TGC → TAC§	Cys → Tyr	11	CCCC, identical
1	Inherited	NC	Normal	592	CAA → TAA	Gln → Stop	12	QQQQ, identical
1	Inherited	NC	Normal	864	delGACA insCAATTAAATGAGAA§	Gly → fs [TAA-867]	14	
1	Sporadic	NC	Normal	948	insA§	Lys → fs (TGA-984)	14	
1	Sporadic	NC	Intron 1	1107	AGG → TGG§	Arg → Trp	14	RGKK, dissimilar
1	Sporadic	NC	Normal	1107	AGG → TGG§	Arg → Trp	14	RGKK, dissimilar
1	Inherited	NC	Normal	1191–1194	delA	Ile → fs (TAG-1198)	14	
1.40	Sporadic	NC	Normal	1191–1194	insA	Ile → fs (TAA-1220)	14	
1	Sporadic	C	Normal	1227	delC§	Leu → fs (TGA-1231)	14	
2.10	Sporadic	NC	Normal	1241	GAC → GAG	Asp → Glu	14	DGGE, similar
1	Sporadic	NC	Normal	1392	1392del1418§	Pro → fs (TAG-1446)	14	
1	Inerited	C	Normal	1392	1392del1418§	Pro → fs (TAG-1446)	14	
1	Sporadic	NC	Normal	1441	insA§		14	
1	Inerited	C	Normal	1441	insA§			
1	Inherited	NC	Normal	1502	CAG → TAG§	Gln → Stop	14	QREQ, dissimilar
1	Inherited	NC	Normal	1504	delGT§	Val → fs (TGA-1517)	14	
1	Sporadic	NC	Normal	1535	TGG → TGA	Trp → Stop	14	WLWM, dissimilar
hibitor 96 BU								
1	Sporadic	NC	Normal	1571	TAT → TAA§	Tyr → Stop	14	Y-YY, dissimilar
1	Sporadic	NC	Normal	1581	AAA → TAA§	Lys → Stop	14	KEKK, dissimilar
0.20	Sporadic	NC	Normal	1696	CGA → GGA	Arg → Gly	14	RRRR, identical
1.80	Sporadic	NC	Normal	1729	delA§	Gln → fs (TAA-1752)	15	
1	Inherited	NC	Normal	1751	GAA → AAA§	Glu → Lys	15	EEEE, identical
1	Sporadic	NC	Normal	1775	TTC → TCC§	Phe → Pro	16	FFFF, identical
1	Sporadic	NC	Normal	1835	TGG → TGA§	Trp → Stop	16	WWWW, identical
7.60	Sporadic	C	Normal	1882	ATC → ATA§	Ile → Ile	17	IIII, identical
3	Inherited	C	Normal	1966	CGA → CAA	Arg → Glu	18	RRRR, identical
1	Sporadic	NC	Normal	1966	CGA → TGA	Arg → Stop	18	RRRR, identical

FVIII GENE MUTATIONS IN INDIAN PATIE

Need To Screen Across the Gene for Markers -- Family Specific

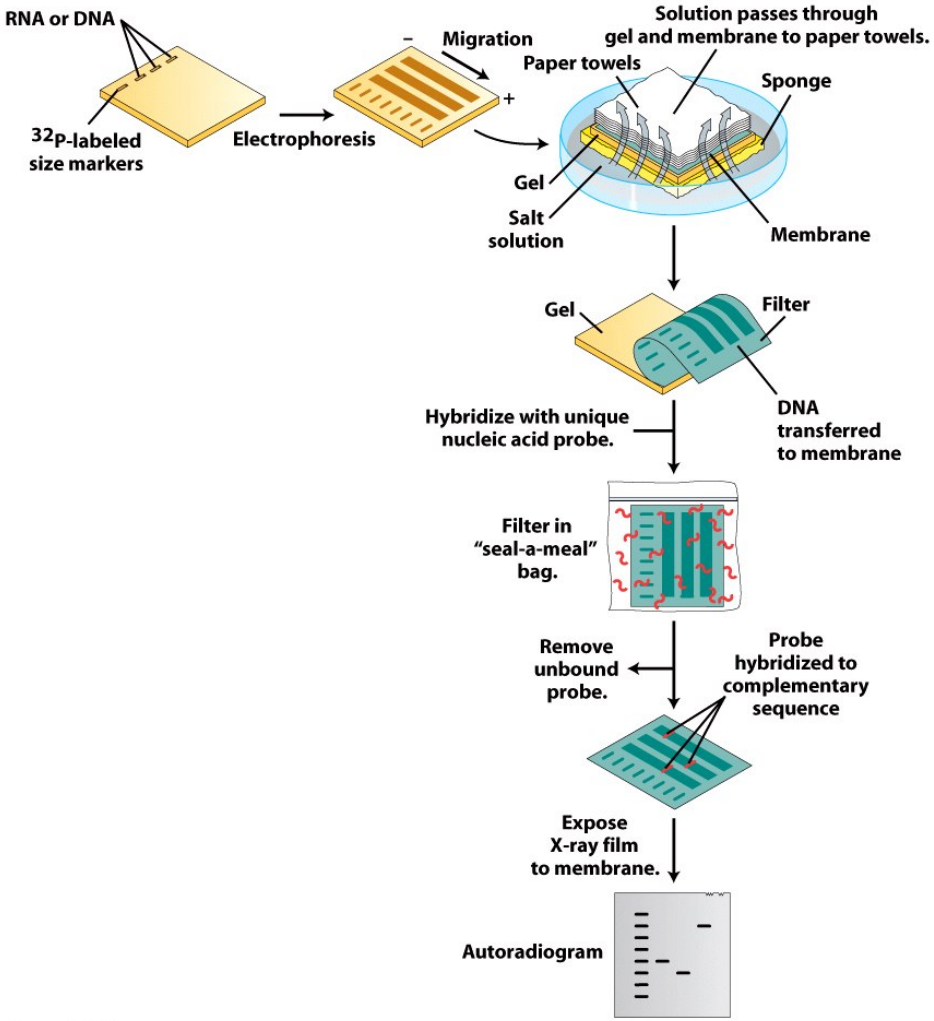
Factor VIII Protein Structure & Positions Where Mutations Disrupt Protein Function and Lead to Hemophilia



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!

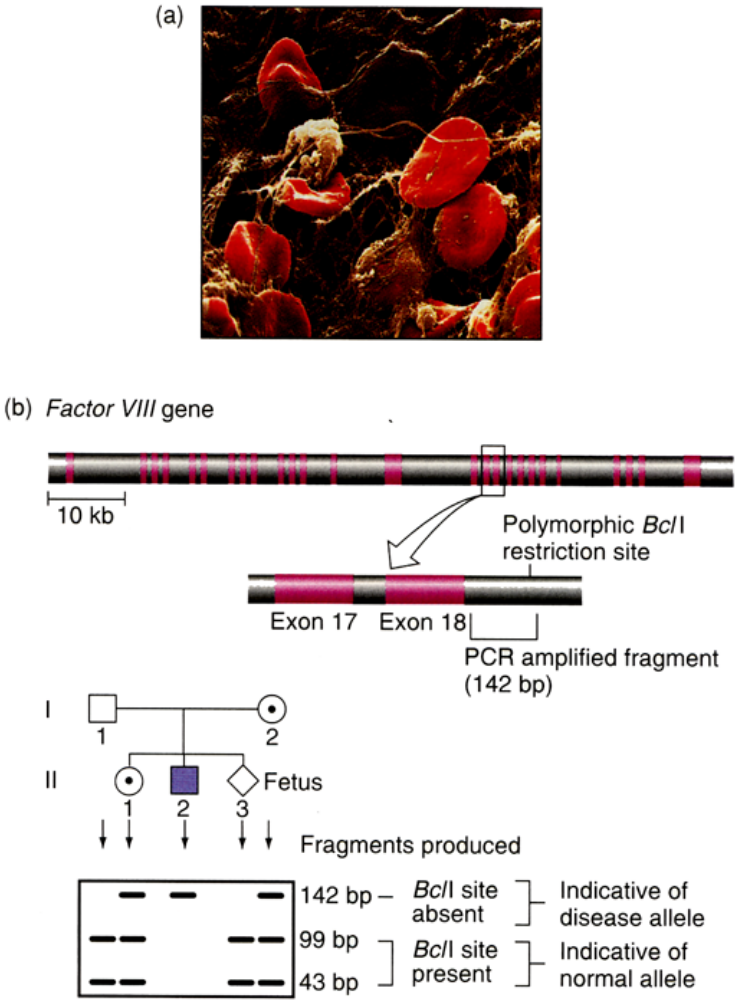
Figure 20-12
Introduction to Genetic Analysis, Ninth Edition

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

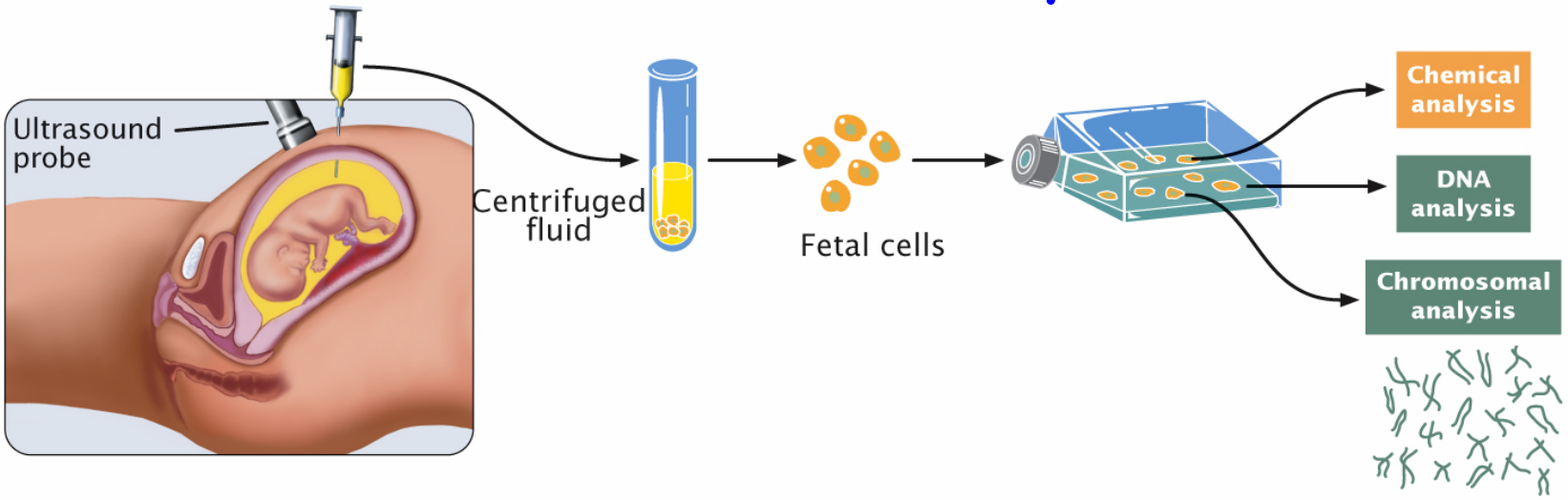


The 21st Century Approach!

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)

Use Gene Probe to Test for Disease Gene Prenatally



Ultrasound Picture

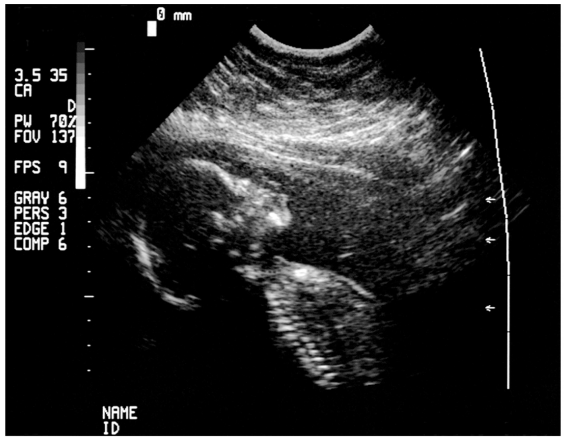
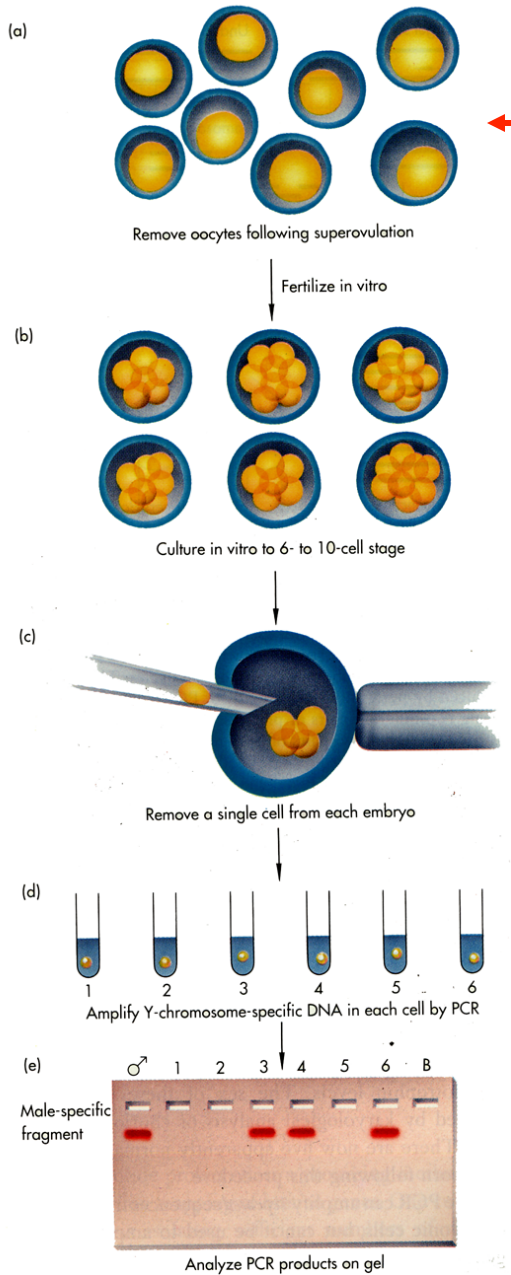


Fig_06-15 *Genetics, Second Edition* © 2005 W.H. Freeman and Company

Table 6.5 Examples of genetic diseases and disorders that can be detected prenatally and the techniques used in their detection

Disorder	Method of Detection
Chromosome abnormalities	Examination of a karyotype from cells obtained by amniocentesis or CVS
Cleft lip and palate	Ultrasound
Cystic fibrosis	DNA analysis of cells obtained by amniocentesis or CVS
Dwarfism	Ultrasound or X-ray; some forms can be detected by DNA analysis of cells obtained by amniocentesis or CVS
Hemophilia	Fetal blood sampling* or DNA analysis of cells obtained by amniocentesis or CVS
Lesch-Nyhan syndrome (deficiency of purine metabolism leading to spasms, seizures, and compulsory self-mutilation)	Biochemical tests on cells obtained by amniocentesis or CVS
Neural-tube defects	Initial screening with maternal blood test, followed by biochemical tests on amniotic fluid obtained by amniocentesis and ultrasound
Osteogenesis imperfecta (brittle bones)	Ultrasound or X-ray
Phenylketonuria	DNA analysis of cells obtained by amniocentesis or CVS
Sickle-cell anemia	Fetal blood sampling or DNA analysis of cells obtained by amniocentesis or CVS
Tay-Sachs disease	Biochemical tests on cells obtained by amniocentesis or CVS

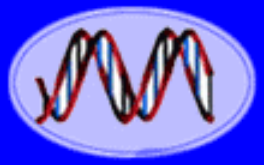
Using PGD to Detect Hemophilia A Disease Alleles



Mother is a Carrier X^HX^h

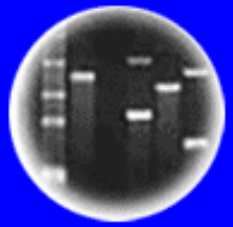
1. Test for Male Embryos
2. Test for Presence of Hemophilia A Disease Alleles!

X^hY



DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

Plants of Tomorrow

Genetic Screening Issues

- Why Screen For Genes?
- When is a Test Accurate Enough?
- Mandatory or Voluntary Screening?
- Who Should Be Tested?
- Employer & Insurance Company Testing?
- Protection From Genotype Discrimination?
- Testing for Genetic Diseases With No Cures??
- How Ensure Privacy & Confidentiality?
- Obligations to Inform Others (Spouse/Sibling) of Genetic Disorder Knowledge?
- Genetic Databases??

Step Four

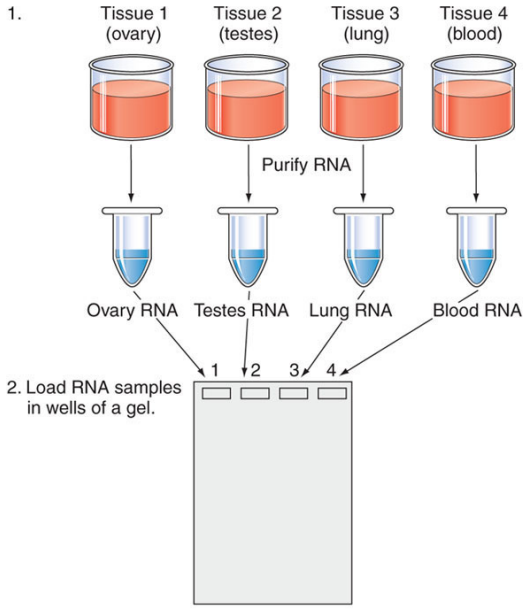
How Find Factor VIII mRNA to Generate a cDNA for Protein Production in Host Cells?

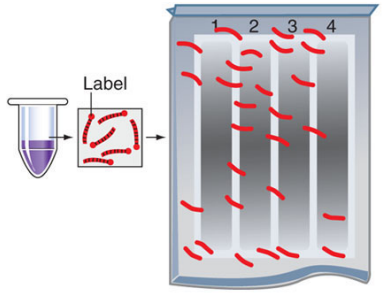
Recall: Eukaryotic Genes Provide Obstacles for Efficient Protein Production in Genetically Engineered Cells! Reasons???

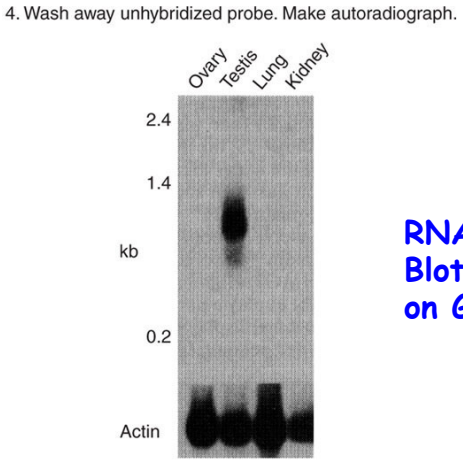
Making the Drug

Need cDNA Not Gene

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





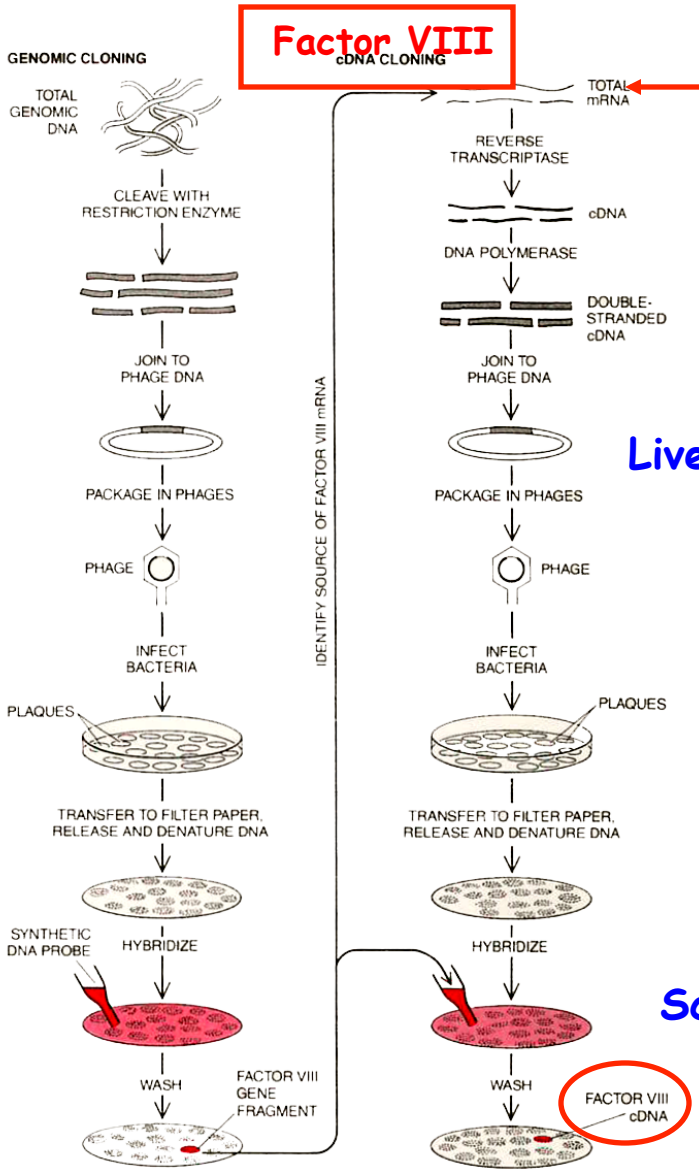


RNA Blot Is Like a DNA Blot Except That RNA is on Gel & Blotted

Factor VIII Gene Is Highly Active in Liver!

Could Also Use PCR (RT-PCR)

Using Factor VIII Gene Probe to Identify Factor VIII cDNA clone



Liver mRNA

Liver cDNA Library

Factor VIII cDNA →

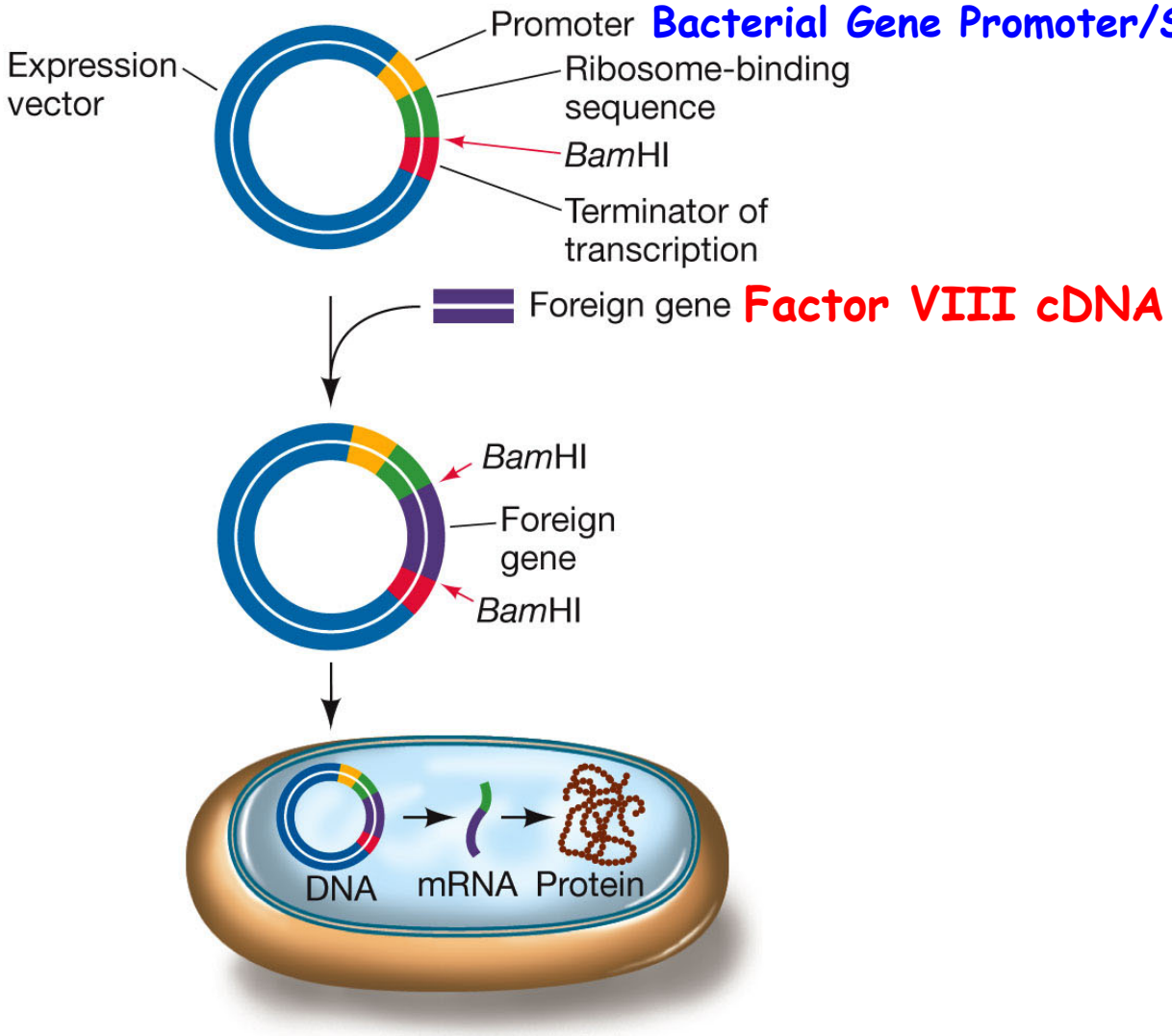
Used to Make Drug !

Screen Liver cDNA Library

The sequence of a cDNA clone is the same as:

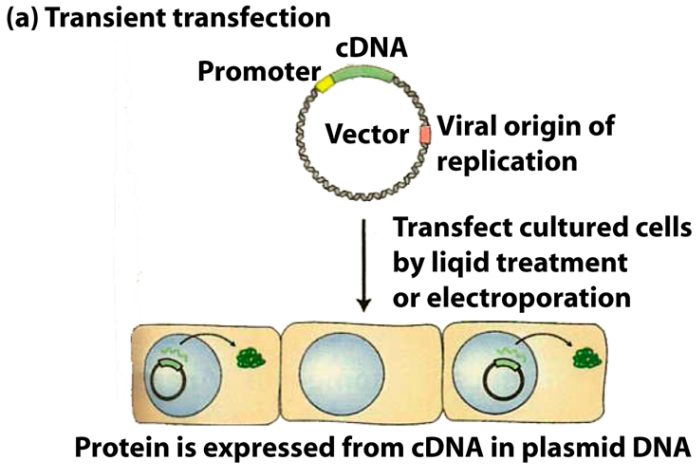
a. The Sense Strand of the Corresponding Gene
b. The mRNA Template
c. The Antisense (Template Strand) of the Corresponding Gene
d. The Sense and Antisense Strands of the Corresponding Gene Minus Introns

Use Expression Vector to Allow cDNA to Produce Protein in Host Cell

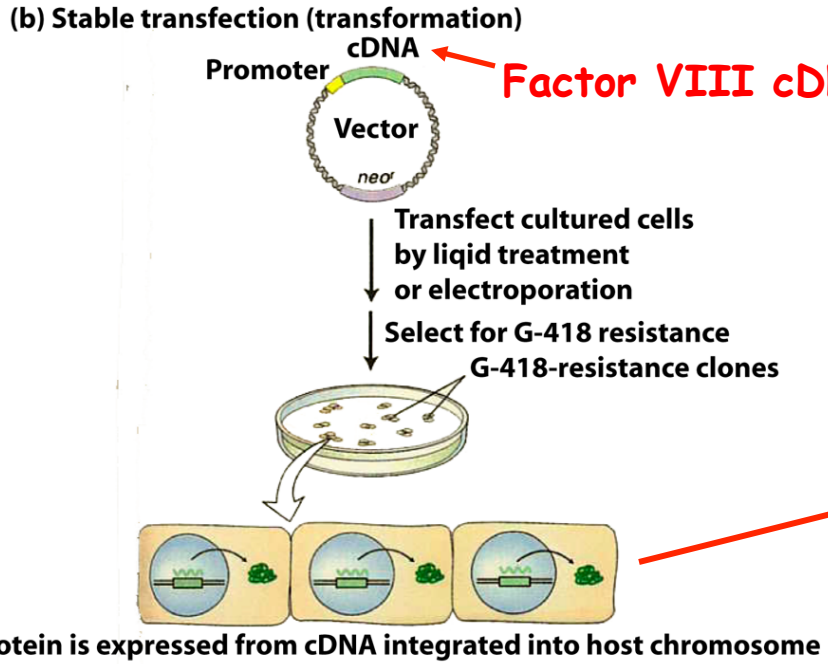


LIFE 8e, Figure 16.16

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



[Why Mammalian Cells?]



Purify Factor VIII Protein!

Recombinant Factor VIII

Bayer Biological Products EU

Bayer HealthCare
Biological Products Division
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Home | About Us | About Haemophilia | For Kids | Research & Development | Press Releases

- Recombinant Factor VIII
- More Resources
- Haemophilia Centres in Europe

Related Links

- Haemo-QoL Project
- Hemophilia Research Awards

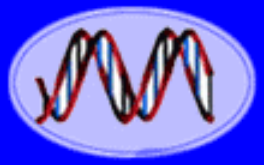
Recombinant factor VIII

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.

Prophylactic Treatment Costs $300,000/ Year!
Most Hemophiliacs Use "On Demand" or As Needed

Factor VIII gene cloned in 1983

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



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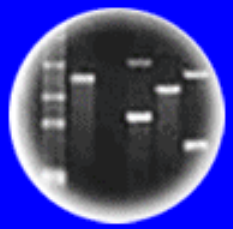

DNA Fingerprinting

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Plants of Tomorrow

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

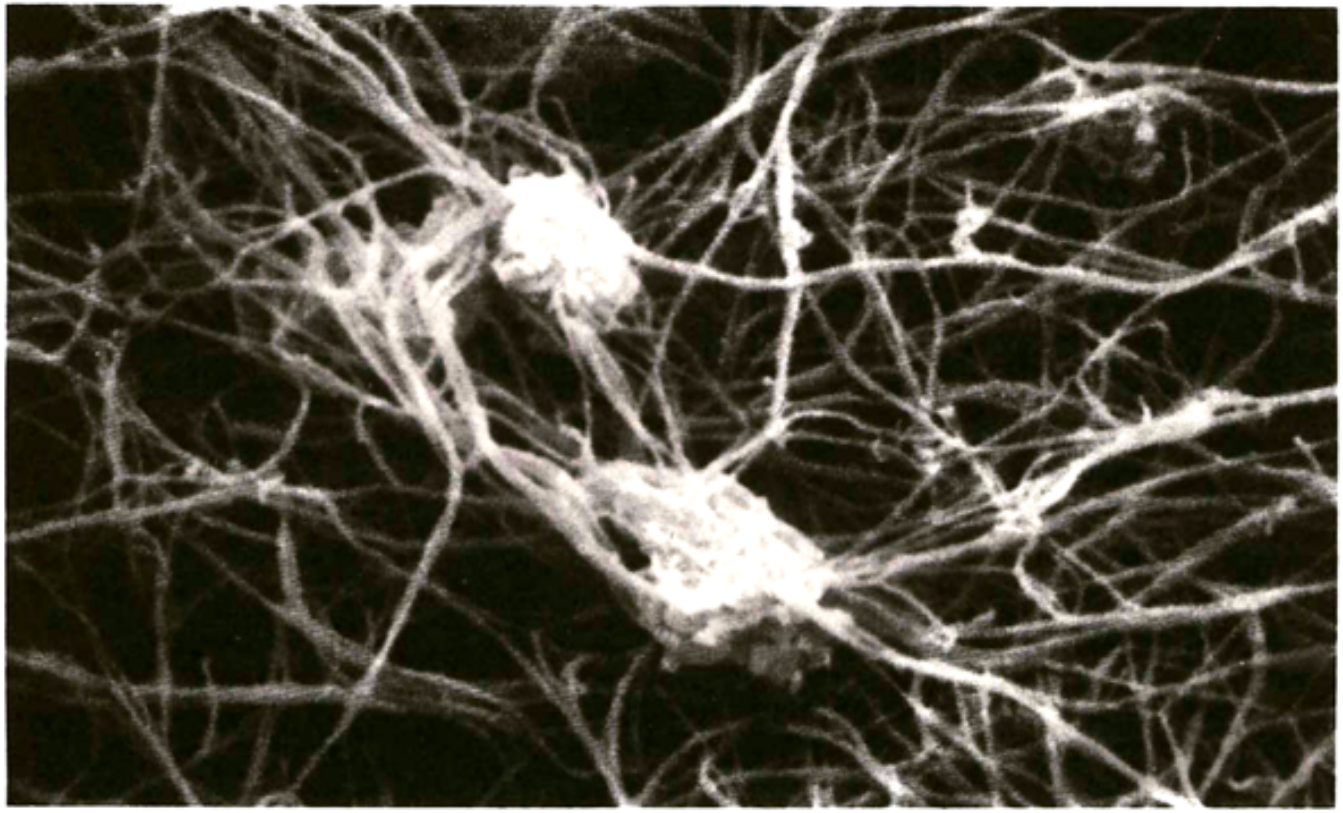
Functional human factor VIII produced recombinantly is used in the treatment of human beings diagnosed to be deficient in factor VIII coagulant activity. Also provided are DNA solates and expression vehicles encoding functional human factor VIII, as well as transformed host cells and processes for producing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

Patent Expired Last Year!

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

The Future: Gene Therapy – A Permanent "Cure"

December 10, 2011

Treatment for Blood Disease Is Gene Therapy Landmark

By NICHOLAS WADE

TIME Partners with CNN

Gene Therapy Shows Promise for Treating Hemophilia

By ALICE PARK Monday, December 12, 2011

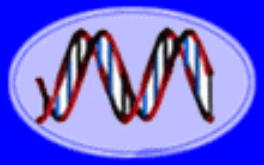
The NEW ENGLAND JOURNAL of MEDICINE

December 12, 2011

ORIGINAL ARTICLE

Factor IX

Adenovirus-Associated Virus Vector–Mediated Gene Transfer in Hemophilia B



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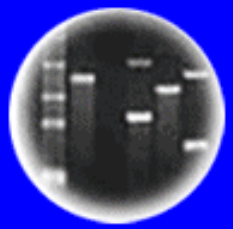
Entire Genetic Code of a Bacteria



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)