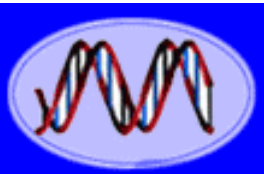
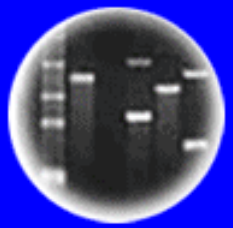


DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

Plants of Tomorrow

HC70A & SAS70A Winter 2010
Genetic Engineering in Medicine, Agriculture, and Law

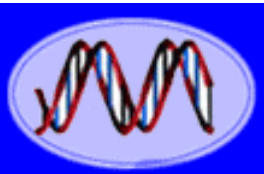
Professors Bob Goldberg & John Harada

Lecture 4

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

UCLA

UCDAVIS
UNIVERSITY OF CALIFORNIA



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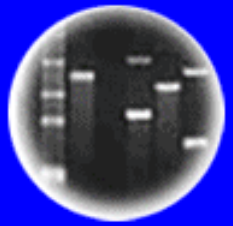

DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

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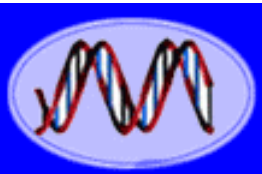
THEMES

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



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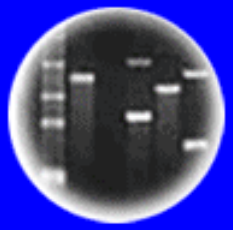

DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

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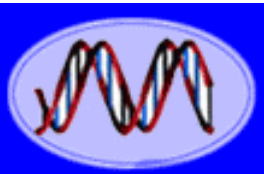
Gene Function Lectures-1/12,1/19,& 1/26

1. What is the Anatomy of a Gene?
2. How Does the Sequence of DNA Correlate With mRNA & Protein Sequences (i.e., colinearity of DNA and protein sequences)?
3. How Are Genes Regulated?
4. How Does DNA Replicate & Pass On the Genotype?
5. What Is Needed For DNA Replication?
6. How Can DNA Replication Be Used to Amplify Specific DNA Sequences in Whole Genomes? What is PCR?
7. What Are Some PCR Applications and How Has it Revolutionized Our Ability to Study Genes and Created New Fields of Investigation?
8. How Do Mutations Occur During DNA Replication and How Do Mutations Lead to Genetic Diversity?
9. How Are Mutations Followed Generation to Generation?
10. Large Changes in Chromosomal Structure and Number Can Cause Mutations as Well as Small Base-Pair "Point" Mutations
11. How Are Karyotypes Used to Detect Large Chromosomal Changes?
12. How Do Mutations Lead to Phenotypic Changes?



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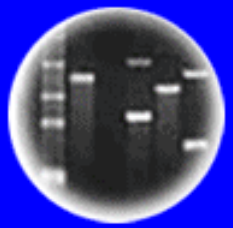

DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

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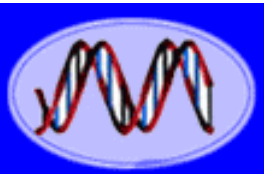
Experiments Discussed

1. Design an Experiment to Show that DNA is the Genetic Material?
2. How Do the Experimental Results Correlate With Properties Expected of the Genetic Material?
3. Design an Experiment to Clone an Origin of Replication (i.e., ori)?
4. How Do the Experimental Results Correlate With the Properties Expected of an Origin of Replication Sequence?



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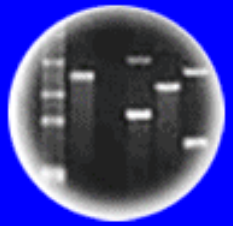

DNA Fingerprinting

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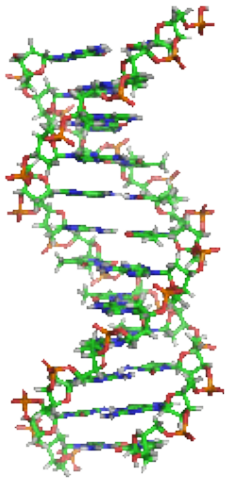
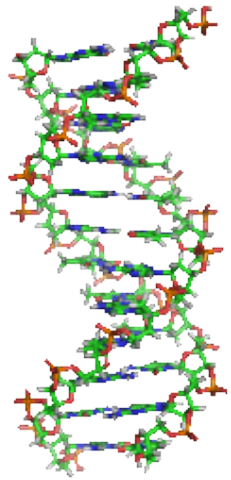
Discussion Section Questions?

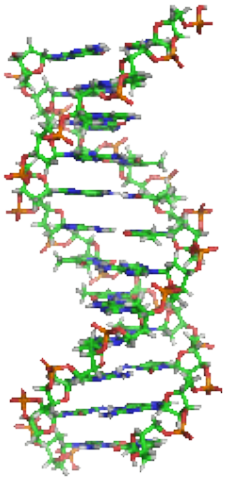
1. How Can a "Foreign" DNA Sequence Be Cloned Into a Bacteria?
2. What Was the Nature of the Controversy When Genetic Engineering Was Invented and How Were Concerns Mitigated?
3. How Can "Foreign" Genes Be Expressed in Bacterial Cells and Manufacture New Drugs?
4. How Can Plants Be Genetically Engineered For New Agriculturally-Important Traits?
5. What is the Nature of the "GMO Controversy," Are Claims Valid, and How Have They Been Mitigated?
6. What are Markers and How Can They Be Used to Obtain DNA Fingerprints?
7. What is the Frye Standard and How Used in DNA Fingerprinting Cases?
8. What are VNTRs (STRs) and How Used in DNA Fingerprinting Analysis?

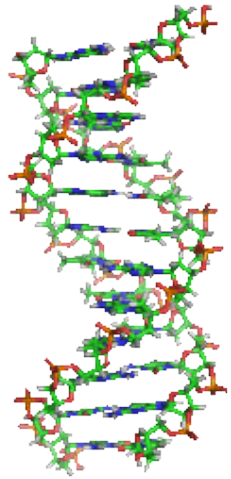




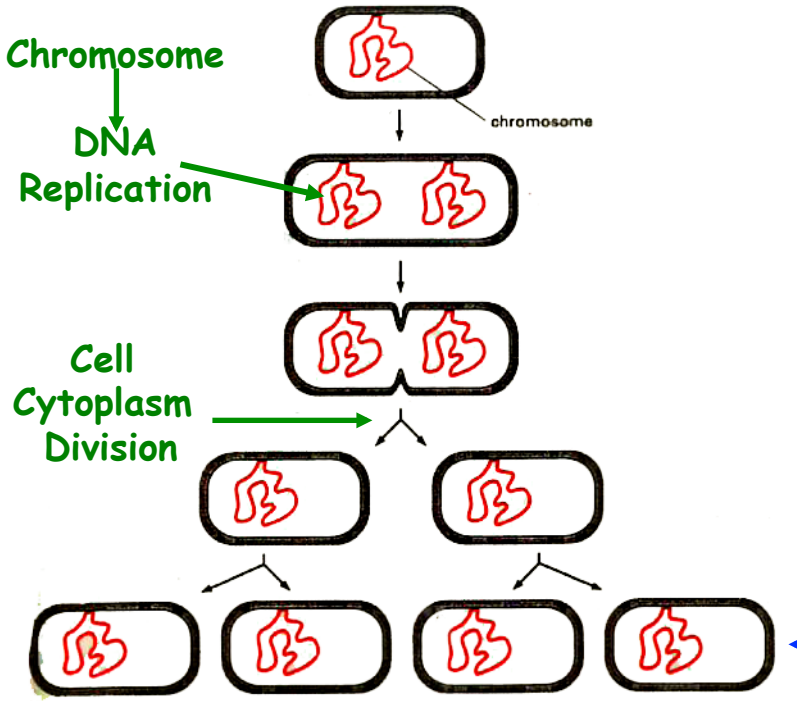
What Are Genes & How Do They Work?

A Short Review





Genes Are Replicated During Each Cell Division

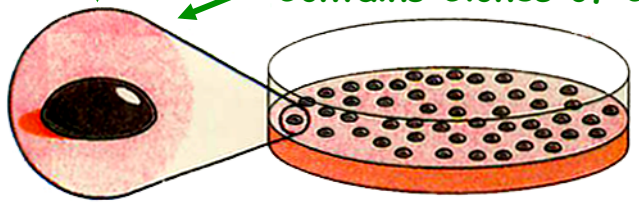


Clones

A Bacterial Colony Contains Many Copies of Same Cell, or Clones, Which are Genetically Identical!

A "Clone"

Note - Each Clone of Bacteria Contains Clones of Cells



-10^6 cells per colony ~50 colonies per dish

Each Daughter Cell Contains The Same Collection of Genes

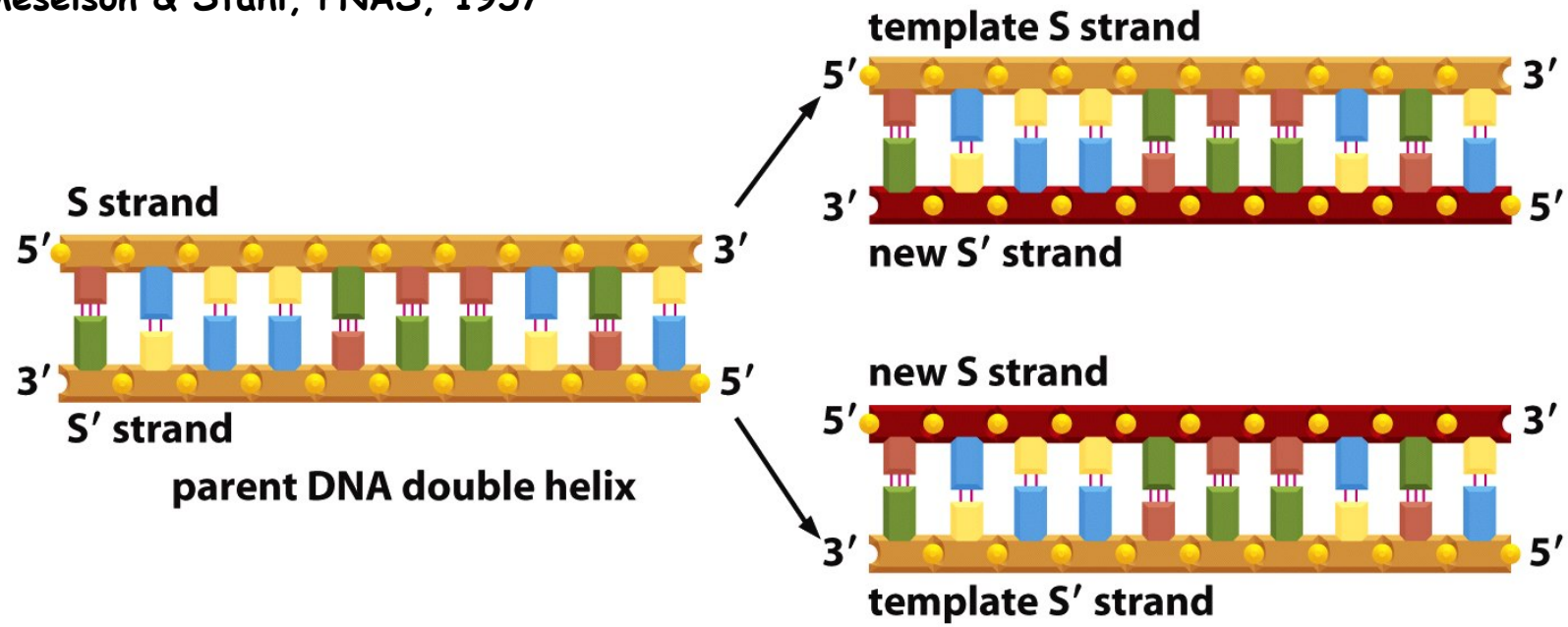
Clones!

Major Properties of Genetic Material

Replication & Stability

DNA Replication Occurs Semi-Conservatively

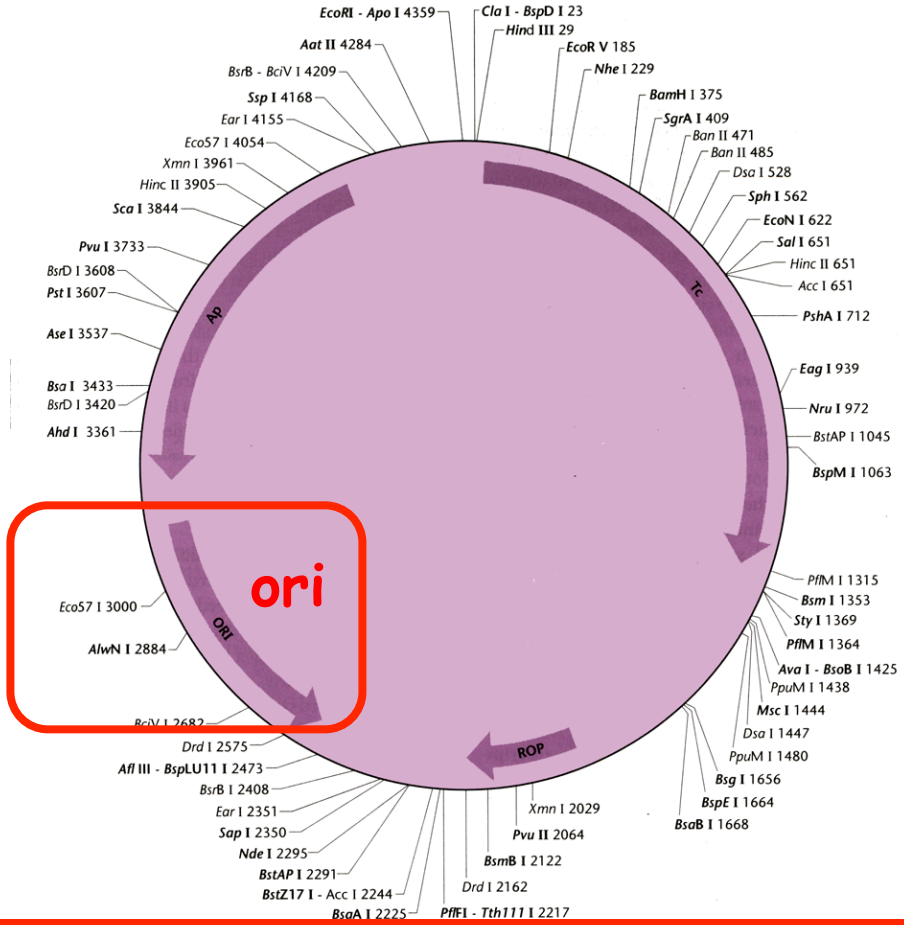
Meselson & Stahl, PNAS, 1957



1. DNA Structure Allows DNA Sequence to Be Maintained by Complementary Base Pairing
2. Each Strand Serves as a Template for the Synthesis of a Complementary Strand
3. New DNA Molecules are Precise Copies of Parental DNA - Each Containing One Newly Synthesized Complementary Strand

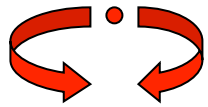
Ori

DNA Replication Starts at The Origin of Replication



DNA Replication is <u>Bidirectional</u> From the Ori!!!

Ori



Hypothesis For Two Direction Synthesis?

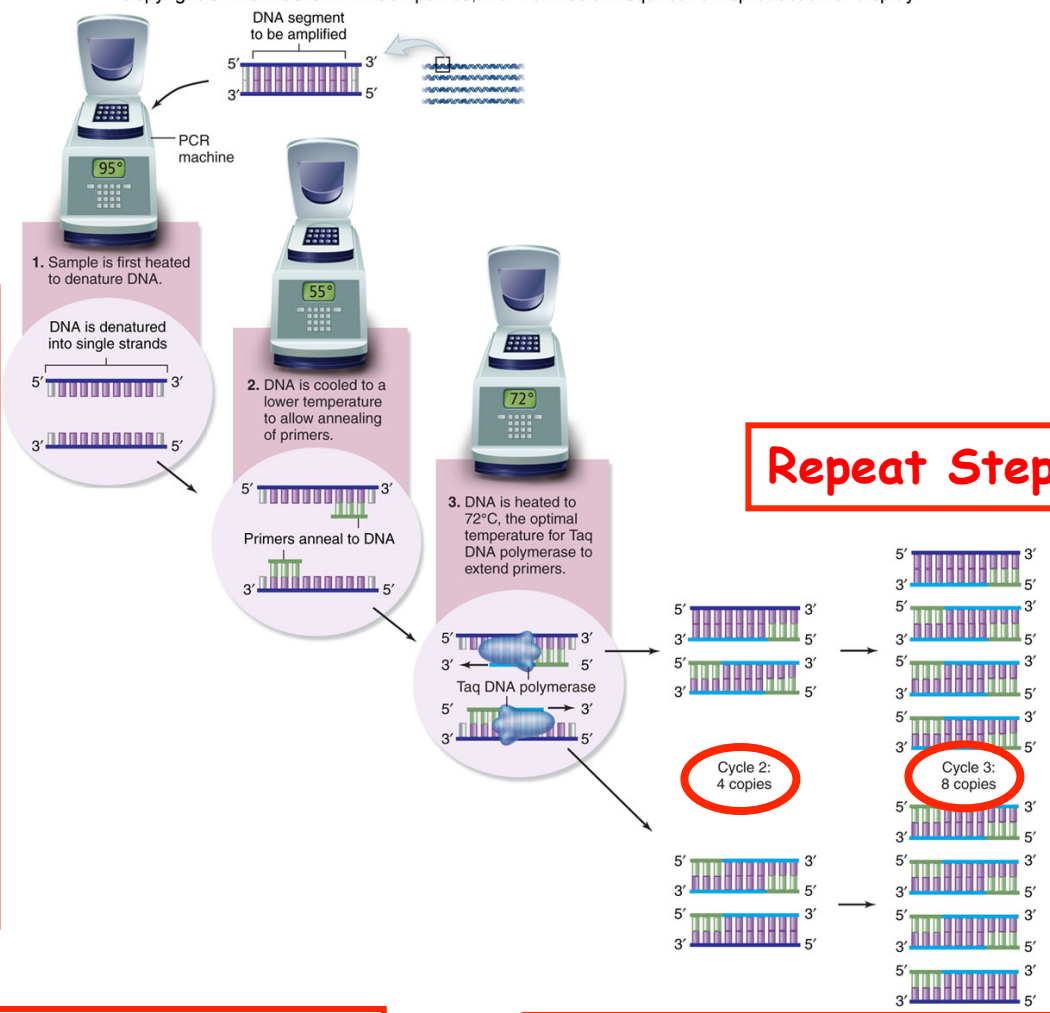
DNA Polymerase Binds to The Origin of Replication (Ori) to Begin DNA Synthesis

How Control Division?

PCR is A Cyclical Process of DNA Replication

Requires

1. Template
2. Primers
3. Knowledge of Specific Sequence
4. Nucleotides
5. Heat-Stable DNA Polymerase
6. Cycler

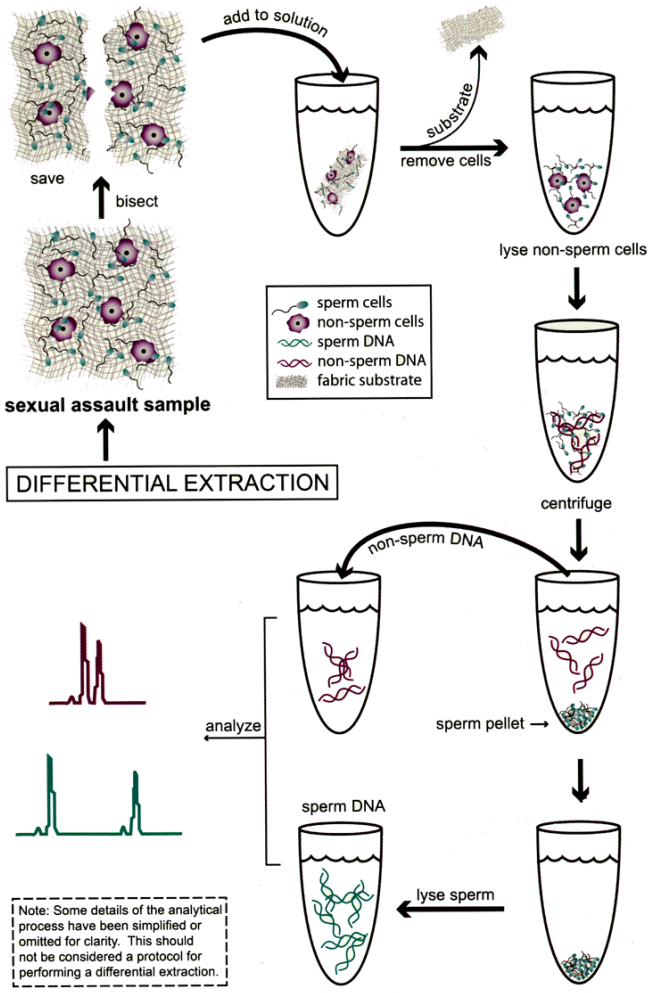


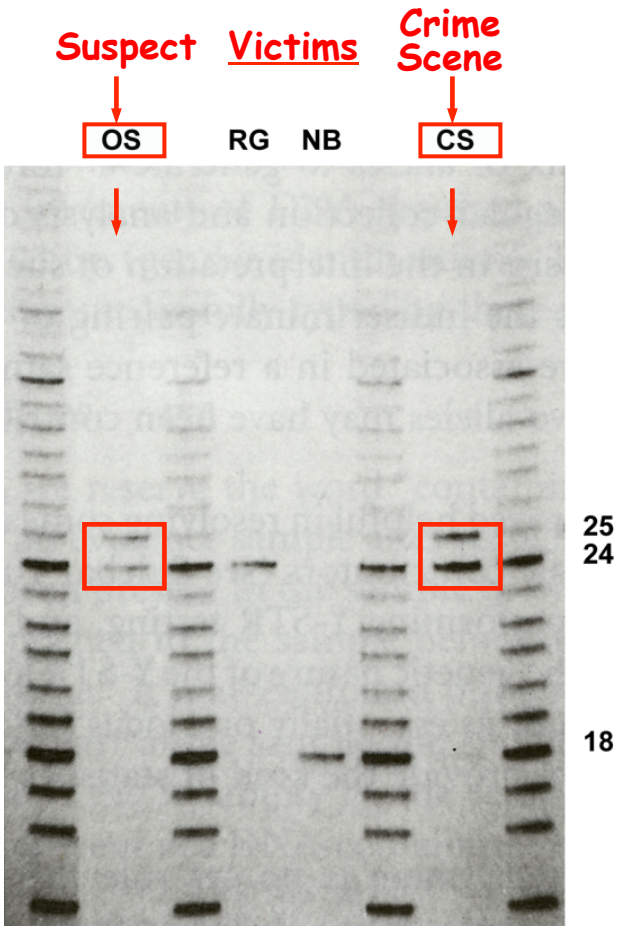
Repeat Steps or Cycle

2^n Molecules of DNA where n = Number of Cycles

Diagnostic For Amplified DNA Sequence → **DNA Fragments All The Same Size Primer-Sequence-Primer**

Using PCR in Crime Scenes



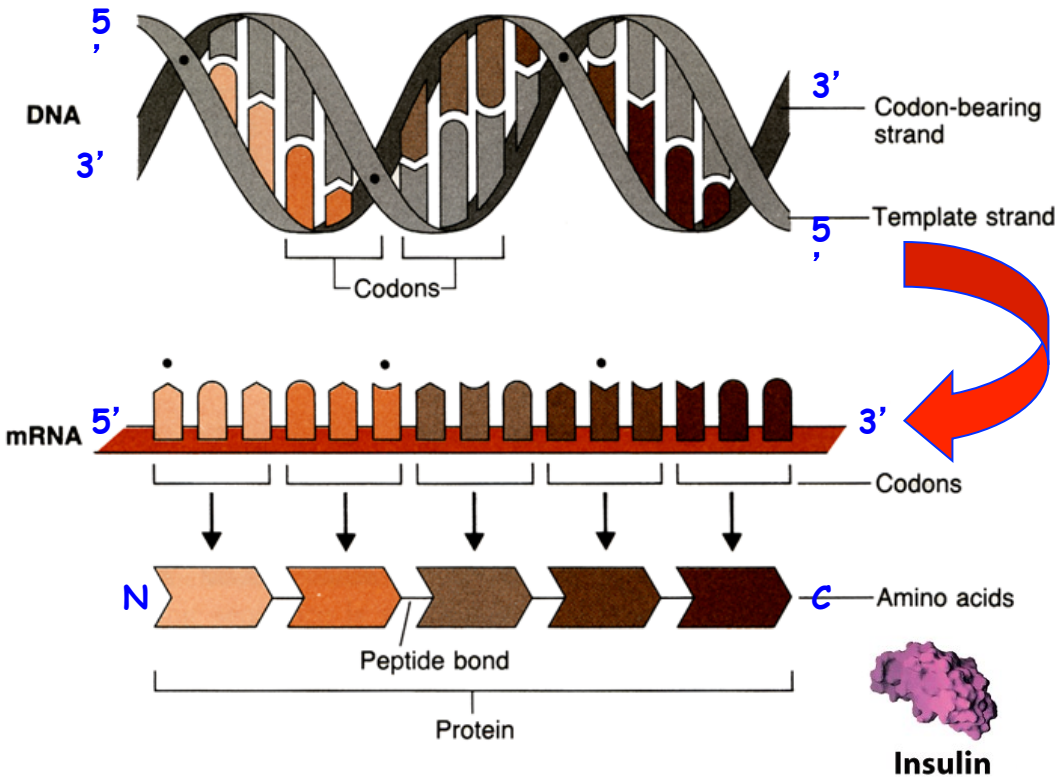


OS = Suspect
CS = Crime Scene
RG & NB = Victims

"Match" What is Probability That This Will Occur by Chance?

DNA Doesn't "Lie" !!

How Does A Gene Lead To A Phenotype?



1. mRNA Synthesized by Transcription

   a. Complementary to Transcribed, Non-Sense Strand

   b. Same Sequence As Sense Strand

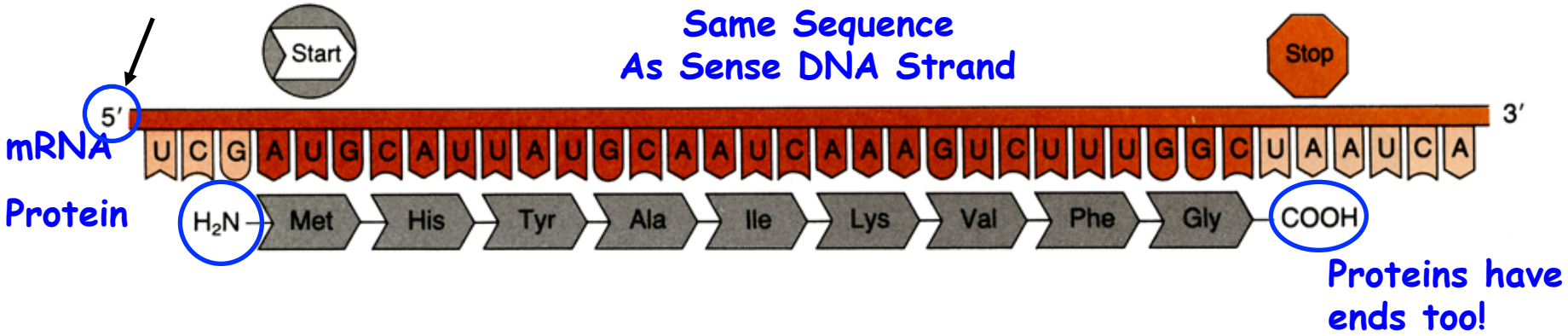
2. mRNA Translated into Protein by Translation of The Genetic Code

   a. Genetic Code on mRNA Translated to Protein Sequence

∴ Sequence of Gene
↓
Sequence of mRNA
↓
Sequence of Protein

Know Sequence
Know Protein
→ Engineer New Protein

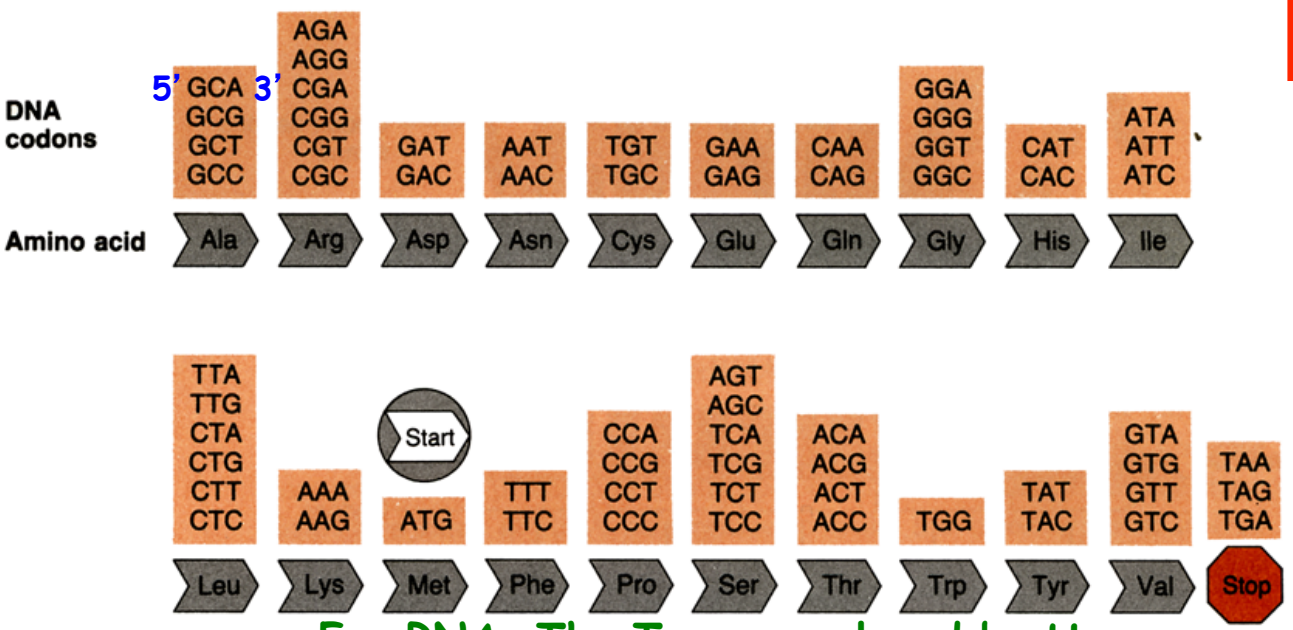
Genetic Code Allows The Sequence of Nucleotides in mRNA/ sense strand of Gene to be Translated into Sequence of Amino Acids in Proteins



Note: Sequence in mRNA (= Sense Gene Strand) is translated 5'→3' (= beginning of sense strand to end) & Protein made in N→C direction therefore order Nts in gene = order amino acid in protein!

The Genetic Code is Universal!

How Know?



For RNA, The Ts are replaced by Us.

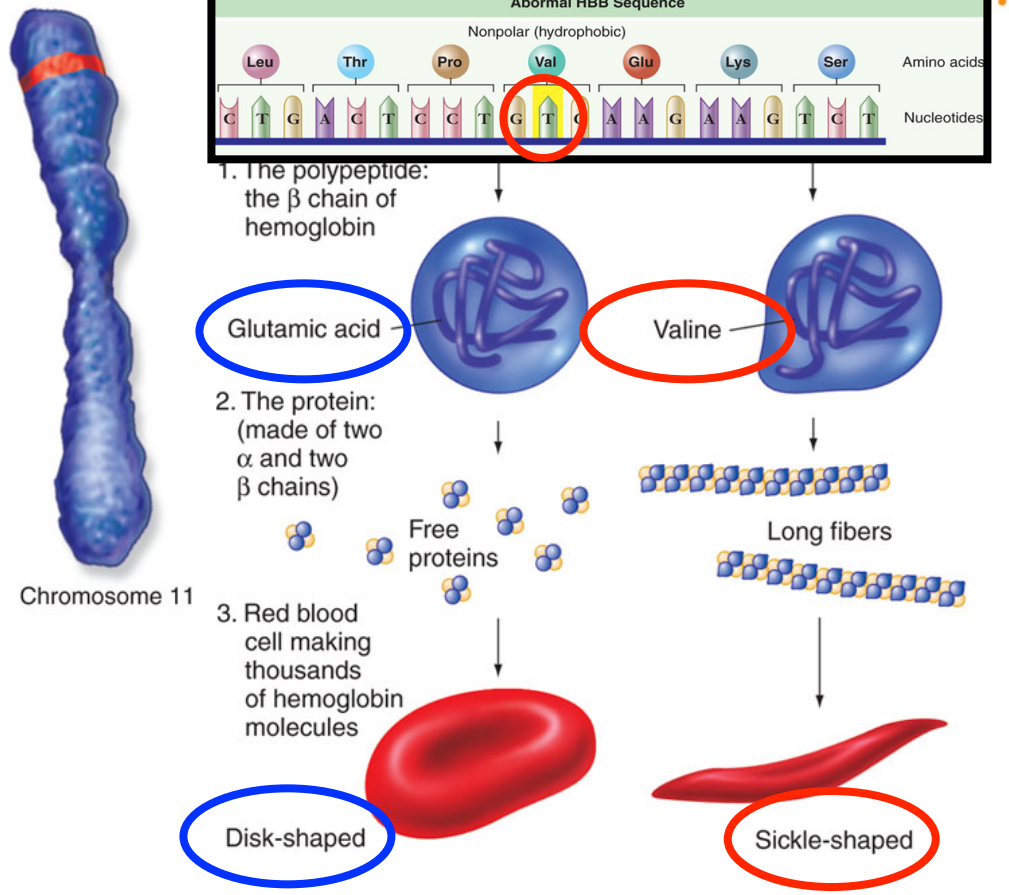
1. Universal
2. Triplet
3. Punctuation
4. Degenerate

Know Sequence of Gene-Know Sequence of Protein Using Genetic Code

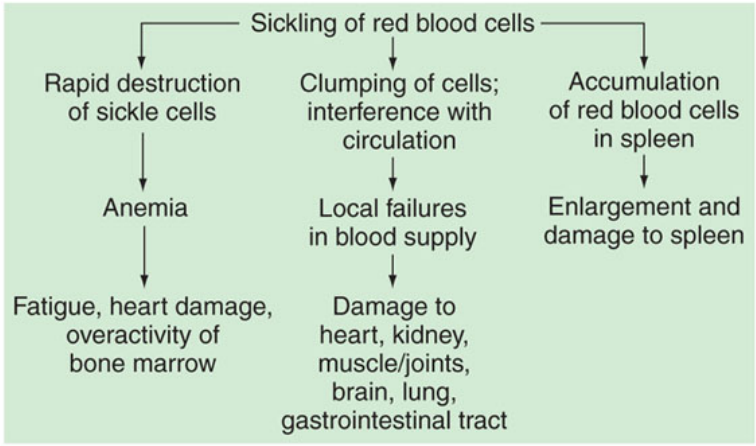
Big Implication For Genetic Engineering! Can Make Genes, Genomes & Specify Proteins Wanted! Can Express Genes From One Organism in Another!

Design An Experiment to Show Code is Universal!

Human Genetic Disorders Occur As A Result of Mutations That Change the Genetic Code



(b) Sickle-cell anemia is pleiotrophic



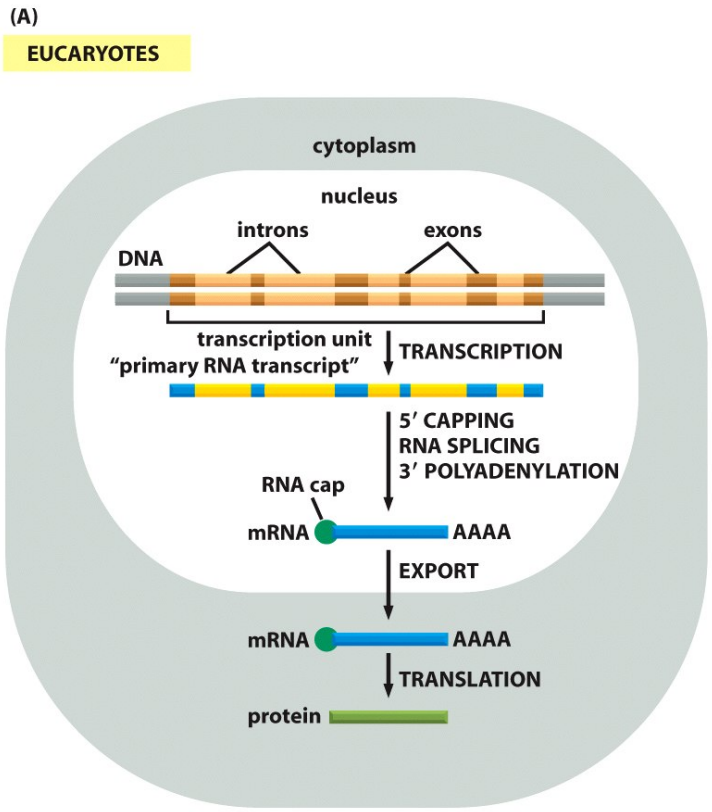
(c) β-chain substitutions/variants Note: Different Alleles!

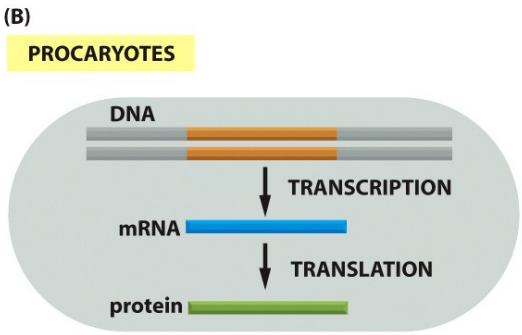
Amino-acid position

	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

Eukaryotic and Prokaryotic Gene Expression Processes Differ Slightly

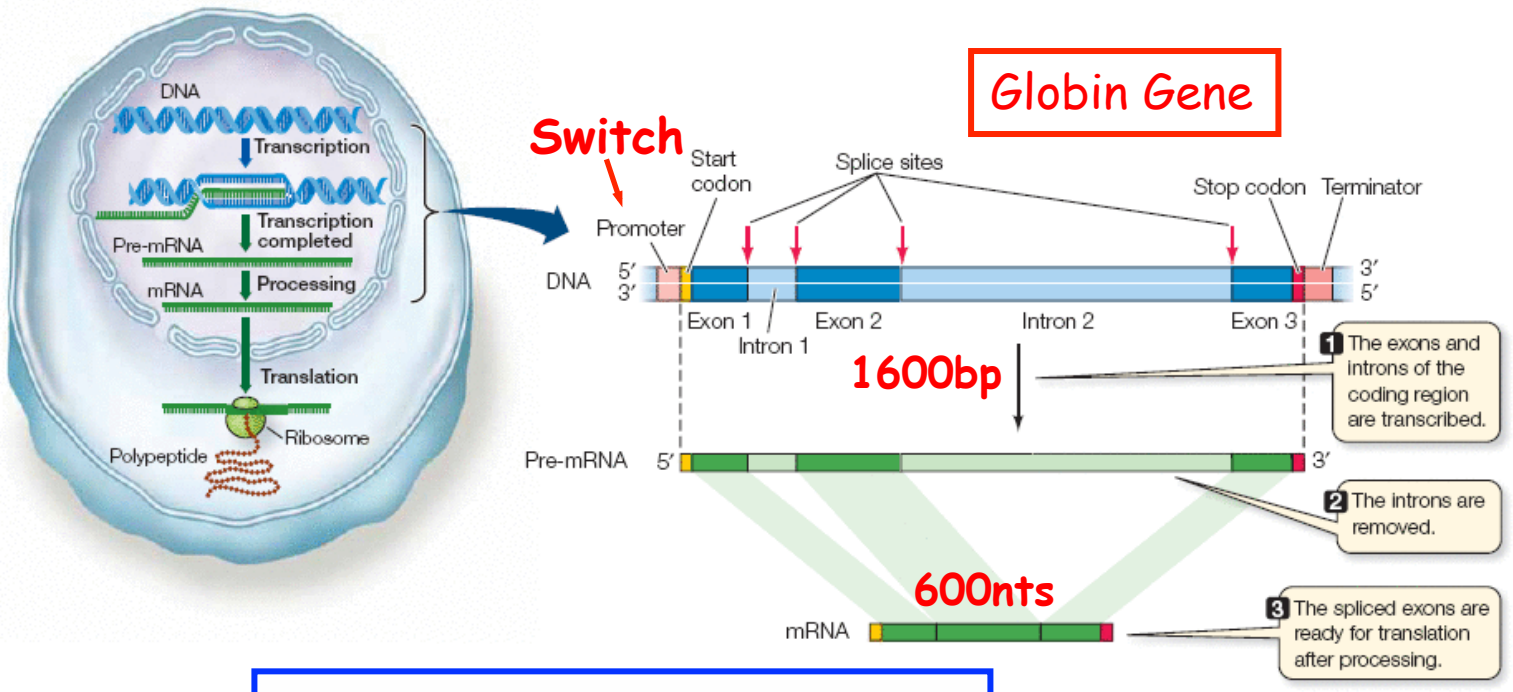




Genes Differ
Switches Differ
Genetic Code the Same
General Processes Same
Eukaryotic Gene Have Introns & Non-Coding Regions in Gene!

Eukaryotic Cells Must Remove Non-Coding Region of RNA Before Genetic Code Can Be Translated Continuously!

RNA Splicing- Removing Non-Coding Sequences From Primary Transcripts & Generating Functional mRNAs



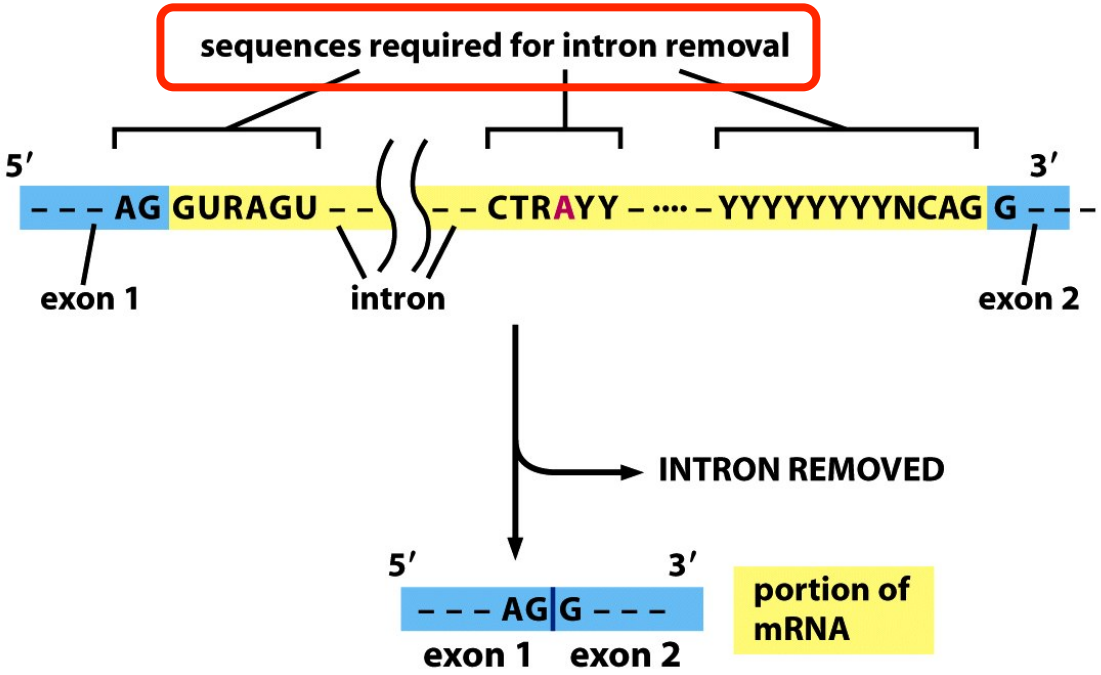
Mutations→ Blood Disorders
Where can these occur?

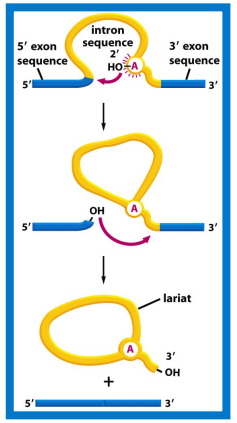
Mutations Can Occur in Coding Region, Switch, & RNA Splice Sites

→ Mutant Phenotype

Implications For Engineering Eukaryotic Gene in Bacterial Cell For Expression?

Yo! It's In The Sequences!

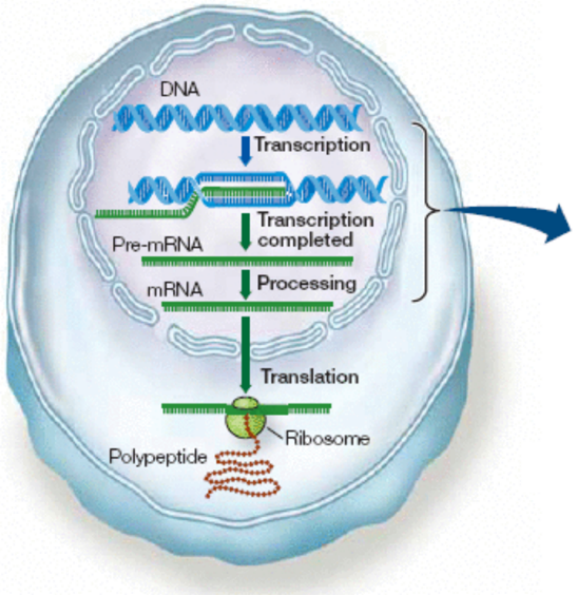




Specific Sequences Required For RNA Splicing!

What Happens If These Sequences Are Mutated in A Gene?

Alternative Splicing- One Gene → Several mRNAs & Proteins



Gene Activity in Varity of Cells, But…..!!!

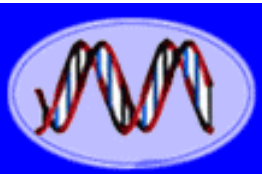
5 Different mRNAs!

Different mRNA = Different Proteins = Different Functions!

Implication- Human Genome Has Only 25,000 Genes But Can Give Rise to Many More Proteins which Are Responsible For Producing the Phenotype

Reason Why Human Genome Can Contain Same Number of Genes as Fly and Plant Genomes!!

Implications for Genetic Engineering? Use Specific cDNA!



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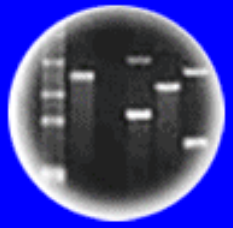

DNA Fingerprinting

Cloning: Ethical Issues
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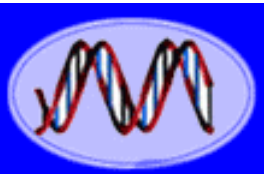
Implications For "Yo - Its in The DNA!!"

Modular Organization of Sequences

1. DNA Replication
   - Ori
2. Transcription
   - Switch/Regulator
   - Terminator
3. Processing of RNA (Eukaryotes)
   - Splicing Sites
4. Translation
   - Start
   - Stop
   - Genetic Code/Codons
5. Coding Sequence
   - Genetic Code

Modules → Anything You Want To Do Using Genetic Engineering!



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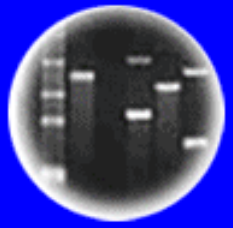

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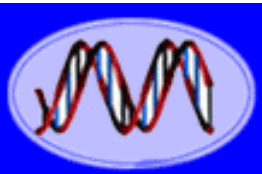
Engineering Genes Requires:

1. The Gene & Its DNA Sequences
2. A Roadmap of Where Coding Sequence & all Switches Located (Sequence, Restriction Site Map)
3. Transcription Start And Stop Switches
4. Coding Region of Gene (genetic code part)
5. Translation Start And Stop Switches
6. Kingdom-Specific Switches/ Signals

Note: The General Process of Gene→Protein is the same in ALL organisms, but the Specific Switches & Enzymes (e.g., RNA Polymerase) are Kingdom Specific

Bacteria Transcription On Switch + Human Insulin Coding Sequence + Bacteria Transcription Off Switch

→ Human Insulin in Bacteria!!



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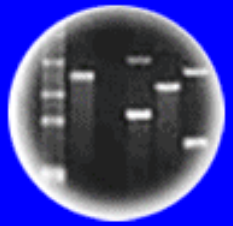

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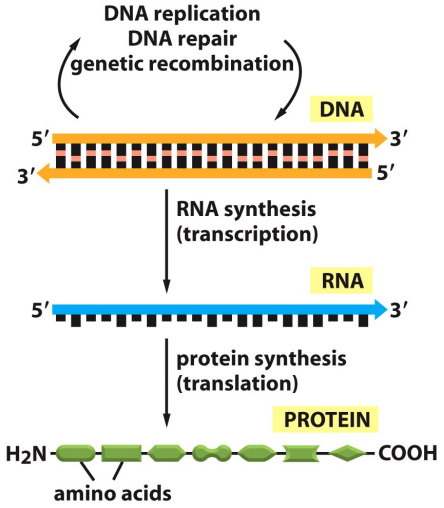
How Do Genes Work & What Are Genes In Context of…

Thinking About The Consequences of GMOs



1. What is a Gene?
2. What is the Anatomy of a gene?
3. How Does the Gene Replicate?
4. How Does the Gene Direct Synthesis of a Protein?
5. Does the Gene Work Independently of other Genes?
6. What is the Sequence & Structure of the Protein?
7. How does it work in cell?
8. Does the Protein Structure imply any Potential "Harm"?
9. Does the Gene Change the organism? Fitness?

Need Science-Based Questions & Science-Based Solutions-NOT OPINIONS!

There's NO HOCUS POCUS all hypothesis are testable!!

"Behind" All Traits!

Same Processes!

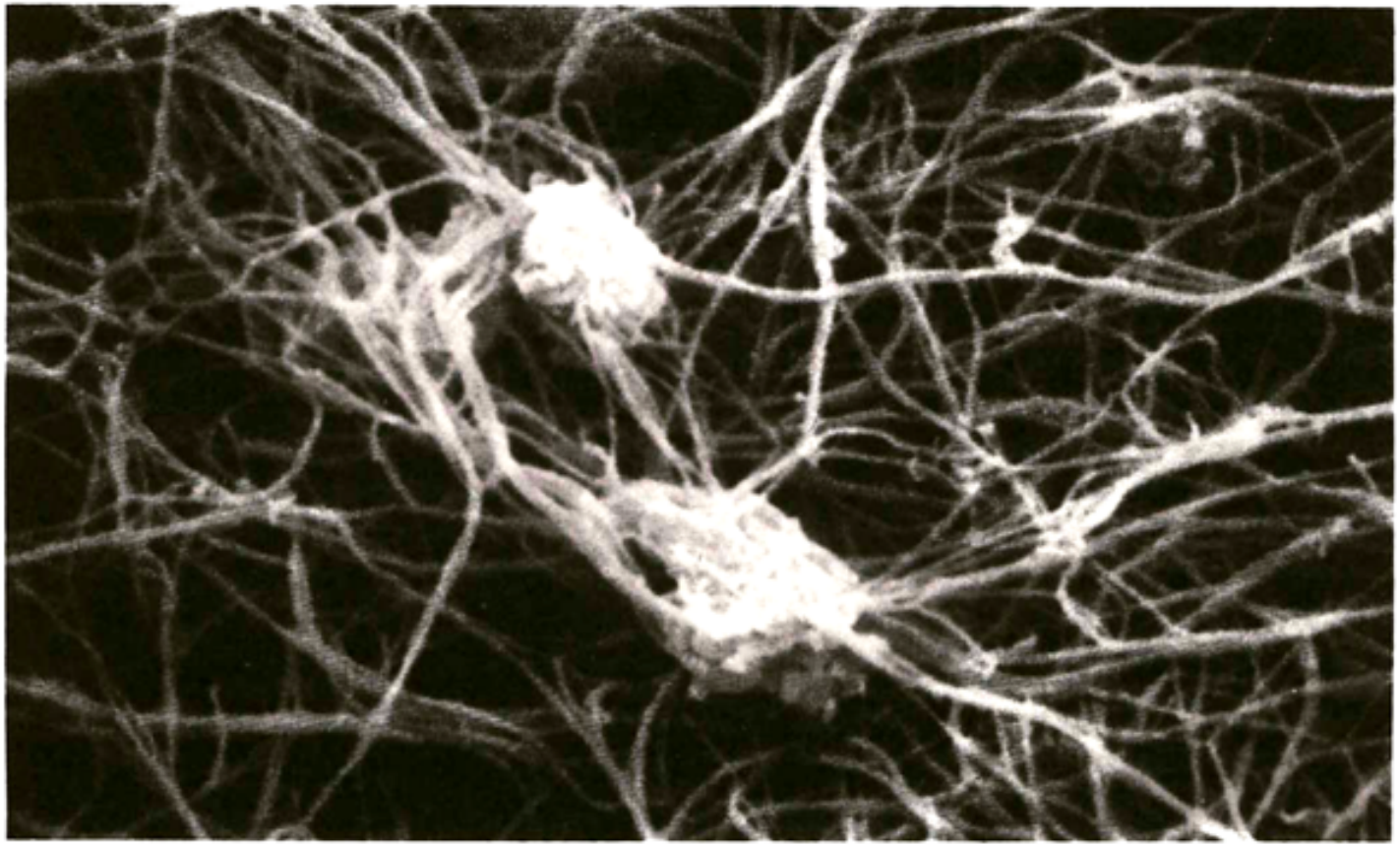
How To Isolate A Human Disease Gene
The Factor VIII Story

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

A "Review of Genetic Engineering"

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.

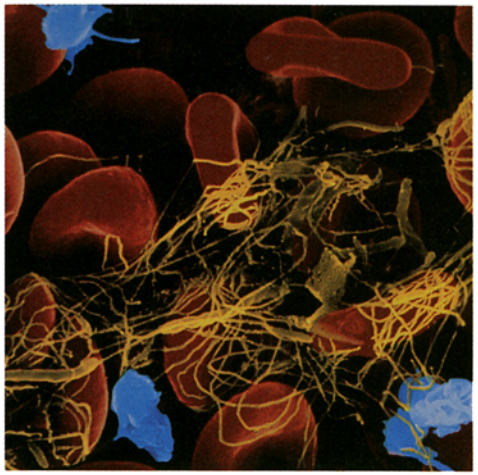
A Case Study of Cloning Genes and mRNAs

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

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

Old Testament-Circumcisions

Royal Family-Europe



First Reference to Hemophilia is in the Old Testament

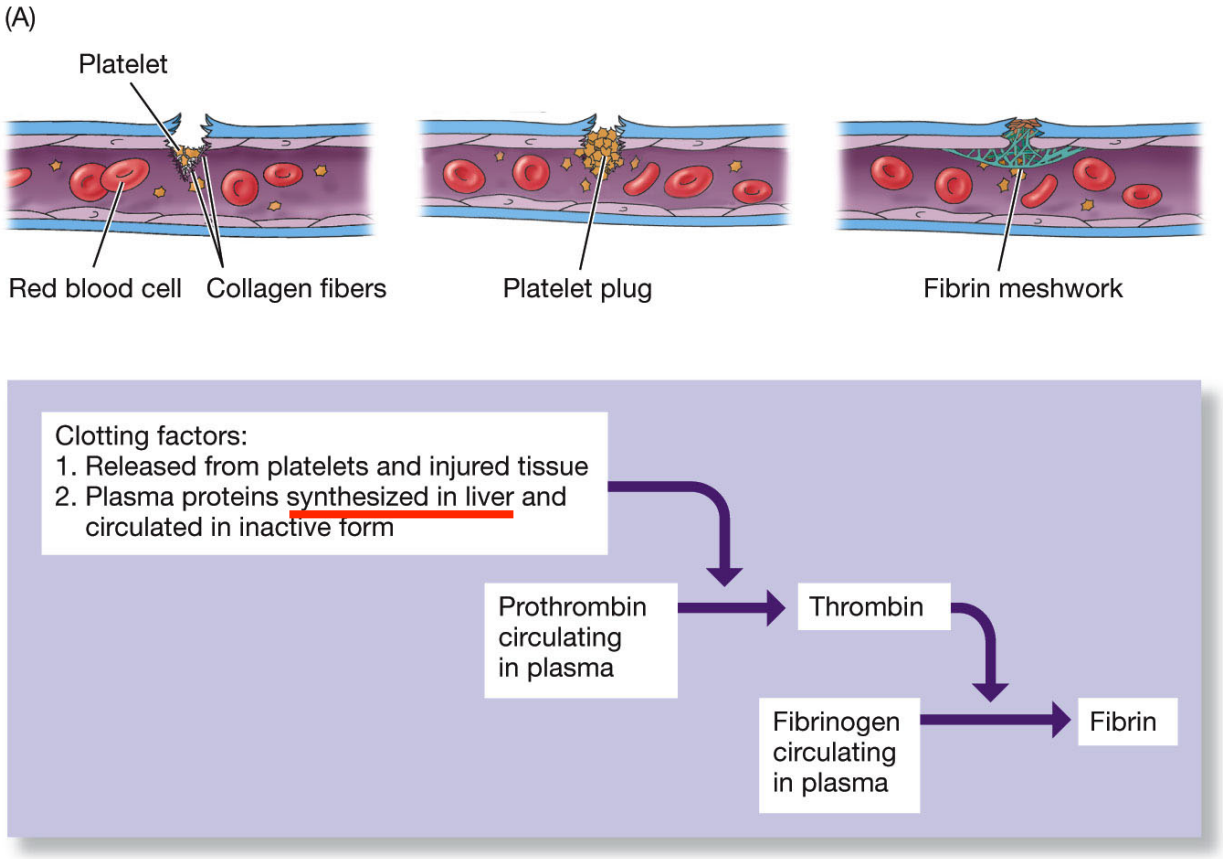
Genesis 17:10-14

'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.'

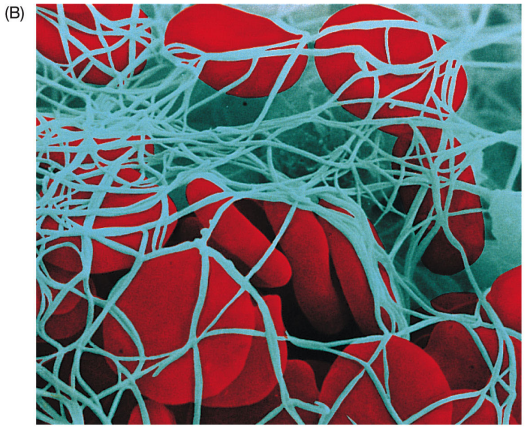
The Talmud also makes reference to families in whom children have died as a result of circumcision (Babylonian Talmud, Chapter *Yevamoth* p64b) [6]. Should a mother lose two children or should two sisters lose a child each after circumcision, subsequent children of the woman, the two sisters or of any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



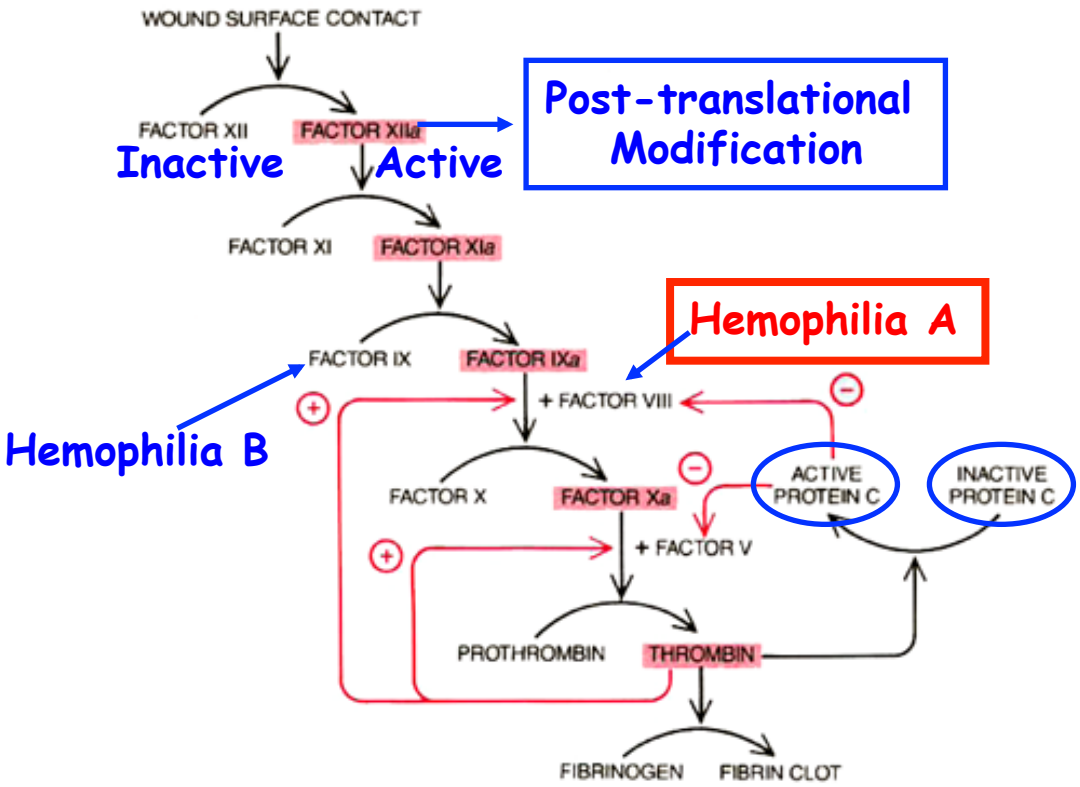
LIFE 8e, Figure 49.10 (Part 1)



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.

ATryn® 2009

Anti-Thrombin??

Cascade

Anti-Thrombin Deficiency (At-III) genetic disease

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!

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

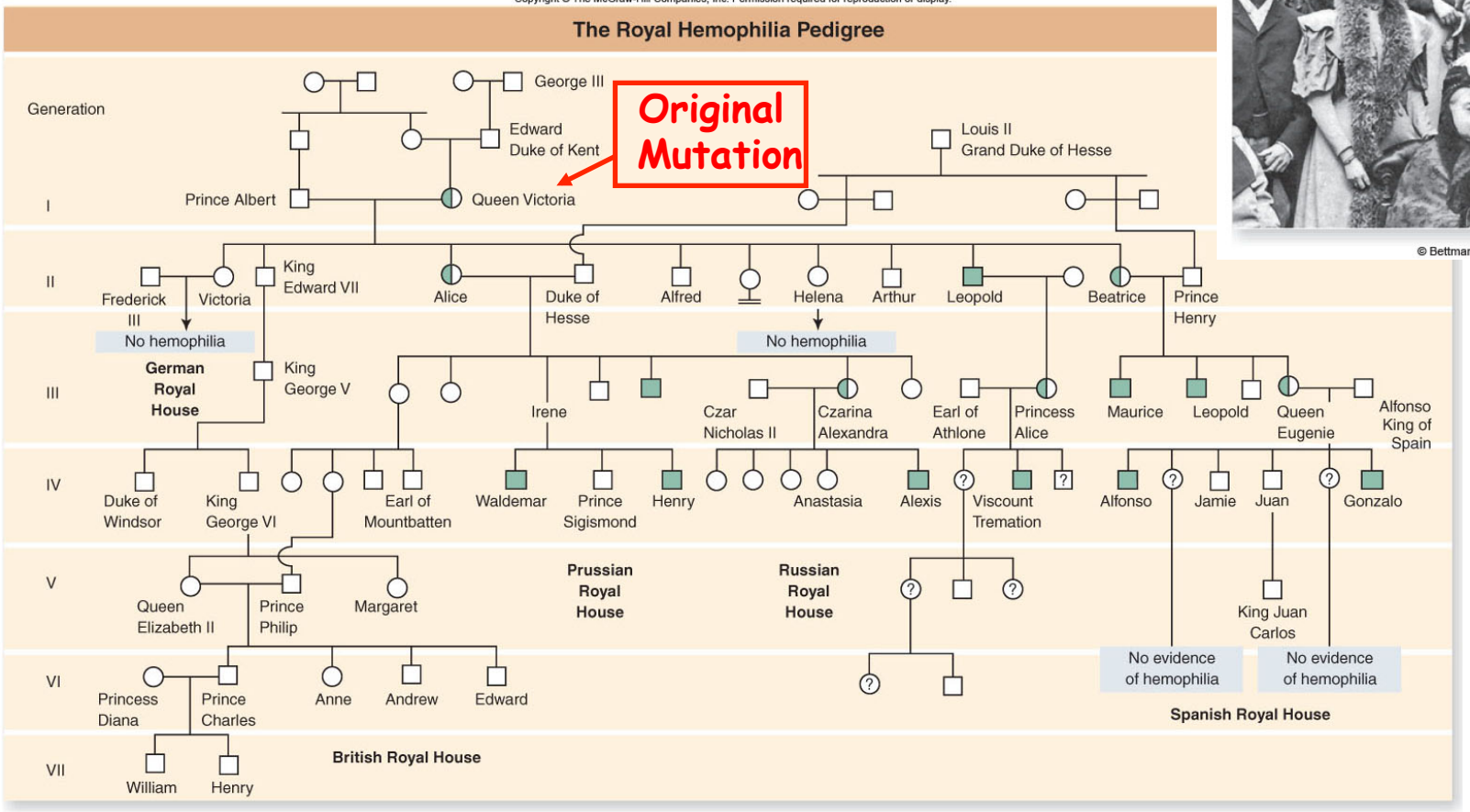
Hemophilia A	Defective Factor VIII Gene	1/10,000 males
Hemophilia B	Defective Factor IX Gene	1/30,000 males

Hypothesis For High Frequency in Males?

Both Genes On X-Chromosome ♀→♂'s

Hemophilia A and B Genes (Traits) Are Sex Linked

© Bettmann/Corbis



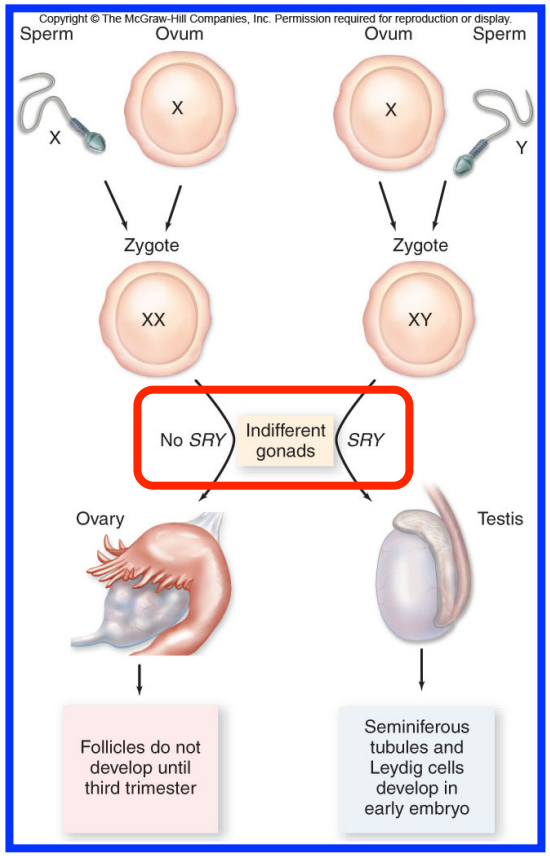
Note: 1. Males Obtain Detective Gene From Mothers

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

Human X and Y Chromosomes Control Gender

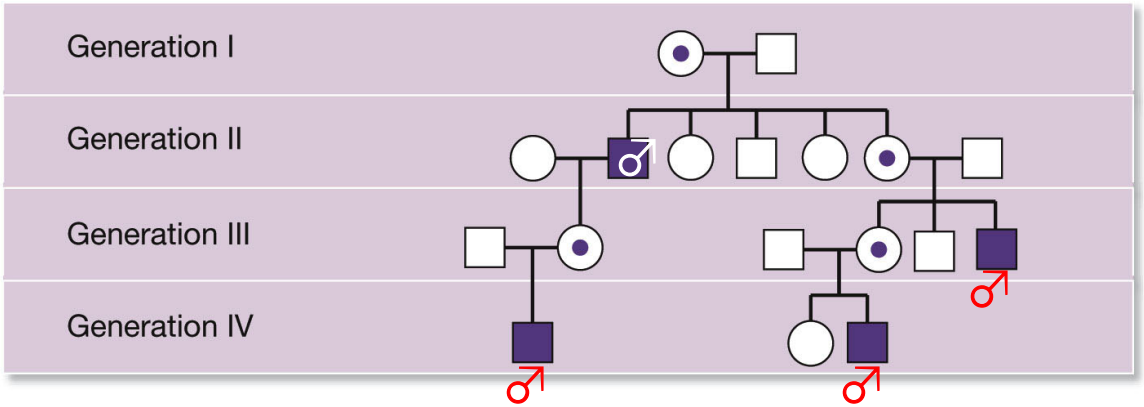
TABLE 13.1 Sex Determination in Some Organisms

	Female	Male
Humans, *Drosophila*	XX	XY
Birds	ZW	ZZ
Grasshoppers	XX	XO
Honeybees	Diploid	Haploid

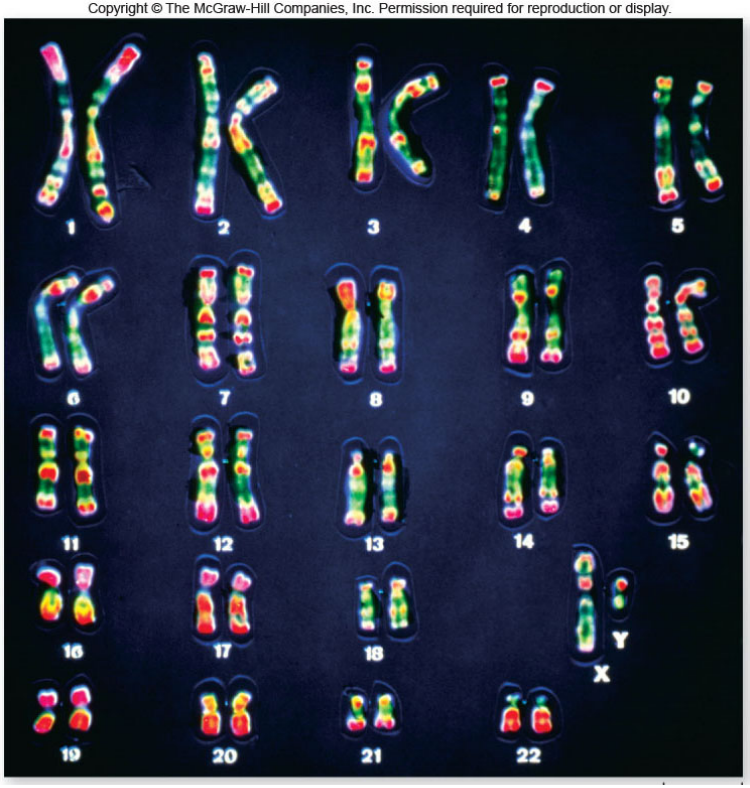


Sex-Linked Inheritance Pattern Follows X-Chromosome Distribution To Gametes

Female who carries gene for phenotype of interest on one X chromosome



Note: 1/2 Sons of Carrier Mothers Have the Disease!



Human Diploid Karyotype

A Male XY

Hemophilia A Disease Alleles Can Arise Because of:

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

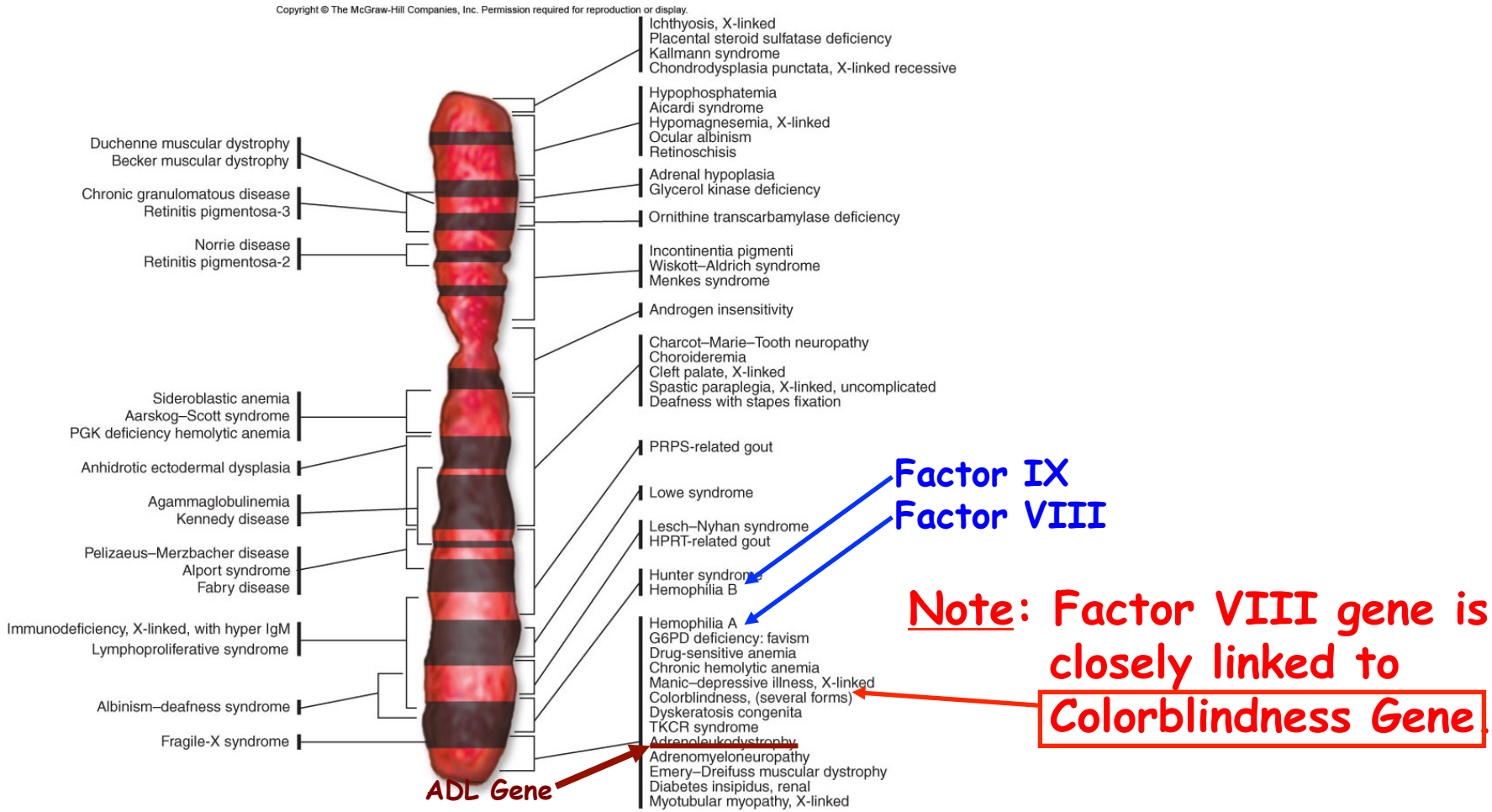
The Hemophilia A Disease Allele Resides at the Same X-Chromosome Locus as the Normal (Wild Type) Hemophilia A Gene:

a. Yes
b. No

An XY Individual is Always a Male:

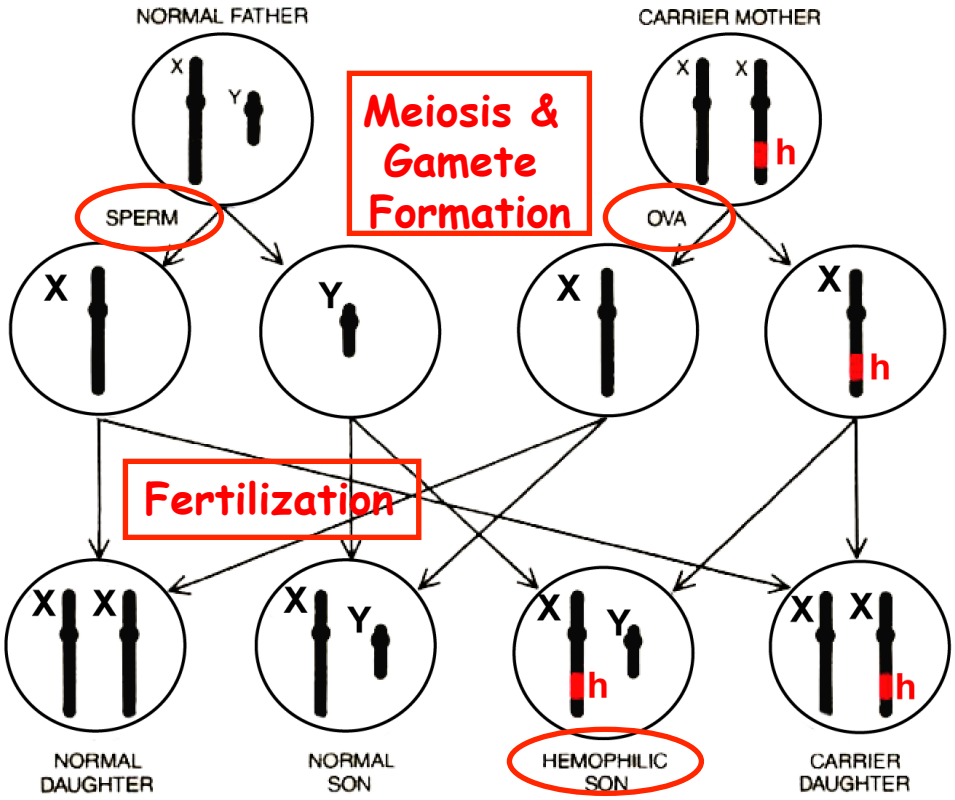
a. Yes
b. No

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



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

Hemophilia A and B Inheritance



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

Parents 2n

Meiosis

Gametes n

Fertilization

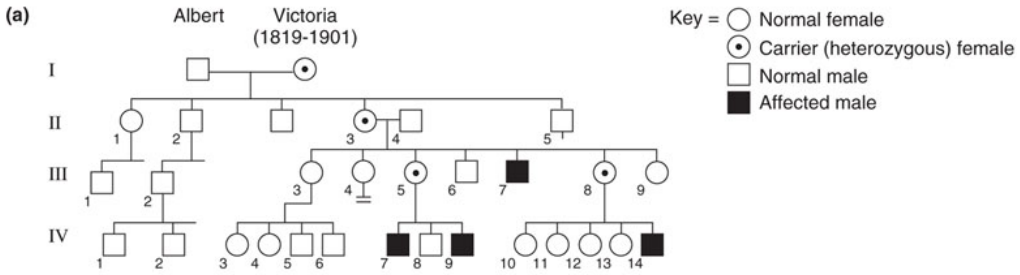
Children 2n

Sex-Linked Inheritance

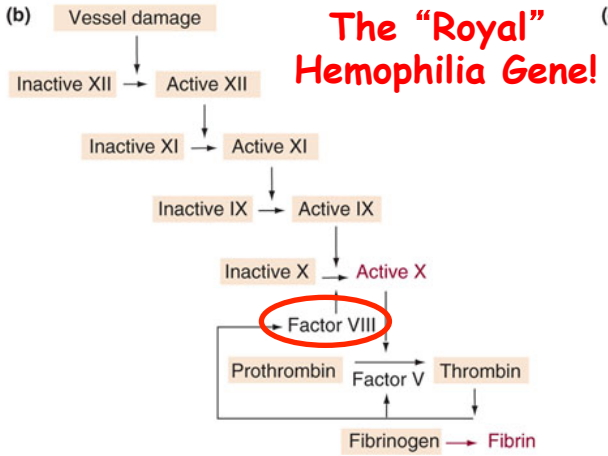
♀ Carriers → 1/2 Sons + No Daughters!

Only One X-Chromosome is ♂

From Disease to Gene- Using Protein to Identify Factor VIII Gene



Note: Pattern of Inheritance

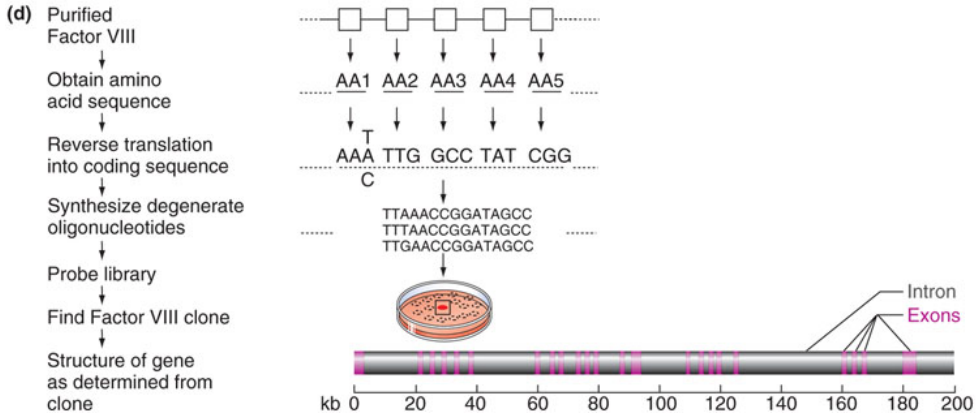


The "Royal" Hemophilia Gene!

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

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

Marker



Key Concept

⬇

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

What Was Known About Factor VIII Before Gene Cloned?

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

∴ How to go From Protein to Gene

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

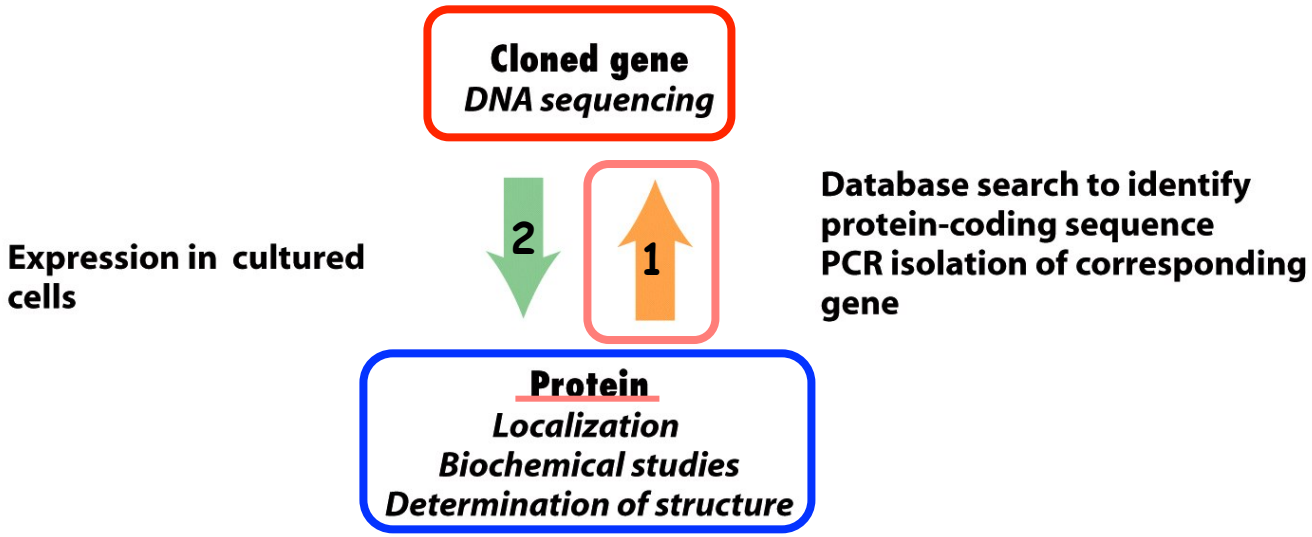


Figure 5-1
Molecular Cell Biology, Sixth Edition

∴ 1. Protein → Gene → Drug

or

2. Gene → Protein Using Sequencing and Genetic Code

Genomics

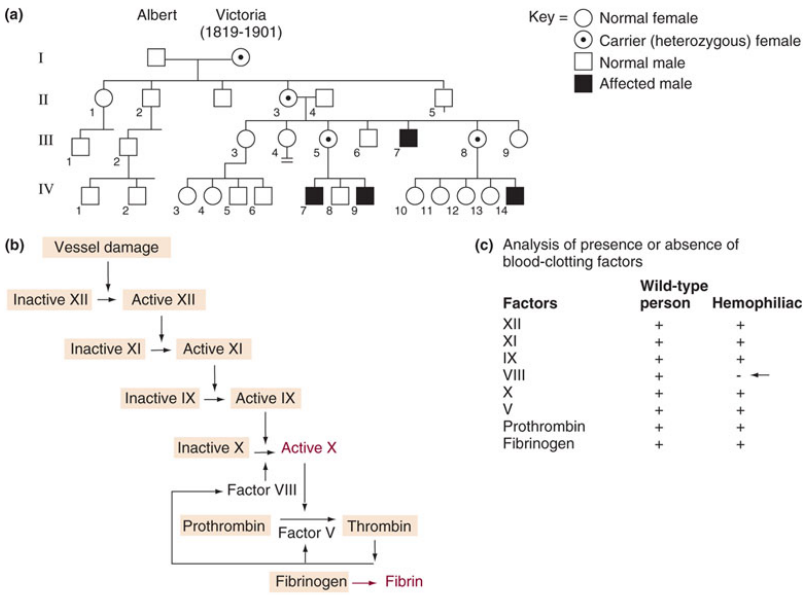
GenBank

2010 → Just Sequence Everything + Identify Protein-GenBank Huge

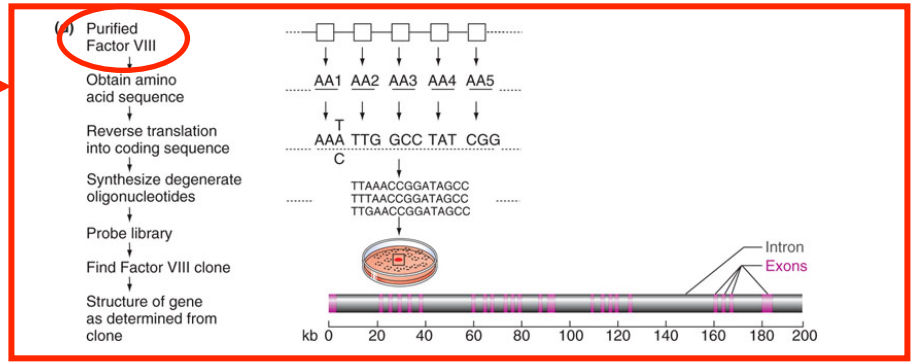
The Problem

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

Early 1980's



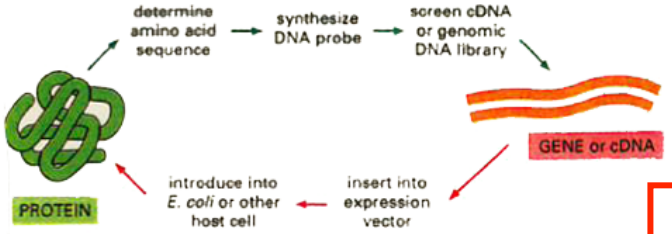
Key:
Protein Sequence Known



How Find Gene & cDNA?

Protein → Gene → mRNA → Drug !

Factor VIII Protein → Gene Using Genome Library (Week One Discussion)



FROM PROTEIN TO GENE

Isolate protein on the basis of its molecular function (e.g., enzymatic or hormonal activity)

↓

Determine partial amino acid sequence of the protein

↓

Synthesize oligonucleotides that correspond to portions of the amino acid sequence

↓

Use oligonucleotides as probes to select cDNA or genomic clone encoding the protein from library

↓

Sequence isolated gene

FROM GENE TO PROTEIN

Isolate genomic clone corresponding to an altered trait in mutants (e.g., nutritional auxotrophy, inherited disease, developmental defect)

↓

Use genomic DNA to isolate a cDNA for the mRNA encoded by the gene

↓

Sequence the cDNA to deduce amino acid sequence of the encoded protein

↓

Compare deduced amino acid sequence with that of known proteins to gain insight into function of the protein

↓

Use expression vector to produce the encoded protein

Pure Protein
↓
Gene From Library

Gene Clone
↓
cDNA
↓
Protein in Expression Vector

Gradually Fill GenBank to Identity by Direct Sequencing

How To Screen Library

Genome Library

1. Sequence → Database
2. Probe from cDNA/ Switch
3. Probe from pure mRNA
4. Synthetic Probe from translated DNA sequence + Genetic Code

cDNA Library

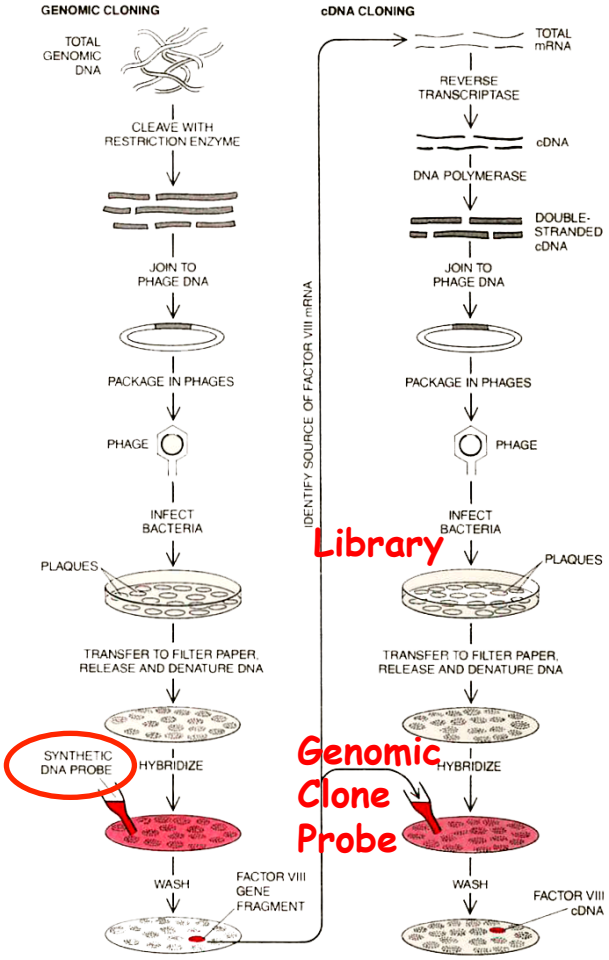
1. Sequence Database
3. Pure mRNA probe
5. Synthetic Probe from translated protein sequence/genetic code
4. Exon Probe
5. Antibody probe using expression vector

Can't use Antibody- Don't know where gene active
∴ Can't Make cDNA Library

Steps Required to Clone Factor VIII Gene and cDNA

Gene

1. Make Genome Library Because Factor VIII Gene in Genome!
3. Purify Protein from Blood- that's where it works (wasn't known where made)
5. Reverse Translate using the genetic code a portion of the protein sequence
7. 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
3. How know what mRNA to use to make cDNA library?
5. Use gene probe to probe RNA blots containing mRNA from all major organs (liver, kidney, blood, etc.)
7. 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!

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

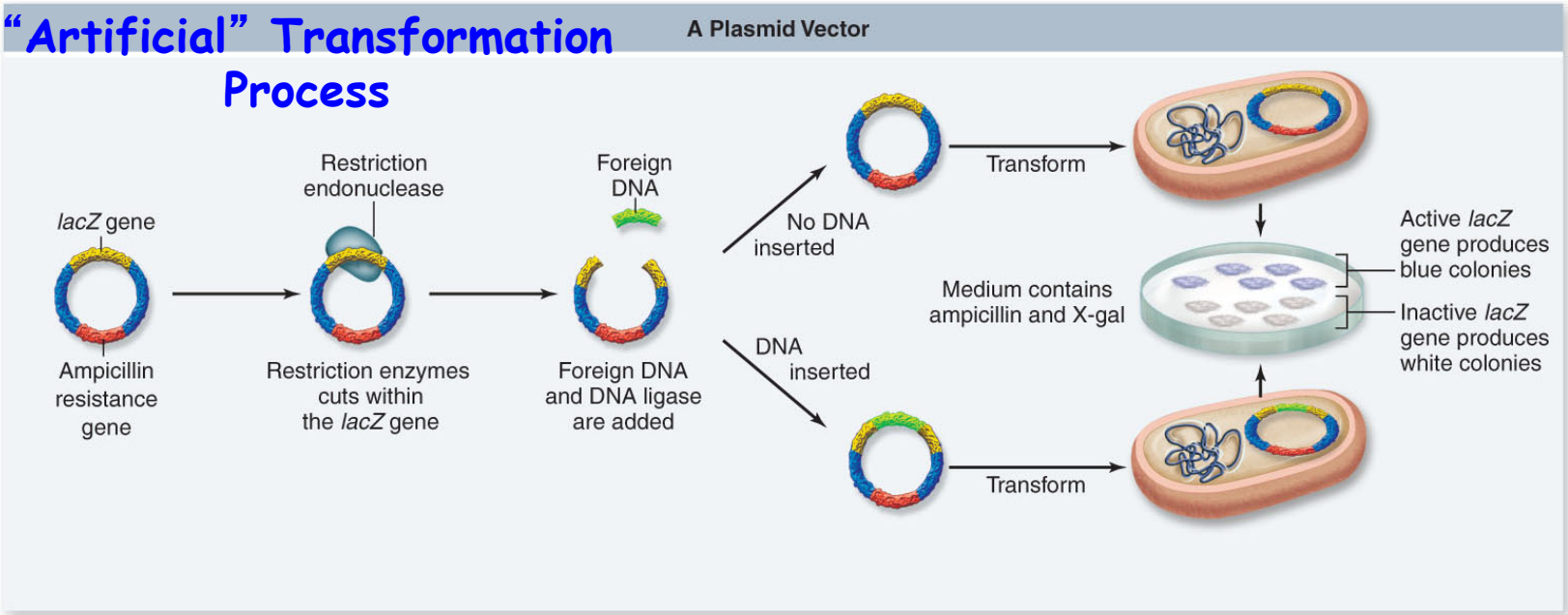
Vector Type	Maximum Insert Size (kb)	Applications	Limitations
Bacterial plasmid vectors (circular)	~6–12	DNA cloning, protein expression, subcloning, direct sequencing of insert	Restricted insert size; limited expression of proteins; copy number problems; replication restricted to bacteria
	DNA	DNA	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

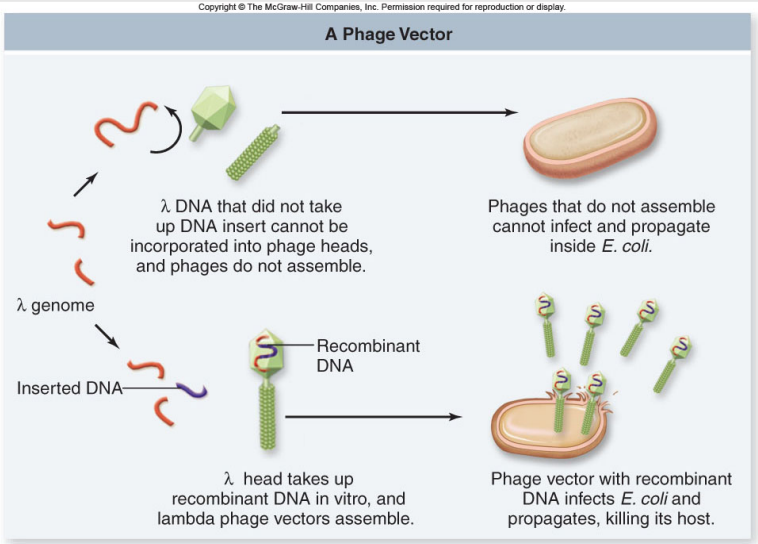
Plasmid vs. Bacteriophage Vectors for Cloning DNA Fragments

"Artificial" Transformation Process



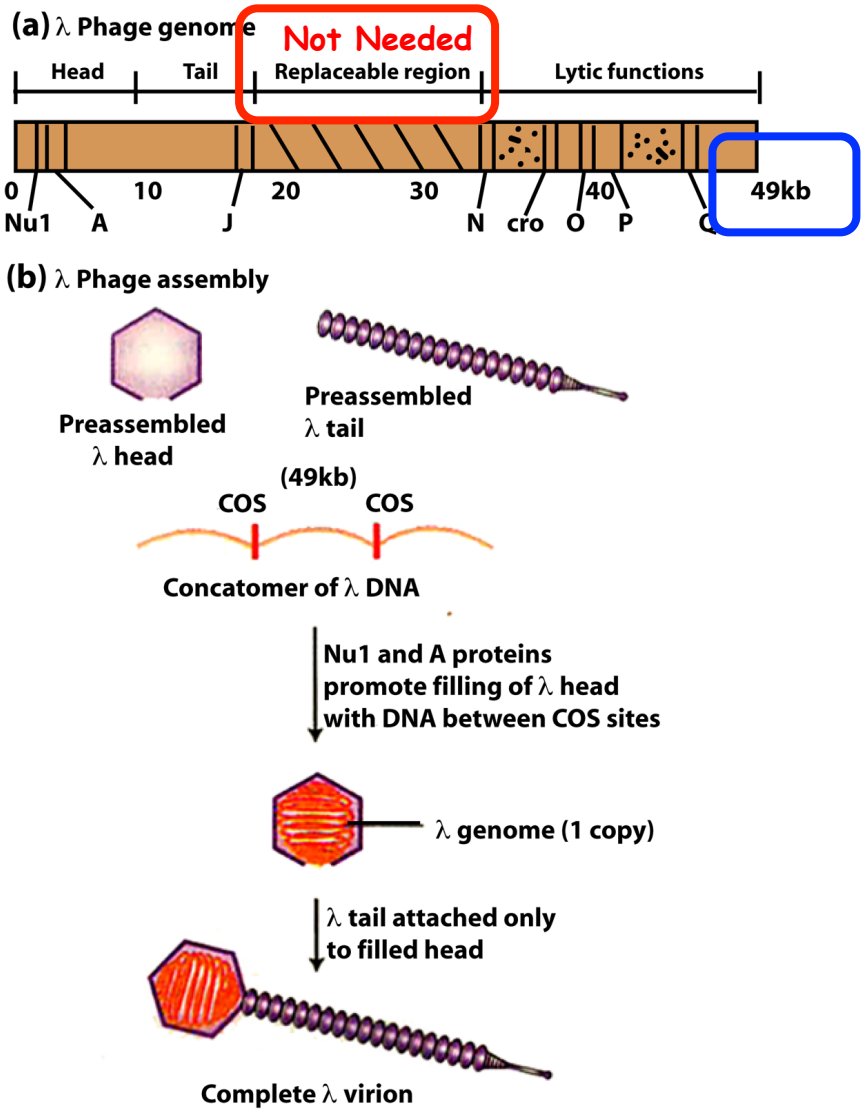
a.

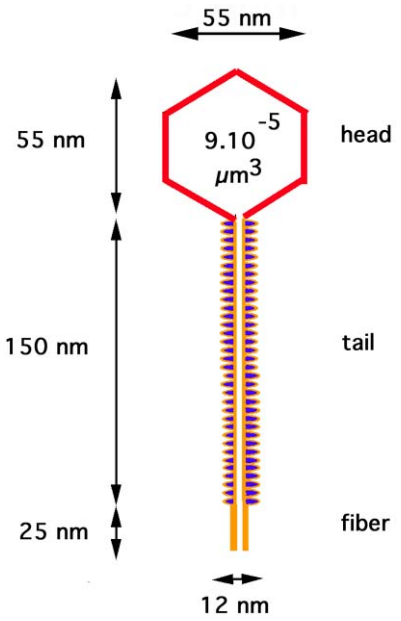
Natural Infection Process



b.

Structure of the λ Phage and Its Genome





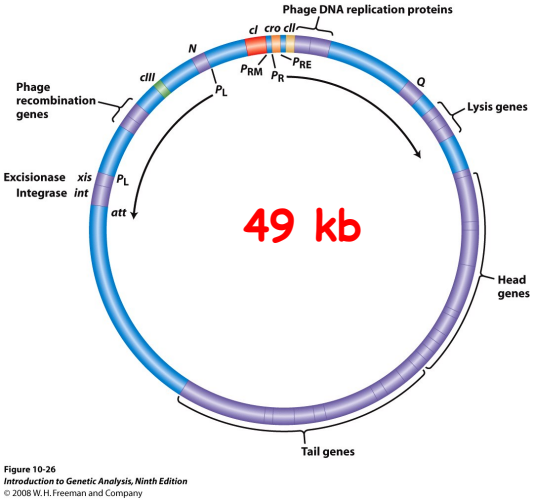
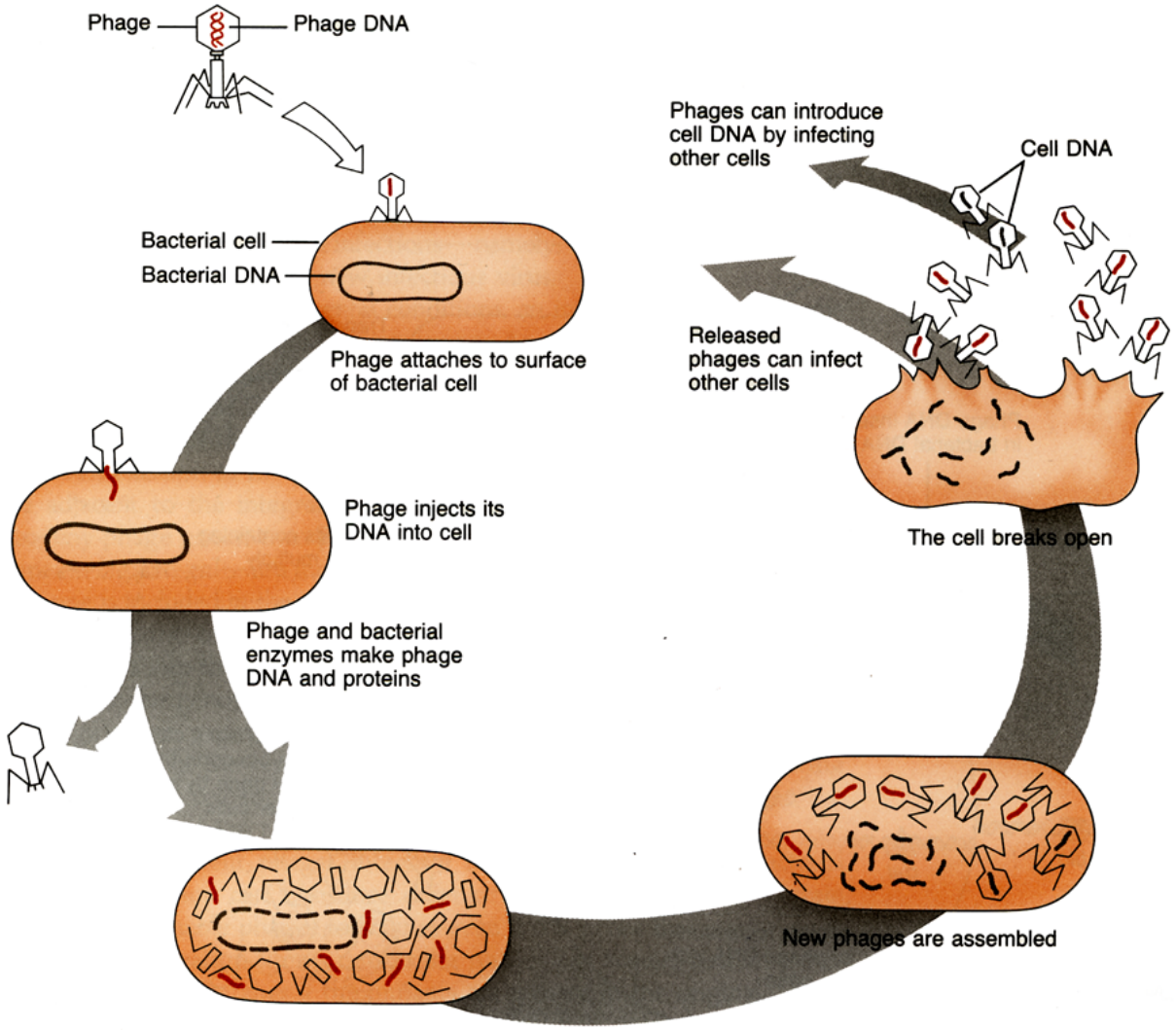


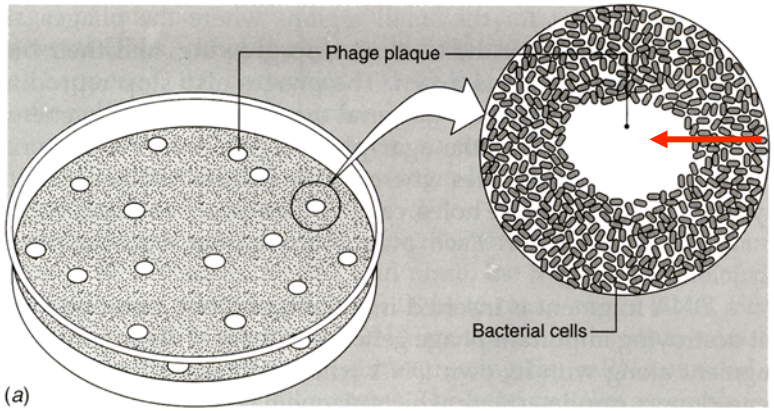
Figure 10-26
Introduction to Genetic Analysis, Ninth Edition

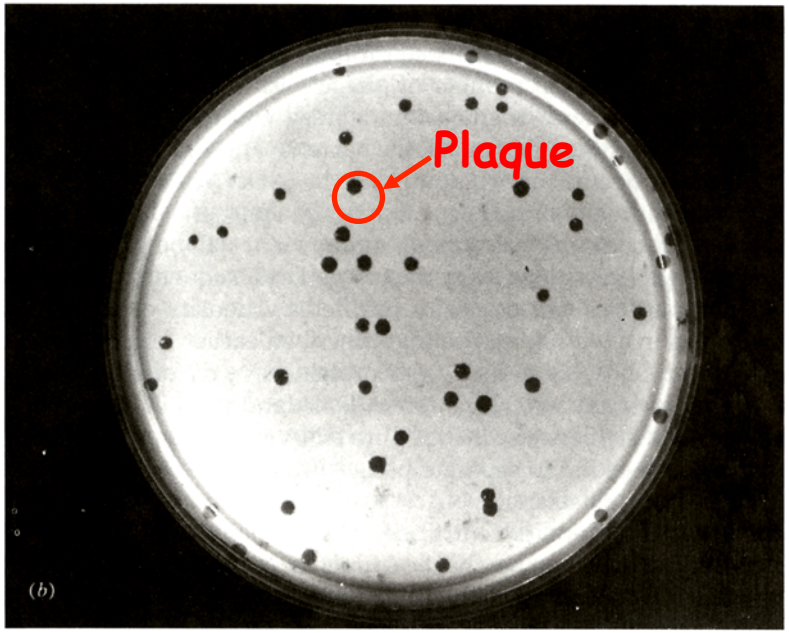
One of First Genome Sequences

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



Lysed Cells Can Be Seen as Clear Plaques on Agar Plates





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!

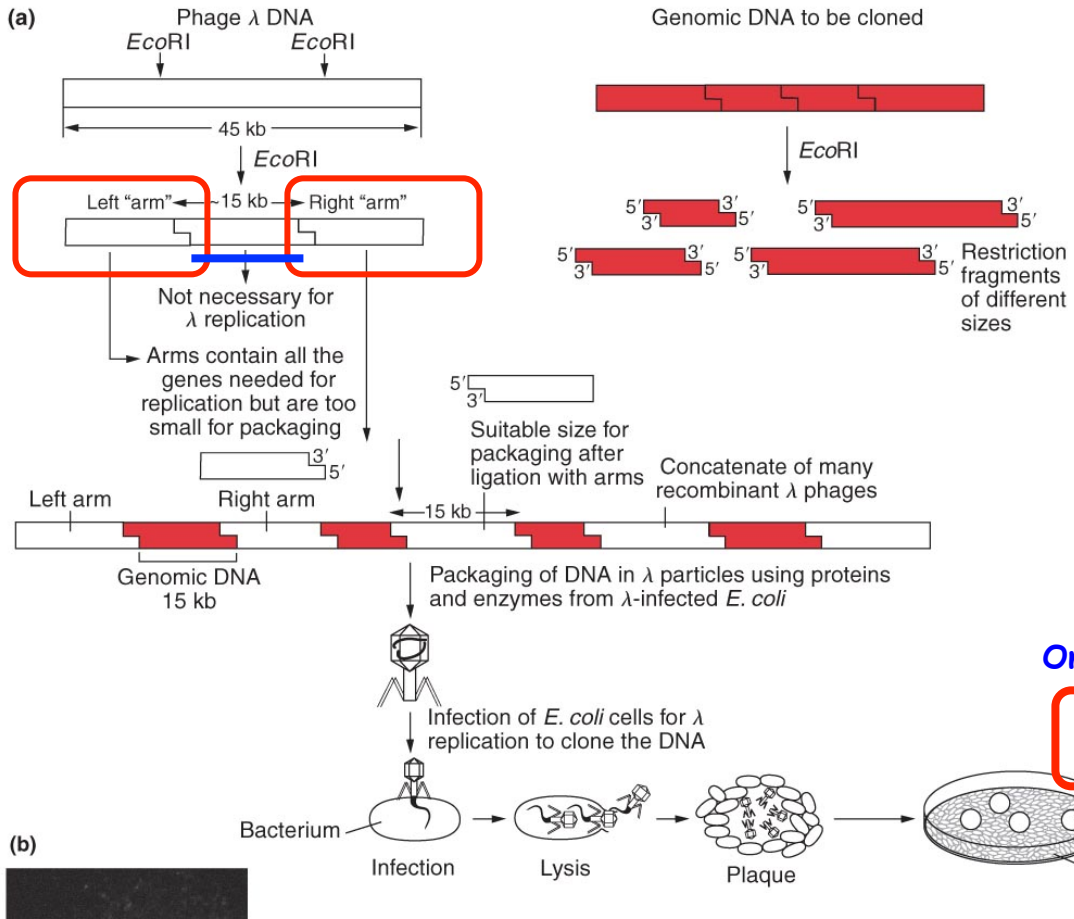
   ∴ Make Recombinant Viruses in the Lab!

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!

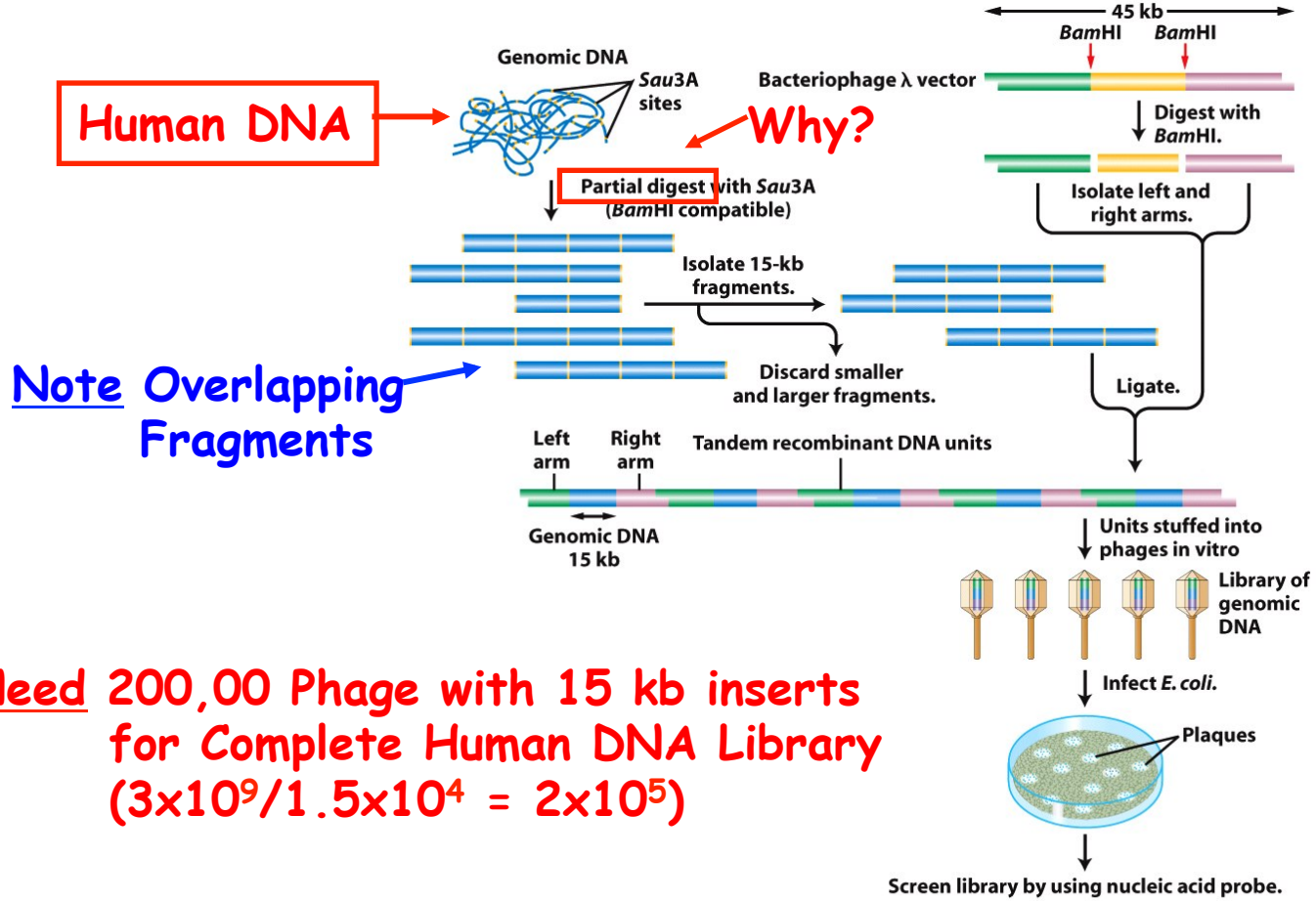
Using a Bacterial Virus To Clone the Human Genome



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



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

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:

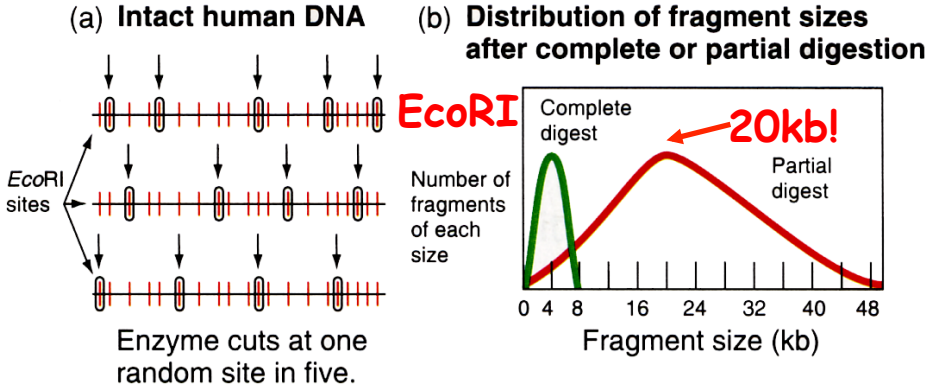
a. Yes
b. No

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

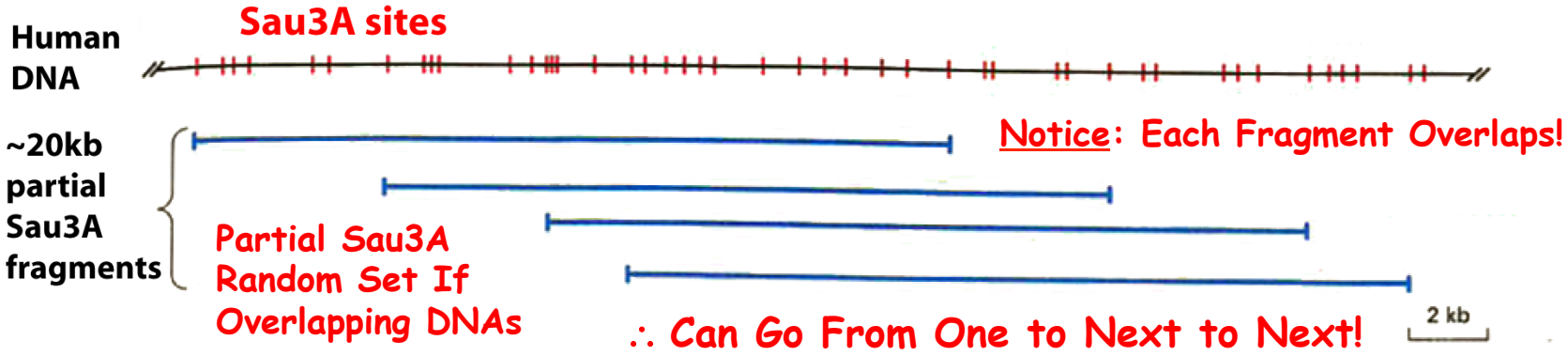
1. Complete Digestion Produces fragments that are too small to clone in λ virus (need 20Kb)
3. Complete Digestion would create huge genome libraries with large # clones to screen
5. Complete Digestion would break up genes of different DNA fragments- particularly if human genes big- ∴ would have one gene 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!



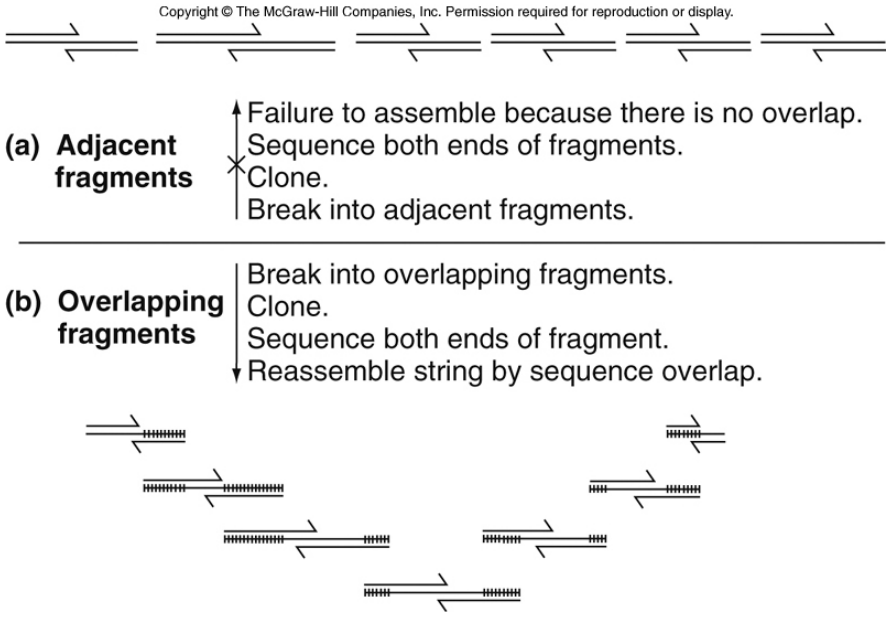
Partial Digestion Produces A series of Large, Overlapping DNA Fragments/ Clones

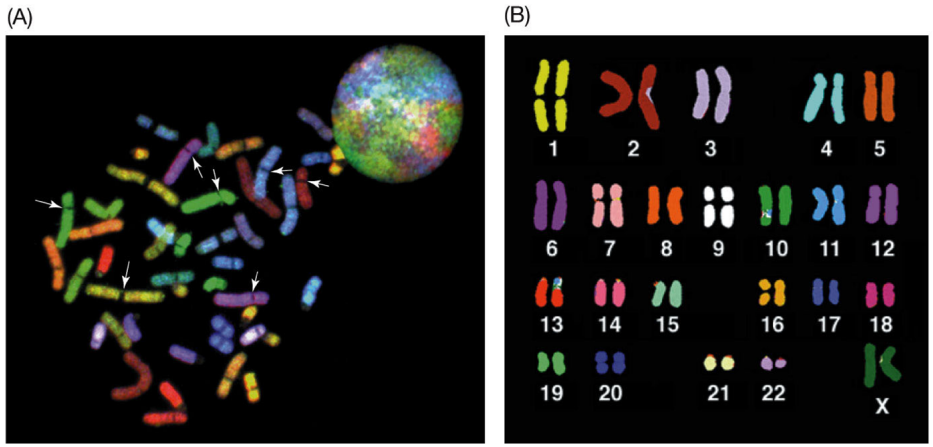
Can connect one clone with another!! Build up clones of each chromosome!!

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



∴ 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 our DNA molecule !





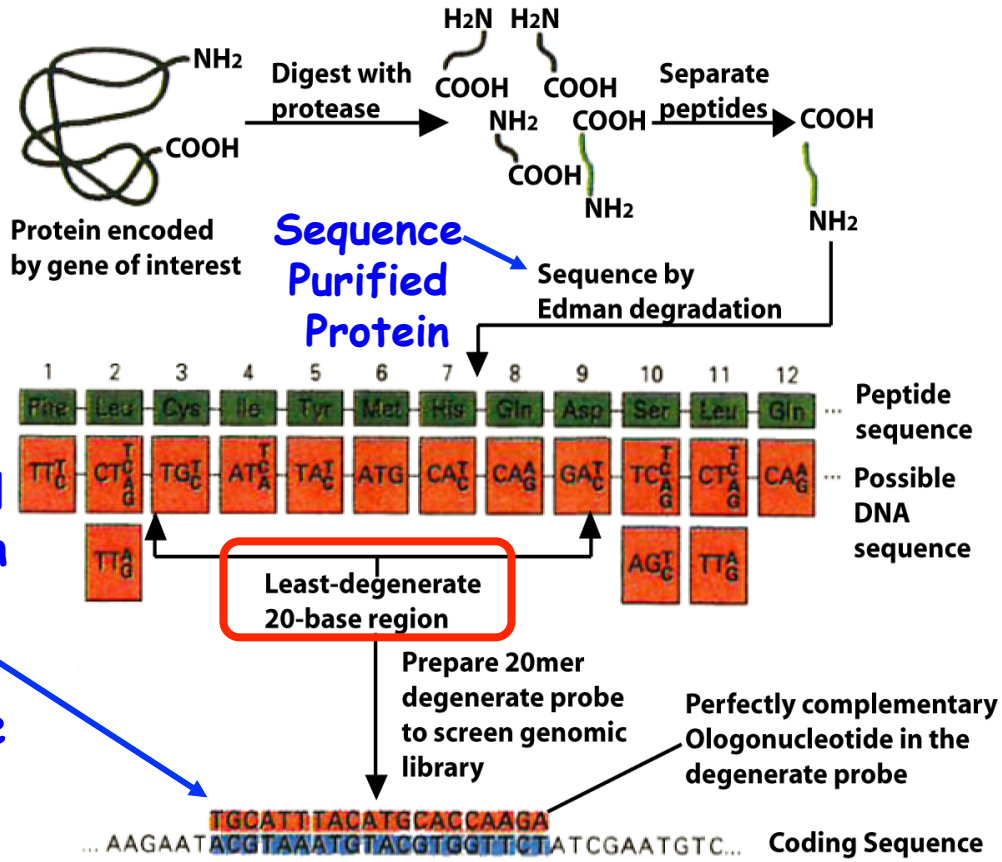
How Find the Factor VIII Gene in a Human Genome Library?

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



2. Make Several Probes All Codon Combinations!

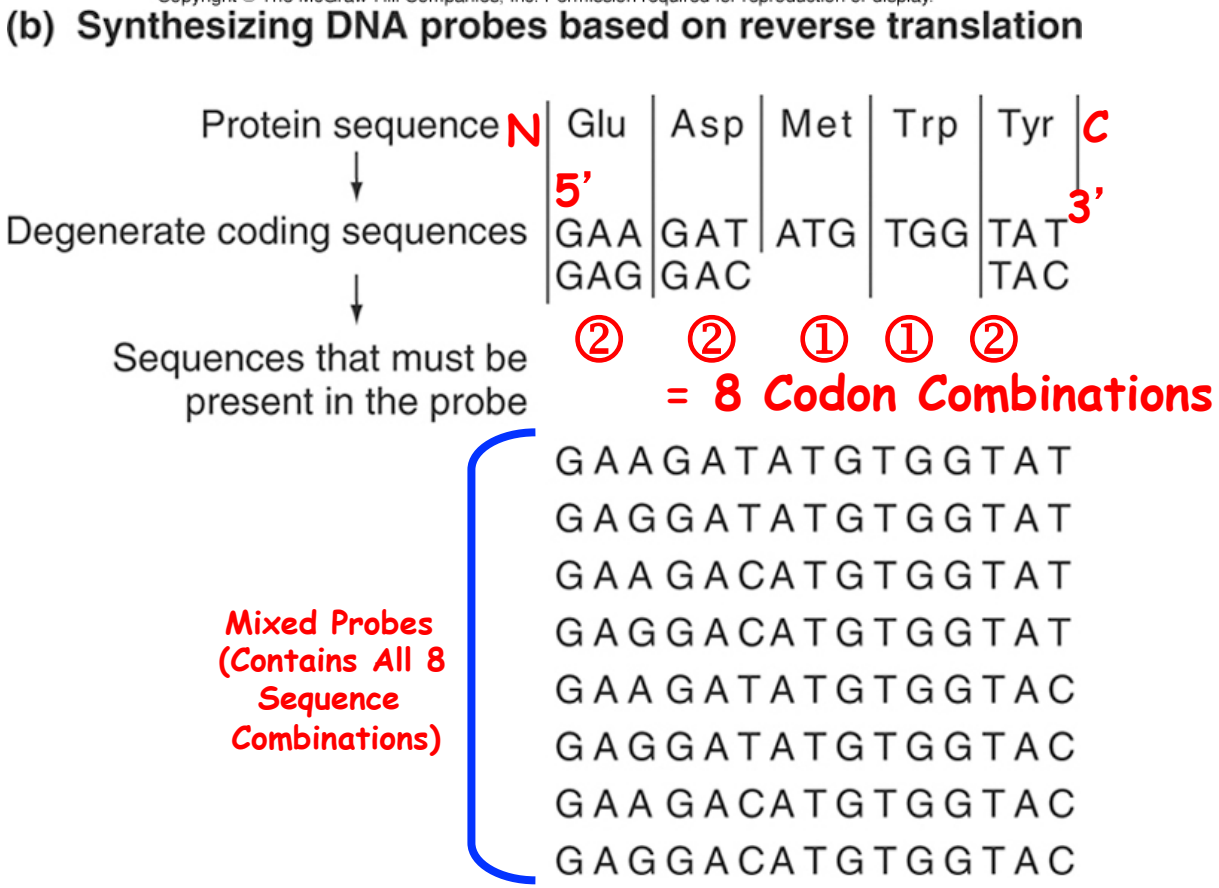
3. One Will Be Correct Probe

1. Use Genetic Code

How many Combinations of <u>Synthetic</u> Probes?

2x3x2x1x2x2x2=96

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

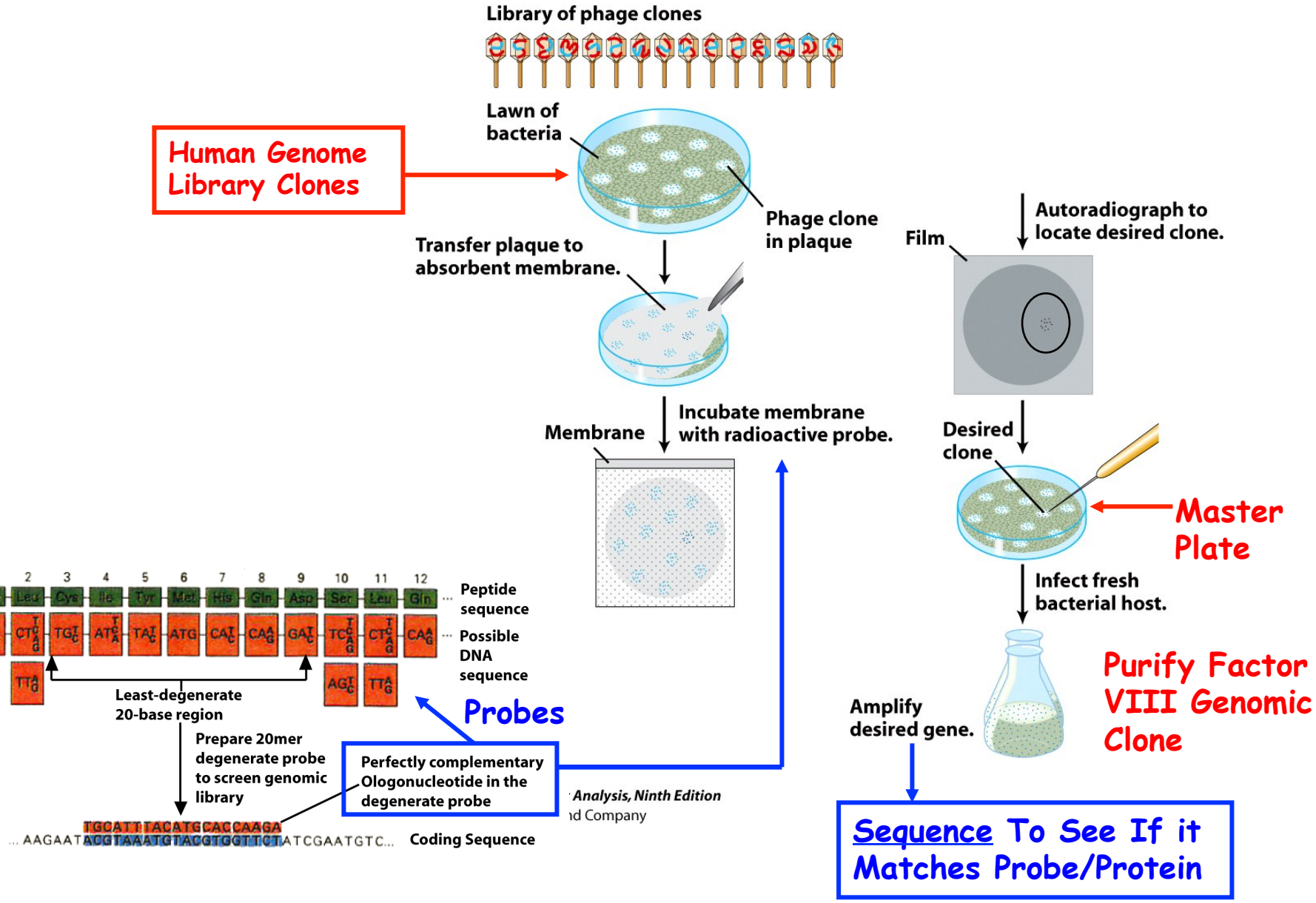


1. Need Amino Acid Sequence of Part of the Protein
2. Need DNA Sequences Representing all Codon Combinations
3. Synthesize DNA Sequence Probes!

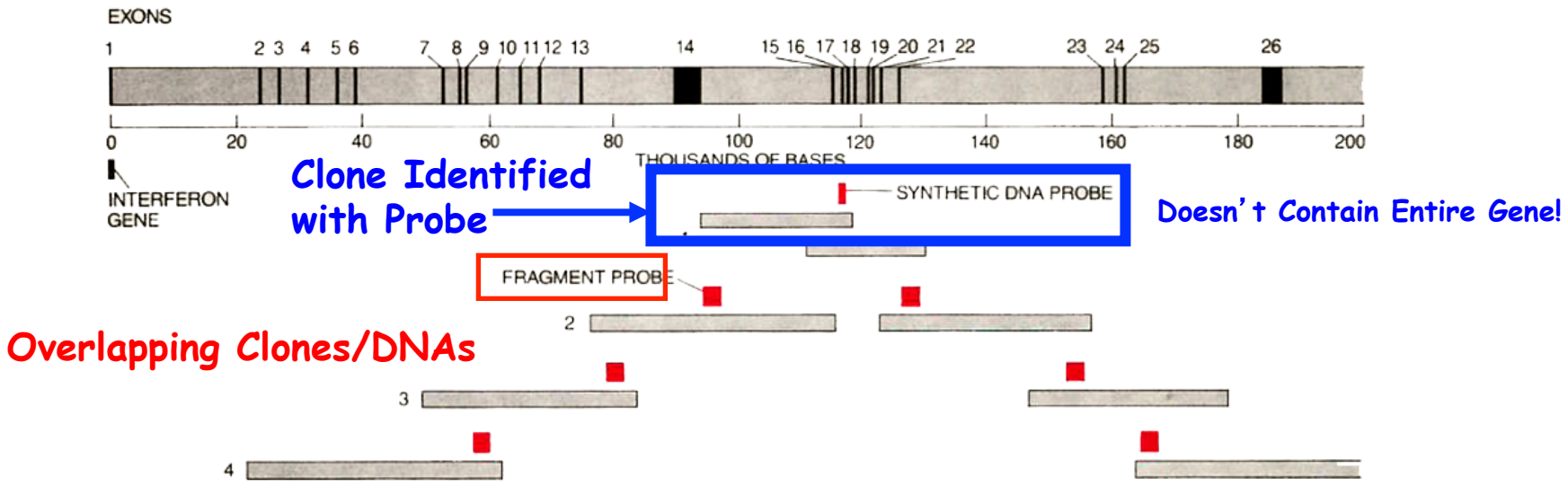
Probes Can Identify Genes in a Genome Library Because They Are: ?

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 !!!



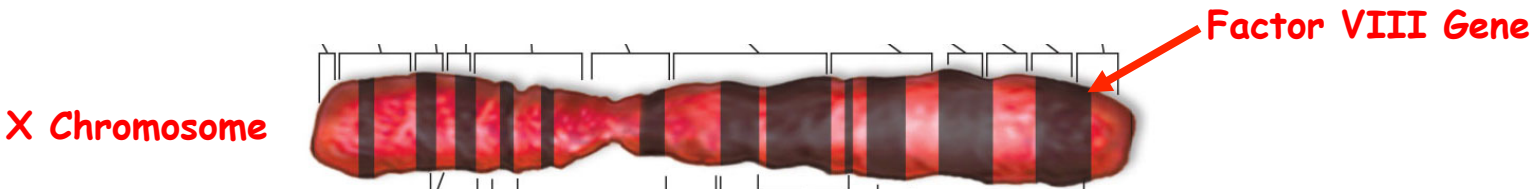
How Find Clones with Rest of Gene? Key Question !

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

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



Finding the Entire Factor VIII Gene? Walking & Sequencing

Walking up and down Genes and Chromosomes

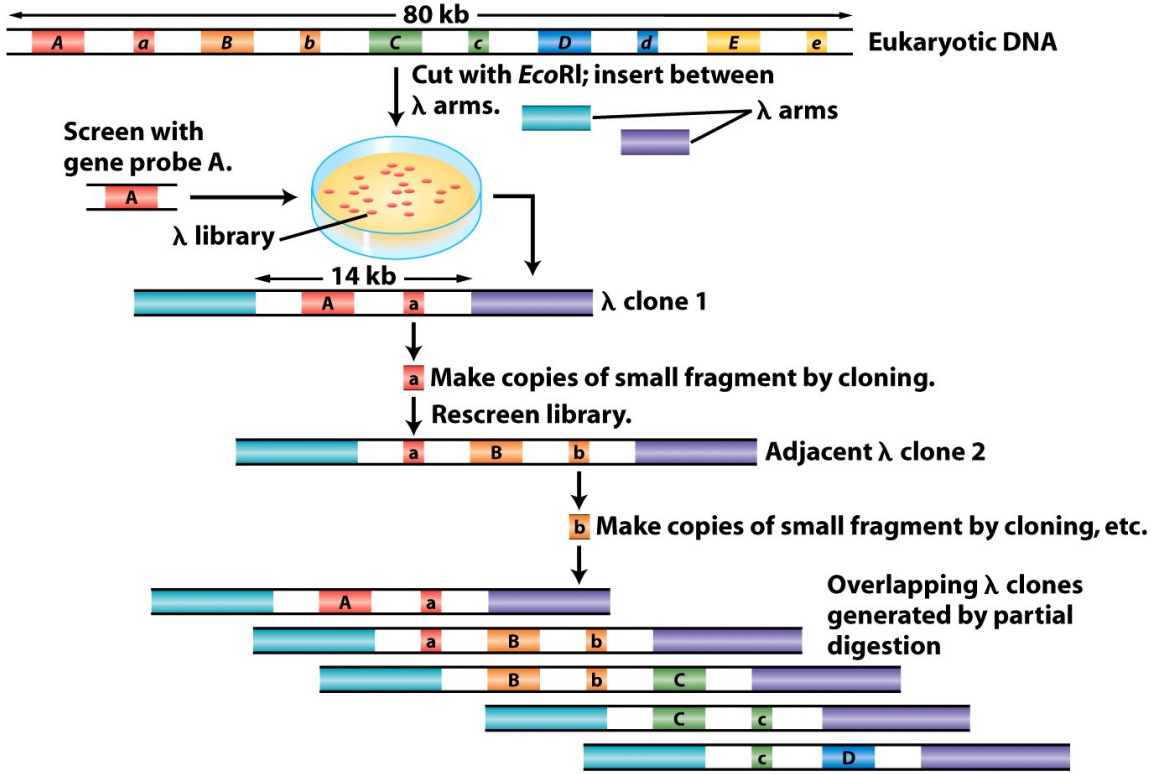


Figure 20-13
Introduction to Genetic Analysis, Ninth Edition

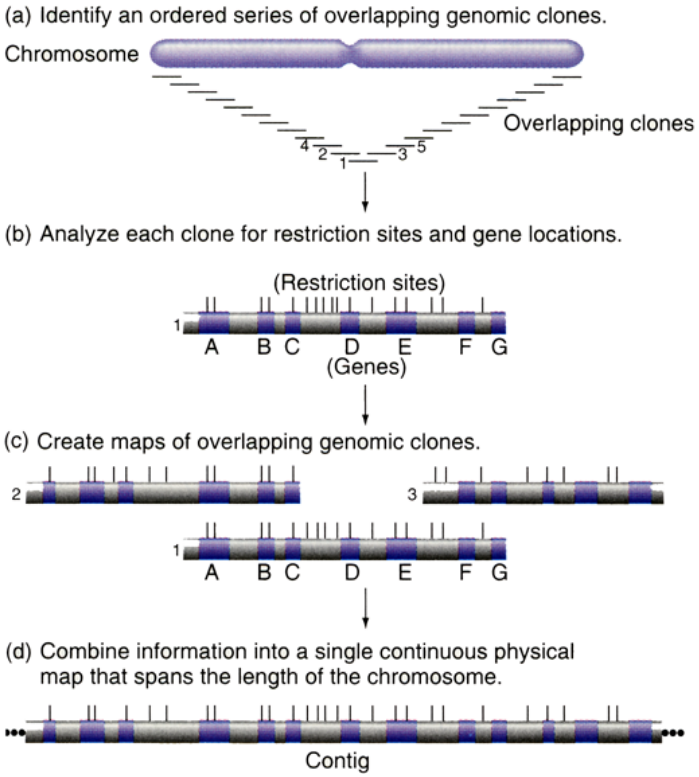
Basis of Genome Projects & Whole Genome Sequencing

Key Concepts → How know Find Complete Factor VIII Gene?

→ Compare Protein & DNA Sequences

Can Walk Down an Entire Chromosome + Obtain an Entire set of Overlapping Clones Containing Every Gene in Chromosome

1. Used to Sequence Human Genome
3. Used to Map Genes to Chromosomes
3. Used for Markers (RFLPs) to Identify & Follow Disease Genes



There are 24 sets of clones for Human Genome

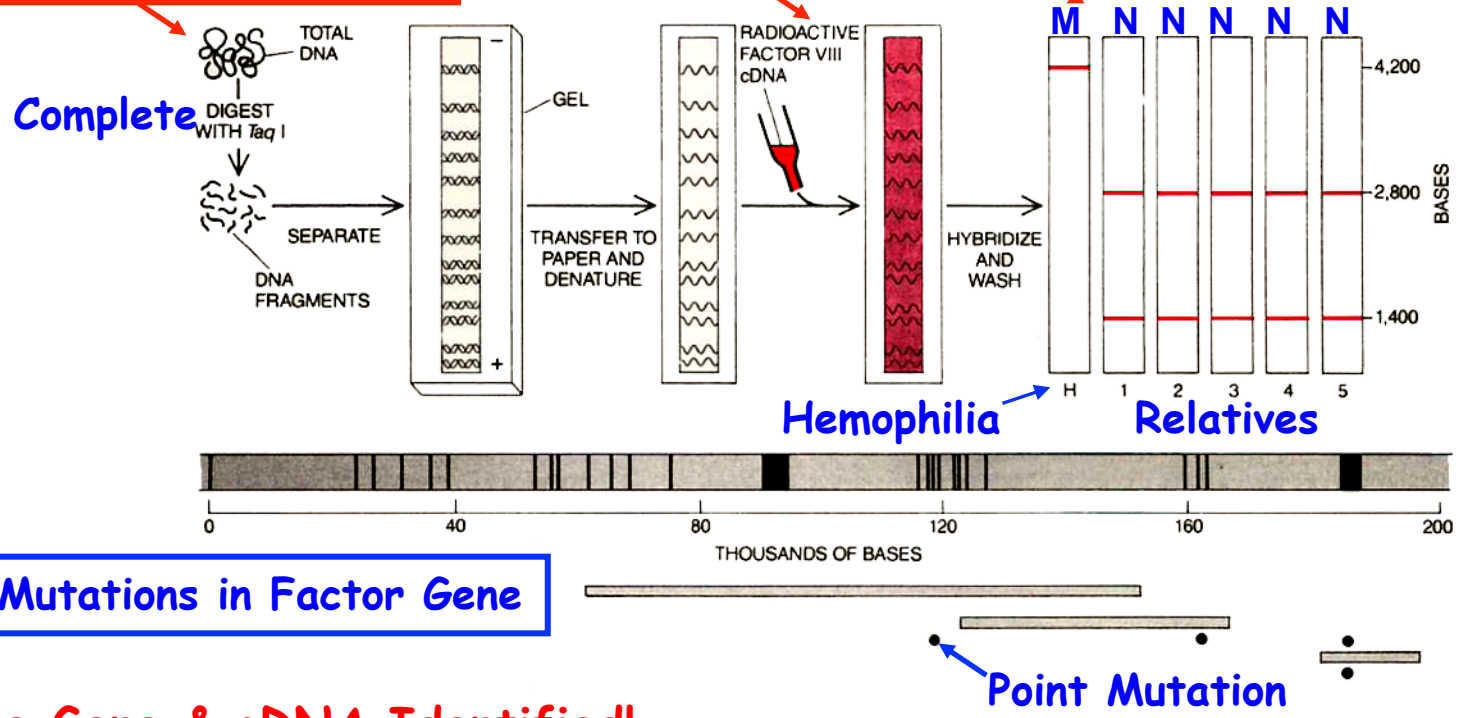
22 Autosomes + X Chromosome + Y Chromosome

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 & 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)]

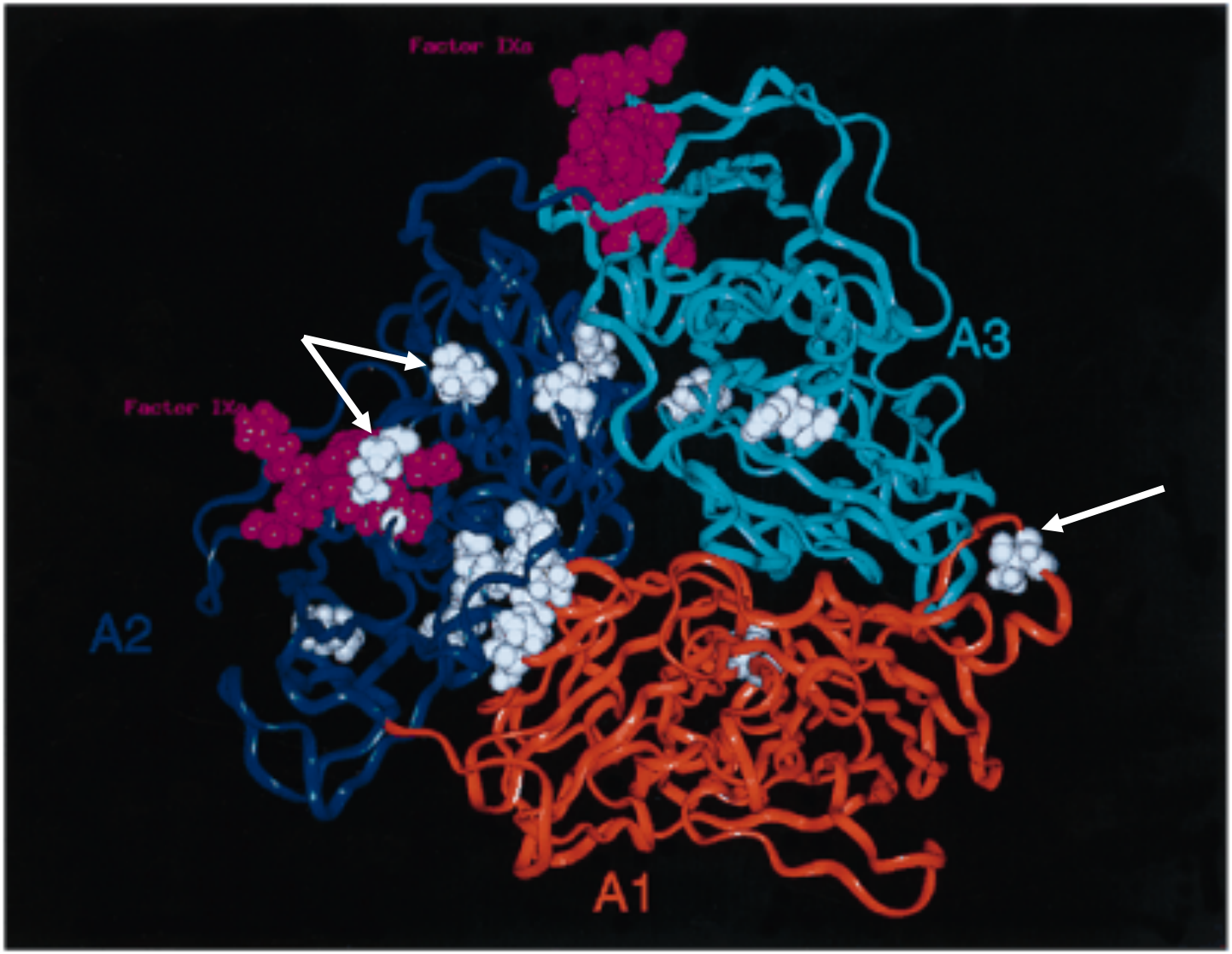
Factor VIII gene mutations in haemophilia A patients without intron 22 inversion.

VIII:C (%)	Family history	Consanguinity*	Inversion	Codon†	Mutation	Amino acid change	Exon	Conservation‡
1	Sporadic	NC	Normal	51	TTT → TCT§	Phe → Ser	2	FFF, 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
nhibitor 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!

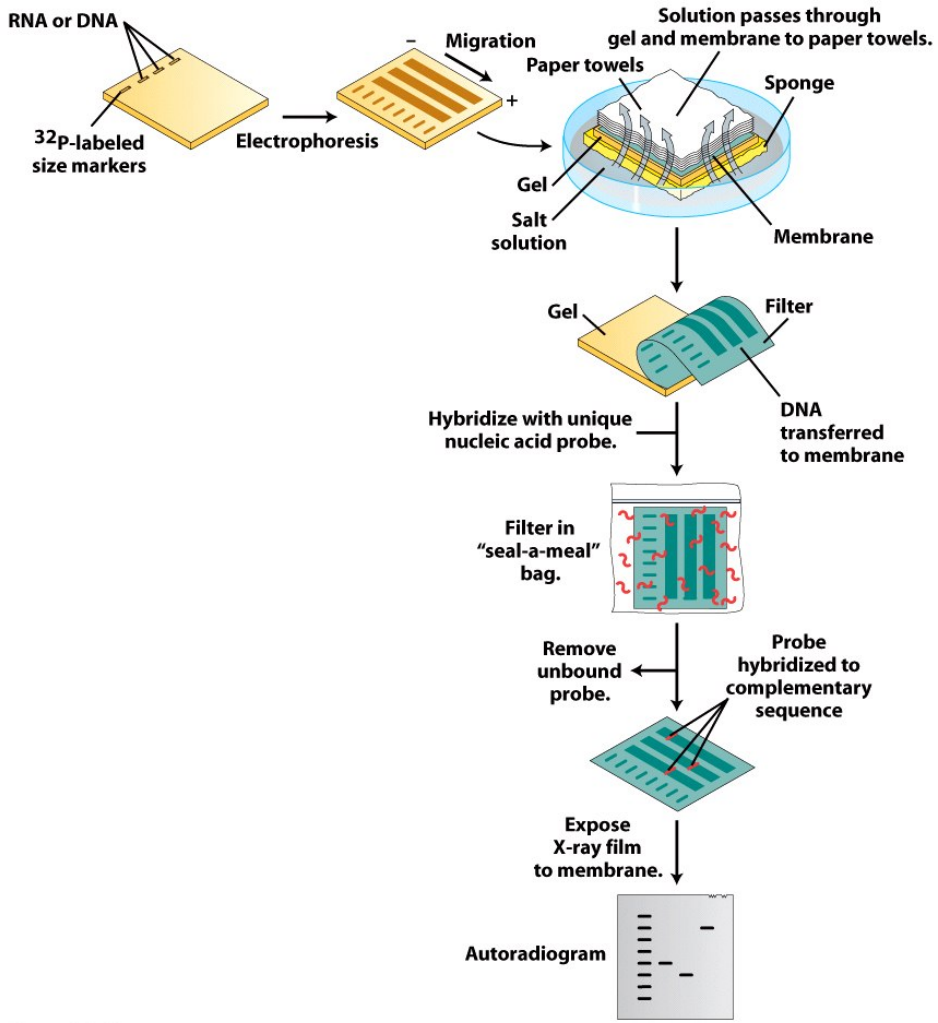


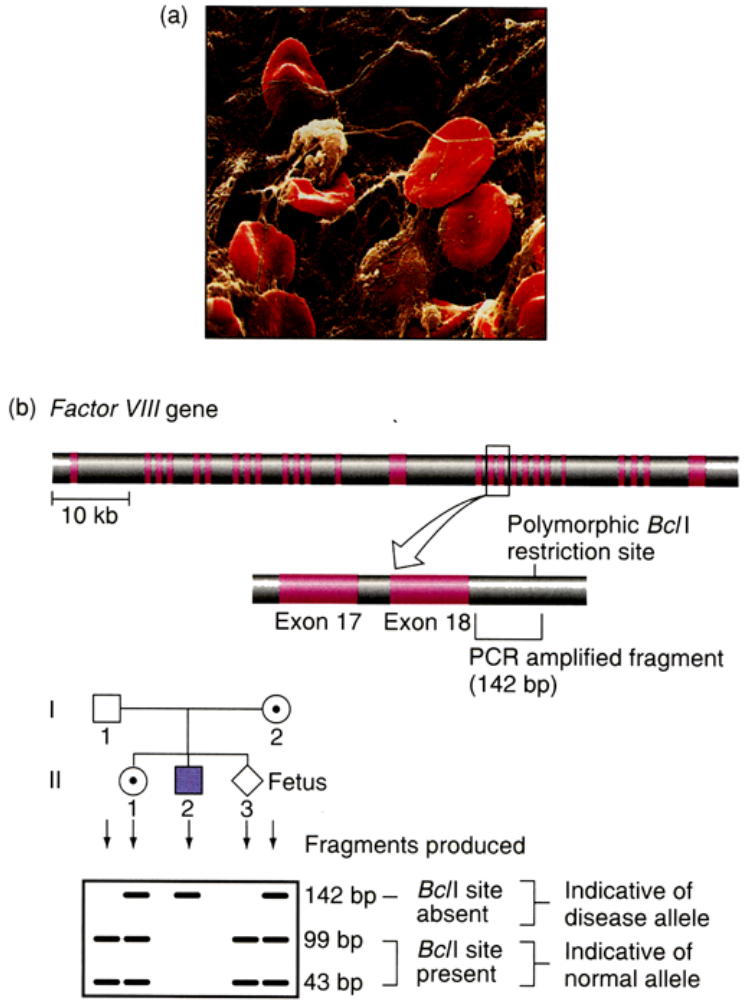
Figure 20-12
Introduction to Genetic Analysis, Ninth Edition

Using PCR and RFLPs 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

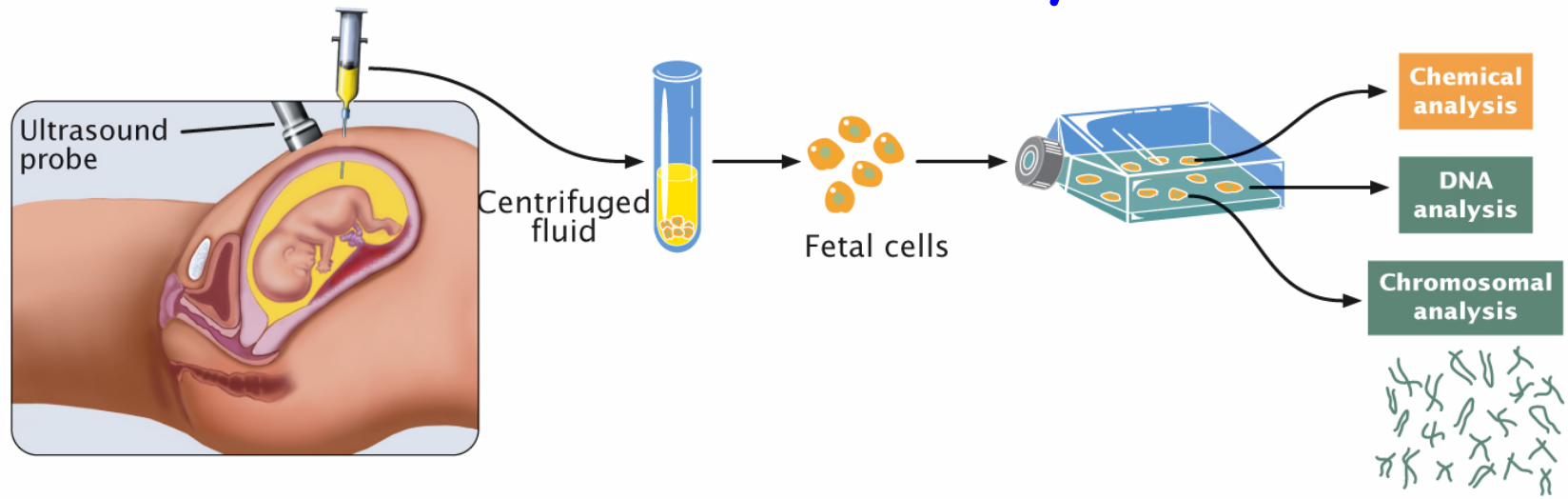


The 21st Century Approach!

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

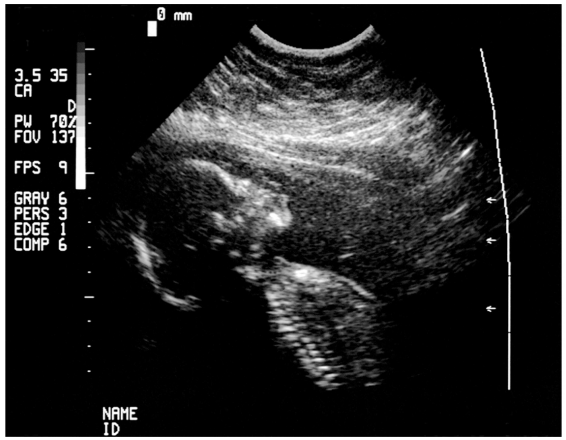
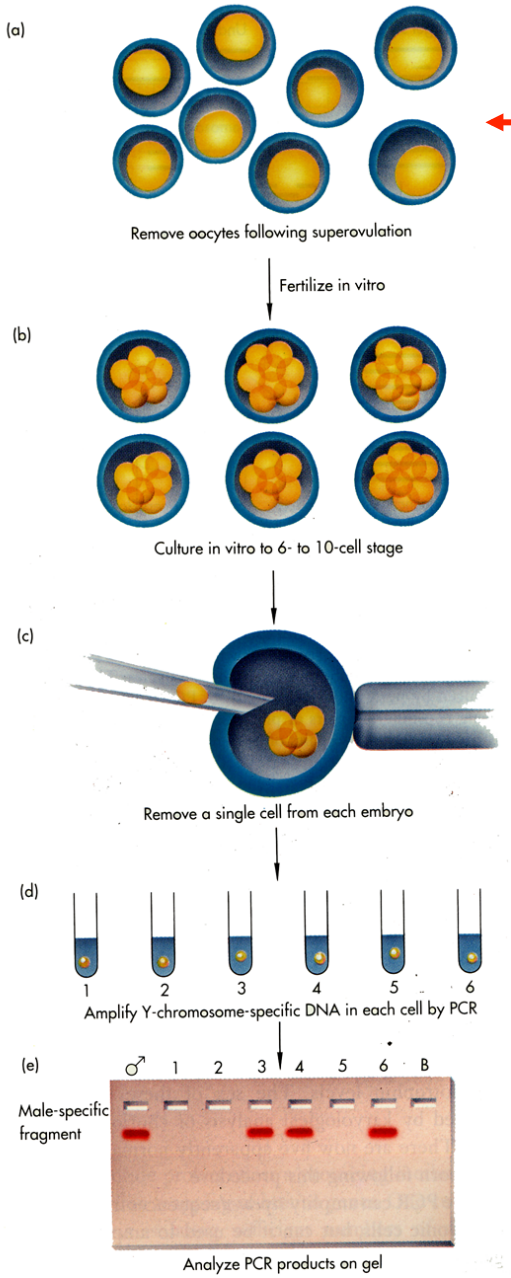


Fig_06-15 *Genetics, Second Edition* © 2005 W.H. Freeman and Company

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

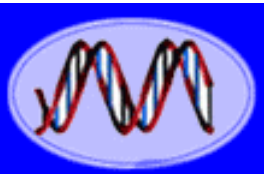
Using PGD to Detect Hemophilia A Disease Alleles



Mother is a Carrier X^HX^h

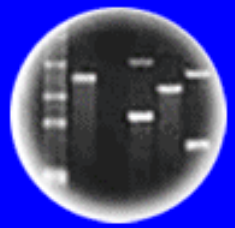
1. **Test for Male Embryos**
2. **Test for Presence of Hemophilia A Disease Alleles!**

   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?
- How Ensure Privacy & Confidentiality?
- Genetic Databases??

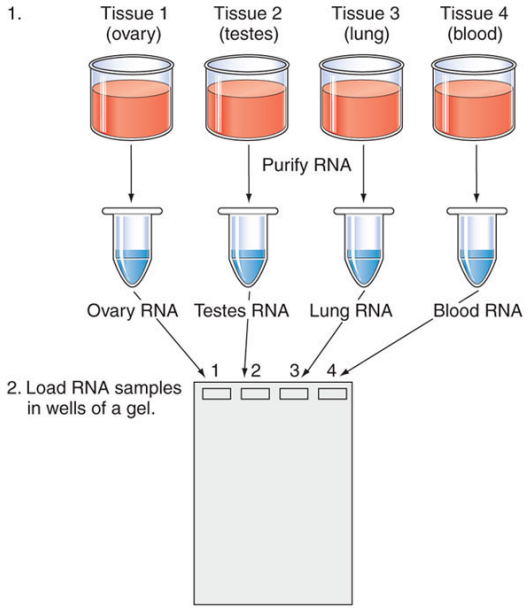
How Find Factor VIII mRNA to Generate a cDNA for Protein Production in Host Cells?

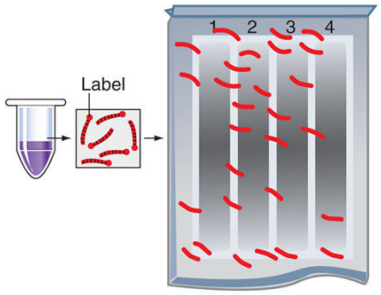
Recall: Eukaryotic Genes Provide Obstacles for Efficient Protein Production in Genetically Engineered Cells! Reasons???

Making the Drug

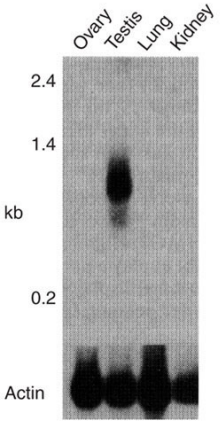
Need cDNA Not Gene

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





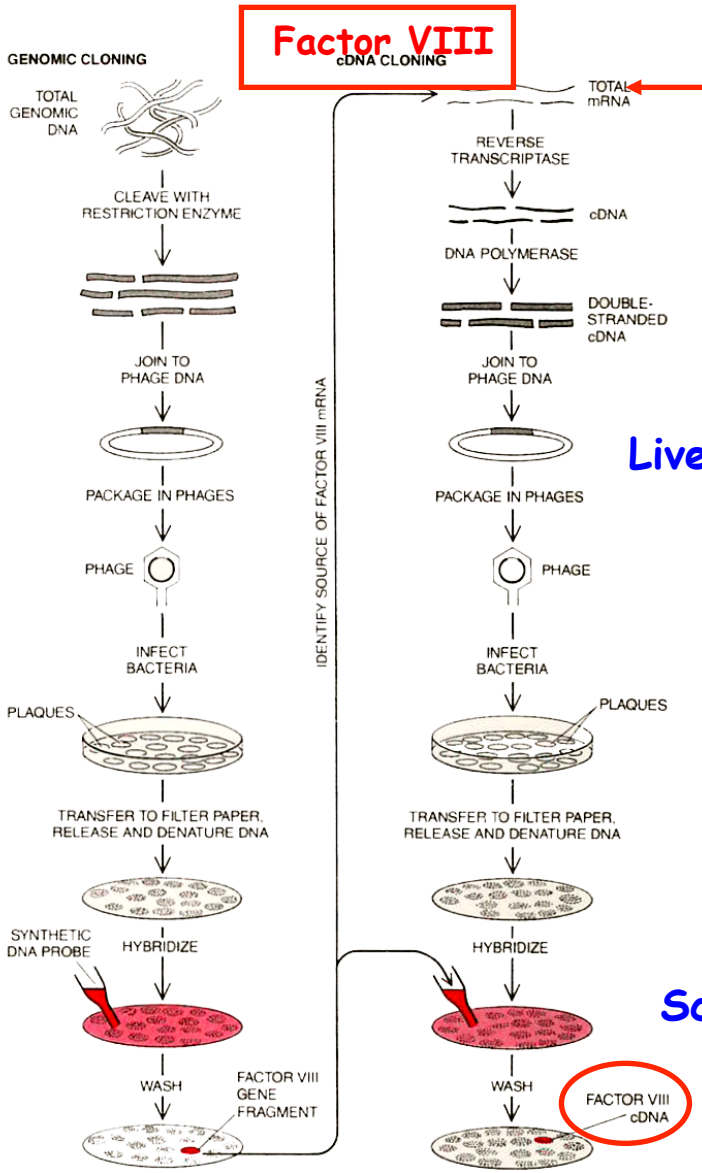
4. Wash away unhybridized probe. Make autoradiograph.



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

Factor VIII Gene Is Highly Active in Liver!

Using Factor VIII Gene Probe to Identify Factor VIII cDNA clone



Liver mRNA

Liver cDNA Library

Factor VIII cDNA →

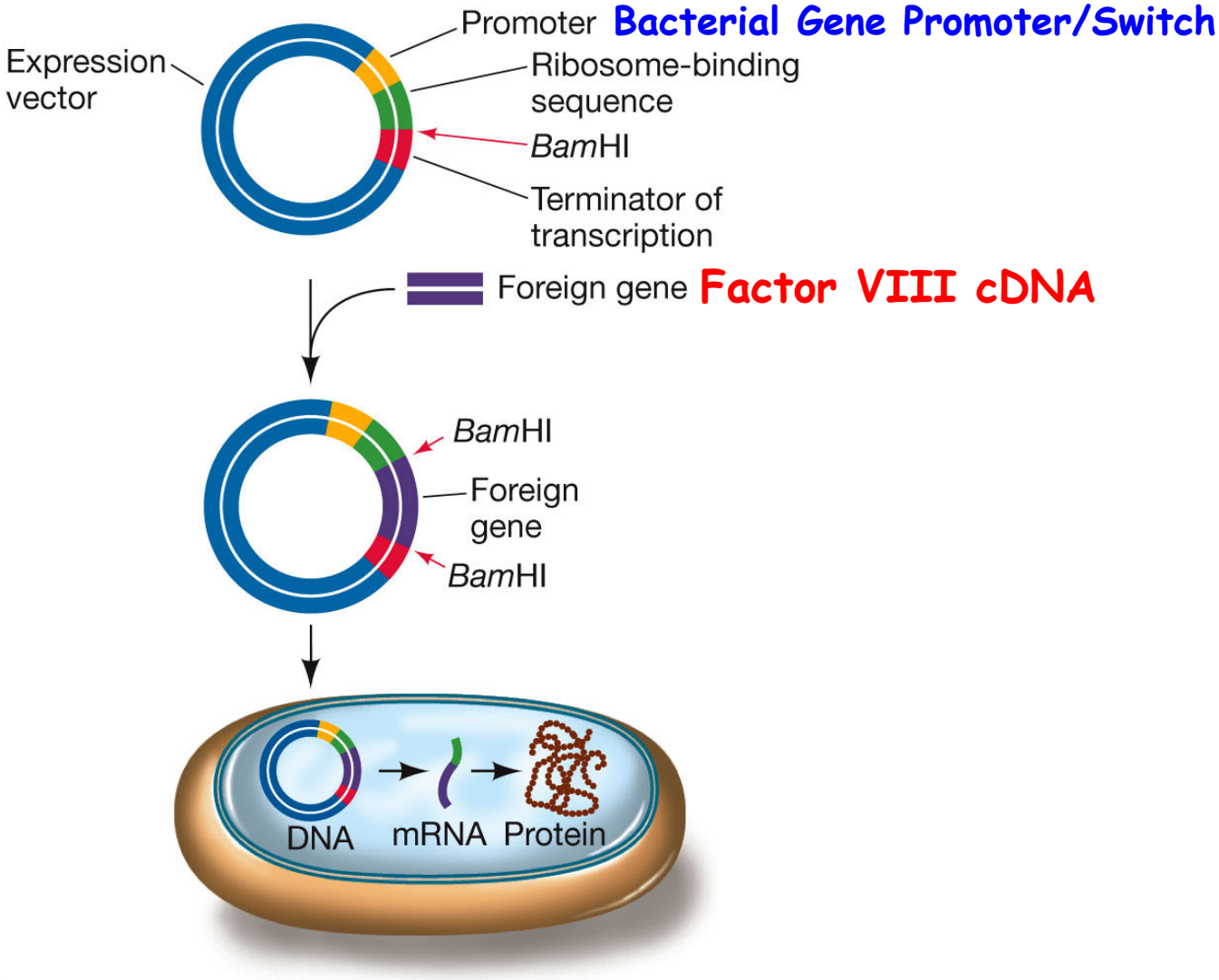
Used to Make Drug !

Screen Liver cDNA Library

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

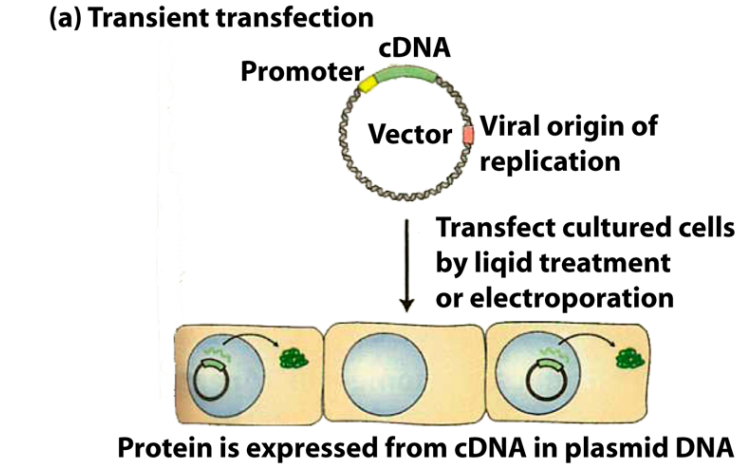
a. Yes
b. No

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

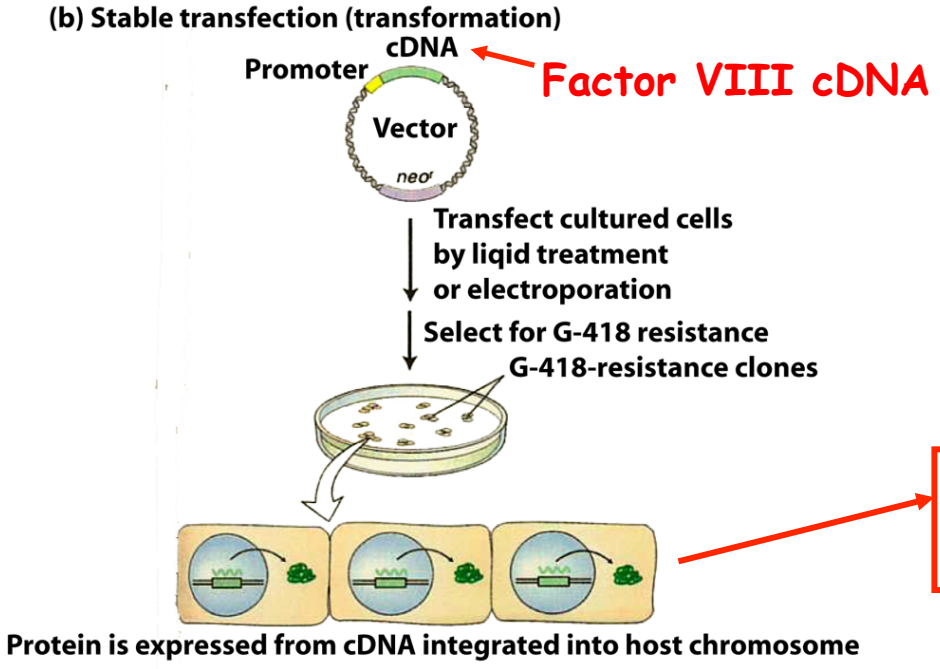


LIFE 8e, Figure 16.16

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



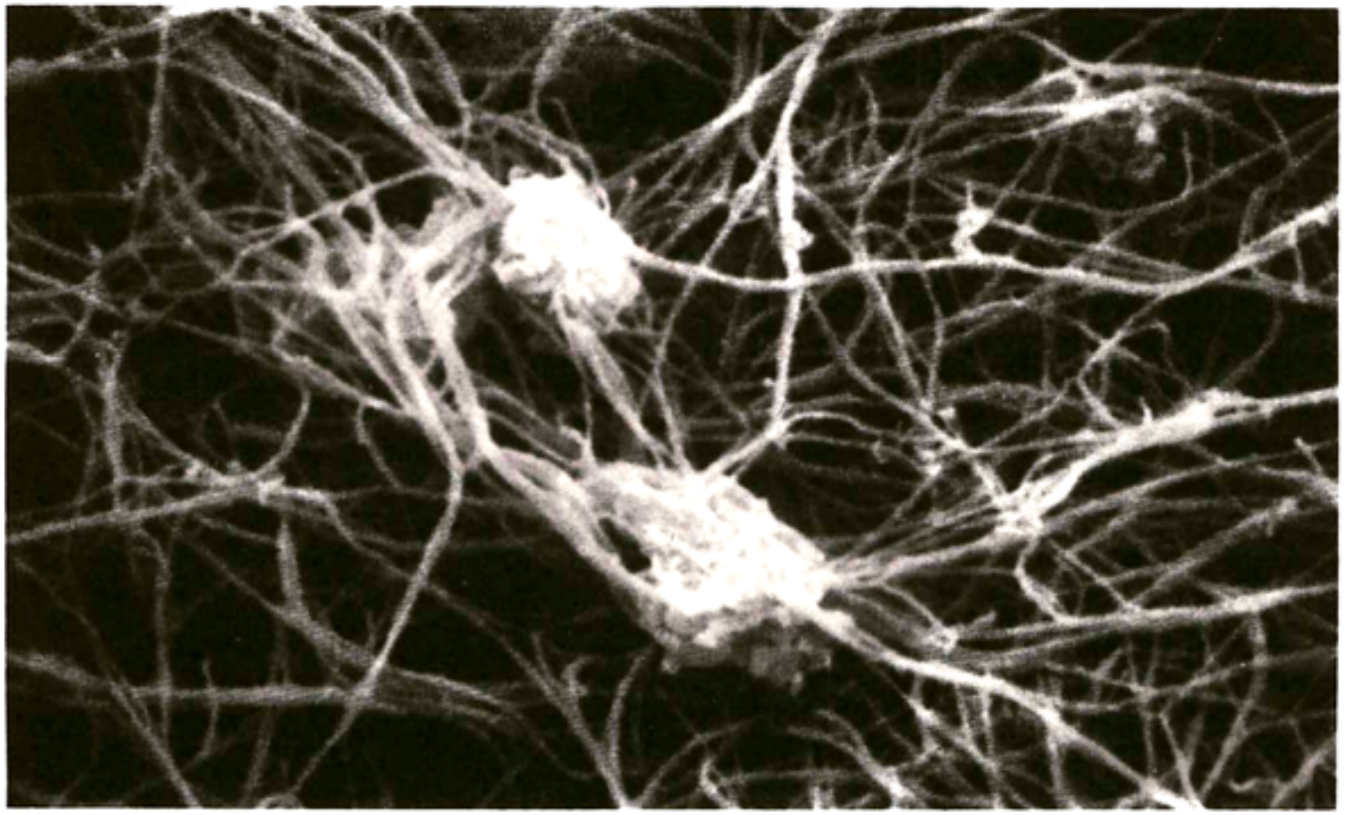
Why Mammalian Cells?



Purify Factor VIII Protein!

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

Bayer Biological Products EU

Bayer HealthCare
Biological Products Division
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- Recombinant Factor VIII
- More Resources
- Haemophilia Centres in Europe

Related Links

- Haemo-QoL Project
- Hemophilia Research Awards

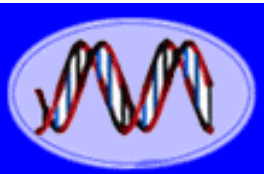
Recombinant factor VIII

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

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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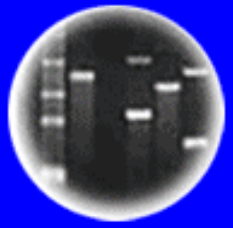
Genetic Code of Life

Entire Genetic Code
of a Bacteria



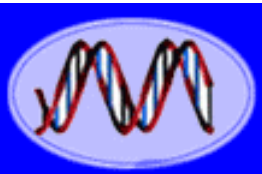
DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

Plants of Tomorrow

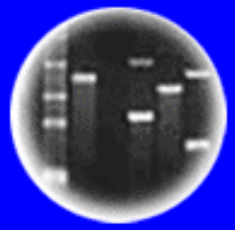
The Factor VIII Story -- A Summary

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



DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

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**

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

a. Yes
b. No

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

a. Yes
b. No