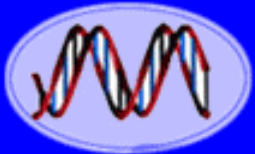
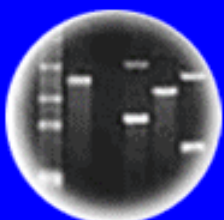

DNA
Genetic Code of Life


Entire Genetic Code
of a Bacteria


DNA Fingerprinting


Cloning: Ethical Issues
and Future Consequences


Plants of Tomorrow

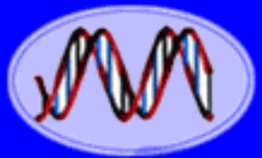
HC70A & PLSS059
Winter 2020
Genetic Engineering in Medicine, Agriculture, and Law

Professors Bob Goldberg & Channapatna Prakash

Lecture 5
How Are Genes Cloned & Engineered?
The Insulin and Factor XIII Stories

UCLA

TUSKEGEE
UNIVERSITY



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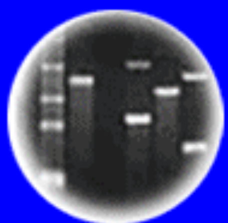

DNA Fingerprinting

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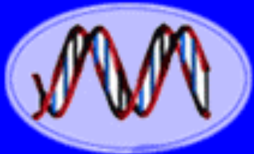
THEMES

1. How Did the Supreme Court Indirectly Give Rise to the Biotechnology Industry?
2. What Strategies Were Developed For Cloning Insulin mRNA and Expressing Insulin in Bacterial Cells? What Strategy "Won" Out?
3. What is Hemophilia and How is it Inherited?
4. How Can a Disease Gene Be Found When It is Not Known Where the Gene is Expressed?
5. What Vectors Can Be Used For Cloning DNA?
6. What is the Advantage of Using a Virus Vector For Constructing Genome Libraries?
7. How To Make a Library of the Human Genome?
8. How Find a Gene With Only a Knowledge of the Protein Sequence?
9. How Use DNA Testing to Detect Factor VIII Disease Alleles?
10. How Isolate a Factor VIII cDNA Clone?
11. Genomic vs. cDNA Libraries
12. How Produce Factor VIII Protein For Use as a Drug



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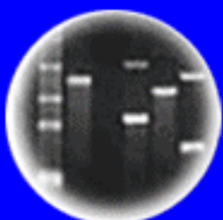

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Drugs Manufactured Using Genetic Engineering

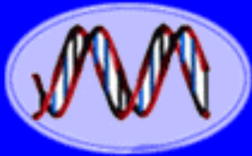
TABLE 1.2 Examples of Recombinant Proteins Manufactured from Cloned Genes

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 *2016—Top 10 Biotechnology Drugs (Each with Worldwide Sales over $5 Billion)

Drug Name	Developer	Drug Type	Function (Treatment of Human Disease Conditions)
Humira	AbbVie	Antibody (monoclonal)	Rheumatoid arthritis, Crohn's disease, Ulcerative colitis
Harvoni	Gilead Sciences	Small molecule	Hepatitis C
Rituxan	Roche	Antibody (monoclonal)	Non-Hodgkin's lymphoma
Revlimid	Celgene	Small molecule	Multiple myeloma
Avastin	Roche	Antibody (monoclonal)	Colorectal cancer; breast cancer; non–small cell lung cancer; ovarian, brain, and cervical cancer
Herceptin	Roche	Antibody (monoclonal)	Breast cancer, gastric cancer
Enbrel	Amgen	Recombinant protein	Rheumatoid arthritis, psoriasis
Prevnar 13	Pfizer	Vaccine	Pneumococcal (*Streptococcus Pneumoniae*) antibacterial vaccine
Lantus	Sanofi	Peptide	Diabetes mellitus types I and II
Neulasta	Amgen	Recombinant protein	Anemia (neutropenia/leukopenia)

*Data based on the most recent source available at the time of publication: Morrison C, Lähteenmäki R. Public biotech in 2016—the numbers. *Nat Biotechnol.* 2017;35:623–629.



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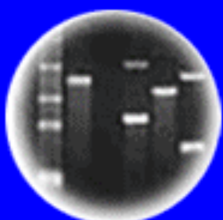

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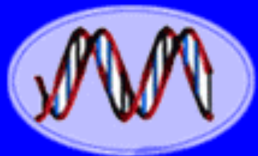
The Origins of the Biotech Industry Started in the Supreme Court

Founded in 1976 By Robert Swanson and Herb Boyer

First IPO in 1980 for $88/share

Purchased by Hoffmann-La Roche in 2009 for $47B

Insulin – The First Biotech Drug



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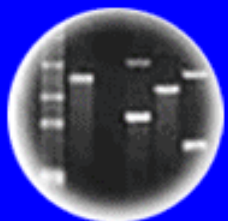

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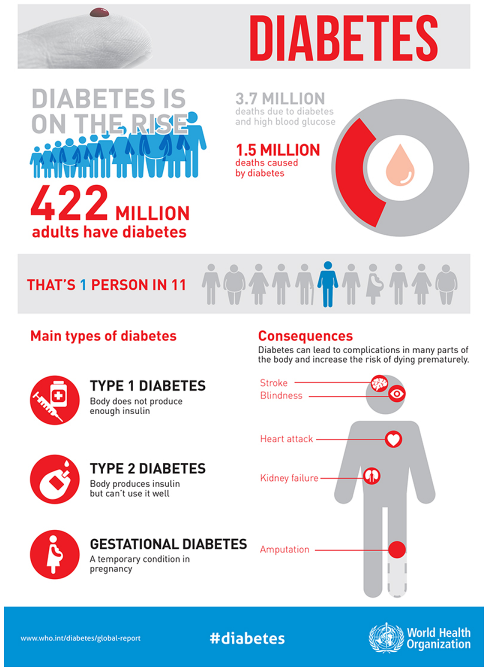


THE TORONTO DAILY STAR

TORONTO DOCTORS ON TRACK OF DIABETES CURE

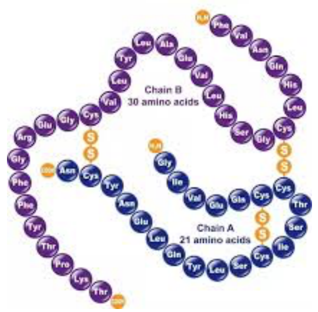
RAILROAD UNIONS IN U.S. MAY JOIN IN MINERS' STRIKE

DIABETES SUFFERERS GIVEN MESSAGE OF HOPE

"DRY" LAWS HERE CAUSE HARDSHIP IN OLD FRANCE

RESERVES OF COAL ARE HIGH IN CITY, PRICE TO GO DOWN

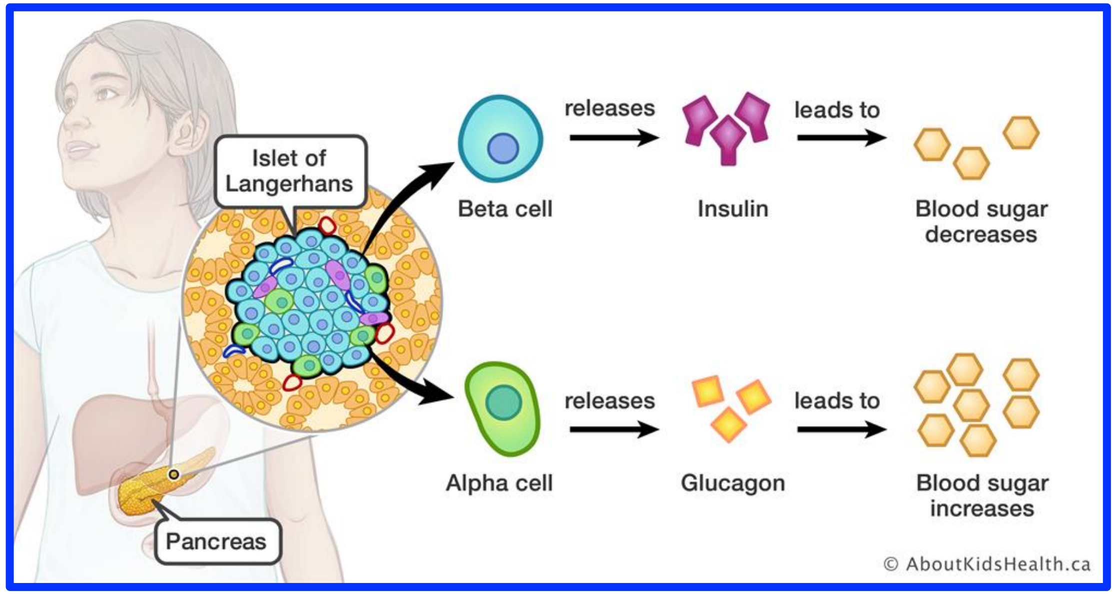
CIVIL WAR STAGE NOT YET REACHED, BUT DANGER GREAT

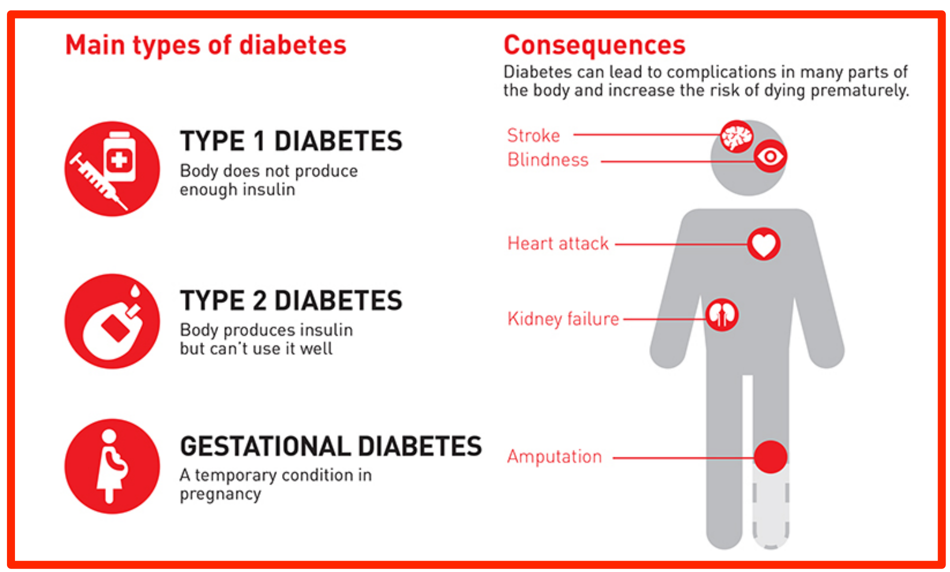


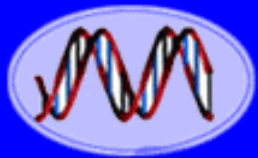
- Sequenced By Fred Sanger 1951-1953
- Nobel Prize in 1958

- Discovered in 1921
- Commercial Production By Eli Lilly in 1923
- Nobel Prize 1923

How Does Insulin Control Sugar Levels?







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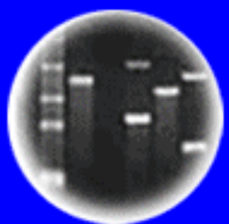

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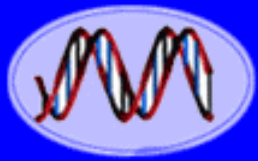

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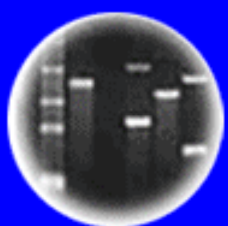

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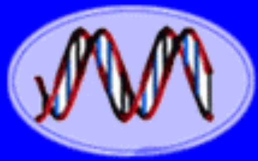
Reasons For Insulin Being the First Biotech Drug

- Diabetes a Major Disease Responsible For Millions of Deaths
- Physiological Basis of the Disease Known
- Site and Mechanism of Insulin Synthesis and Secretion Within the Pancreas Known
- Insulin Was Purified and Amino Acid Sequence Known
- Small Protein Consisting of 51 Amino Acids
- Insulin Protein Structure Understood 110 amino acids Total - A Chain 21 Amino Acids and B Chain 30 Amino Acids)
- Predicted Small Size of mRNA (~390 nts) and Gene
- Insulin Made in Large Quantities in the Pancreas
- Techniques For Cloning mRNA Using Reverse Transcriptase Or Direct DNA Synthesis Known



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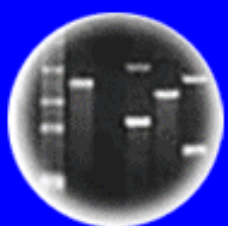

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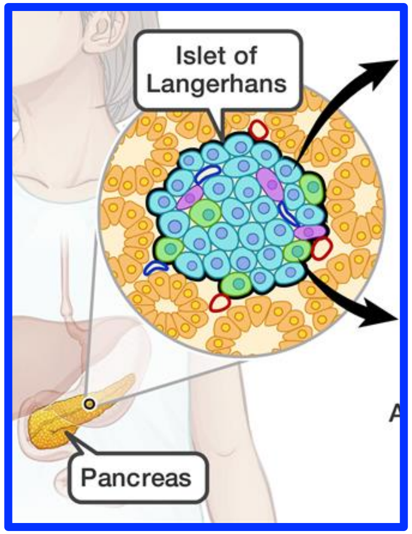
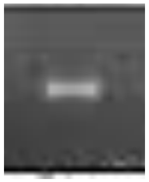
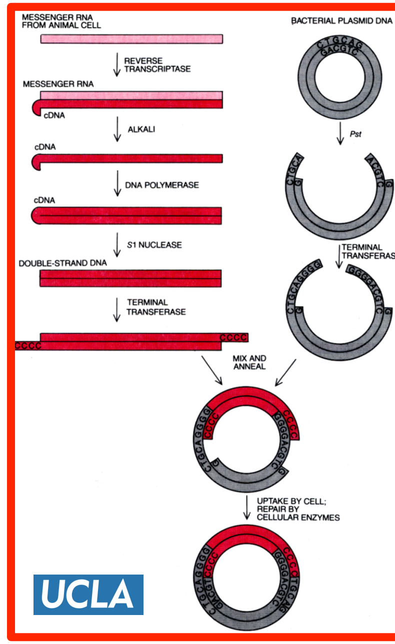
Two Strategies For Isolating the Insulin "Gene" And Engineering *E. coli* Cells to Produce Human Insulin



Purify Insulin mRNA From Pancreas



Synthesize & Clone cDNA



Use cDNA/mRNA Sequence

```
   -310  cctgaatatagccaactaaattctaggaactctaagaggactacgcttgtctccaacatcttatcgtcaa  -240
catcttctgcaagcgataactatatttctggtccgccaaagtagtatacgctaagaacaagaggaagagagtcgtaaggt  -160
ttttattcccagccggcgagagcagaaactgttgttctagctgcctttctggtcttaacaggaccattttgctggccag  -80
tgaaaaactaactcgggtgaaacaacattggtgctaccagcctctcctgactgttccaacggtgccttctcgtagccaga  -1

ATG AGC AAG TTC CTC CTC CAG AGC CAC TCC GCC AAC GCC TGC CTG CTC ACC CTT CTG CTC   60
Met ser lys phe leu leu gln ser his ser ala asn ala cys leu leu thr leu leu leu   20

ACG CTG GCC TCC AAC CTC GAC ATA TCC CTG GCC AAC TTC GAG CAC TCG TGC AAC GGC TAC  120
thr leu ala ser asn leu asp ile ser leu ala*asn phe glu his ser cys asn gly tyr   40

ATG CGG CCC CAC CCG CGG GGT CTG TGC GGC GAA GAC CTG CAC GTC ATC ATT TCC AAC CTG  180
met arg pro his pro arg gly leu cys gly glu asp leu his val ile ile ser asn leu   60

TGC AGC TCT CTG GGG GGC AAC AGG AGG TTC CTG GCC AAG TAC ATG GTC AAA AGA GAC ACG  240
cys ser ser leu gly gly asn arg arg phe leu ala lys tyr met val lys arg asp thr   80

GAA AAT GTG AAC GAC AAG TTA CGA GGG ATC CTG CTC AAT AAG AAA GAA GCT TTC TCC TAC  300
glu asn val asn asp lys leu arg gly ile leu leu asn lys lys glu ala phe ser tyr  100

TTG ACC AAG AGA GAG GCC TCA GGC TCC ATC ACA TGC GAA TGT TGC TTC AAC CAG TGT CGG  360
leu thr lys arg glu ala ser gly ser ile thr cys glu cys cys phe asn gln cys arg  120

ATA TTT GAG CTG GCT CAG TAC TGC CGT CTG CCA GAC CAT TTC TTC TCC AGA ATA TCC AGA  420
ile phe glu leu ala gln tyr cys arg leu pro asp his phe phe ser arg ile ser arg  140

ACC GGA AGG AGC AAC AGT GGA CAT GCG CAG TTG GAG GAC AAC TTT AGT tagacatgttgaggg  483
thr gly arg ser asn ser gly his ala gln leu glu asp asn phe ser                  156

cgtaaatgcttttaaatttttaatttggtgattattattataaaggaggagtccacgtggtgtcagatttagcgggttt  563
tttccacgtgtttgactaaagtttccagatttatttcataccagcgatacccgcaggaatagaaggtcccctaagaagct  643
gaaggcattattgat                                                                  658
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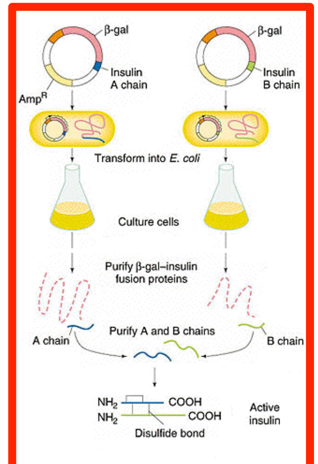
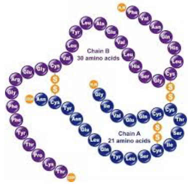
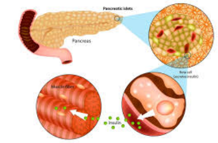
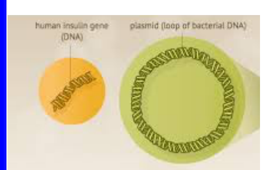
Direct Synthesis and Cloning of A Chain & B Chain mRNAs Separately

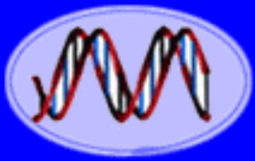




The Race For the Insulin Gene







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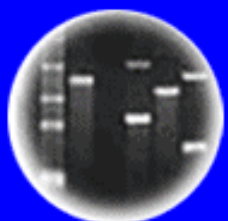

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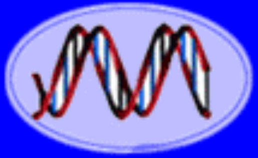

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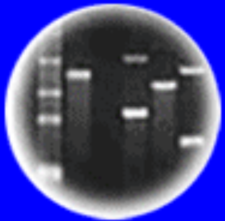

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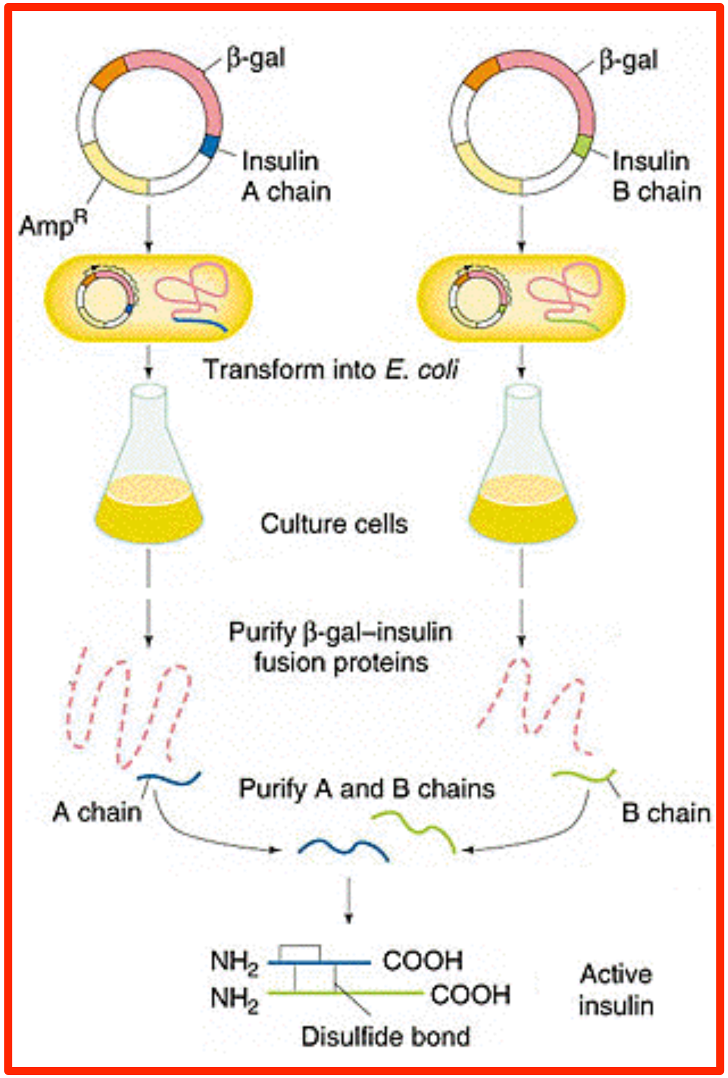
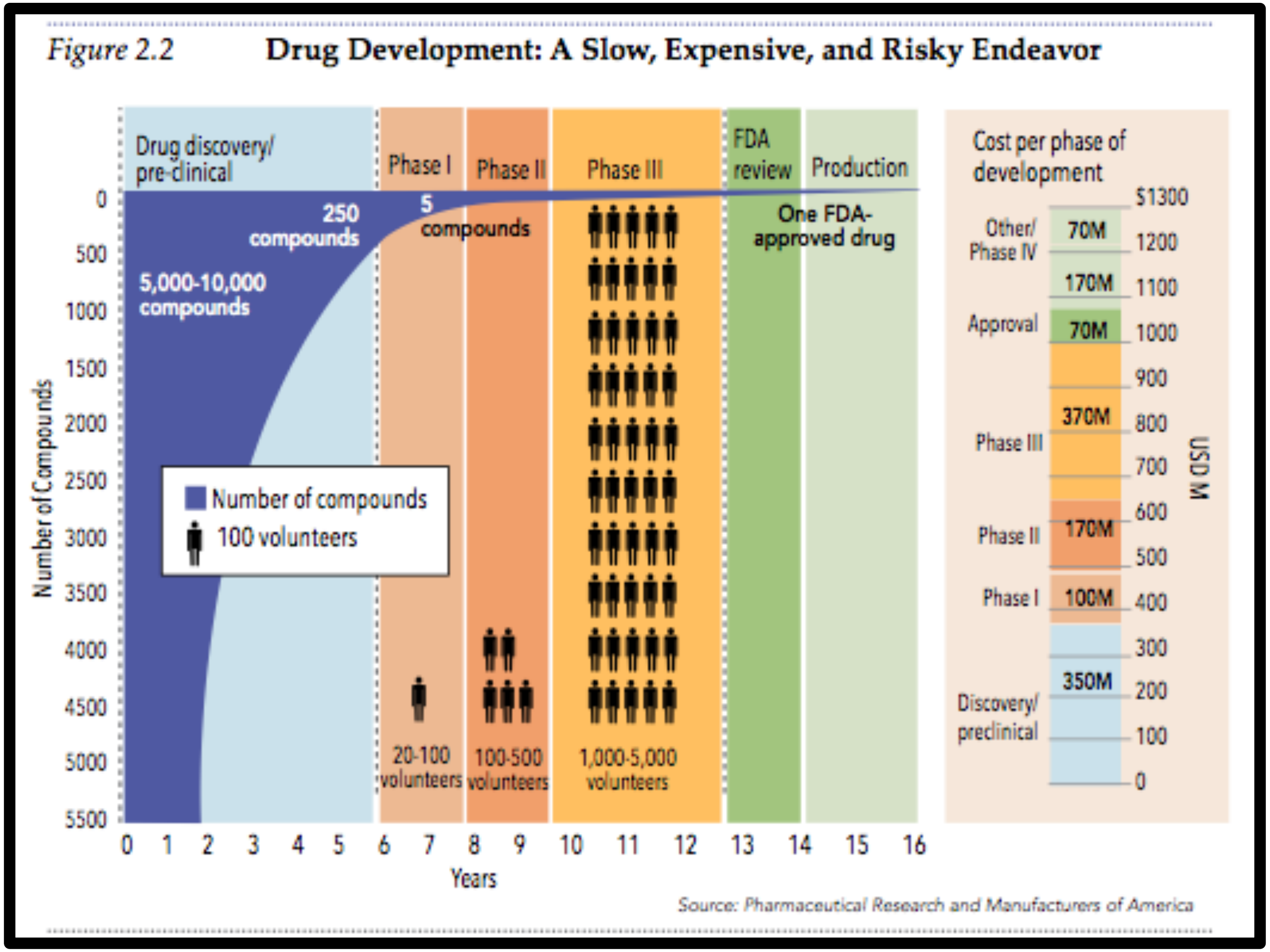
The Winning Strategy Used For Synthesizing Human Insulin in *E. coli* Cells



$30B per Year Market!

Each Chain Made Directly in Separate *E. coli* Cells
Combined After Synthesis to Make Recombinant Insulin
Note: *E. coli cannot process a Pre-Insulin Protein*

Need FDA Approval Before Recombinant DNA Drug Can Be Marketed and Used to Treat Patients



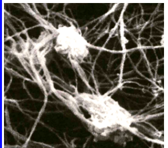
Insulin Was the First Recombinant DNA Drug and Got FDA Approval in 1982 - ~10 Years After Cohen and Boyer's Experiments

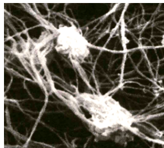
The Factor VIII Story is Different and More Complex Than the Insulin Story

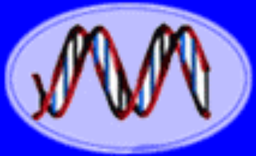
The Molecular Genetics of Hemophilia

Hemophiliacs bleed because a defective gene deprives them of a key blood-clotting protein. The protein has now been made artificially by isolating the normal gene and then inserting it into cultured cells

by Richard M. Lawn and Gordon A. Vehar







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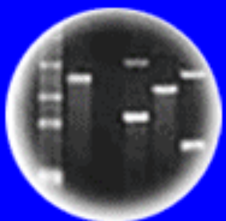

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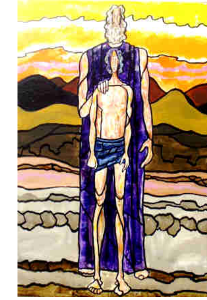
Hemophilia Has Been Known As An Inherited Disease For >2500 Years!



First Reference to Hemophilia is in the Old Testament

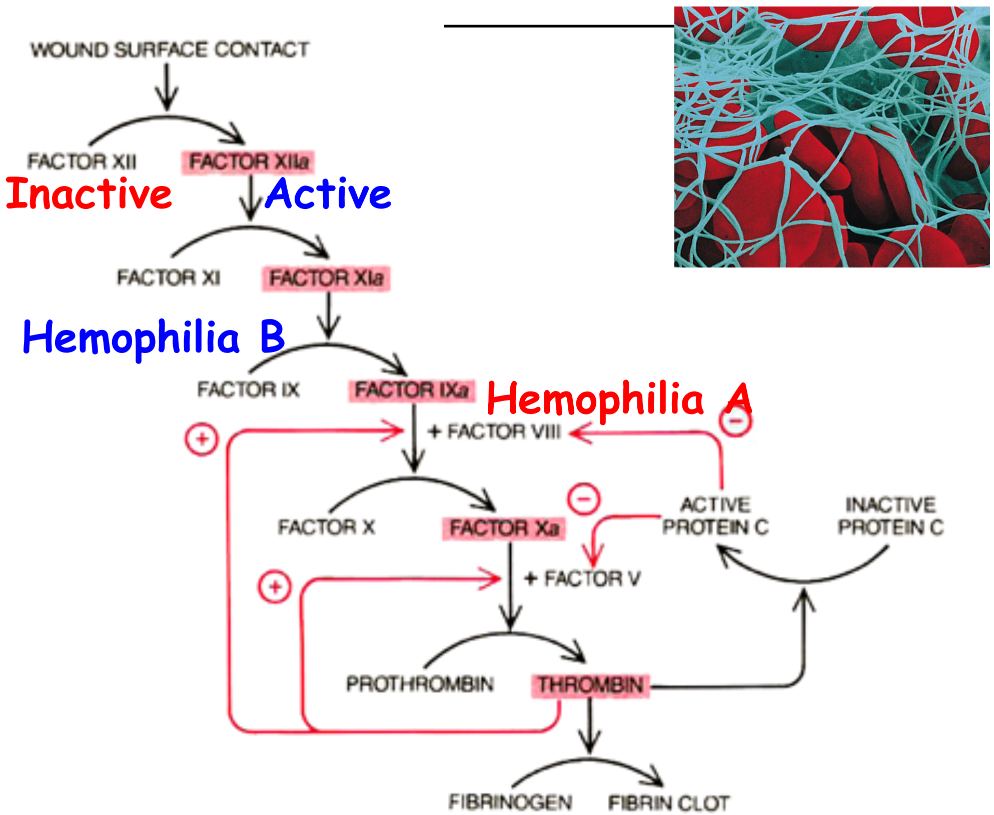
Genesis 17:10-14

'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!

Protein Factors in Blood Lead To Clotting



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!

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

TABLE 13.2 Some Important Genetic Disorders

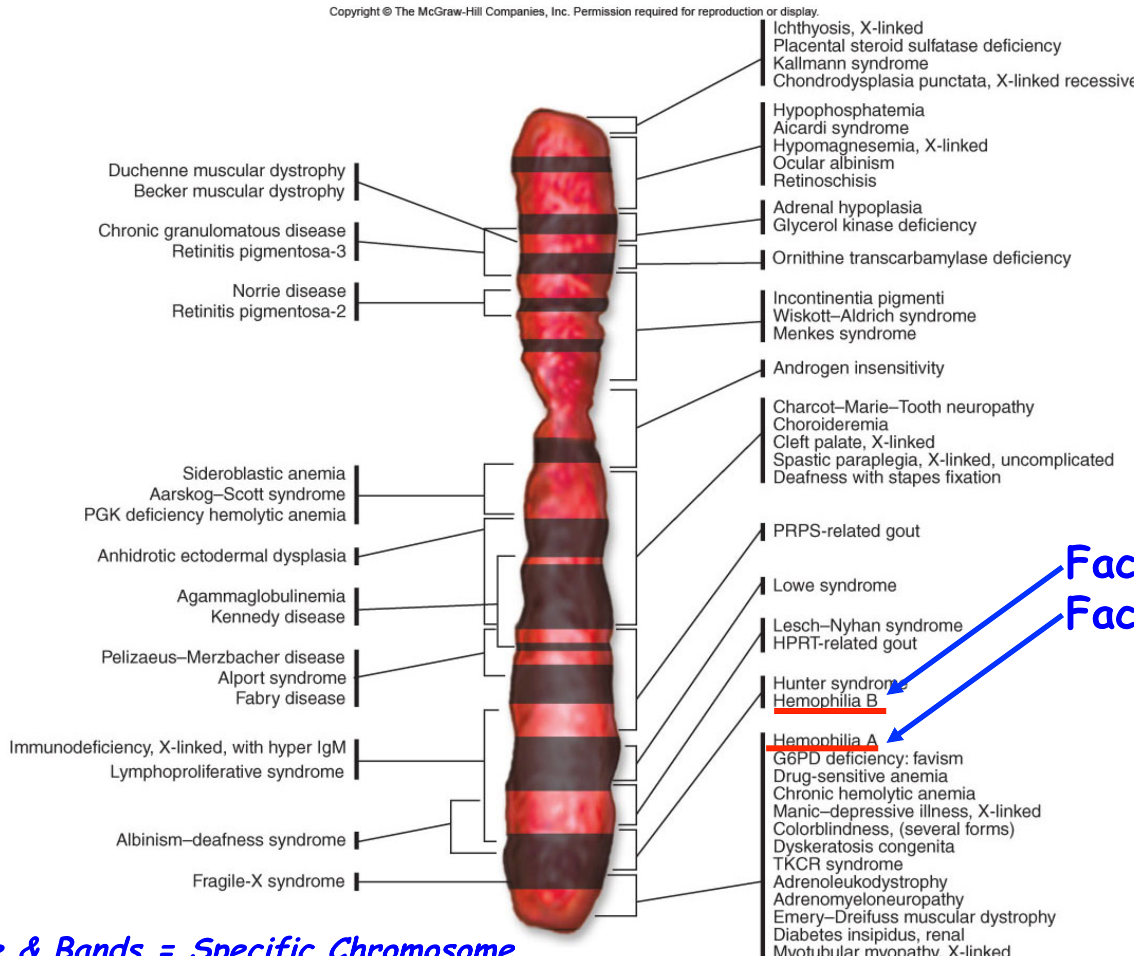
Disorder	Symptom	Defect	Dominant/ Recessive	Frequency Among Human Births
Cystic fibrosis	Mucus clogs lungs, liver, and pancreas	Failure of chloride ion transport mechanism	Recessive	1/2500 (Caucasians)
Sickle cell anemia	Blood circulation is poor	Abnormal hemoglobin molecules	Recessive	1/600 (African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

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

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



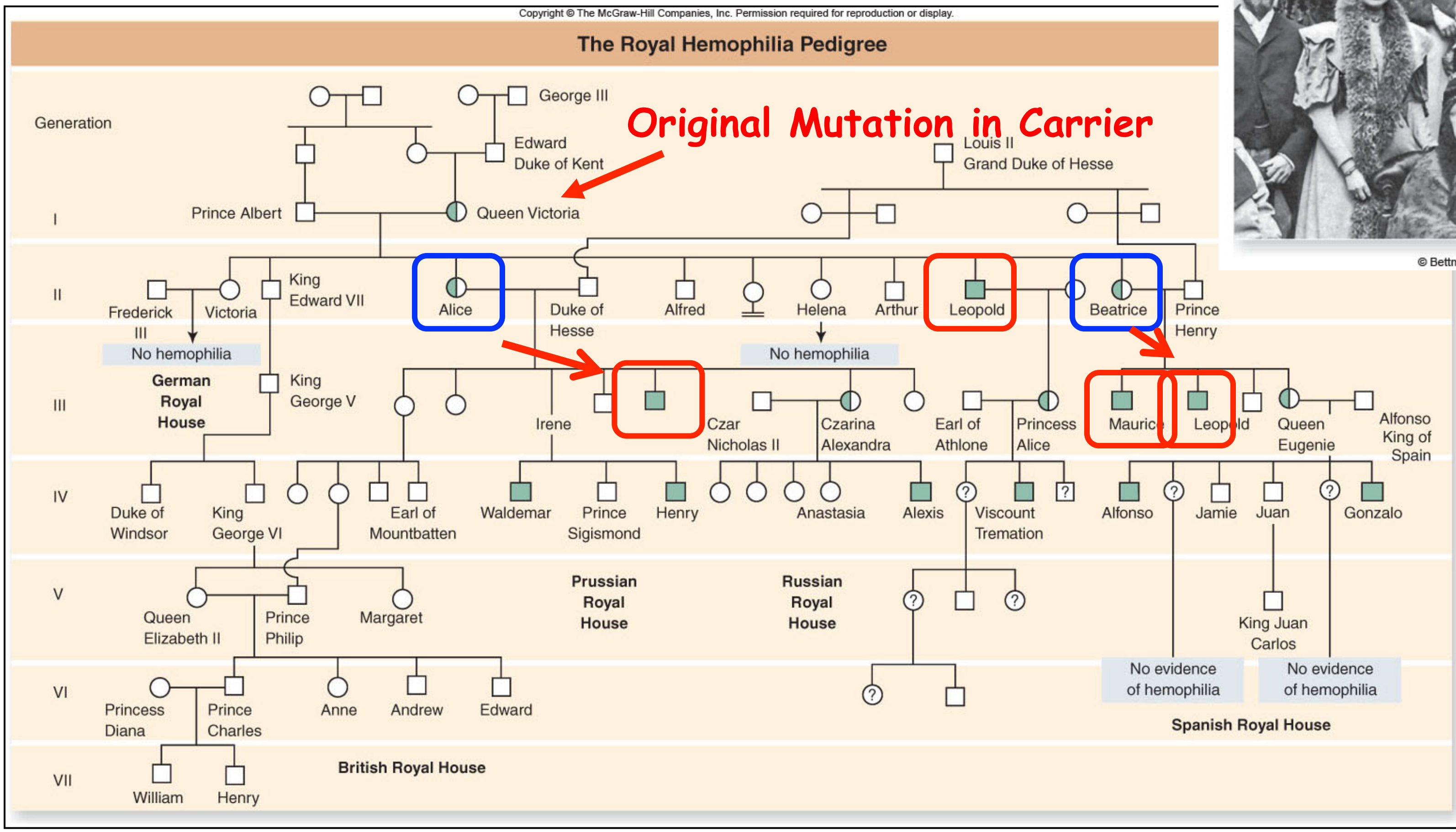
Factor IX
Factor VIII

Size & Bands = Specific Chromosome

Nature, March, 2005

The X chromosome has ~1098 Genes and 150,000,000 bp (150 Mb). ***168 Mendelian Diseases Explained by 113 X-Linked Genes***

Hemophilia A and B Genes Are Sex Linked & Recessive Traits



Note: 1. Males Obtain Detective Gene From Mothers

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

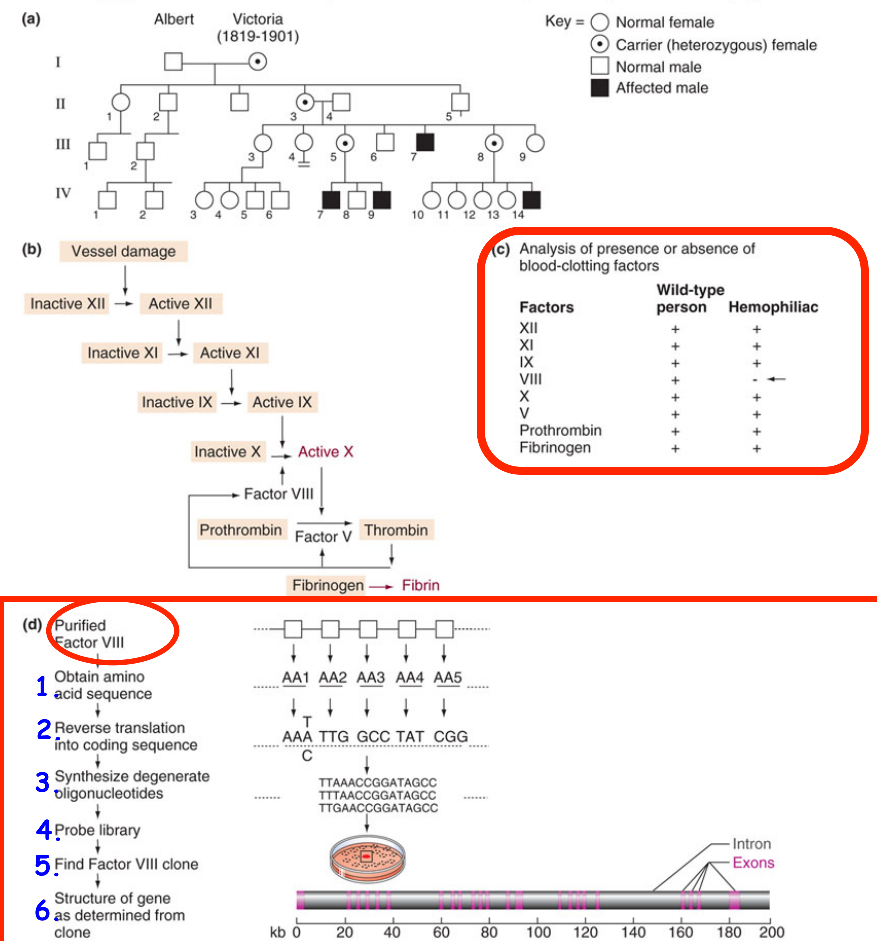
What Was Known About Factor VIII
Before Gene Cloned?

- Blood Protein (But Perhaps Synthesized Elsewhere!)
- Not Known Where Site of Synthesis Was
- Could Be Purified In Small Amounts From >20 Liters Of Human Blood + Cow Blood + Pig Blood
- Short Stretch Of Protein Sequenced = Known Protein Sequence!
- Hemophilia A Could Be Treated By *Blood Transfusions* From Normal Individuals, ∴ Clotting Factor In Blood
- 1980s Aids Epidemic Caused Many Hemophiliacs To Get HIV/AIDs (~50% Of Hemophiliacs Got Aids In 1985)
    - ∴ How To Go From Protein To Gene

The Problem!!

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

Early 1980's



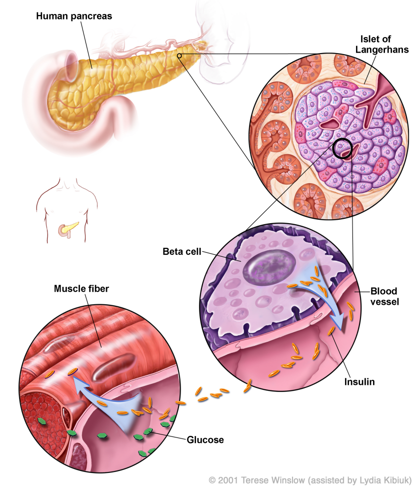
Key Concept

↓

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

Key: Protein Sequence Known →

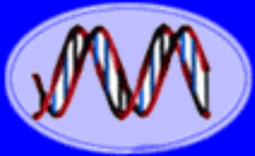
Different Than Insulin Knew Where Protein Made!



mRNA → Drug

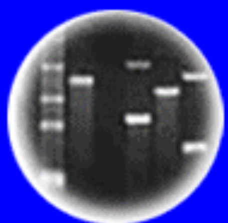
How Find Gene & cDNA?

Protein → Gene → mRNA → Drug !



DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

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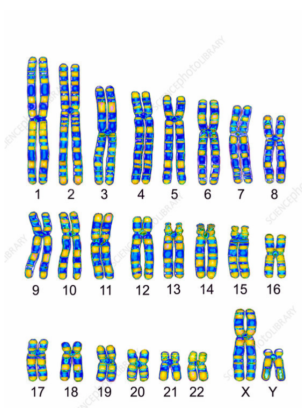
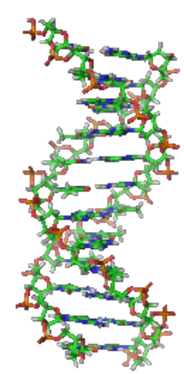
Step One

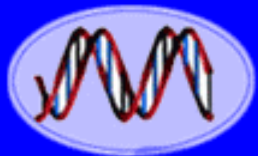
If It is Not Known Where Gene is Active Can "Look" to Genome Instead of mRNA to Find + Clone Gene!

How to Construct a Human Genome Library to Find the Factor VIII Gene?





Genomic Libraries vs. cDNA Libraries



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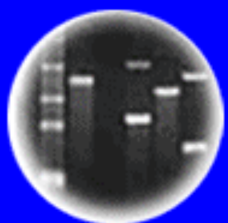

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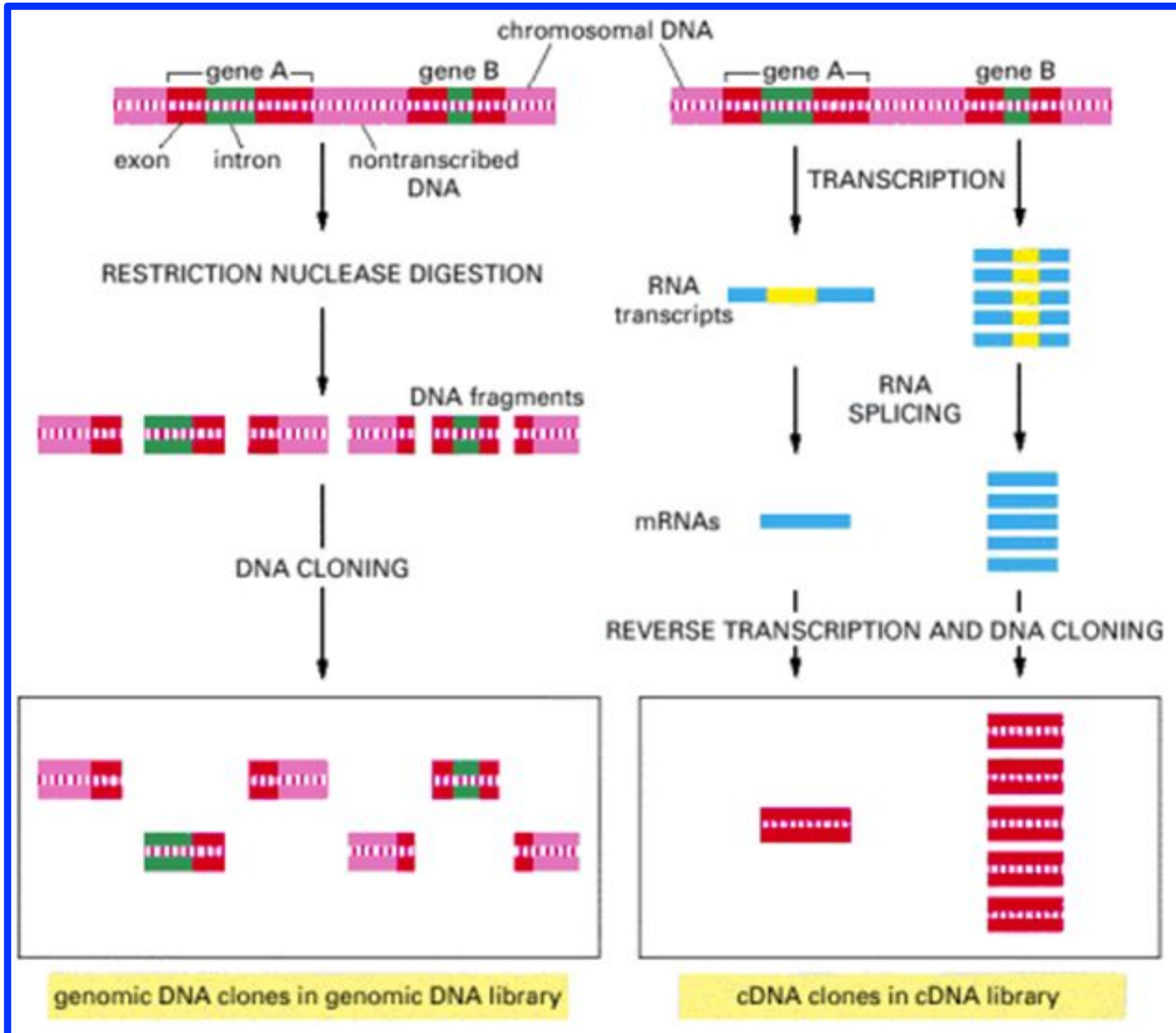

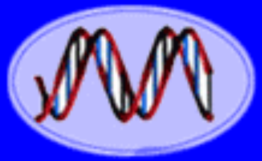

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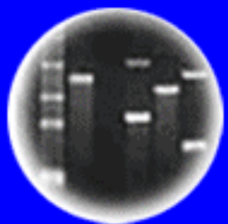

DNA Fingerprinting

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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

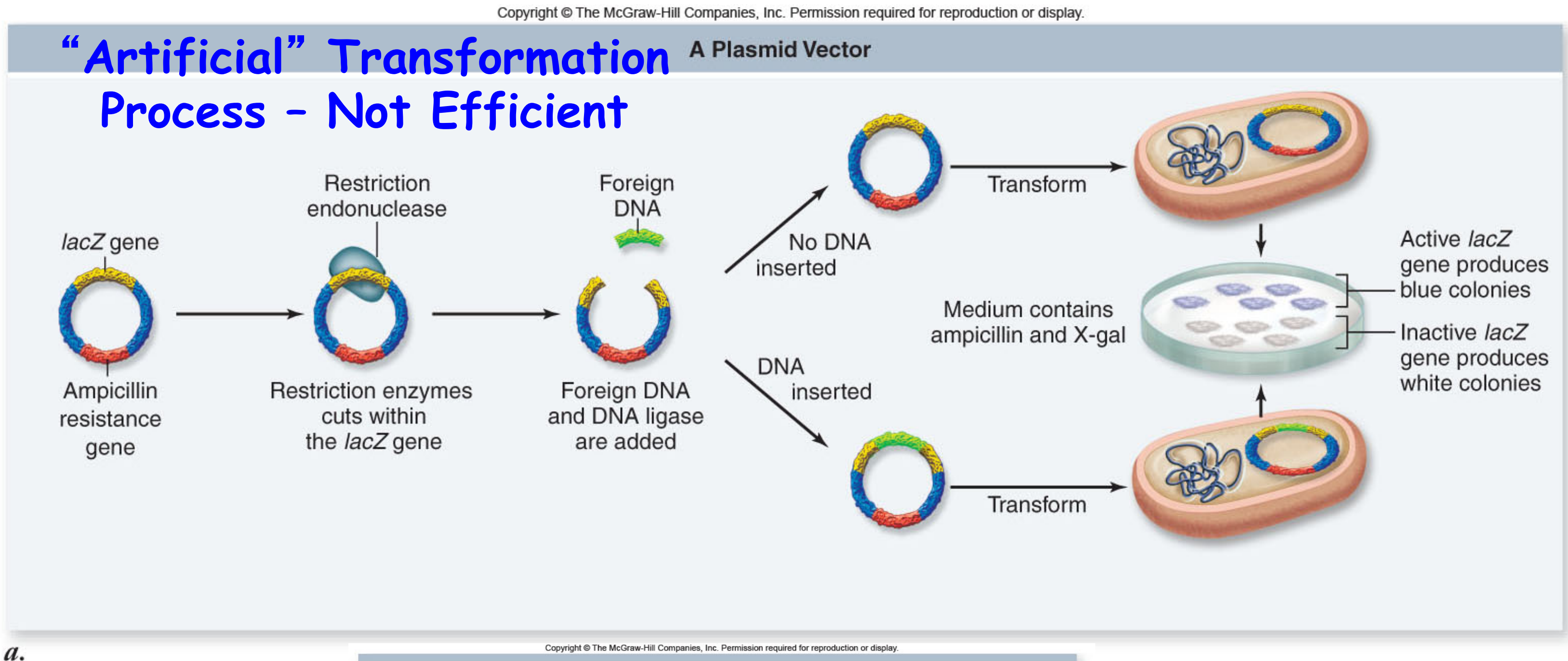
Vector Type	Maximum Insert Size (kb)	Applications	Limitations
Bacterial plasmid vectors (circular)	~6–12	DNA cloning, protein expression, subcloning, direct sequencing of insert	Restricted insert size; limited expression of proteins; copy number problems; replication restricted to bacteria
	DNA	DNA	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

Properties of All Vectors

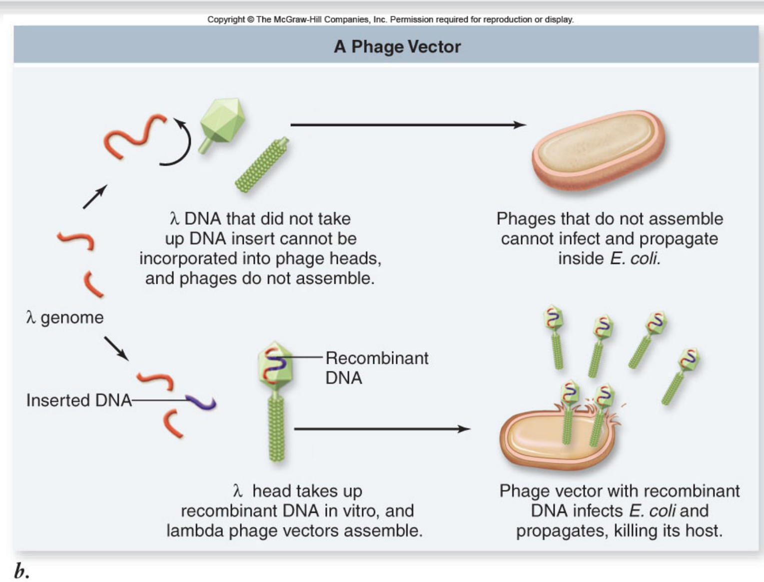
1. Replicate
2. Selectable
3. Can Be Used To Insert Foreign Genes/Restriction Sites
4. Easily Isolated + Transferred Back To Cells

Plasmid vs. Bacteriophage Vectors for Cloning DNA Fragments

“Artificial” Transformation Process – Not Efficient



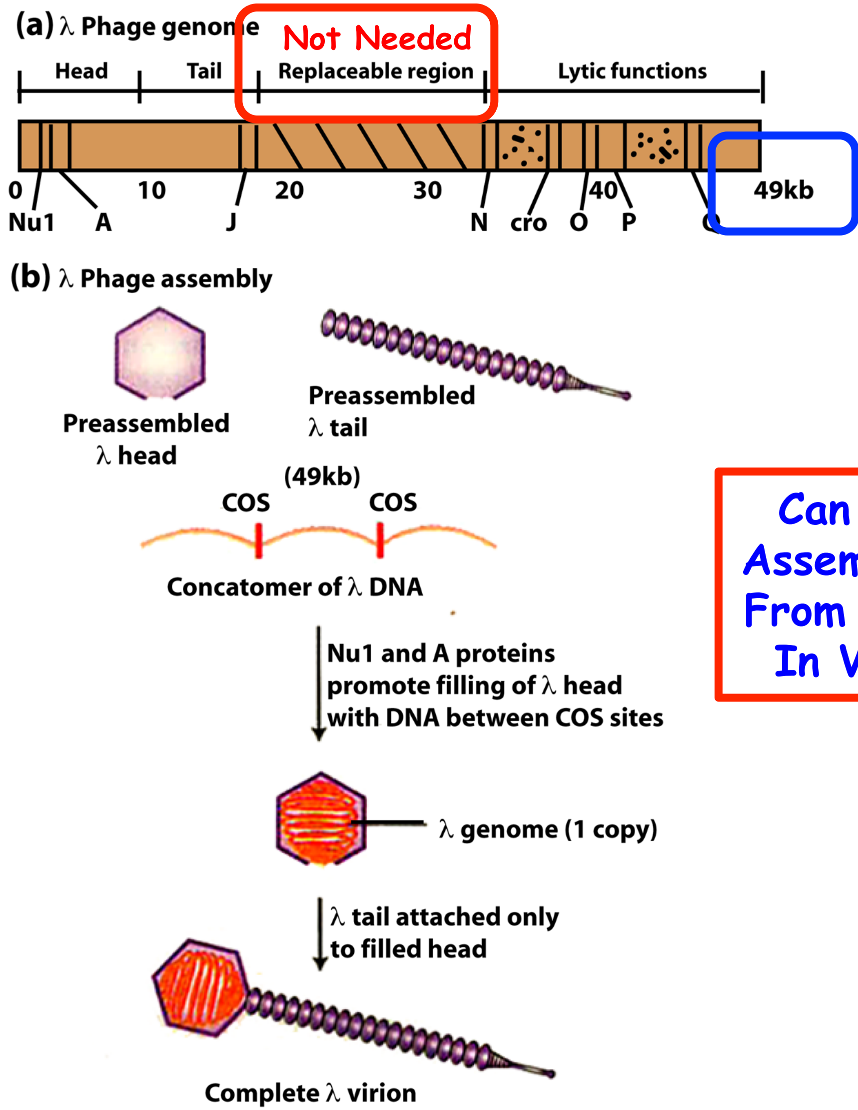
“Natural” Infection Process

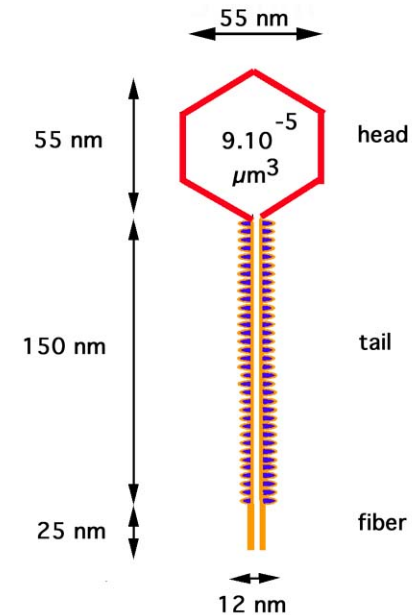


Advantages

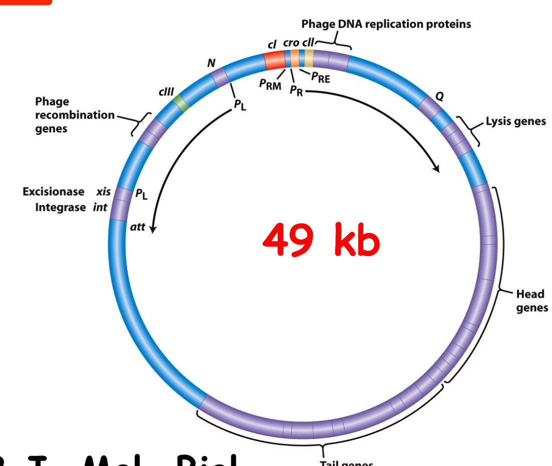
- Much More Efficient
- Can Use Less DNA
- Get Lots More Clones
- Need Lots of Clones For Large Genome

Structure of the λ Phage and Its Genome



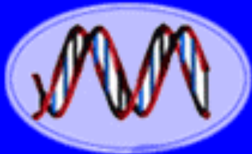


Can Be Assembled From Parts In Vitro



First Genome Sequence

Sanger et al. 1982 J. Mol. Biol. 162: 729-773.



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Entire Genetic Code
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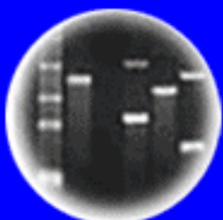

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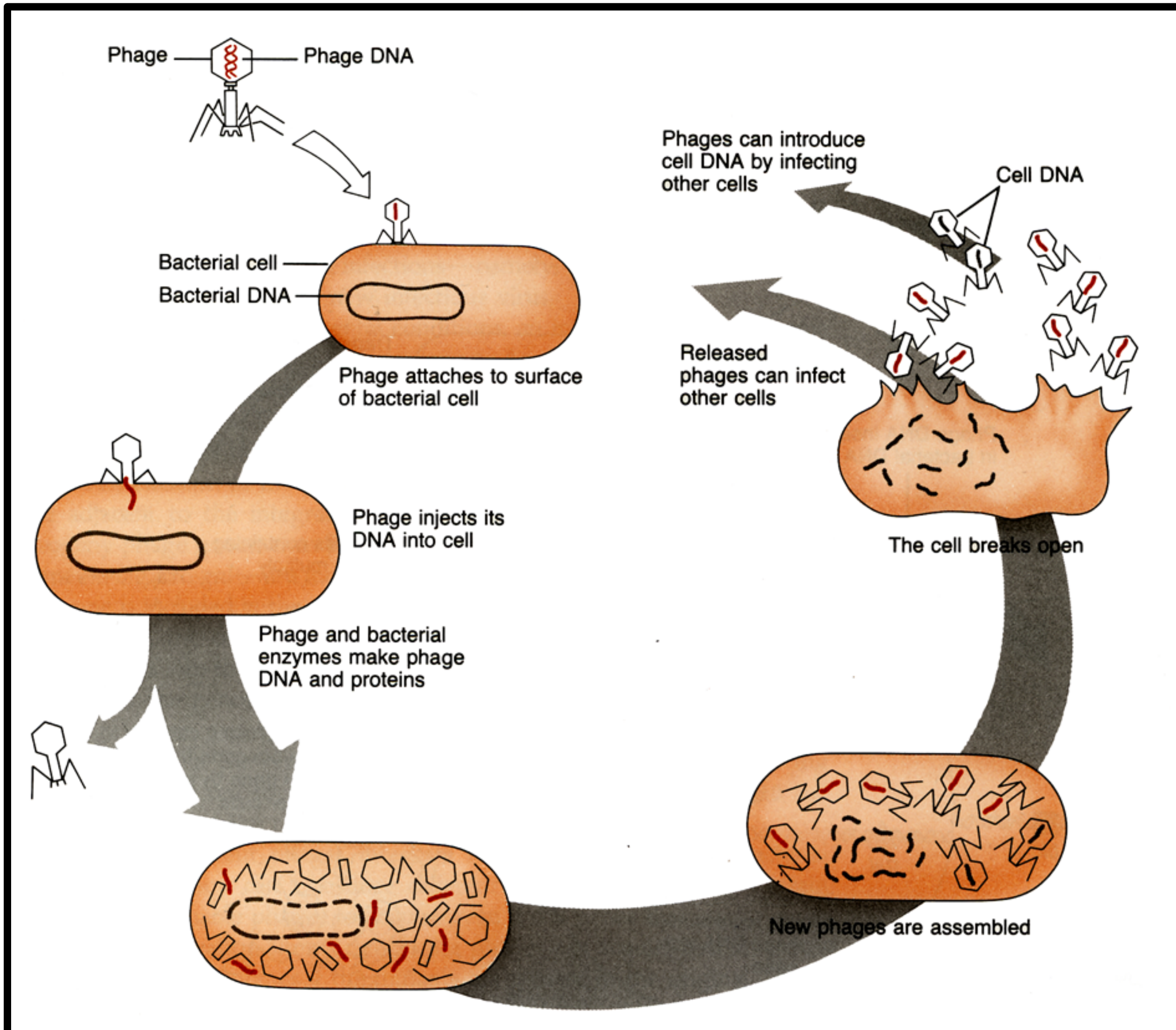
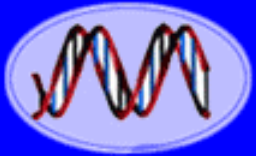
λ Phage Infects *E.coli* & Destroys (Lyses) Cells





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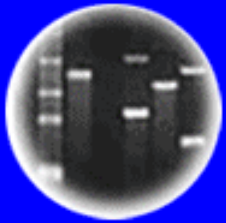

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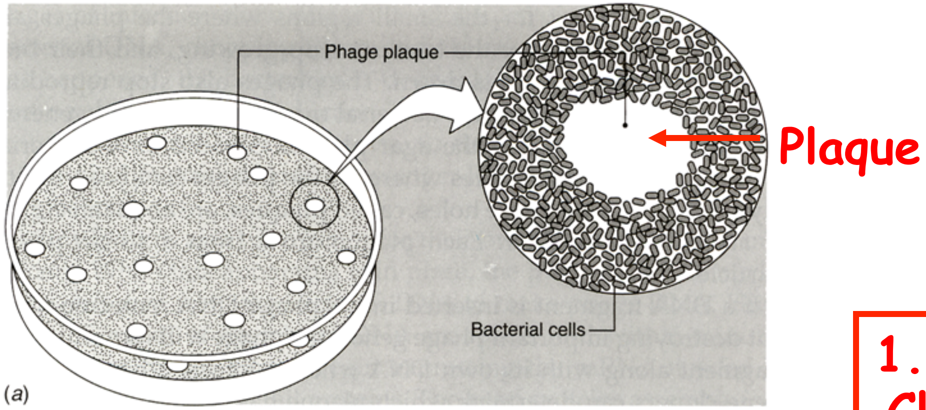
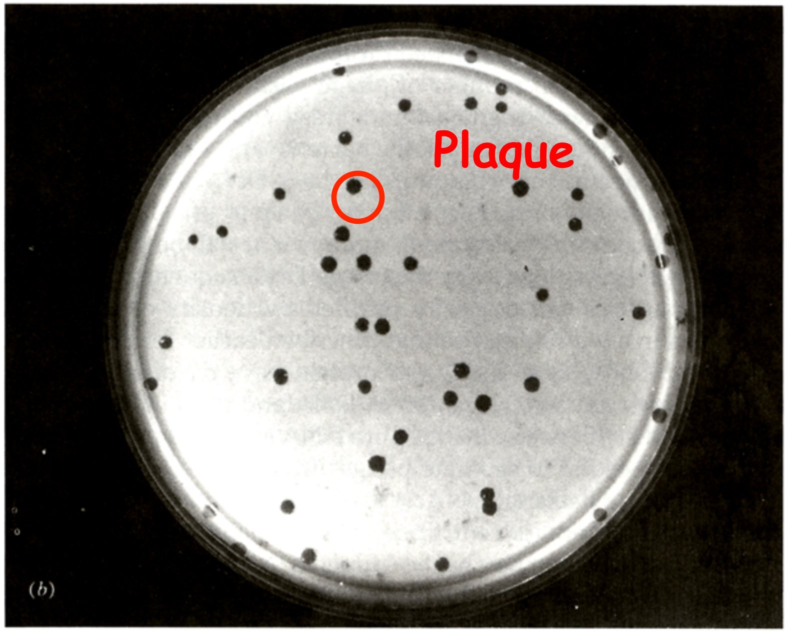
Lysed Cells Can Be Seen as Clear Plaques on Agar Plates





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!

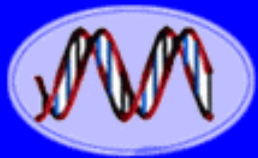
2. Can Clone DNA Segments In Viral Genome & Self-Assemble With Viral Proteins Into Virus In A Test Tube!

∴ 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!



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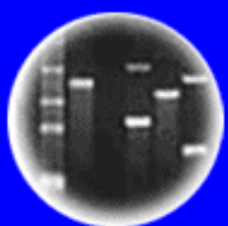

DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

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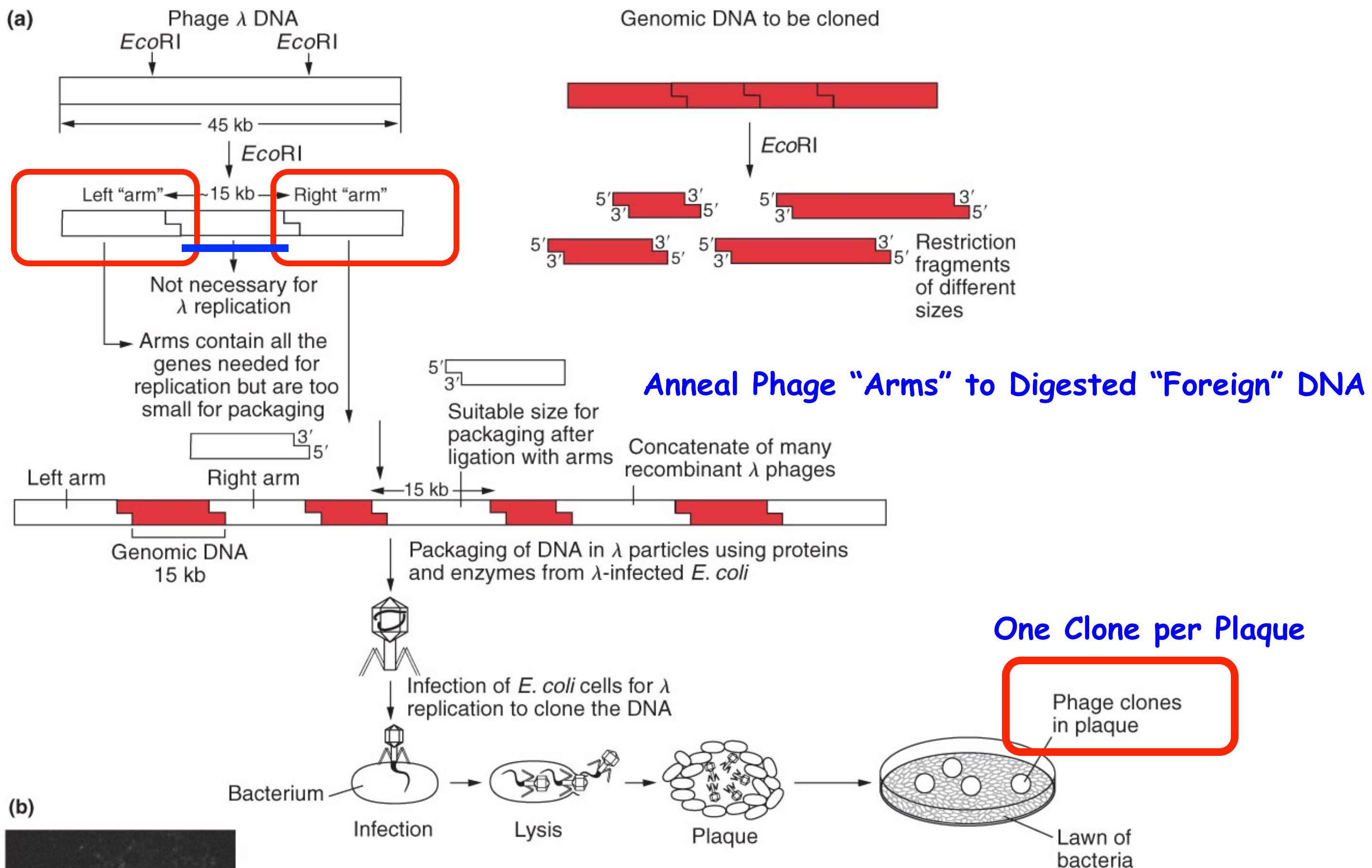
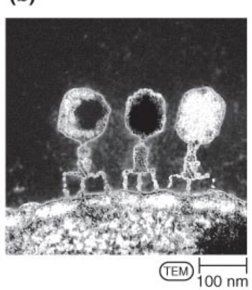
Using a Bacterial Virus To Clone the Human Genome



Anneal Phage "Arms" to Digested "Foreign" DNA

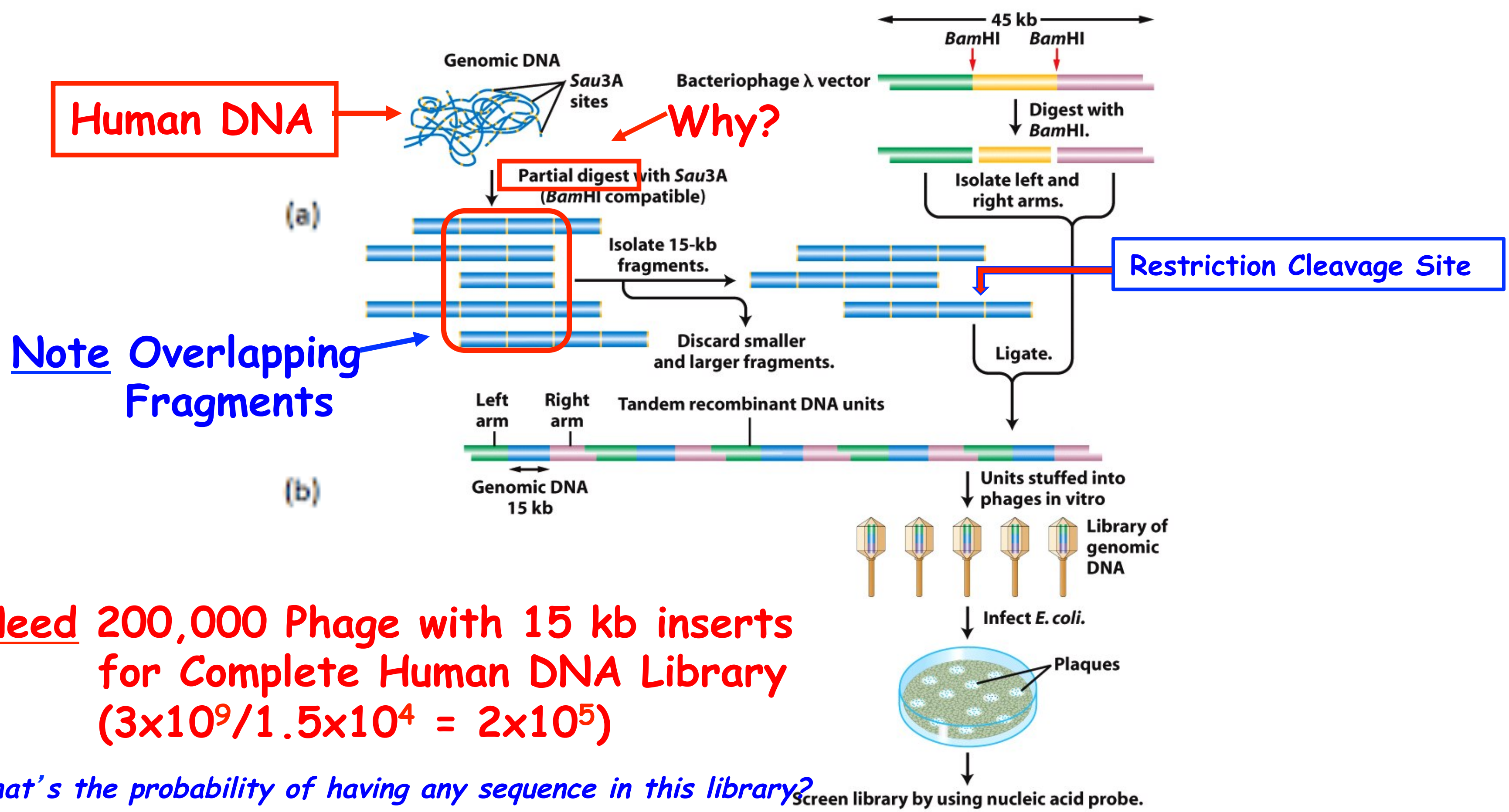
One Clone per Plaque

(b)



Mixture of Plaques = Library With All Human DNA Sequences Represented

Cloning the Human Genome and Screening for the Factor VIII Gene



Need 200,000 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
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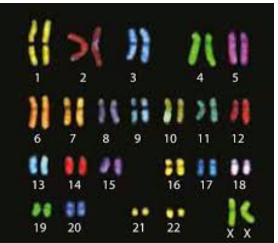
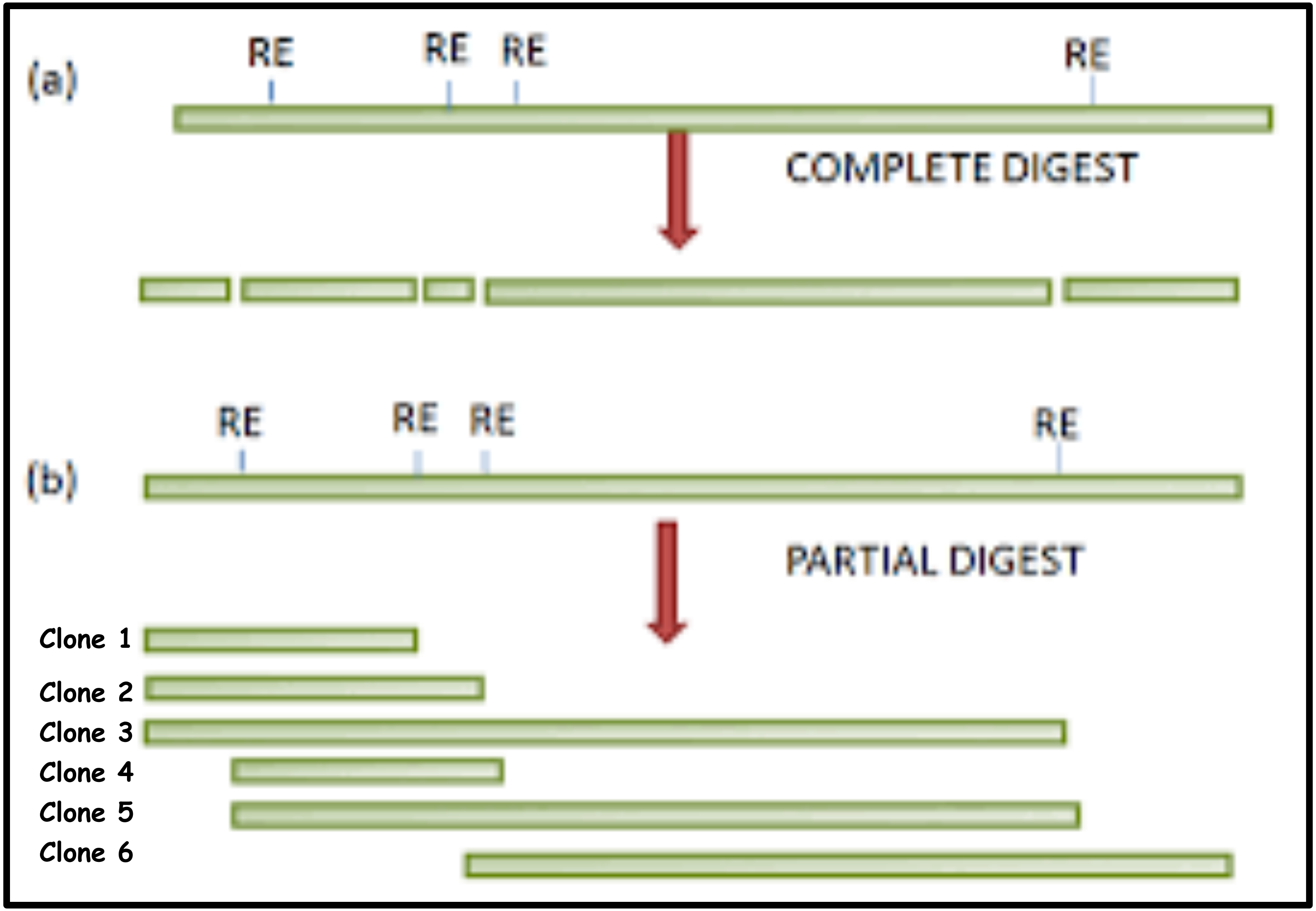


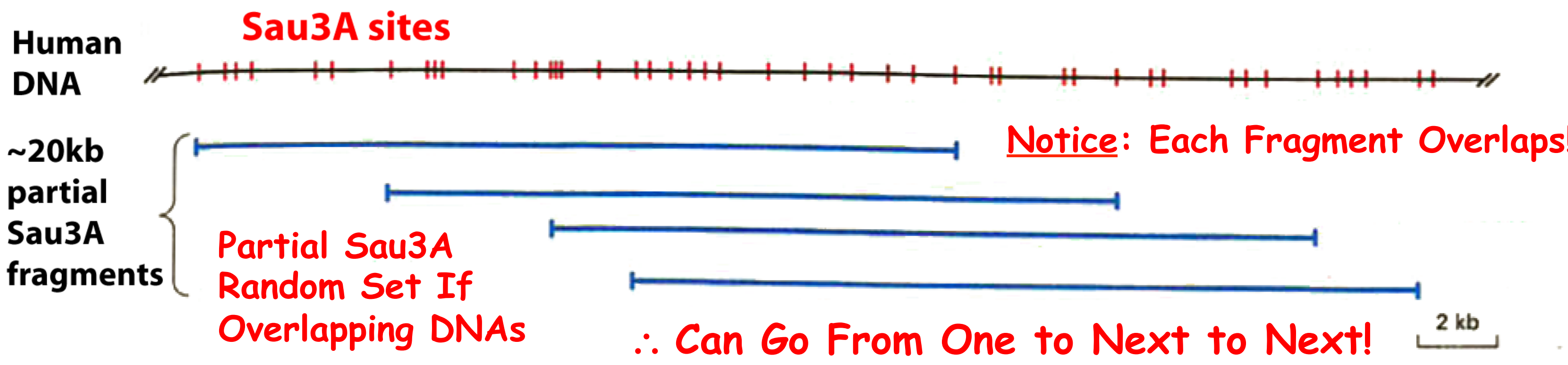
Why Partial Digestion? An Important Concept!

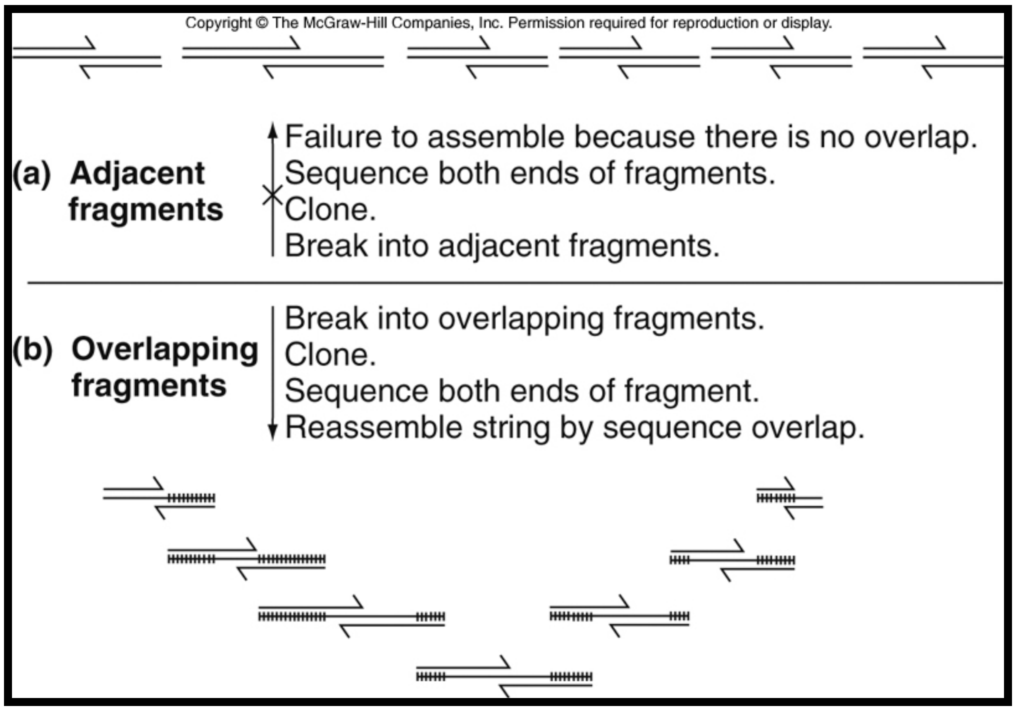
What is Complete & Partial Digestion?

Partial Digestion Permits "Walking" From One DNA Region to the Next



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





Step Two

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

The Genetic Code

Second Letter

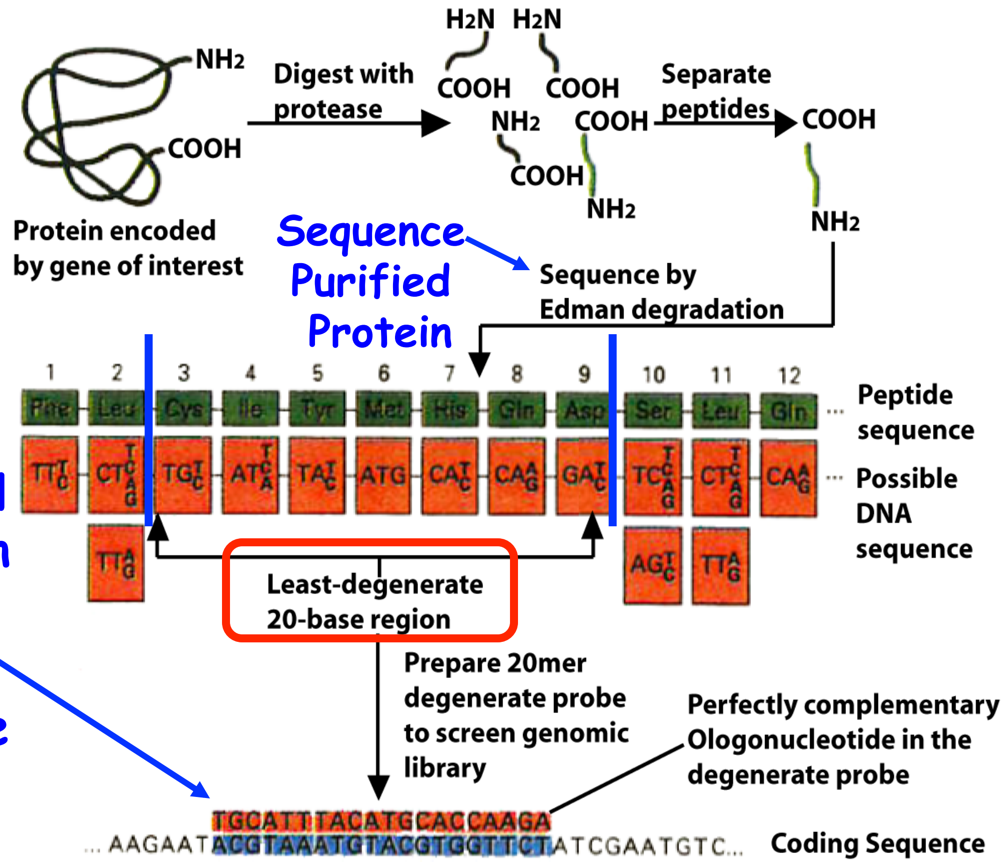
1st letter	U		C		A		G		3rd letter
U	UUU	Phe	UCU		UAU	Tyr	UGU	Cys	U
	UUC		UCC	Ser	UAC		UGC		C
	UUA	Leu	UCA		UAA	Stop	UGA	Stop	A
	UUG		UCG		UAG	Stop	UGG	Trp	G
C	CUU		CCU		CAU	His	CGU		U
	CUC	Leu	CCC	Pro	CAC		CGC	Arg	C
	CUA		CCA		CAA	Gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU		ACU		AAU	Asn	AGU	Ser	U
	AUC	Ile	ACC	Thr	AAC		AGC		C
	AUA		ACA		AAA	Lys	AGA	Arg	A
	AUG	Start Met	ACG		AAG		AGG		G
G	GUU		GCU		GAU	Asp	GGU		U
	GUC	Val	GCC	Ala	GAC		GGC	Gly	C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

Properties

- Universal
- Three Nucleotides
- Punctuation
- Degenerate

Factor VIII Protein → Gene

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



Sequence Purified Protein

1. Use Genetic Code

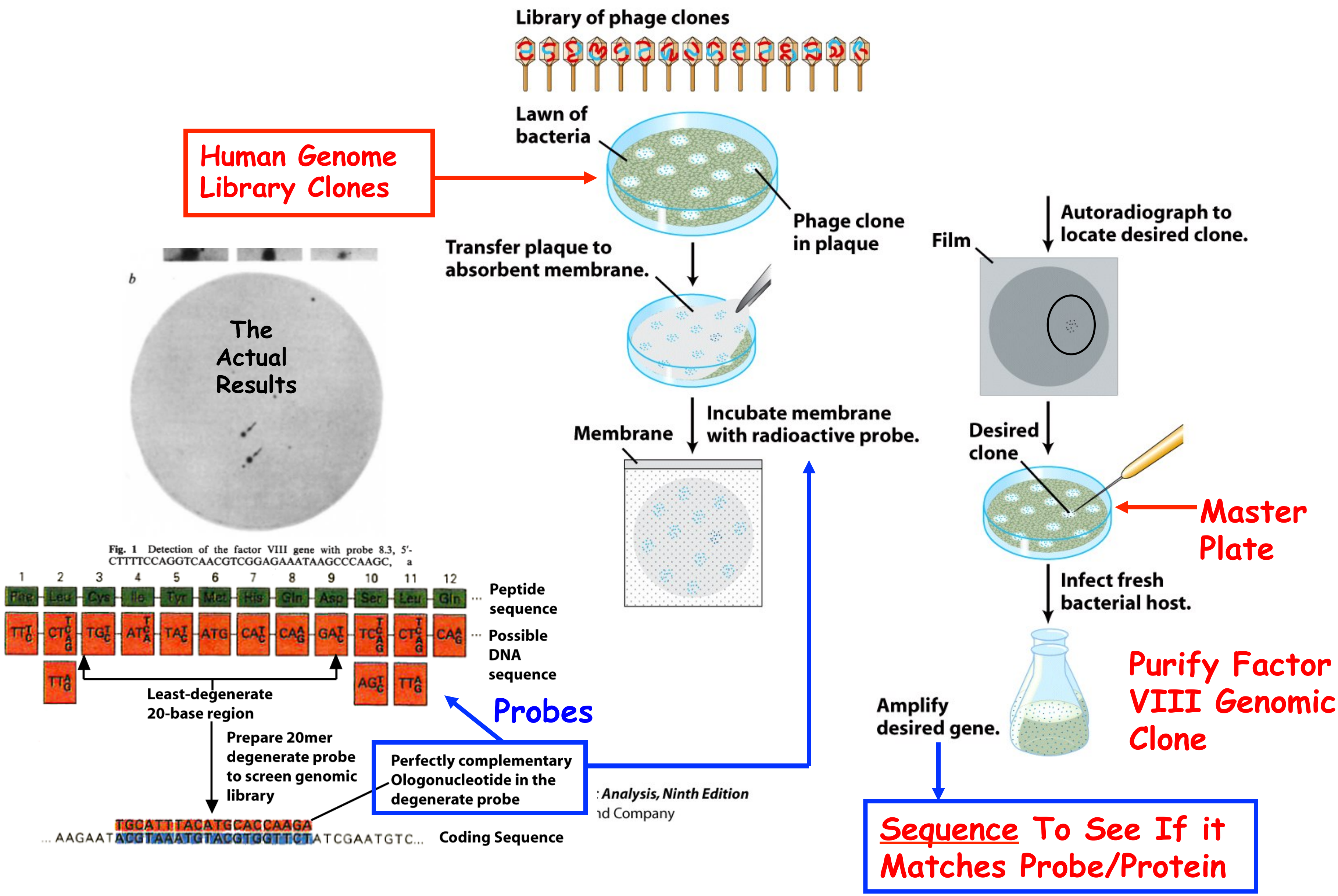
2. Make Several Probes All Codon Combinations!

3. One Will Be Correct Probe

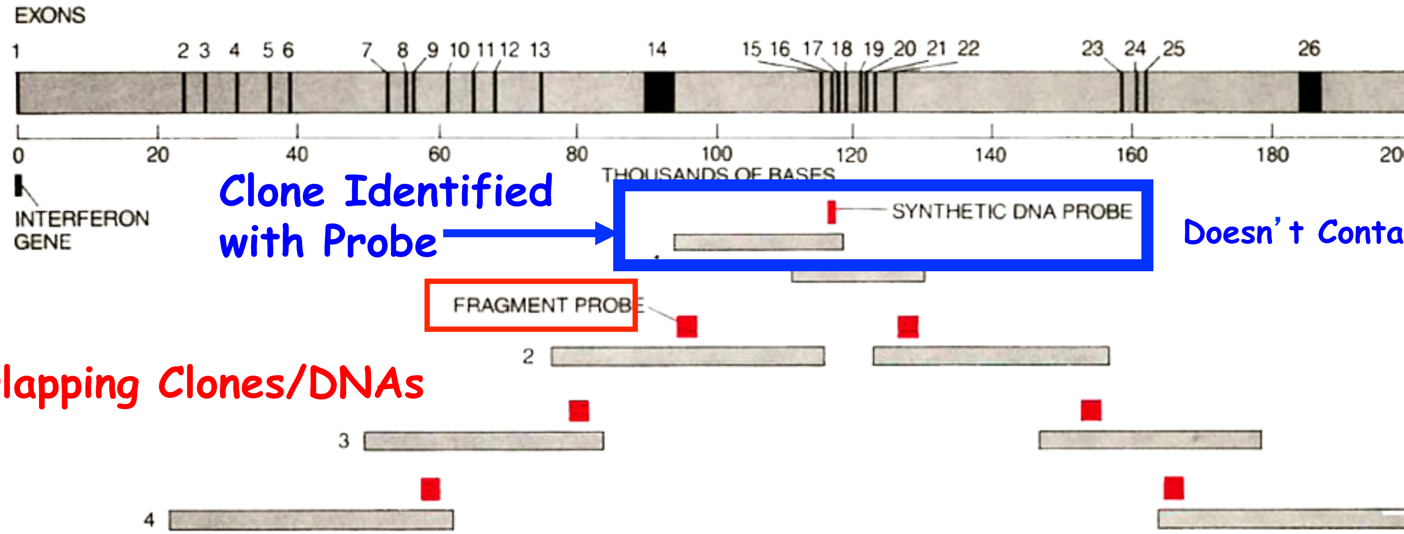
How many Combinations of Synthetic Probes?

2x3x2x1x2x2x2=96

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?



Clone Identified with Probe

Doesn't Contain Entire Gene!

Overlapping Clones/DNAs

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

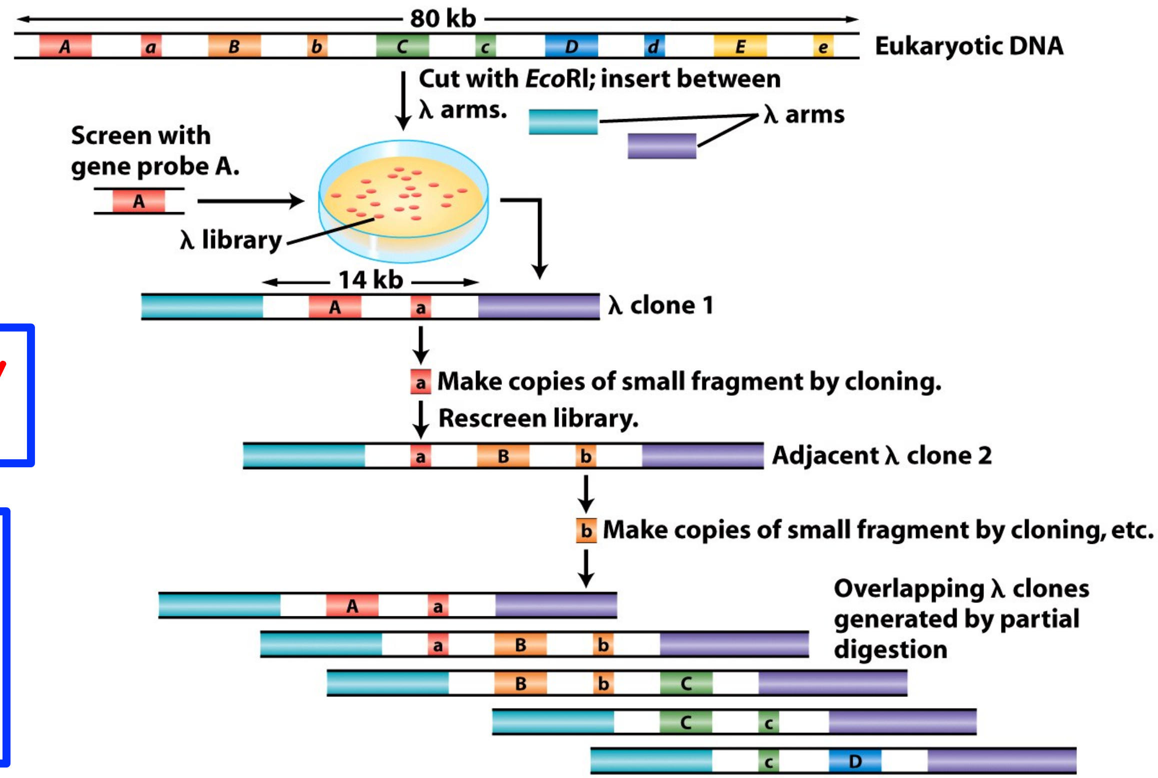


Figure 20-13
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Reiterative Library Screening Process

Find Overlapping Clones By Restriction Site Mapping

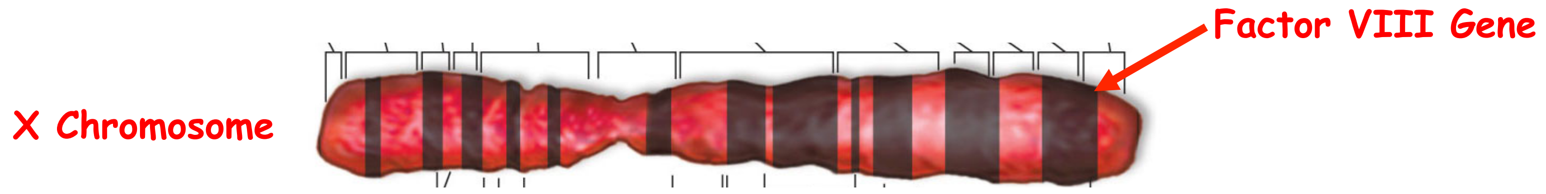
Basis of Genome Projects & Whole Genome Sequencing

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 Mutations Occur Throughout the Gene

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

actor 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
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

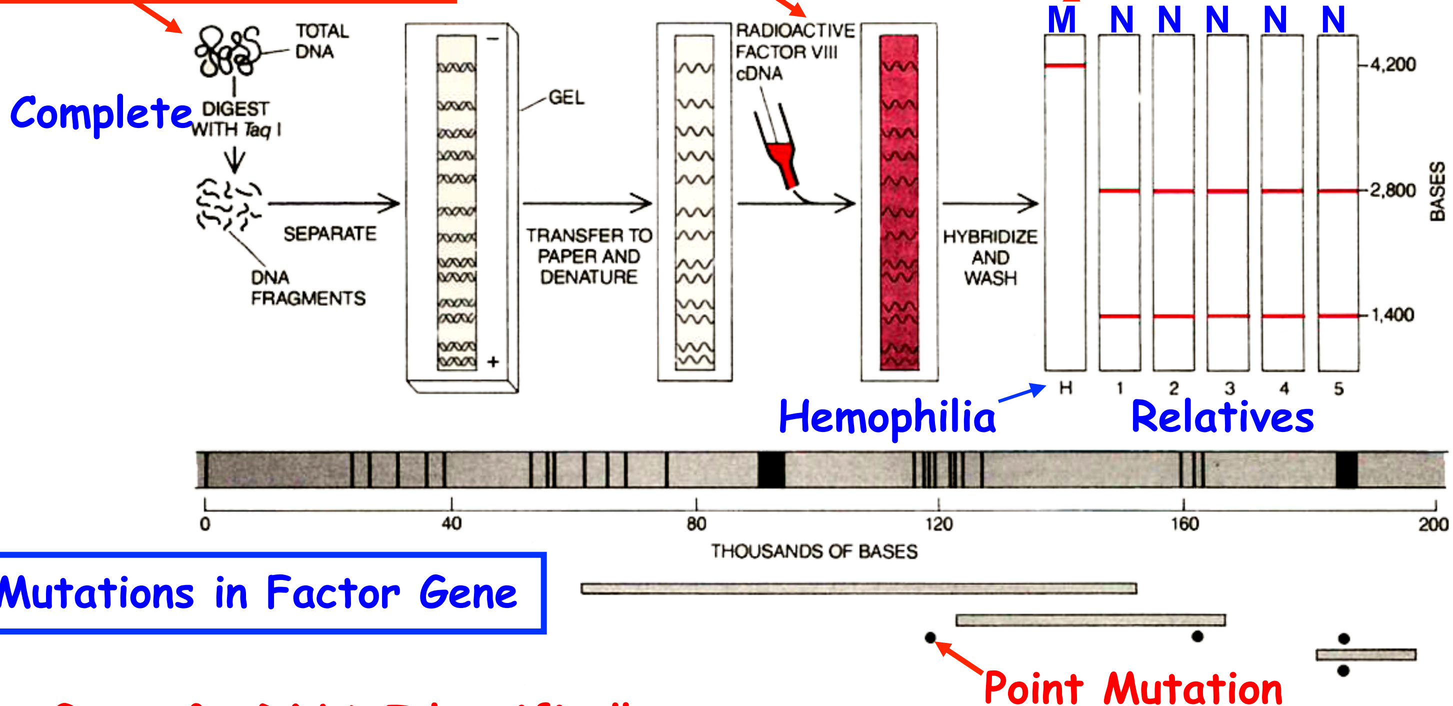
Need To Screen Across the Gene for Markers -- Family Specific

Factor VIII 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



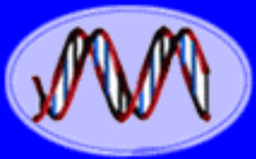
RFLP

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



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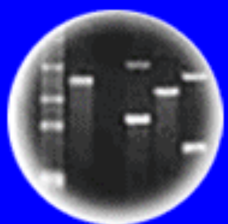

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Step Four

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

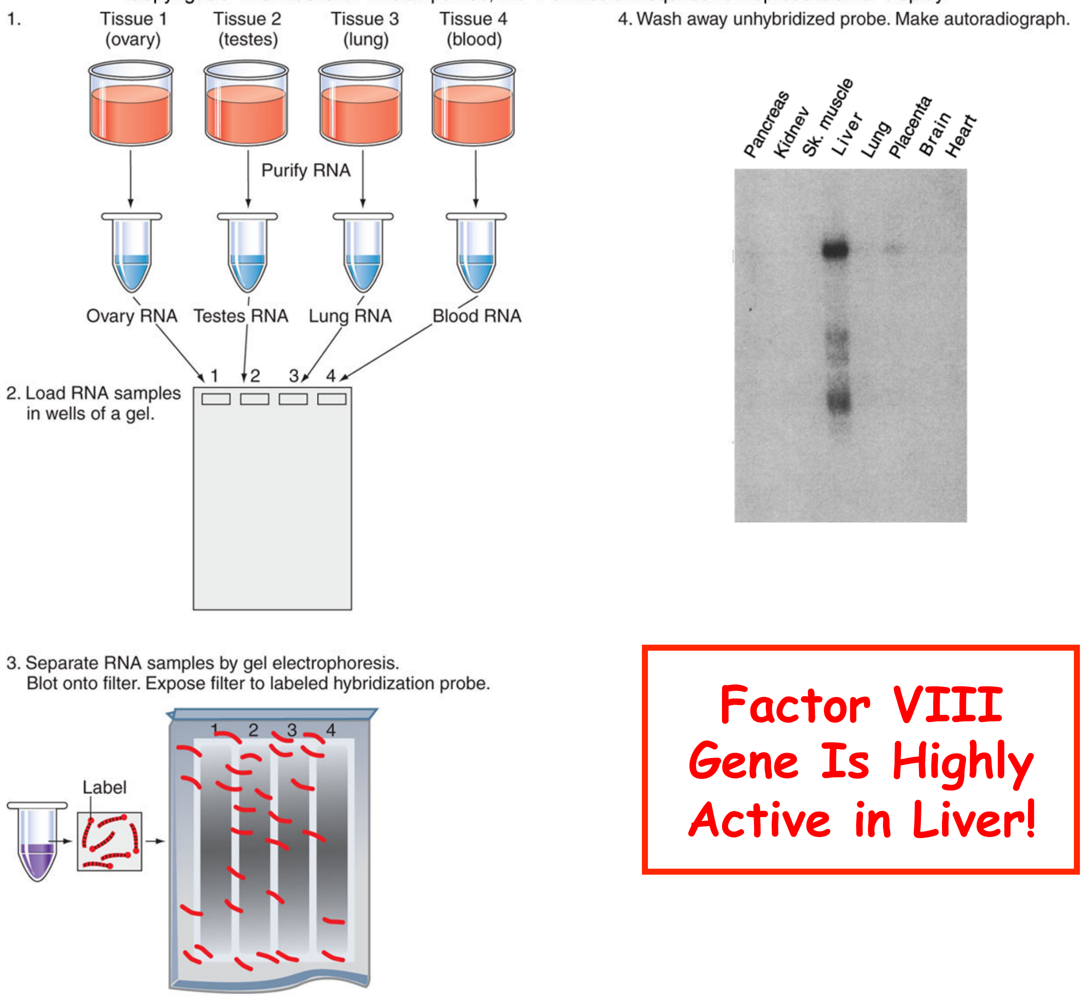
Recall: Eukaryotic Genes Provide Obstacles for Efficient Protein Production in Genetically Engineered Cells!

Introns! Switches!

Making the Drug

Need cDNA Not Gene

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

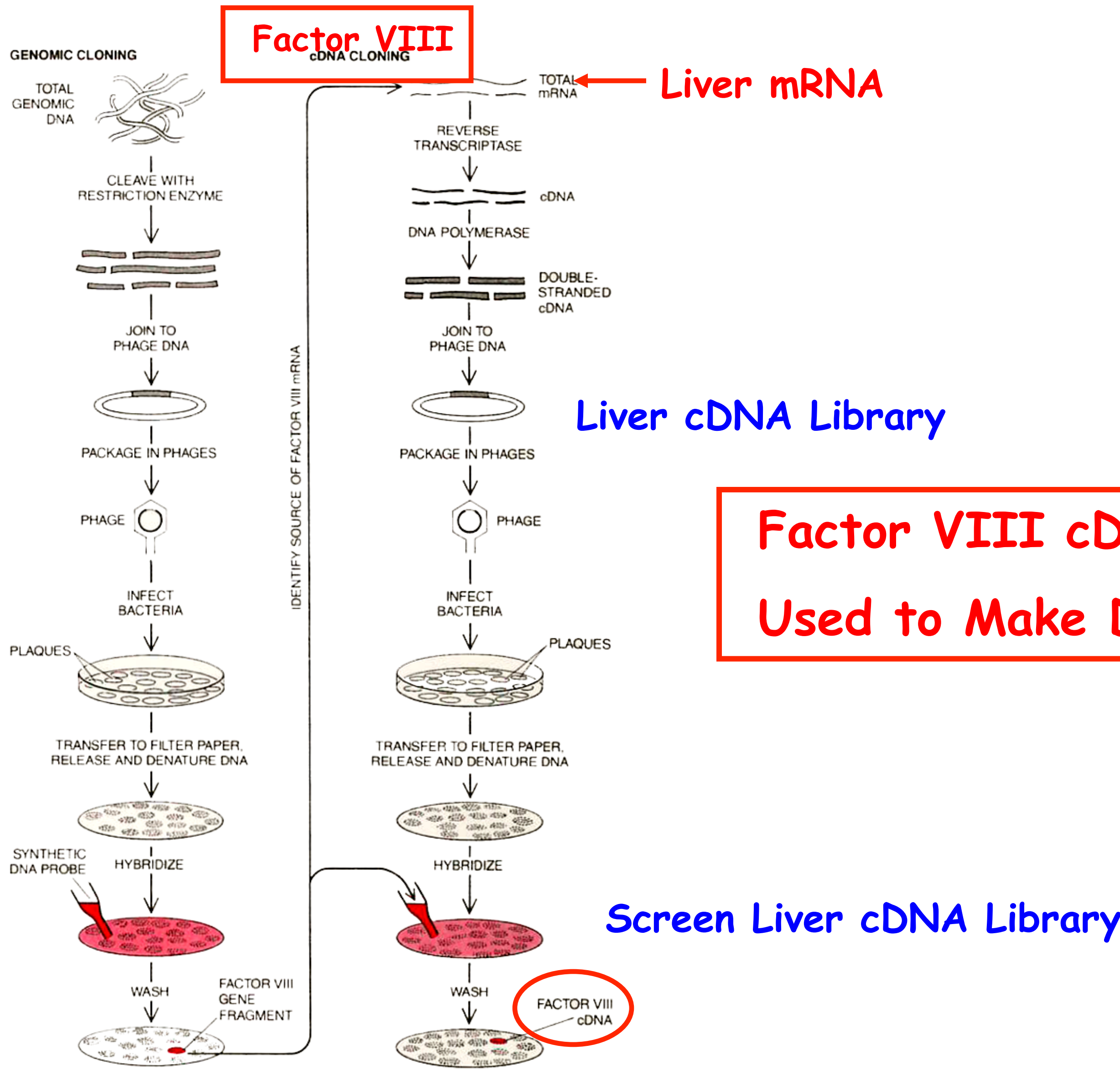


RNA Blot Is Like a DNA Blot Except That RNA is on Gel & Blotted

Factor VIII Gene Is Highly Active in Liver!

Can Also Use PCR (RT-PCR)

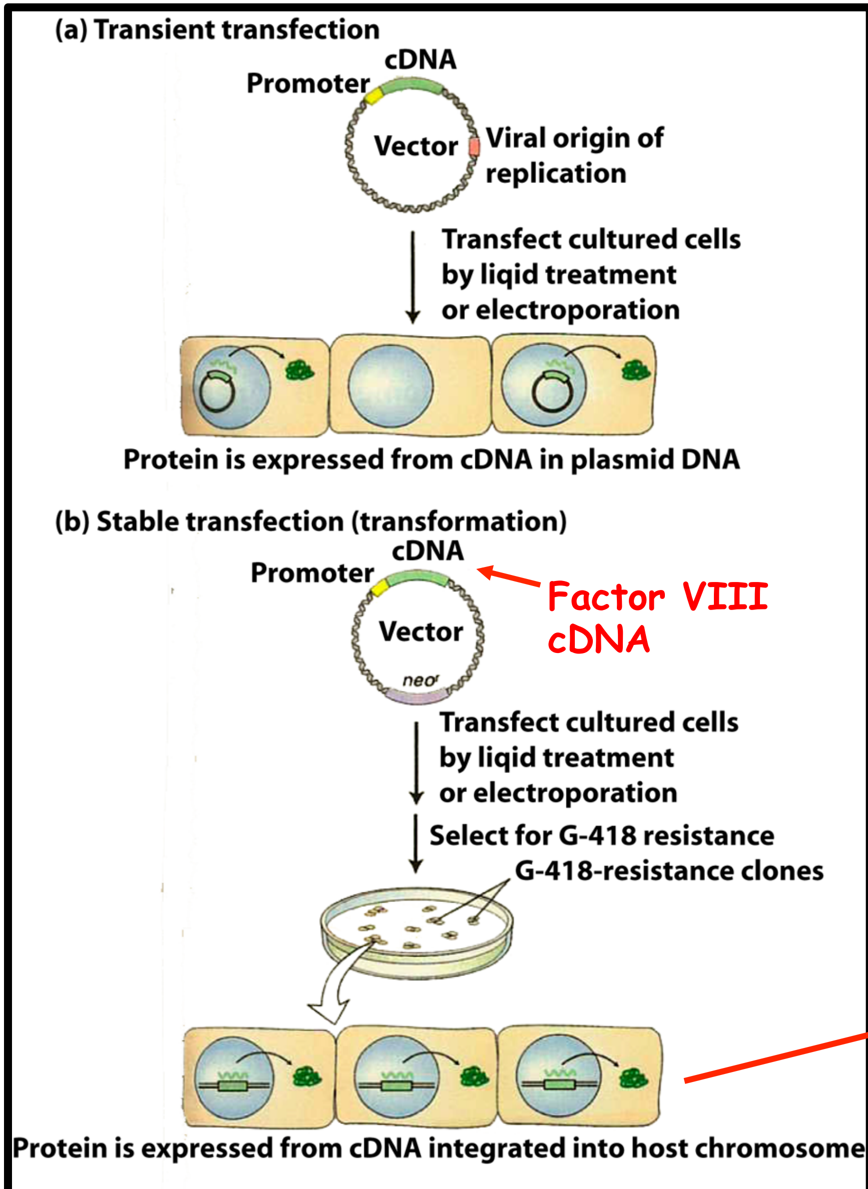
Using Factor VIII Gene Probe to Identify Factor VIII cDNA clone



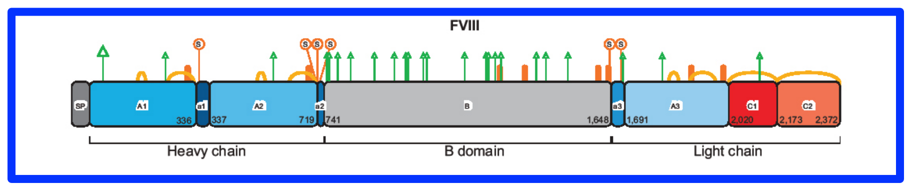
Factor VIII cDNA →

Used to Make Drug !

Engineer Factor VIII cDNA to Produce Protein in Host Cell & Synthesize 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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- 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.

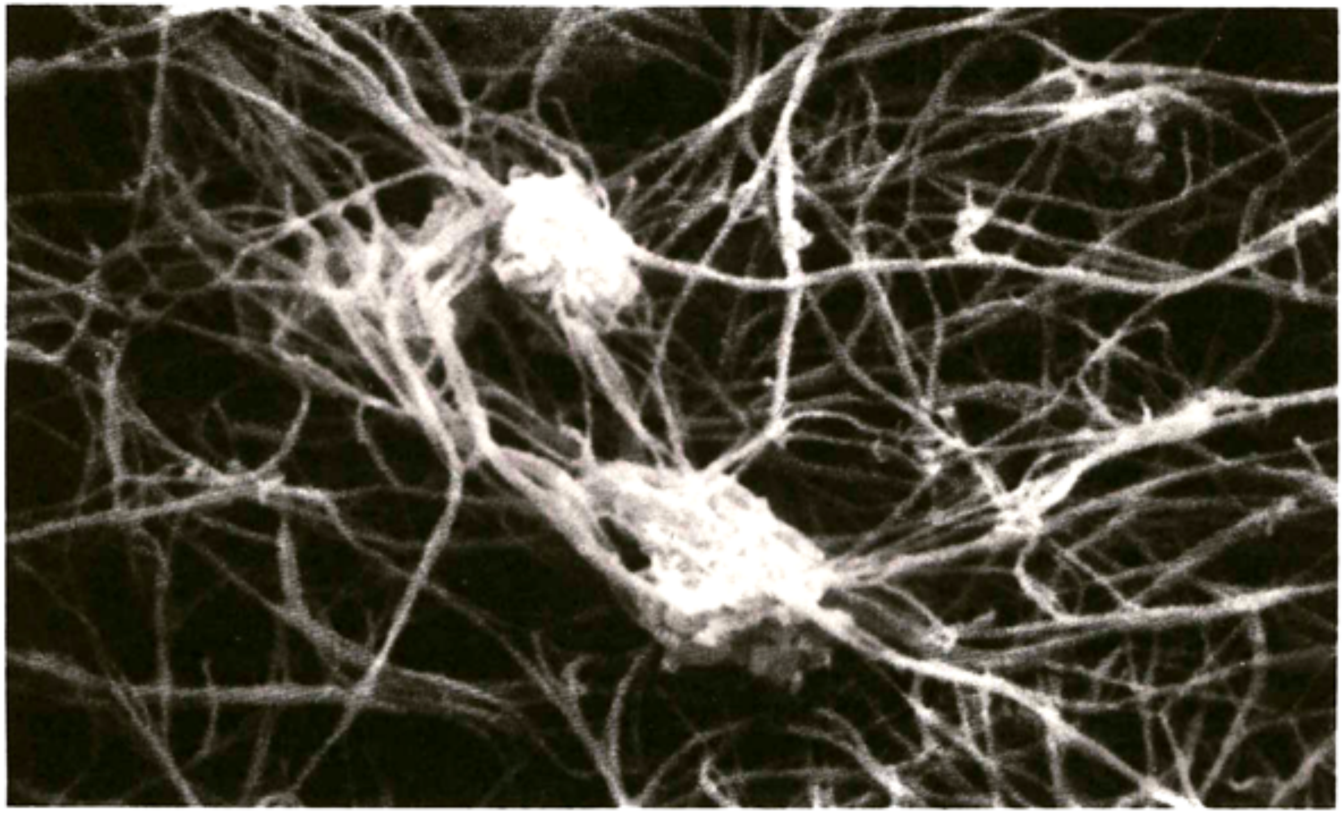
Prophylactic Treatment Costs $300,000/ Year!
Most Hemophiliacs Use "On Demand" or As Needed

Factor VIII Gene Cloned In 1983

Factor VIII (Recombinant) Approved As Drug In 1993! Ten Years From Gene → Drug! (Off Patent In 2011)

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 First Ever In-Human Gene Editing Will Try and Combat Hemophilia

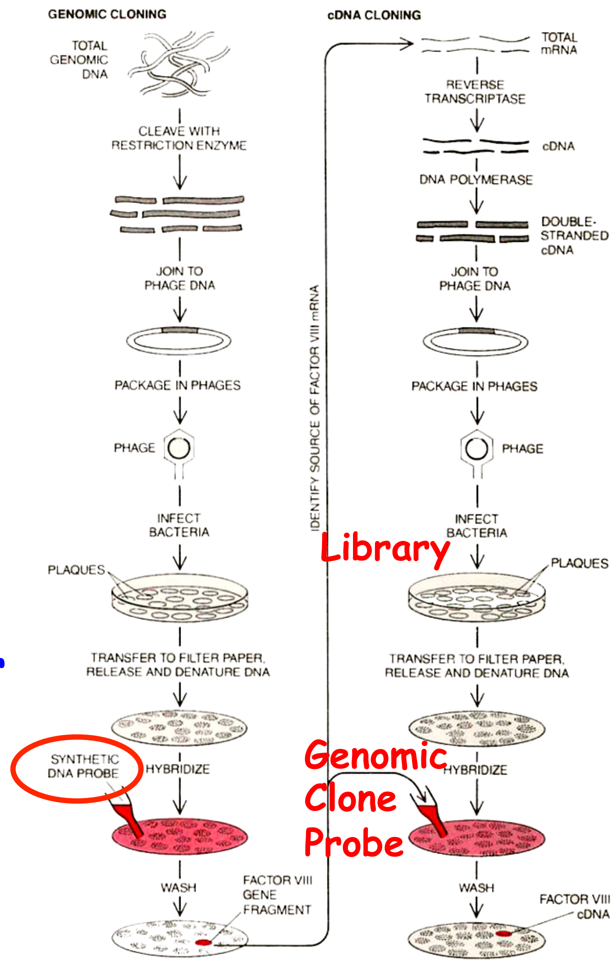
Factor IX – Hemoglobin B
FDA-Approved Clinical Trial
2016

Summary of 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?



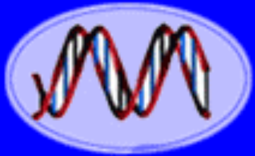
cDNA

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?

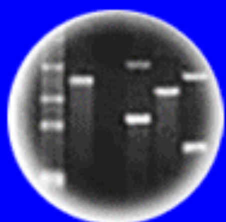
Story continued

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



DNA
Genetic Code of Life

Entire Genetic Code of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

Plants of Tomorrow

The Factor VIII Story - A Summary

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