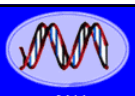


DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria

DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

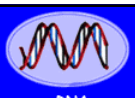
Plants of Tomorrow

HC70A
Spring 2021
Genetic Engineering in Medicine, Agriculture, and Law

Professor Bob Goldberg

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

1



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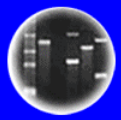

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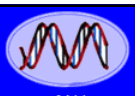
THEMES

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

2



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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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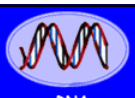

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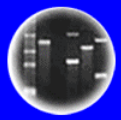

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The Origins of the Biotech Industry Started in the Supreme Court

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

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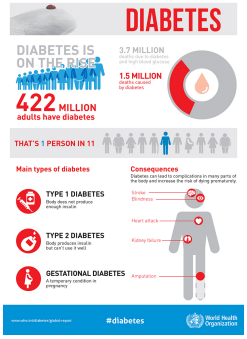
Insulin – The First Biotech Drug



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

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

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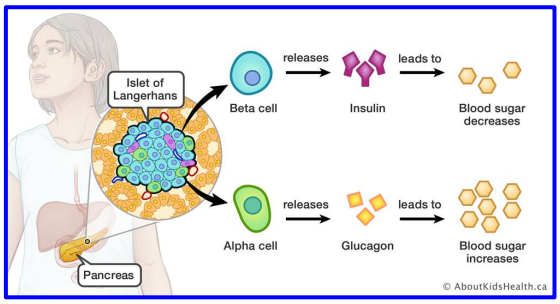
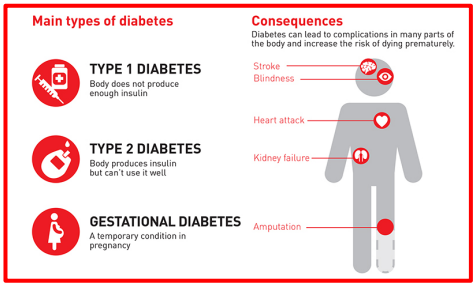
How Does Insulin Control Sugar Levels?





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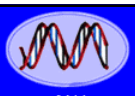

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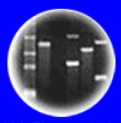

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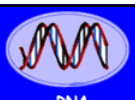
Reasons For Insulin Being the First Biotech Drug

- 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

7



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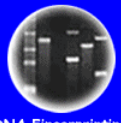

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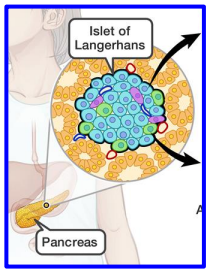
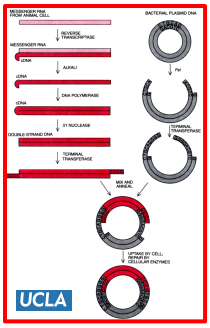
Two Strategies For Isolating the Insulin "Gene" And Engineering *E. coli* Cells to Produce Human Insulin



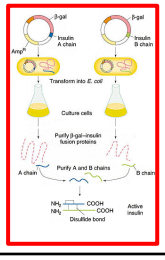
Purify Insulin mRNA From Pancreas

Synthesize & Clone cDNA



Use cDNA/mRNA Sequence

Direct Synthesis and Cloning of A Chain & B Chain mRNAs Separately



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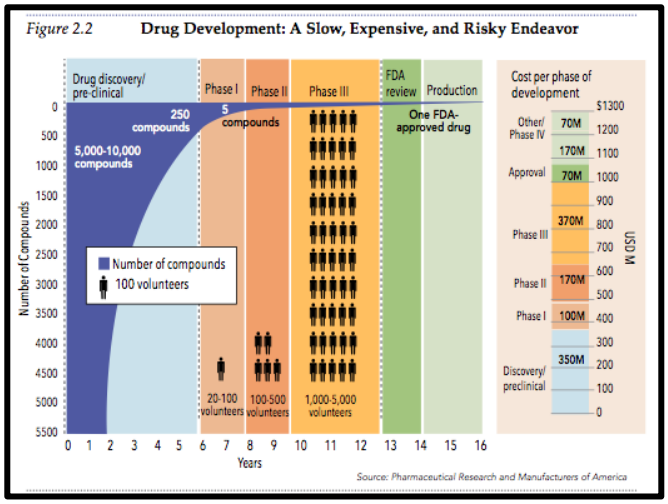
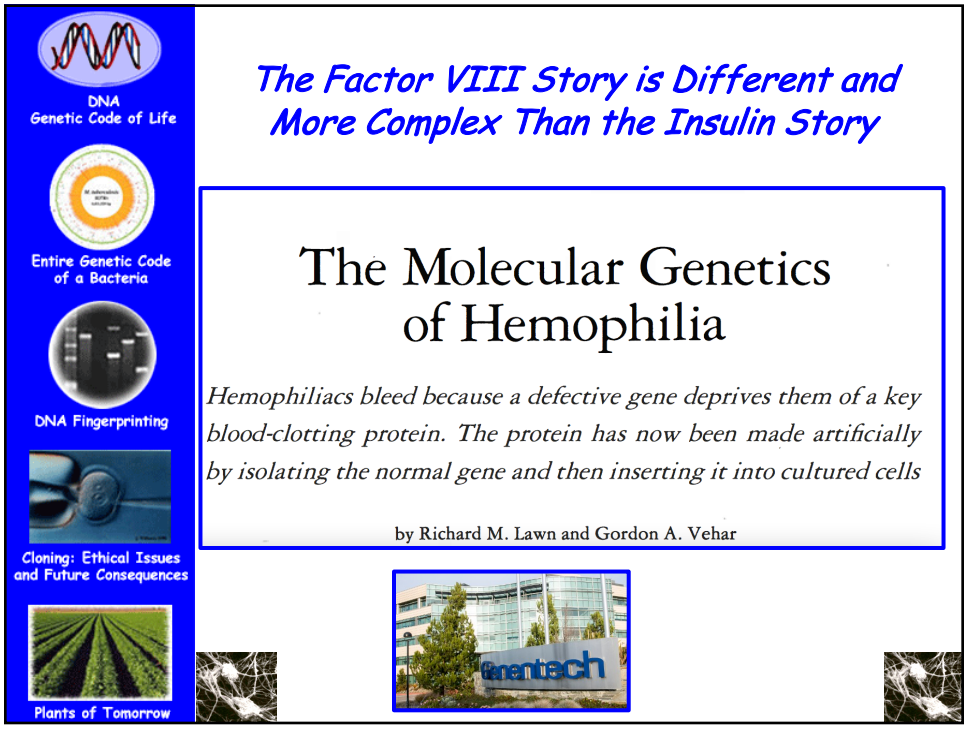
9

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

10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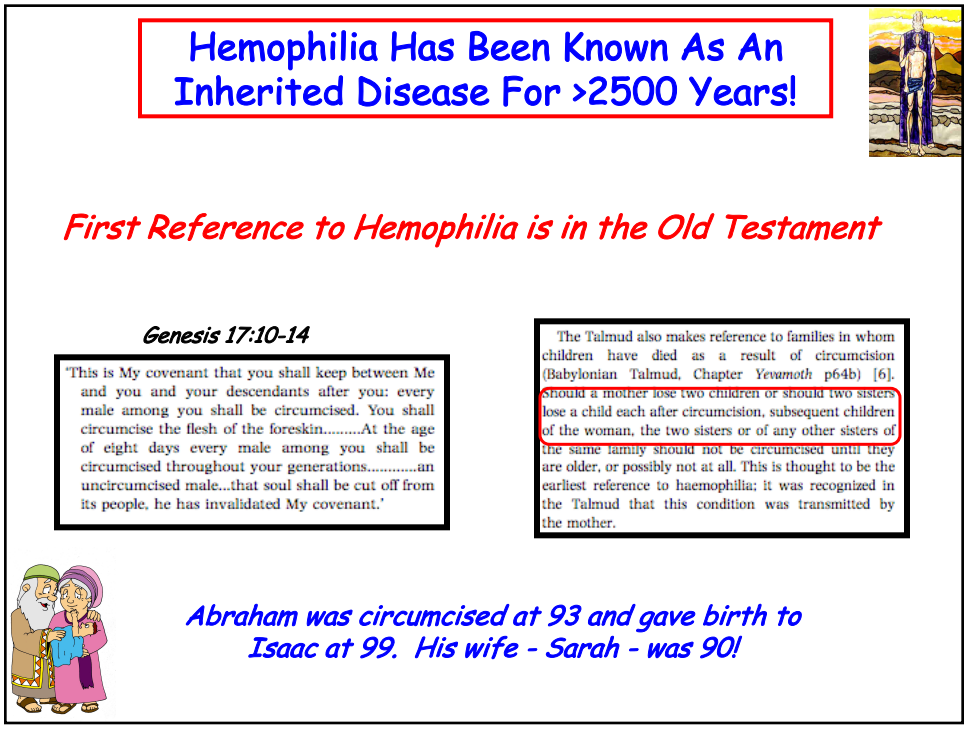
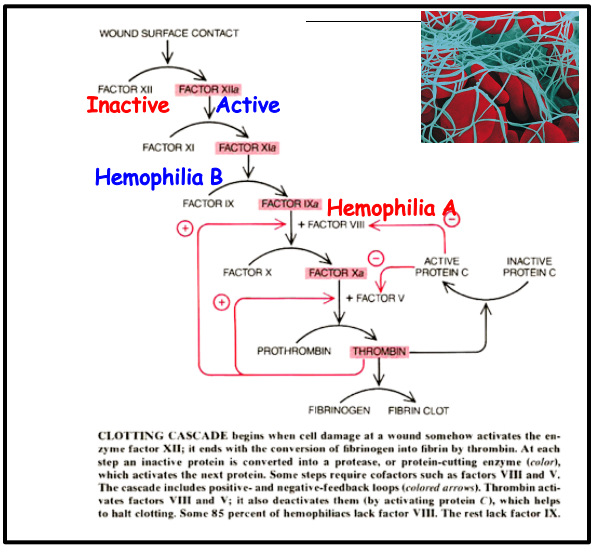
The Factor VIII Story is Different and More Complex Than the Insulin Story
The Molecular Genetics of Hemophilia
Hemophiliacs bleed because a defective gene deprives them of a key blood-clotting protein. The protein has now been made artificially by isolating the normal gene and then inserting it into cultured cells
by Richard M. Lawn and Gordon A. Vehar
Genentech

11


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!

12

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!

13

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

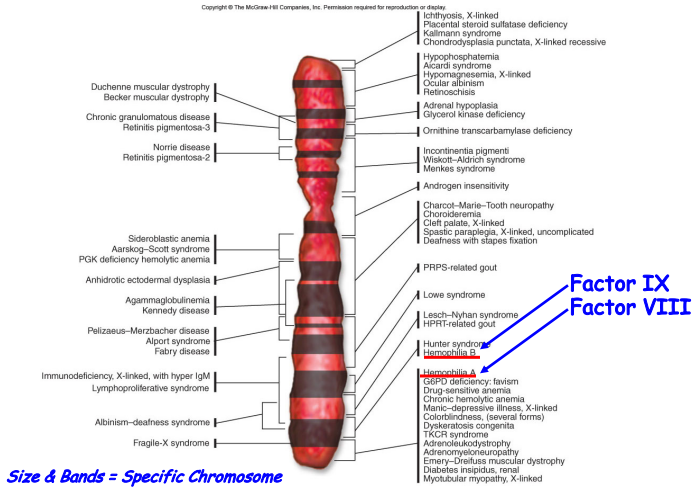
18,000 People in US Have Hemophilia & 400 Babies/Year Are Born With Disorder
Prior to 1960s – Average Life Span Was 11 Years

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)

14

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

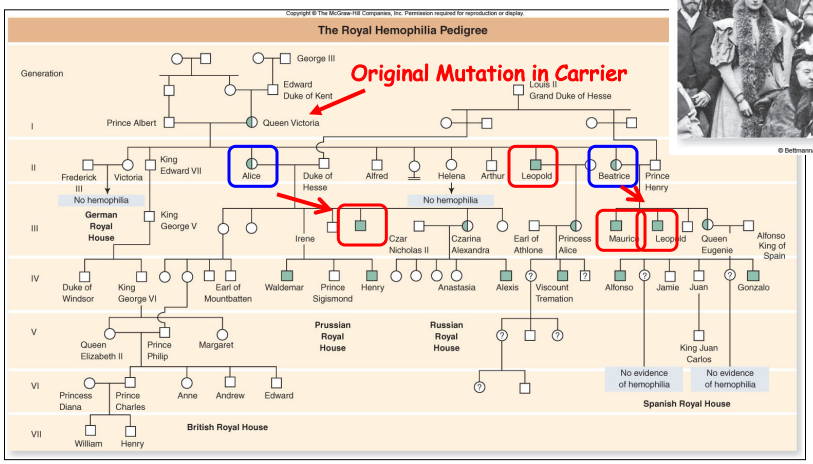


Nature, March, 2005

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

15

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

16

What Was Known About Factor VIII
Before Gene Cloned?

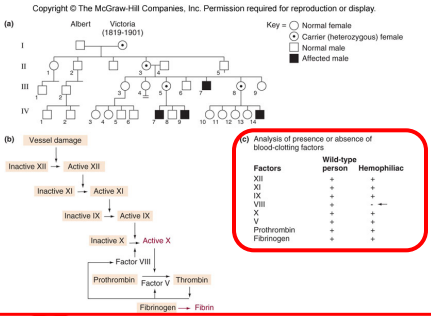
- Blood Protein (But Perhaps Synthesized Elsewhere!)
- Not Known Where Site of Synthesis Was
- Could Be Purified In Small Amounts From >20 Liters Of Human Blood + Cow Blood + Pig Blood
- Short Stretch Of Protein Sequenced = Known Protein Sequence!
- Hemophilia A Could Be Treated By *Blood Transfusions* 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

17

The Problem!!

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

Early 1980's

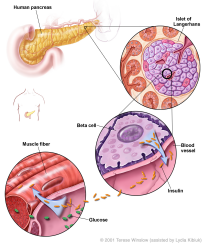


Key Concept

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

Key:
Protein Sequence Known

Different Than Insulin Knew Where Protein Made!

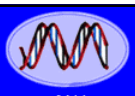


How Find Gene & cDNA?

Protein → Gene → mRNA → Drug !

mRNA ⟶ Drug

18



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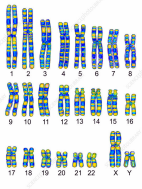
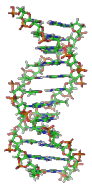
Step One

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?





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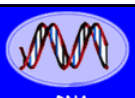

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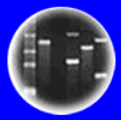

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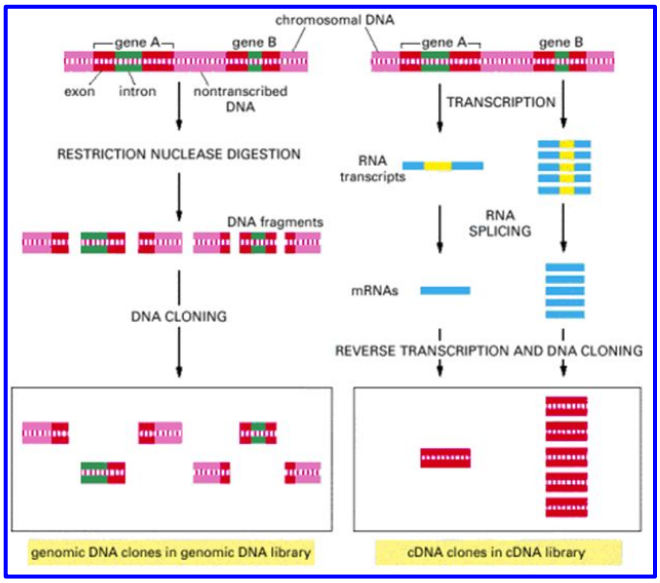
Genomic Libraries vs. cDNA Libraries



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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

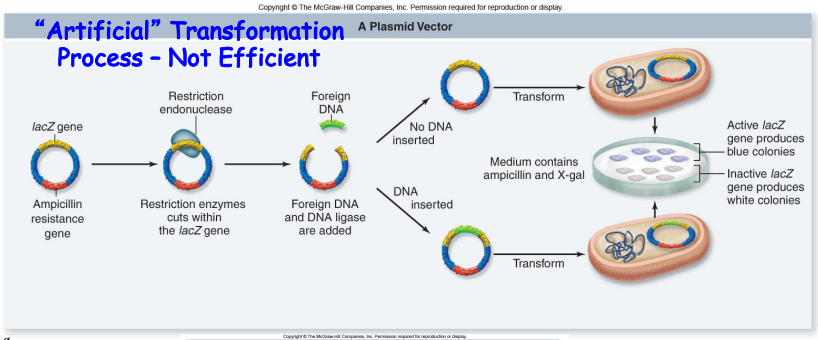
Properties of All Vectors

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

21

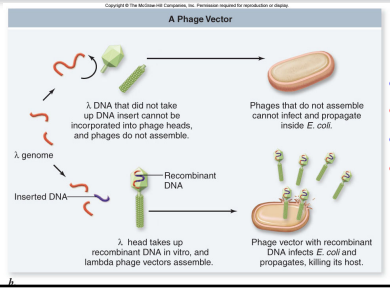
Plasmid vs. Bacteriophage Vectors for Cloning DNA Fragments

"Artificial" Transformation Process – Not Efficient



a.

"Natural" Infection Process



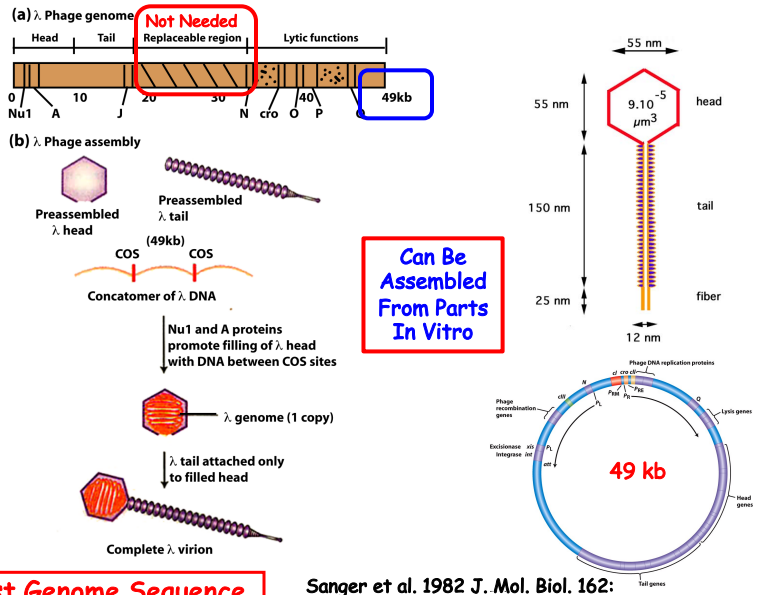
b.

Advantages

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

22

Structure of the λ Phage and Its Genome

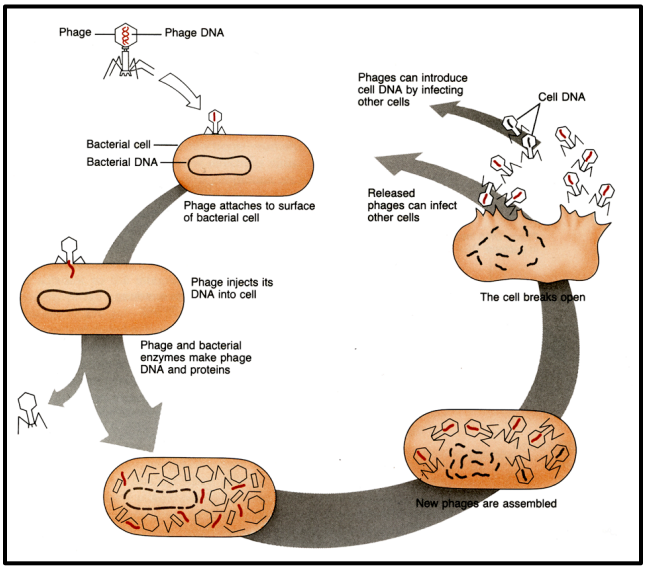


First Genome Sequence

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

23

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



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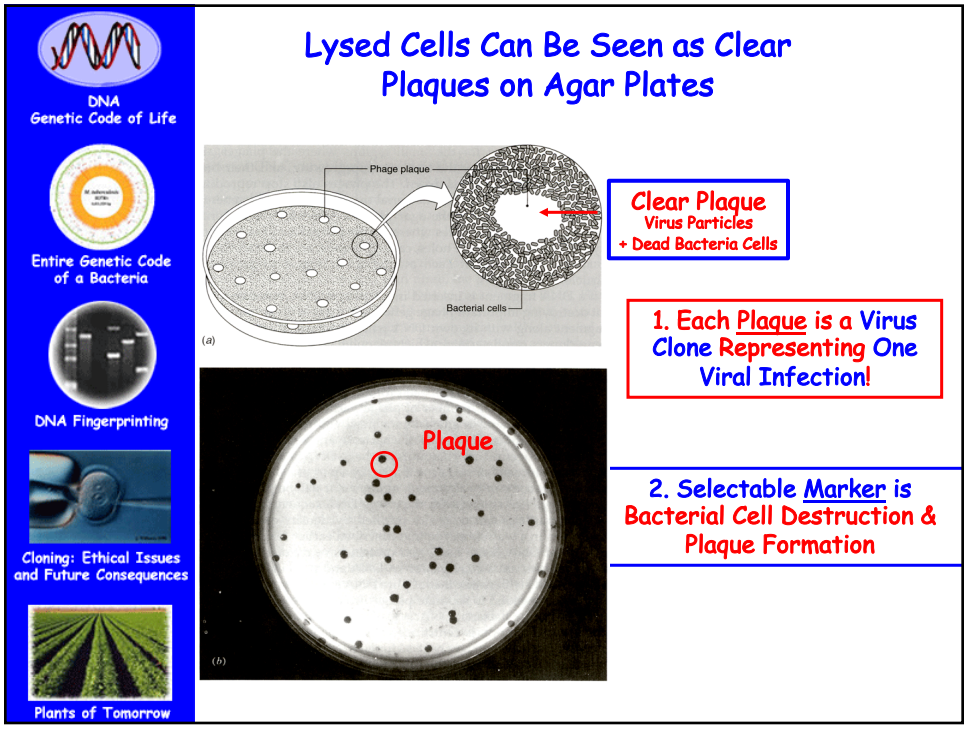


25

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!

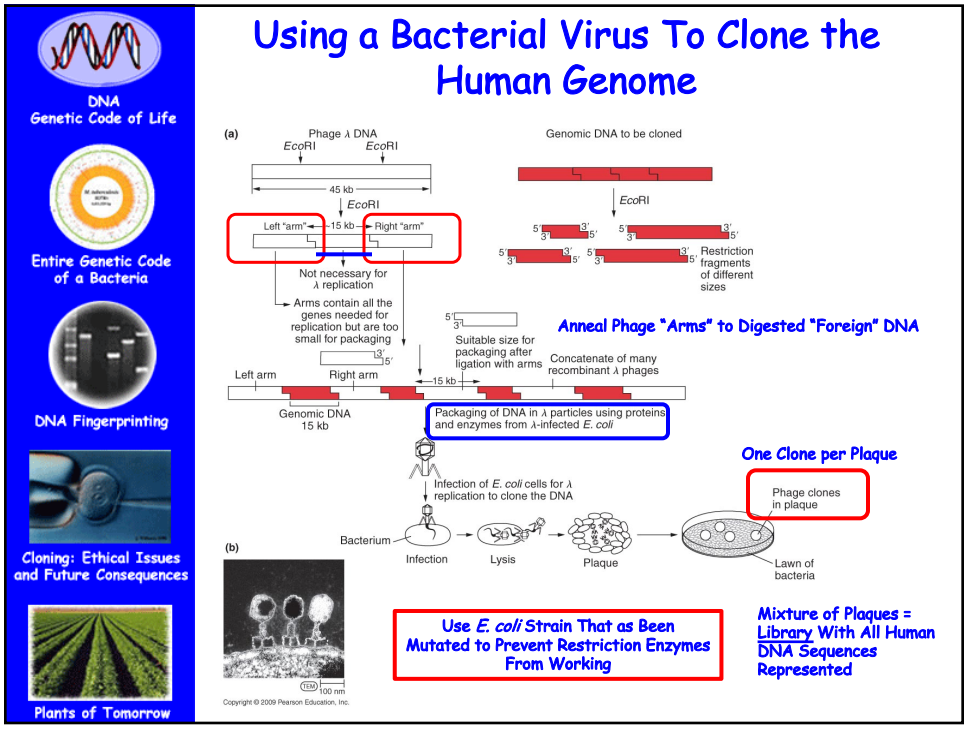
∴ Make Recombinant Viruses In The Lab!

3. Use "Natural" Infection Process To Generate Large Number Of Clones For A Eukaryotic Genome Library.

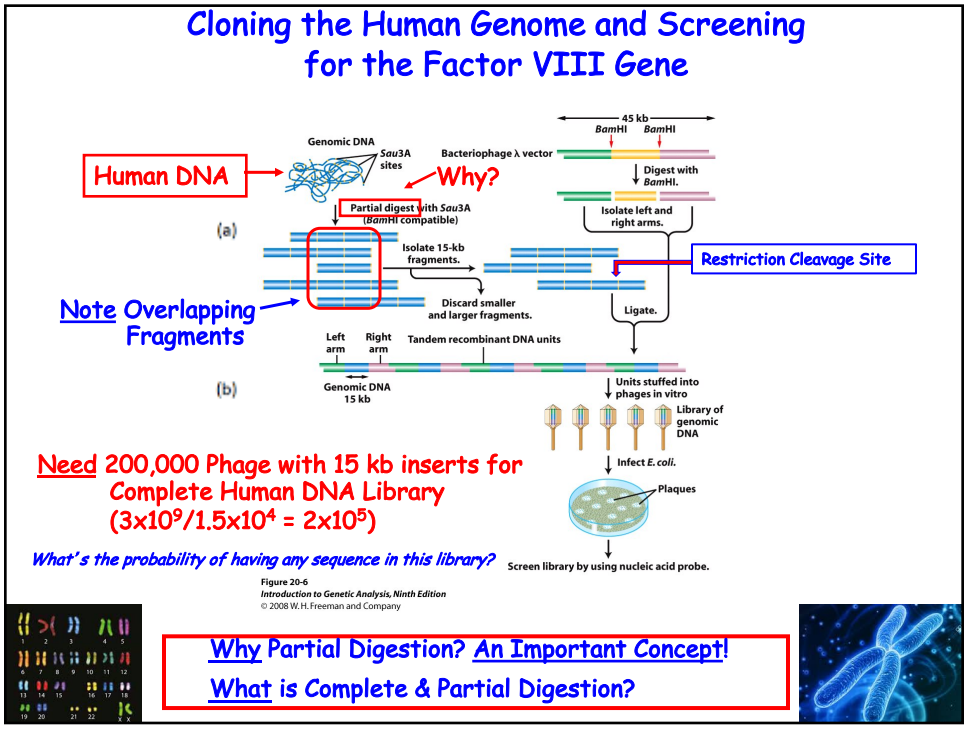
Much Higher Efficiency For Getting Recombinant DNA →Bacterial Cells Compared With Dna Transformation.

∴ Set More Clones Per Amount Of Recombinant DNA!

26


DNA
Genetic Code of Life
Entire Genetic Code of a Bacteria
DNA Fingerprinting
Cloning: Ethical Issues and Future Consequences
Plants of Tomorrow
Using a Bacterial Virus To Clone the Human Genome
(a)
Phage λ DNA
EcoRI
EcoRI
45 kb
EcoRI
Left "arm"
15 kb
Right "arm"
Not necessary for λ replication
Arms contain all the genes needed for replication but are too small for packaging
Genomic DNA to be cloned
EcoRI
Restriction fragments of different sizes
Anneal Phage "Arms" to Digested "Foreign" DNA
Suitable size for packaging after ligation with arms
Concatenate of many recombinant λ phages
Left arm
Right arm
15 kb
Genomic DNA
15 kb
Packaging of DNA in λ particles using proteins and enzymes from λ-infected E. coli
One Clone per Plaque
Infection of E. coli cells for λ replication to clone the DNA
Phage clones in plaque
Bacterium
Infection
Lysis
Plaque
Lawn of bacteria
(b)
100 nm
Copyright © 2009 Pearson Education, Inc.
Use E. coli Strain That as Been Mutated to Prevent Restriction Enzymes From Working
Mixture of Plaques = Library With All Human DNA Sequences Represented

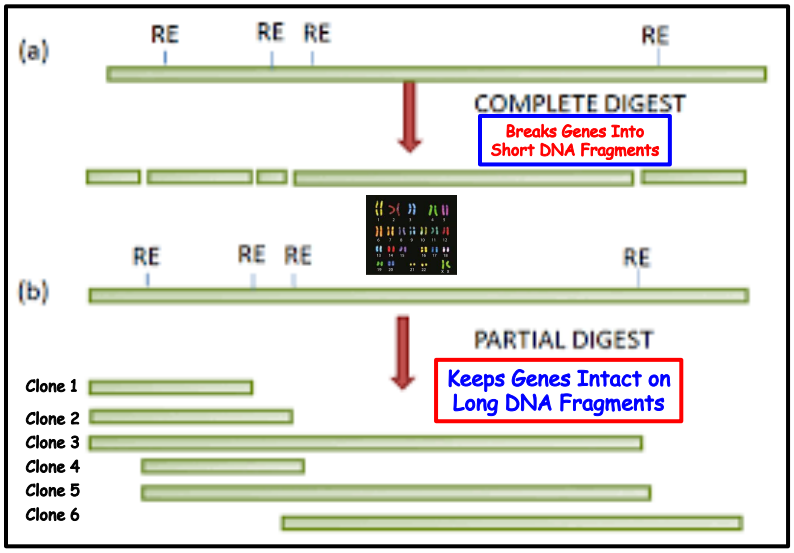
27


Cloning the Human Genome and Screening for the Factor VIII Gene
Human DNA
Genomic DNA
Sau3A sites
Why?
Bacteriophage λ vector
45 kb
BamHI
BamHI
Digest with BamHI.
Partial digest with Sau3A (BamHI compatible)
Isolate left and right arms.
(a)
Isolate 15-kb fragments.
Restriction Cleavage Site
Note Overlapping Fragments
Discard smaller and larger fragments.
Ligate.
Left arm
Right arm
Tandem recombinant DNA units
(b)
Genomic DNA
15 kb
Units stuffed into phages in vitro
Library of genomic DNA
Infect E. coli.
Plaques
Screen library by using nucleic acid probe.
Need 200,000 Phage with 15 kb inserts for Complete Human DNA Library
(3x10⁹/1.5x10⁴ = 2x10⁵)
What's the probability of having any sequence in this library?
Figure 20-6
Introduction to Genetic Analysis, Ninth Edition
© 2008 W.H. Freeman and Company
Why Partial Digestion? An Important Concept!
What is Complete & Partial Digestion?

28

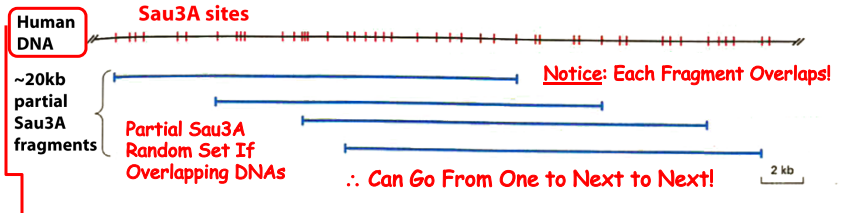
Partial Digestion Permits "Walking" From One DNA Region to the Next

Iterative Process of Screening & Rescreening Human Genome Library



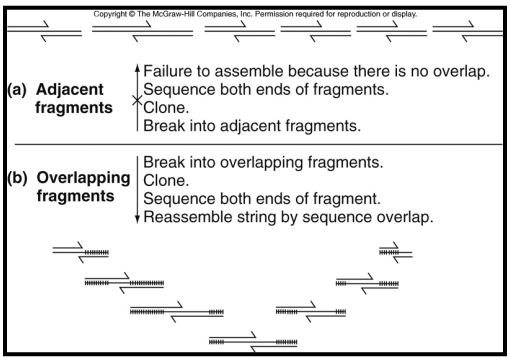
29

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



Genes Connected to Each Other in Long Linear DNA Molecules
How Find Genes Contiguous to Each Other in Cells?

"Walking"



30

Step Two

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

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The Genetic Code

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

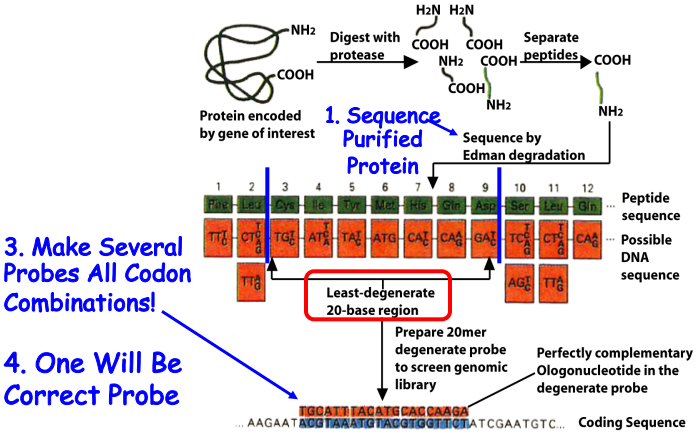
Properties

- Universal
- Three Nucleotides
- Punctuation
- Degenerate

32

Factor VIII Protein → Gene

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

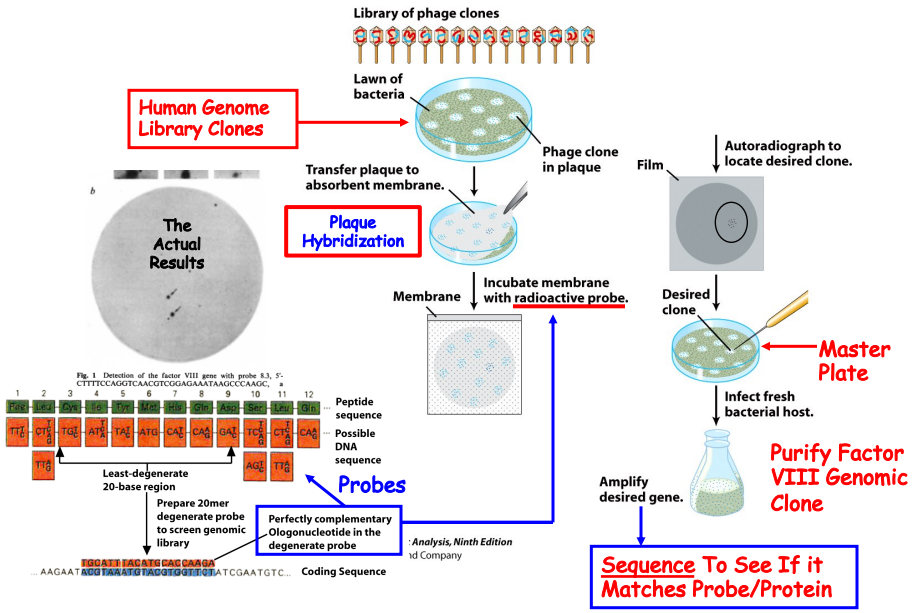


How many Combinations of Synthetic Probes?

2x3x2x1x2x2x2=96

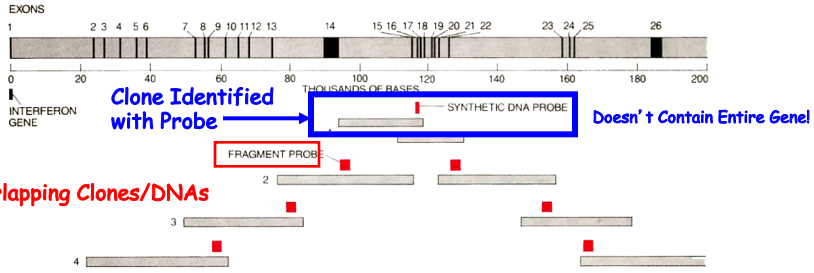
33

Finding The Factor VIII Gene Or Part of Gene!!



34

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



How Find Clones with Rest of Gene? Key Question !

Remember - the Library Contains Overlapping DNA Clones ∴ Can Use One Part of First Clone to Re-Screen Library & "Walk" to Other Gene Regions - Using Restriction Maps & Sequencing (Compare With Protein Sequence) as Guides!

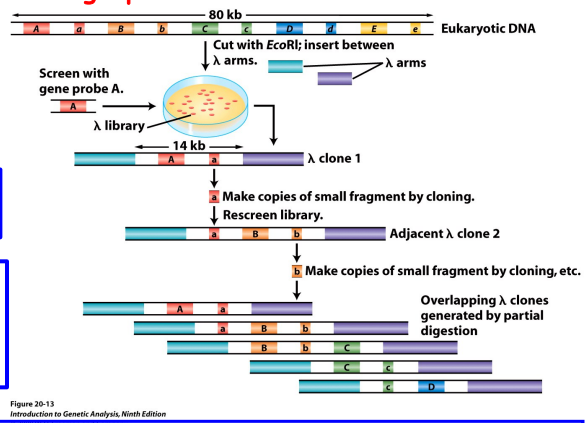
Sequence ----------> GenBank

35

Step Three

Finding the Entire Factor VIII Gene? Walking & Sequencing

Walking Up and Down Genes and Chromosomes



Iterative 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

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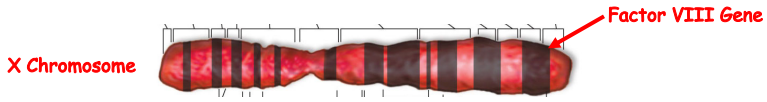
The Factor VIII Gene Was Found To Be Very Large

- 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



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Factor VIII SNP Mutations Occur Throughout the Gene

[*Haemophilia* 11, 481-491 (2005)] *Larger the Gene – Larger Number of Mutations!*

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

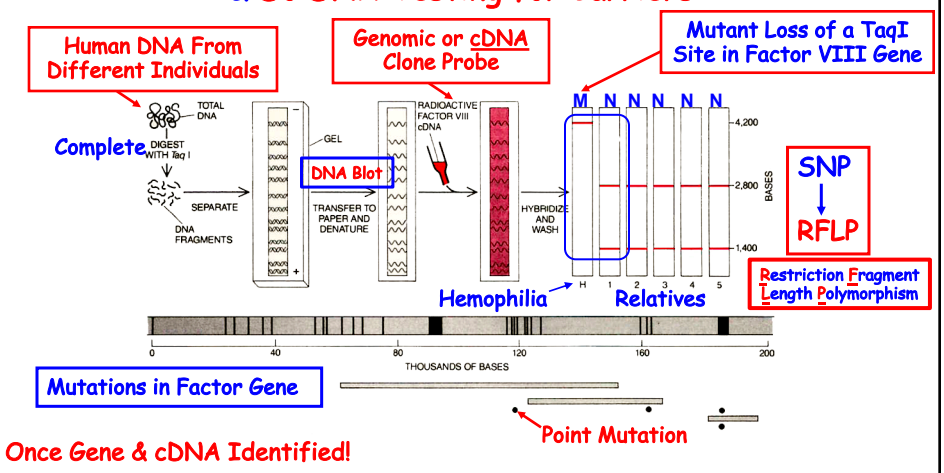
VIII:C (%)	Family history	Consanguinity*	Inversion	Codon†	Mutation	Amino acid change	Exon	Conservation‡
1	Sporadic	NC	Normal	51	TTT → TCT§	Phe → Ser	2	FFFF, identical
1.20	Sporadic	NC	Normal	80	GTT → GAT	Val → Asp	3	VVVV, identical
1	Sporadic	NC	Normal	102	GGT → GTT§	Gly → Val	3	GGGG, identical
2	Sporadic	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
3	Sporadic	NC	Normal	372	CGC → CAC	Arg → His	8	RRRR, identical
1	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	1804	delGT§	Val → fs (TGA-1517)	14	
1	Sporadic	NC	Normal	1535	TGG → TGA	Trp → Stop	14	WLWM, dissimilar
hibitor 96 BU								
1	Sporadic	NC	Normal	1571	TAT → TAA§	Tyr → Stop	14	Y-YY, dissimilar
1	Sporadic	NC	Normal	1581	AAA → TAA§	Lys → Stop	14	KEKK, dissimilar
3.20	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

38

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



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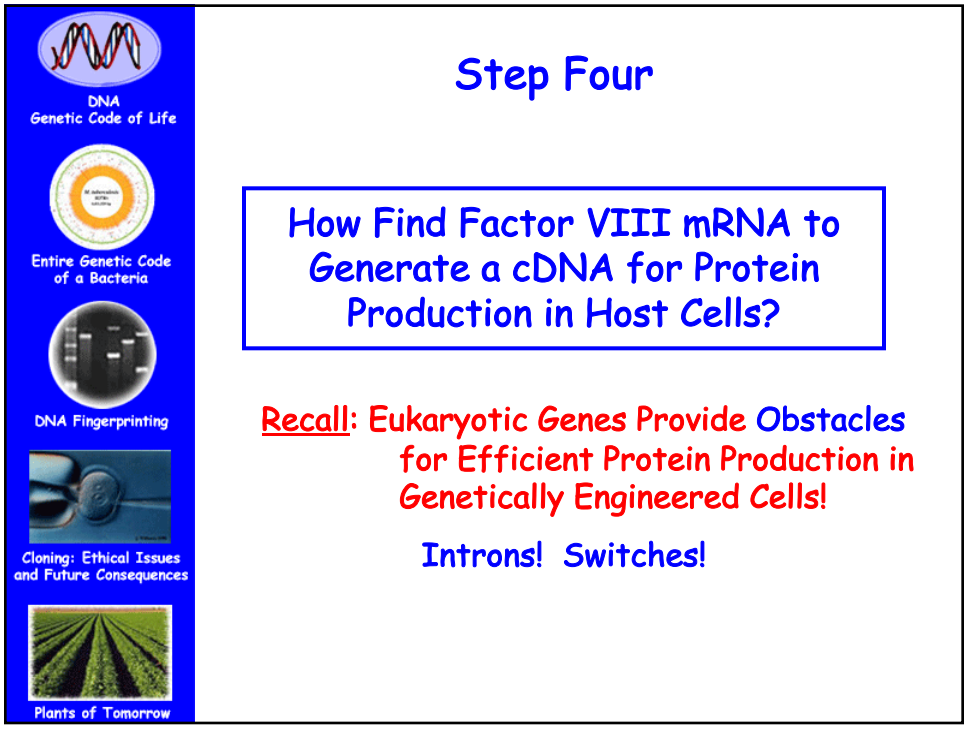


Step Four

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!

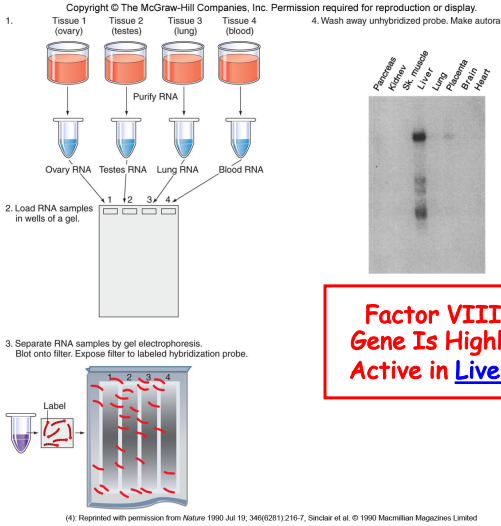
Introns! Switches!

40

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)

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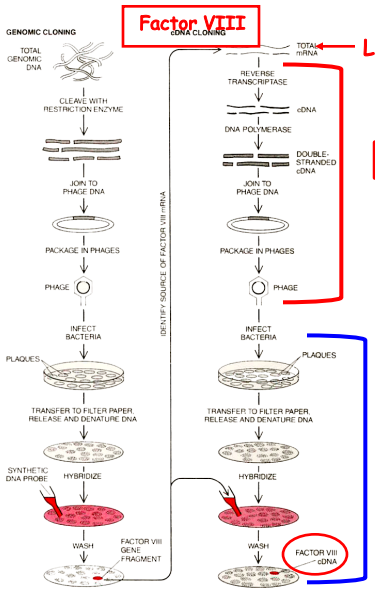
Using Factor VIII Gene Probe to Identify Factor VIII cDNA clone



Construct Liver cDNA Library

Factor VIII cDNA → Used to Make Drug! Not Gene!!

Screen Liver cDNA Library

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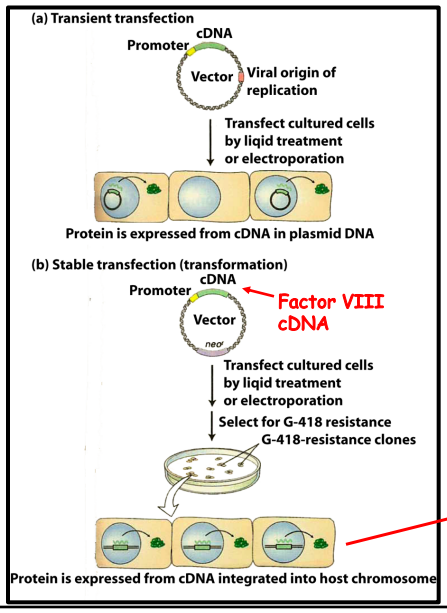
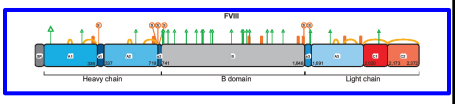
Engineer Factor VIII cDNA to Produce Protein in Host Cell & Synthesize Factor VIII in Mammalian Cells



Why Mammalian Cells?



Purify Factor VIII Protein!

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Recombinant Factor VIII

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

Factor VIII Gene Cloned In 1983

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

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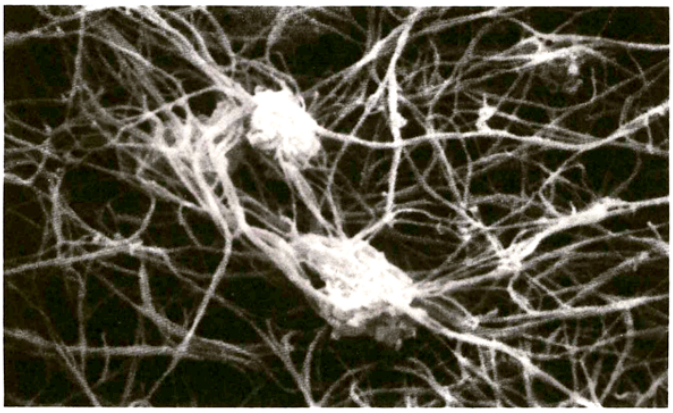
Using Factor VIII to Treat Hemophilia

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

45

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

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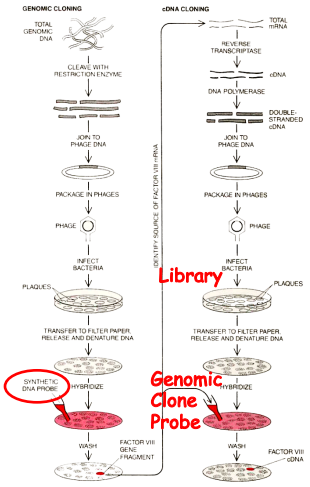
Summary of Steps Required to Clone Factor VIII Gene and cDNA

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!

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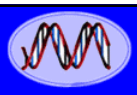
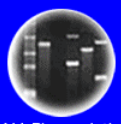


DNA
Genetic Code of Life

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)

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