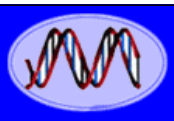
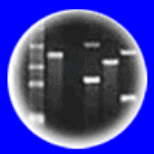


DNA
Genetic Code of Life

Entire Genetic Code
of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues
and Future Consequences

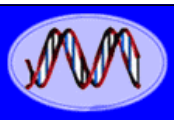
Plants of Tomorrow

HC70A, SAS70A, & PLSS059
Winter 2018
Genetic Engineering in Medicine, Agriculture, and Law

Professors Bob Goldberg, John Harada, & Channapatna Prakash

Lecture 5
The Nuts & Bolts of Genetic Engineering:
From Mutations to Pedigrees to Drug
The Factor XIII Story

UCLA TUSKEGEE UNIVERSITY UCDAVIS UNIVERSITY OF CALIFORNIA



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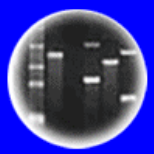

DNA Fingerprinting

Cloning: Ethical Issues
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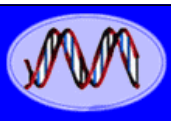
Plants of Tomorrow

THEMES

1. PCR
2. What Causes Gene Mutations?
3. How Do Gene Mutations Lead to Genetic Variability?
4. How Can We Test For Gene Mutations at the DNA Level?
5. What is Hemophilia and How is it Inherited?
6. How Can a Disease Gene Be Found When It is Not Known Where the Gene is Expressed?
7. What Vectors Can Be Used For Cloning DNA?
8. What is the Advantage of Using a Virus Vector For Constructing Genome Libraries?
9. How To Make a Library of the Human Genome?
10. How Find a Gene With Only a Knowledge of the Protein Sequence?
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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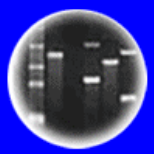

DNA Fingerprinting

Cloning: Ethical Issues
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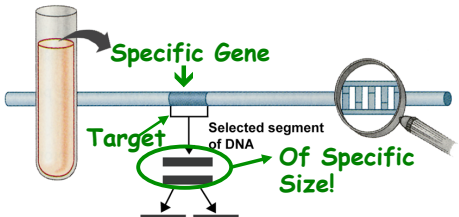
Plants of Tomorrow

WHAT ARE THE PROPERTIES OF A GENE?

1. Replication
2. Stability (Mutations)
3. Universality
   a) All Cells
   b) All Organisms
4. Direct Cell Function/ Phenotype

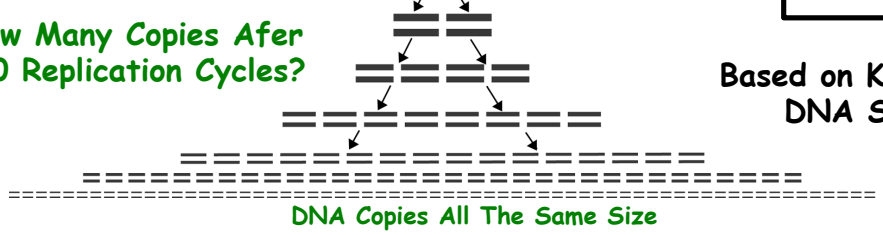
The Second Genetic Engineering Revolution - The Polymerase Chain Reaction (PCR) is a Molecular Xerox Machine That Can Amplify DNA Sequences in a Test Tube Without Cloning!



Kary Mullis

1993

How Many Copies Afer 10 Replication Cycles?



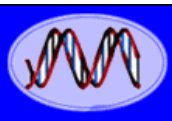
DNA Copies All The Same Size

Based on Knowledge of DNA Synthesis

1. PCR Has Revolutionized DNA Analysis!
Specific DNA Sequences/Genes Can Be "Copied" Directly From "Tiny" Amount of DNA!

2. No Cloning Needed!

3. But Need Sequence! ⇨ Have to Clone "Gene" First



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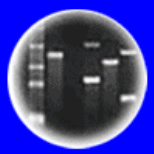


DNA Fingerprinting

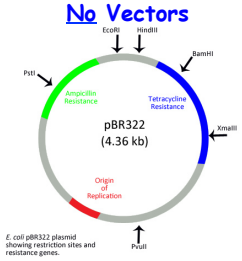
Cloning: Ethical Issues and Future Consequences

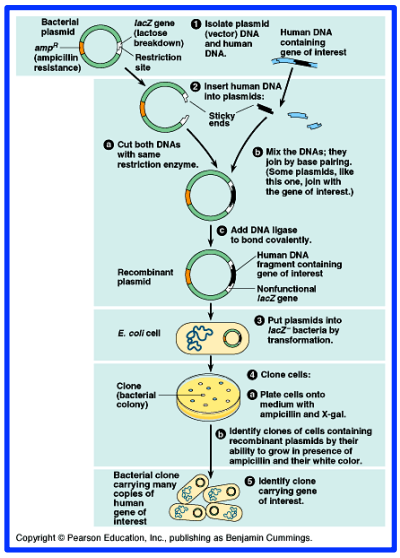
Plants of Tomorrow

What Are the Advantages of PCR?

No Cloning Steps – RE Digestion, Annealing, Ligation, Transformation, & Sorting Clones

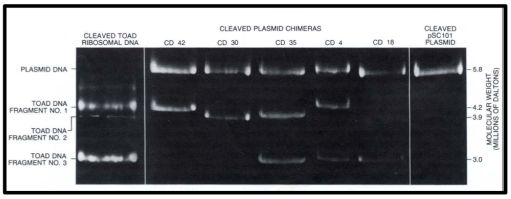
No Vectors





No Bacteria

No Finding the Correct Clone



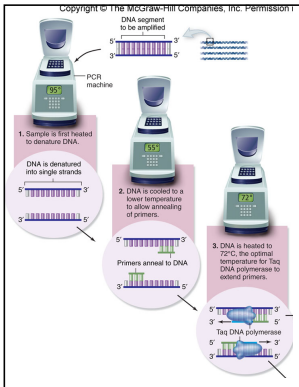
Less Labor & Expertise

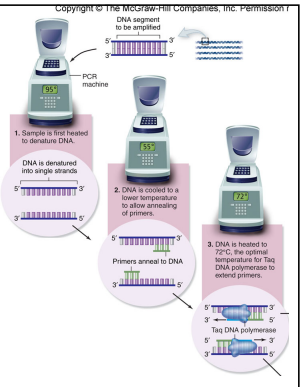
Several Hours vs. Weeks!!

PCR Has Many Uses, Has Changed Many Fields, and Lead To New Ones That Have Had a Big Impact On Our Lives

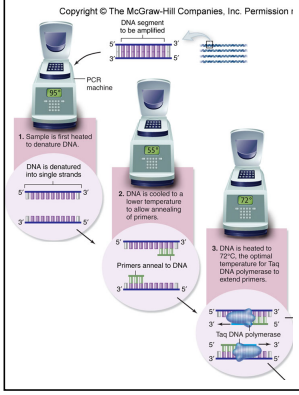
1. Amplify Any DNA Sequence, or Gene, From "Tiny" Amounts of DNA or Biological Materials IF ORIGINAL SEQUENCE KNOWN
2. Study DNA From Limited and/or Degraded Sources Such As:
   1. A Single Human Hair or Cheek Cell
   2. An Ancient Fossil (e.g., Neanderthal Bone or Mammoth Hair)
   3. An Ancient Insect Trapped in Amber
   4. Human Remains (e.g., 9/11 Victims)
   5. *A Single Human Embryo Cell*
   6. Contaminated Meat To Determine the Causal Organism
3. Used In:
   1. DNA Fingerprinting-Individual Identification-Genetic Disease Screening
   2. Forensics (Crime Scenes, Mass Graves, Criminal Suspects, Wrongfully Convicted)
   3. Paternity & Family Relationships (e.g., Immigration, Tracing Lost Children)
   4. Disease Diagnosis & Pathogen Identification (Humans, Animals, & Plants)
   5. Human Origins & Migrations
   6. Ancient Genome Sequences & Evolutionary Studies
   7. Specific mRNA Detection
   8. "Cloning" Specific DNA Sequences
   9. Tracing Plant & Animal Sources (e.g., Poaching Stolen Cattle, Cactus)
4. Need as Little as One Molecule of DNA & Can Replicate an ∞ Amount of Specific Sequences

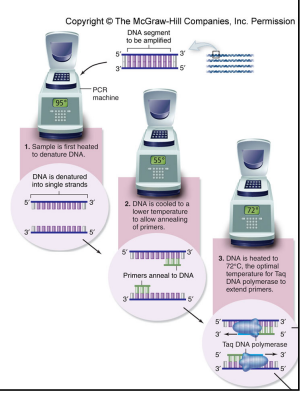
Revolutionized How To Study & Manipulate DNA



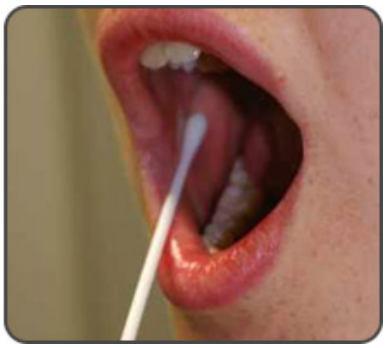


Examples of PCR Applications

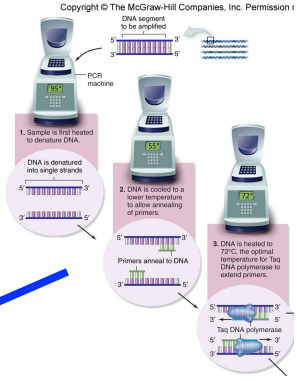


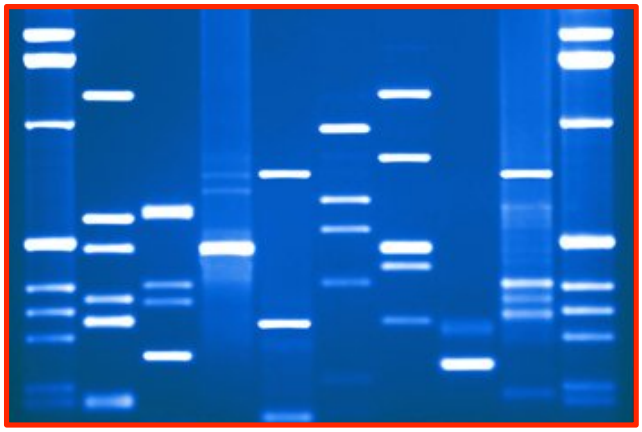


Using PCR to Determine Your DNA Fingerprint & Identity



What is YOUR DNA Fingerprint?

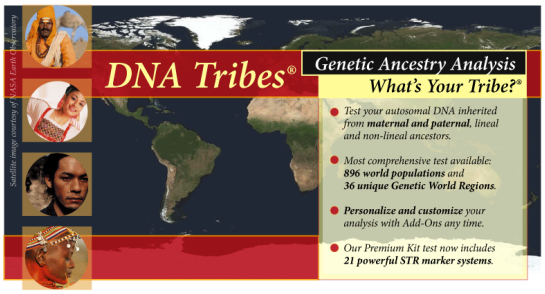


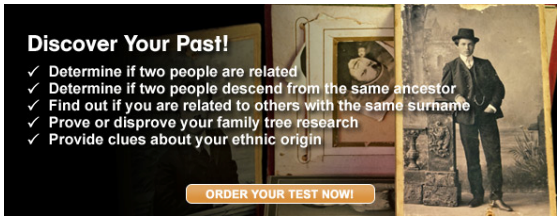


Unique Pattern of DNA Bands = Fingerprint

Using DNA Fingerprints to Identify Individuals & Genes They Don't "Lie"

Using PCR To Determine an Individual's Ancestry





PCR Started a New Industry

DNA can reveal ancestors' lies and secrets

LA Times, January 18, 2009

Using PCR to Amplify Neanderthal Bone DNA & Sequence The Entire Genome!

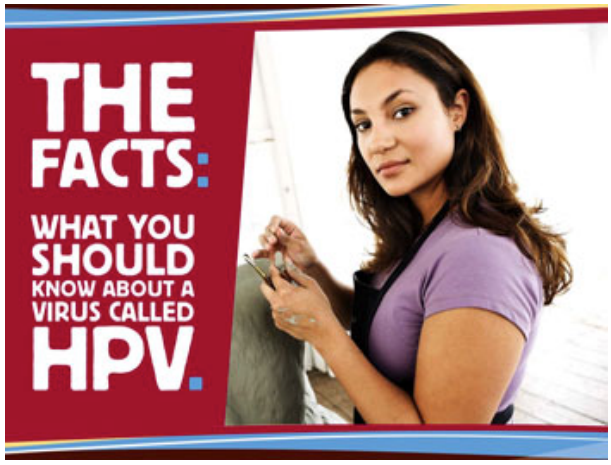
Analysis of one million base pairs of Neanderthal DNA

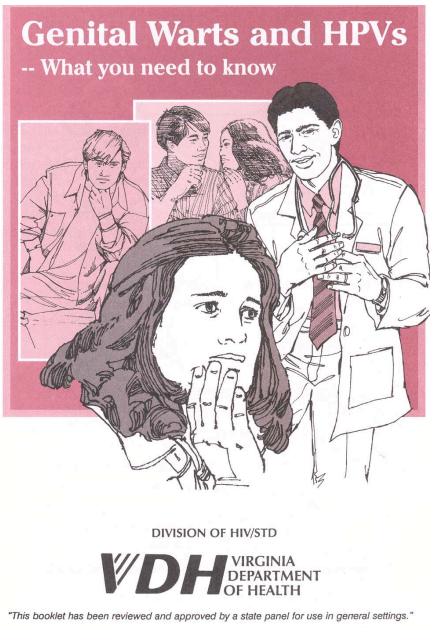
From a 45,000 Year-Old Bone

Richard E. Green[1], Johannes Krause[1], Susan E. Ptak[1], Adrian W. Briggs[1], Michael T. Ronan[2], Jan F. Simons[2], Lei Du[2], Michael Egholm[2], Jonathan M. Rothberg[2], Maja Paunovic[3]‡ & Svante Pääbo[1]

Nature, November, 2006

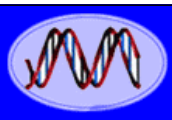
Using PCR To Detect Human Pathogens
(Viruses, Fungi, Bacteria)





ViroSeq™
HIV-1 Genotyping System

Each Genome Has Specific DNA Sequences That Can Be Used For Screening And Diagnosis Using PCR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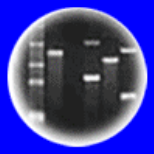
DNA Fingerprinting


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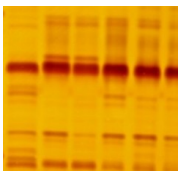
Plants of Tomorrow

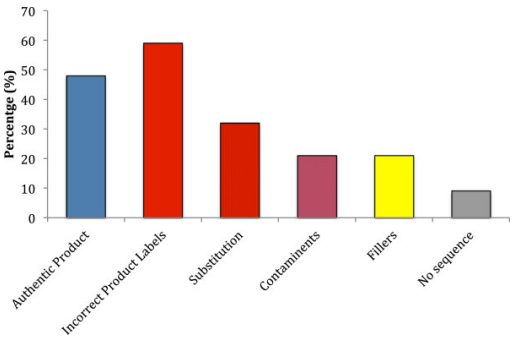
And Consumer Fraud in the Natural Food Industry

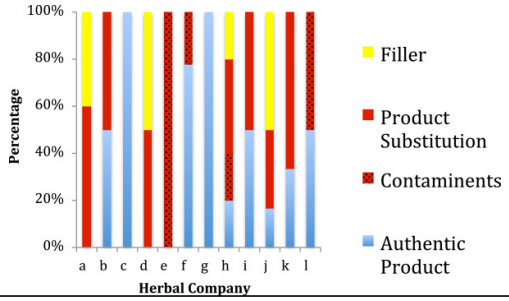
DNA barcoding detects contamination and substitution in North American herbal products

BMC Medicine, 11, 222, 2013

Barcoding = DNA Fingerprinting!

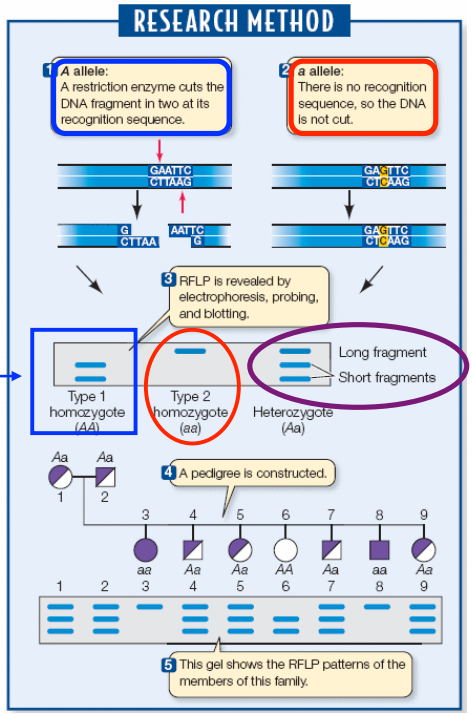




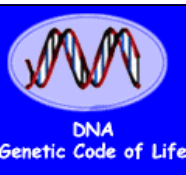


Genetic Diseases Can Also Be Followed in Families Using DNA Methods (e.g., PCR) & Pedigrees – With DNA Markers Linked to the Disease Phenotype

DNA Fingerprints →

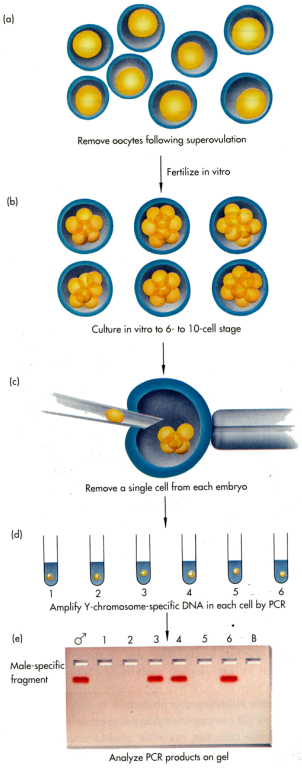


← RFLP – Restriction Fragment Length Polymorphism



PCR Can Be Used To Analyze Gene in A Single Embryo Cell

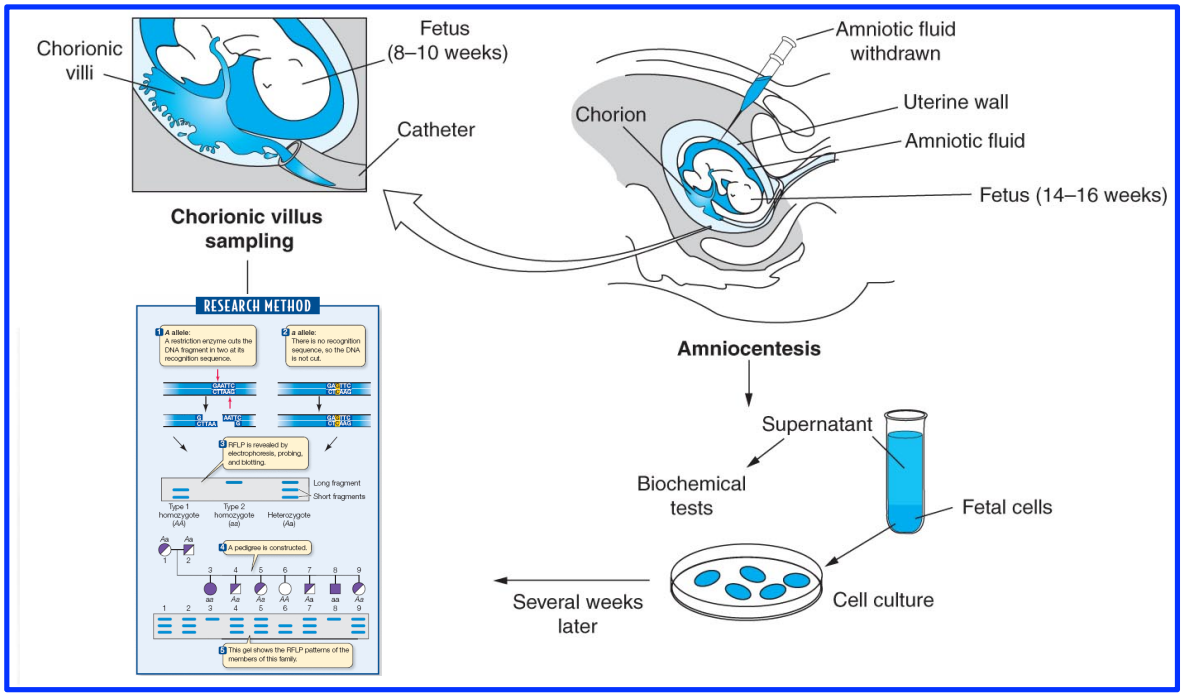
PGD
Pre-Implantation Genetic Diagnosis



What is The Implication of This Procedure Considering That The Human Genome Has Been Sequenced?

Sex Determination in 8-cell Embryo! ←

DNA Testing Can Be Carried Out Before Child Birth During Pregnancy



RESEARCH ARTICLE **New Non-Invasive DNA Tests Are Available Based on PCR**

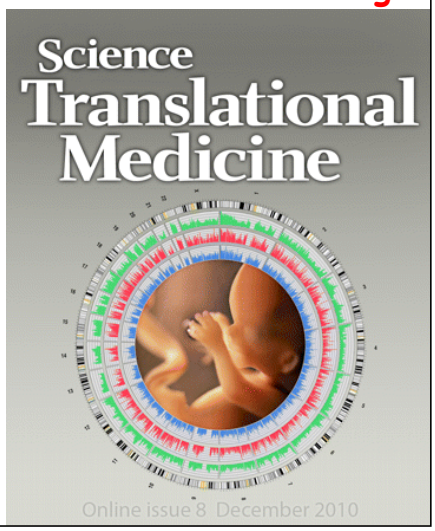
PRENATAL DIAGNOSIS

Maternal Plasma DNA Sequencing Reveals the Genome-Wide Genetic and Mutational Profile of the Fetus

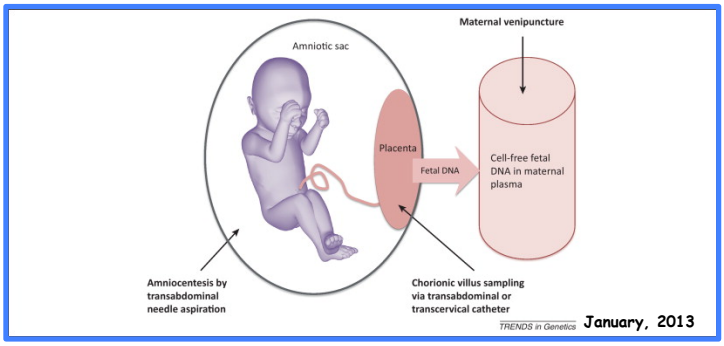
Science Translational Medicine, December 8, 2010 (61,1-12)

Sequencing DNA From the Blood of a Pregnant Woman Allows the Complete Genome Of the Fetus to Be Decoded!

A New Era in DNA Testing!!

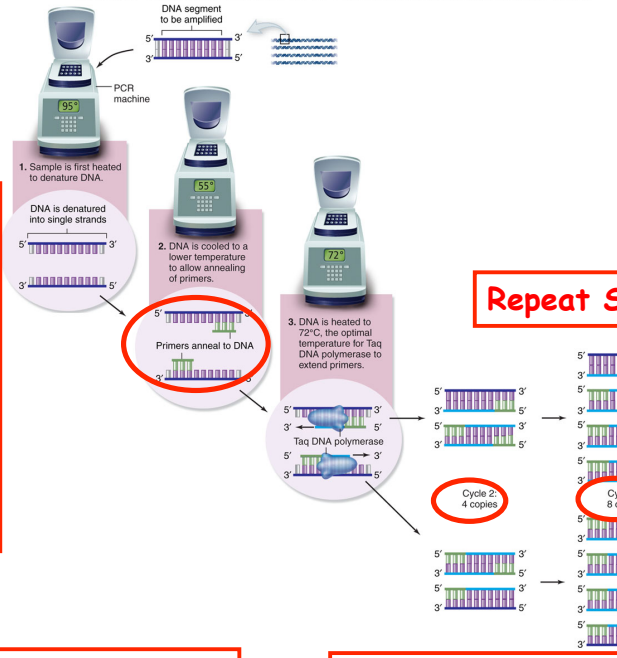


~10% of DNA in Maternal Plasma is From the Fetus



January, 2013

PCR is A Cyclical Process of DNA Replication



Requires

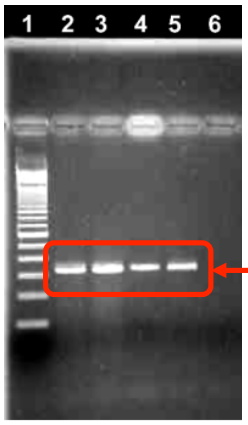
1. Template
2. Primers
3. Knowledge of Specific Sequence
4. Nucleotides
5. Heat-Stable DNA Polymerase
6. Thermo Cycler

Repeat Steps or Cycle

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

Diagnostic For Amplified DNA Sequence (Between Primers) → DNA Fragments All The Same Size Primer-Sequence-Primer

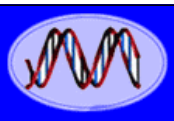
Using Gel Electrophoresis to Visualize PCR Products



Specific Diagnostic DNA Band Unique to DNA Sequence Being Amplified

- Target-Specific Band
- Diagnostic For Specific DNA Sequence
- Band Size Unique For Specific Sequence
- Primers "Surround" the Target Sequence

Can Amplify One DNA Sequence From An Entire Genome *or an Entire Genome*!!!



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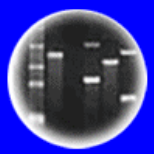

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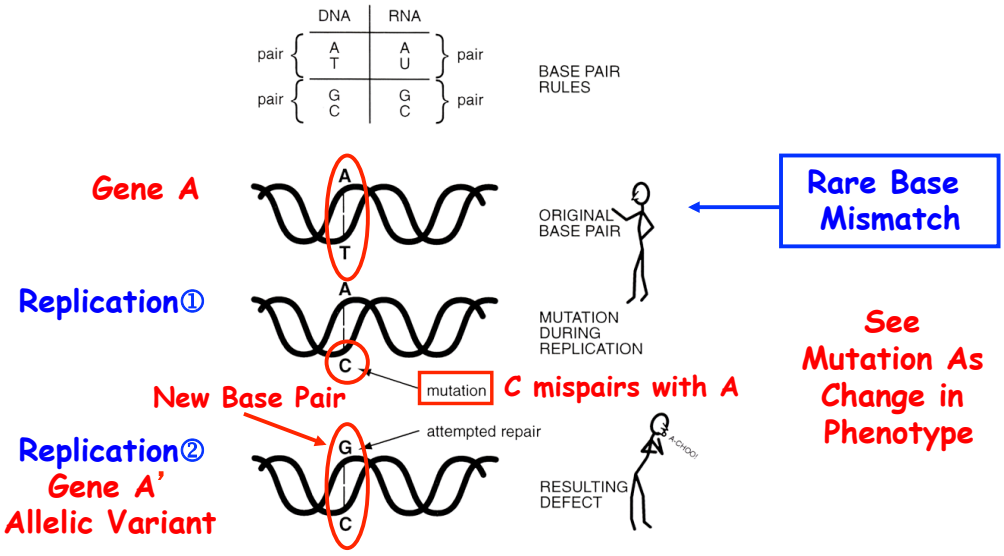
WHAT ARE THE PROPERTIES OF A GENE?

1. Replication
2. Stability (Mutations)
3. Universality
   a) All Cells
   b) All Organisms
4. Direct Cell Function/ Phenotype

DNA Replication is Precise But Mistakes or Mutations Can Occur

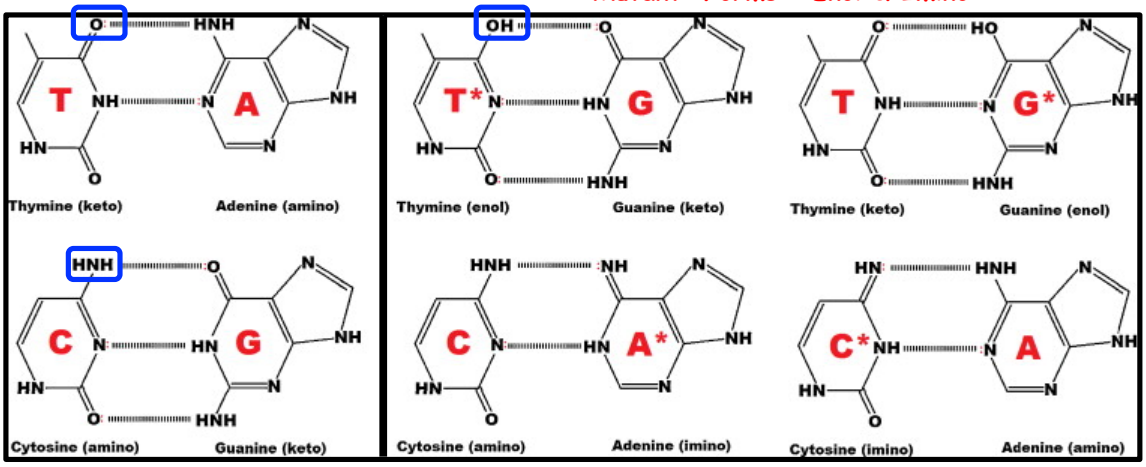


Change DNA Sequence From A-T to G-C

∴ Change Protein Amino Acid Sequence ⇨ Alter Function!

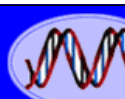
Tautomers Change Base Pairing Rules During DNA Replication & Result in Mutations

Normal Forms – Keto & Amino | **"Mutant" Forms – Enol & Imino**



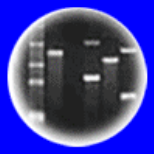
Lead To Mistakes in DNA Replication & Mutations ➤ Genetic Diversity

Chemistry Leads to Biology!!



DNA
Genetic Code of Life

Entire Genetic Code of a Bacteria



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Cloning: Ethical Issues and Future Consequences

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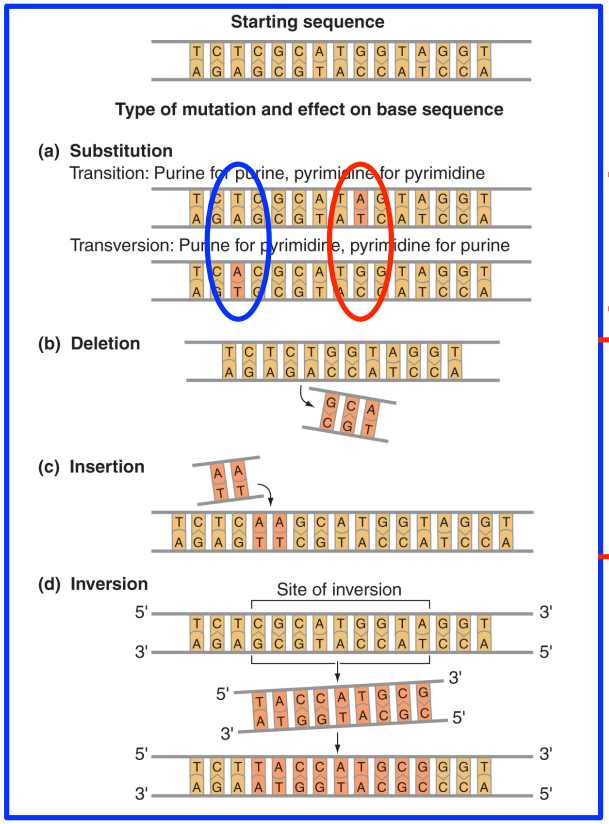
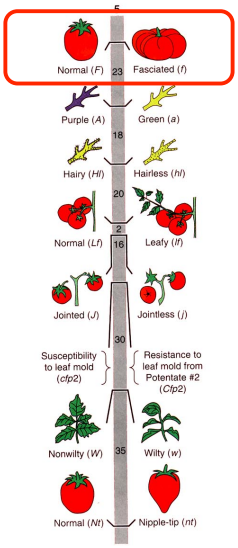
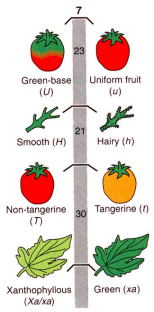
Different Events Cause Gene Mutations

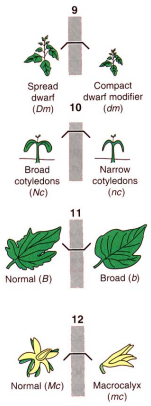


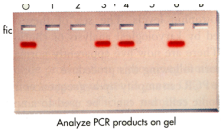
Alternative Forms of the Same Gene Lead to Genetic Diversity

Alleles





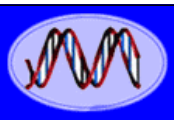




Can Follow These Traits With DNA Markers As Well

mutations result in genetic diversity!!!

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



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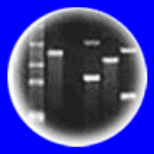

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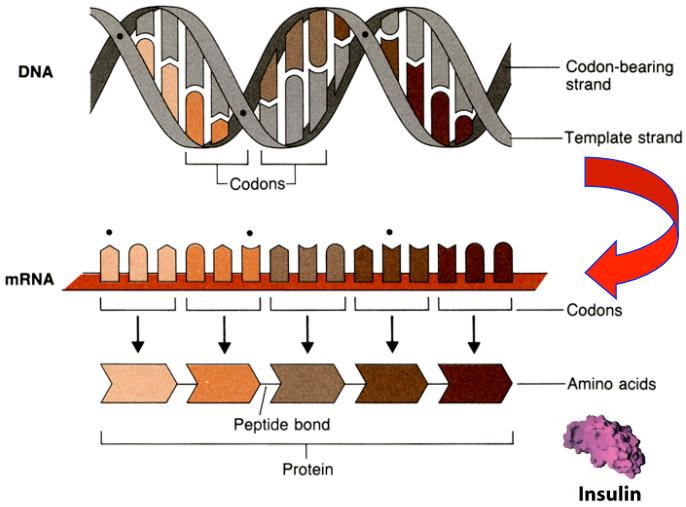
WHAT ARE THE PROPERTIES OF A GENE?

1. Replication
2. Stability (Mutations)
3. Universality
   a) All Cells
   b) All Organisms
4. Direct Cell Function/ Phenotype

2 How Does A Gene Lead To A Phenotype?



① mRNA Synthesized by Transcription

- Complementary to Transcribed, Non-Sense Strand
- Same Sequence As Sense Strand

② mRNA Translated into Protein by Translation of The Genetic Code

Genetic Code on mRNA Translated to Protein Sequence

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

Colinearity of Sequences!

Know Sequence
Know Protein → Engineer New Protein

The Genetic Code is Universal!

How Know?

DNA codons	Amino acid
GCA GCG GCT GCC	Ala
AGA AGG CGA CGG CGT CGC	Arg
GAT GAC	Asp
AAT AAC	Asn
TGT TGC	Cys
GAA GAG	Glu
CAA CAG	Gln
GGA GGG GGT GGC	Gly
CAT CAC	His
ATA ATT ATC	Ile
TTA TTG CTA CTG CTT CTC	Leu
AAA AAG	Lys
Start ATG	Met
TTT TTC	Phe
CCA CCG CCT CCC	Pro
AGT AGC TCA TCG TCT TCC	Ser
ACA ACG ACT ACC	Thr
TGG	Trp
TAT TAC	Tyr
GTA GTG GTT GTC	Val
TAA TAG TGA	Stop

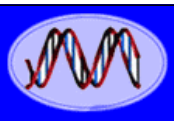
1. Universal
2. Triplet
3. Punctuation
4. Degenerate

For RNA, The Ts are replaced by Us.

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!



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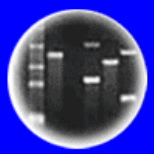

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Cloning: Ethical Issues
and Future Consequences

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Expression of Jellyfish Green Fluorescence Protein (GFP) in Pigs Shows That Genetic Code is Universal!!

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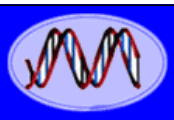

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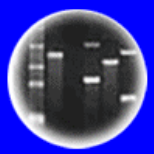

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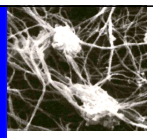
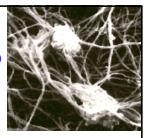


How Do We Treat a Genetic Disease? From Gene To Drug



Due to Mutations in a Different Class of Blood Proteins

The Molecular Genetics of Hemophilia

(Potentially Lethal Disease)

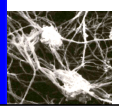
Hemophiliacs bleed because a defective gene deprives them of a key blood-clotting protein. The protein has now been made artificially by isolating the normal gene and then inserting it into cultured cells

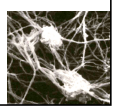
by Richard M. Lawn and Gordon A. Vehar

A Case Study of Cloning Genes and mRNAs

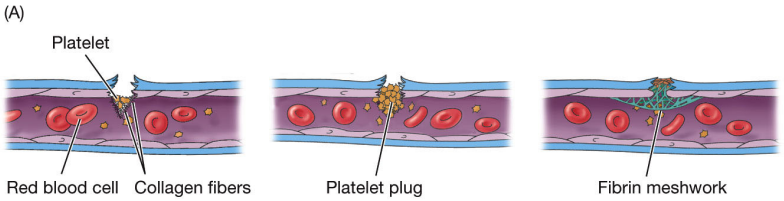
Reference: Scientific American, March 1, 1986

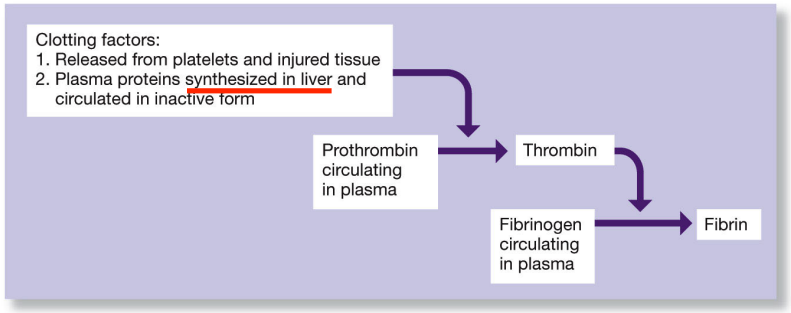
(Pick Up After Class)

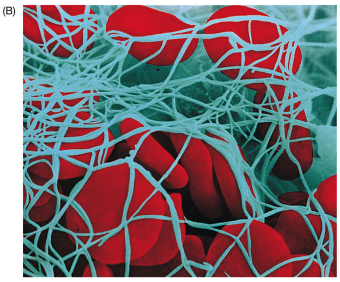




A Cascade Of Events After Wounding Leads to A Fibrin Clot





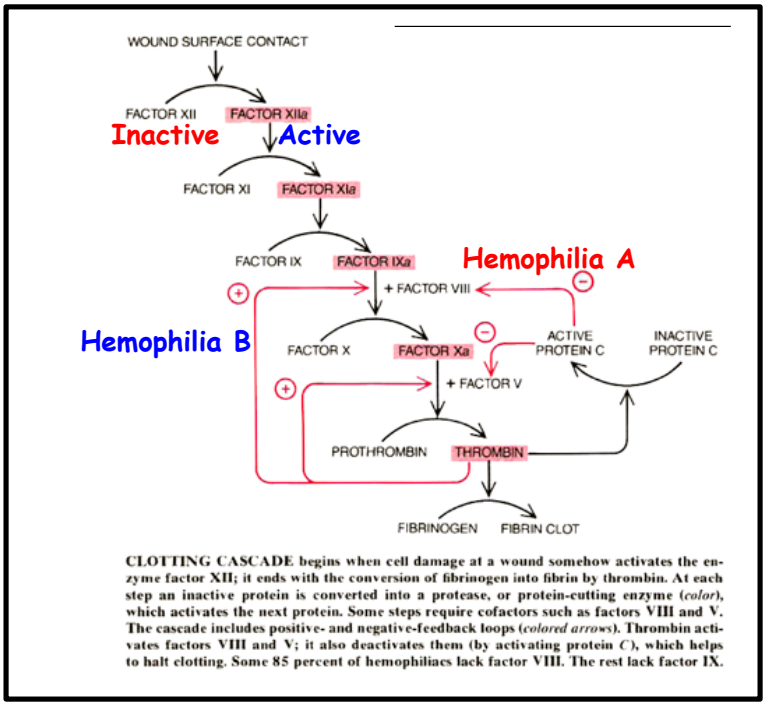


LIFE 8e, Figure 49.10 (Part 2)

LIFE 8e, Figure 49.10 (Part 1)

Clotting Factors Such As Factor VIII Play A Critical Role in This Process

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

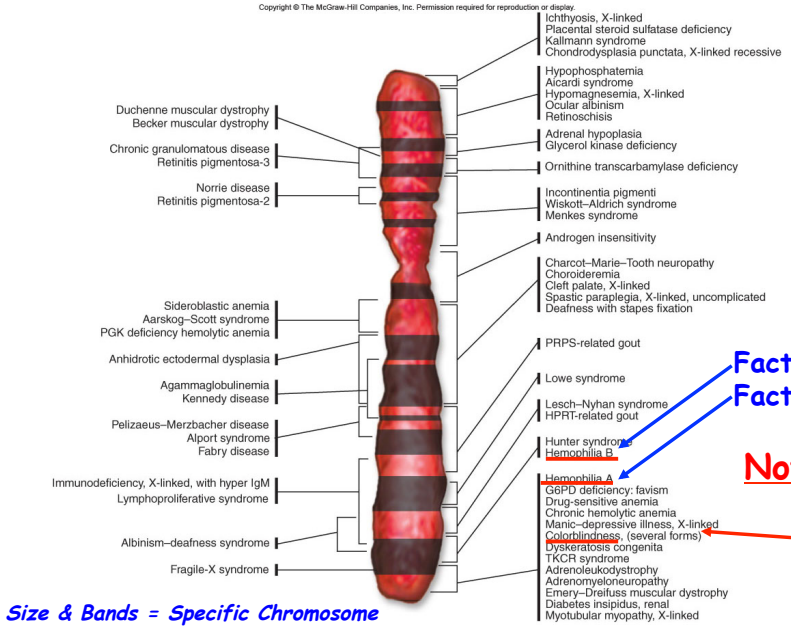
18,000 People in US Have Hemophilia & 400 Babies/Year Are Born With Disorder
Prior to 1960s – Average Life Span Was 11 Years

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%

Hypothesis For High Frequency in Males?

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

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



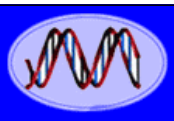
Size & Bands = Specific Chromosome

Nature, March, 2005

Factor IX
Factor VIII

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

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



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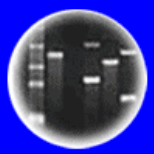

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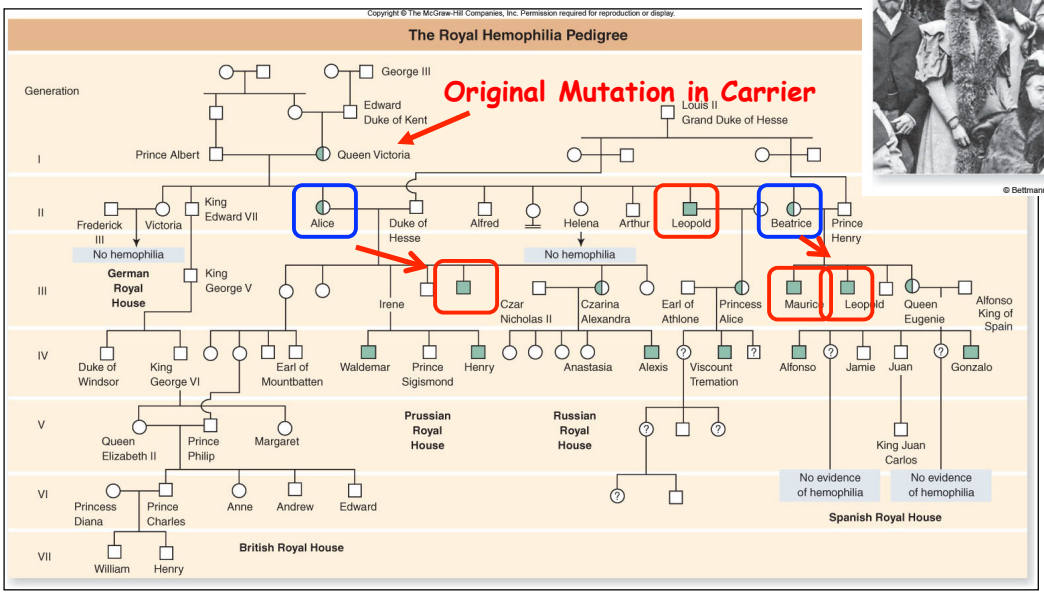
Pedigrees Can Be Used To Determine If a Trait is Dominant or Recessive

Each Type of Inheritance Predicts Specific Results in Each Generation

Hemophilia A and B Genes Are Sex Linked & Recessive Traits When Mutated



Note: 1. Males Obtain Detective Gene From Mothers

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

Hemophilia A and B Sex-Linked Inheritance

Carrier Female

Healthy Male

Egg / Sperm	X	X
X	XX ♀ *Carrier*	XX ♀ *Healthy*
Y	XY ♂ Hemophiliac	XY ♂ *Healthy*

Sex-Linked Inheritance

♀ Carriers → 1/2 Sons Afflicted + No Daughters!

Only One X-Chromosome is in ♂

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

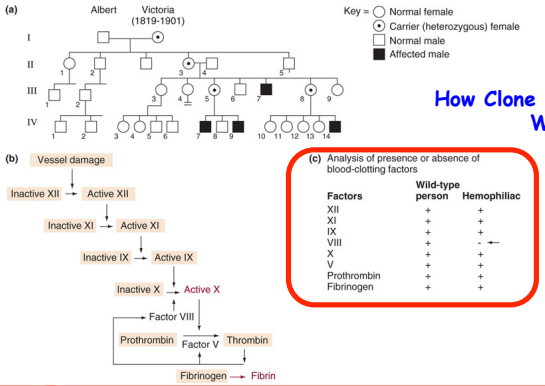
1. Blood Protein (But Perhaps Synthesized Elsewhere!)
2. Could be purified in small amounts from >20 Liters of human blood + cow blood + pig blood
3. Short Stretch of Protein Sequenced = Known Protein Sequence!
4. Hemophilia A could be treated by *blood transfusions* from normal individuals, ∴ clotting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

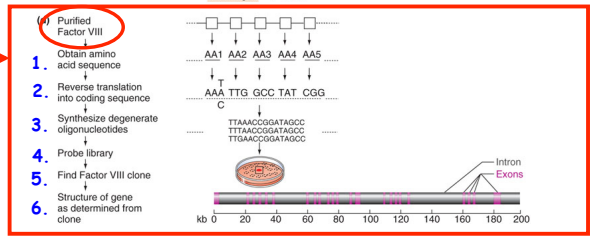
Early 1980's



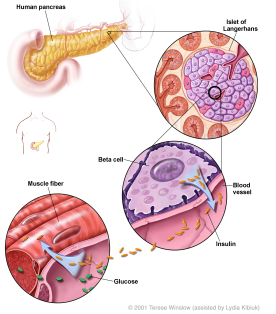
Key Concept

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

Key:
Protein Sequence Known



Different Than Insulin
Knew Where Protein Made!



How Find Gene & cDNA?

mRNA → Drug

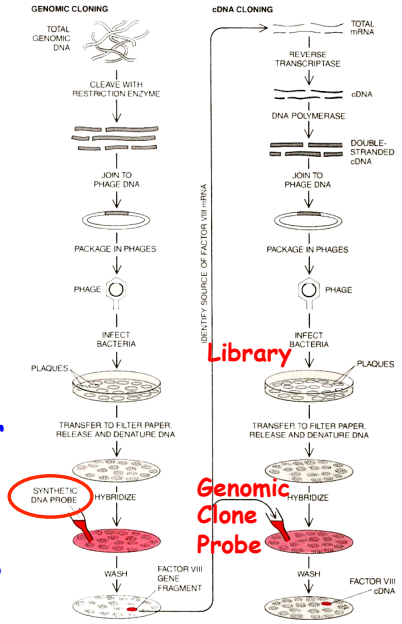
Protein → Gene → mRNA → Drug !

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?



Library

Genomic Clone Probe

cDNA

1. Use Gene probe to screen cDNA library for Factor VIII cDNA clone
2. How know what mRNA to use to make cDNA library?
3. Use gene probe to probe RNA blots containing mRNA from all major organs (liver, kidney, blood, etc.)
4. Find Factor VIII mRNA in liver- male, liver- secrete into blood

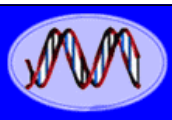
Why Need cDNA?
Story continued

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

Step One

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

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



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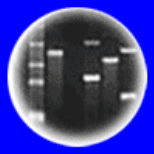

DNA Fingerprinting

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Plants of Tomorrow

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

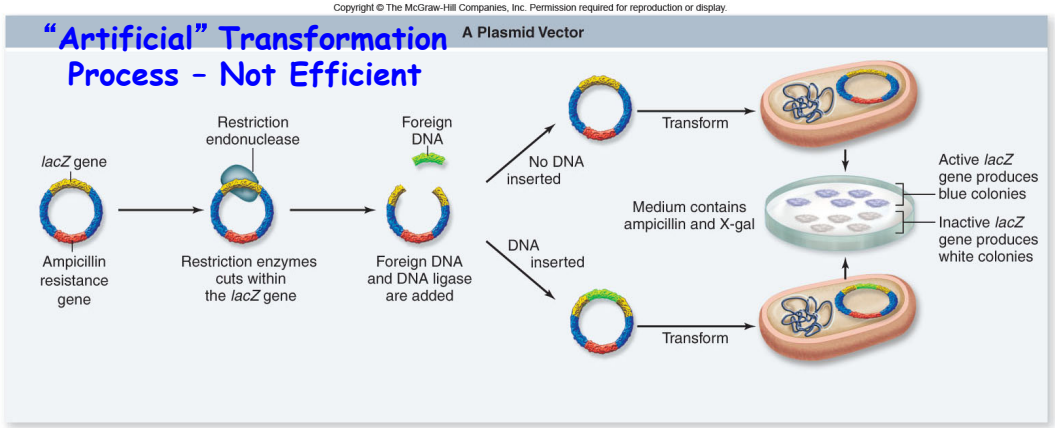
Vector Type	Maximum Insert Size (kb)	Applications	Limitations
Bacterial plasmid vectors (circular)	~6–12	DNA cloning, protein expression, subcloning, direct sequencing of insert	Restricted insert size; limited expression of proteins; copy number problems; replication restricted to bacteria
	DNA	DNA	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

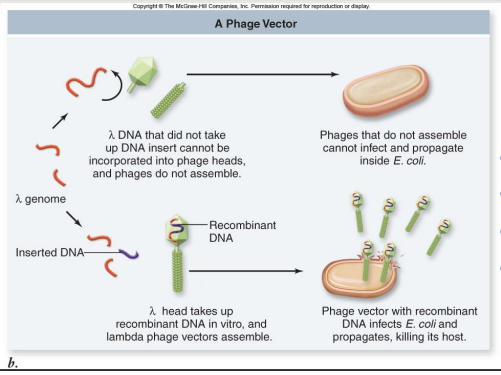
Plasmid vs. Bacteriophage Vectors for Cloning DNA Fragments

"Artificial" Transformation Process – Not Efficient



a.

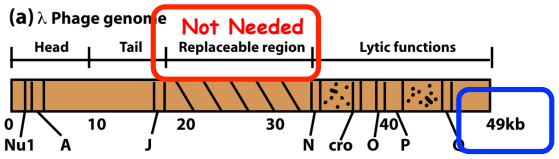
"Natural" Infection Process

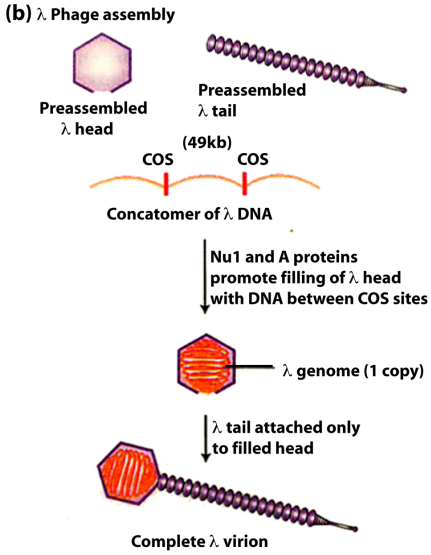


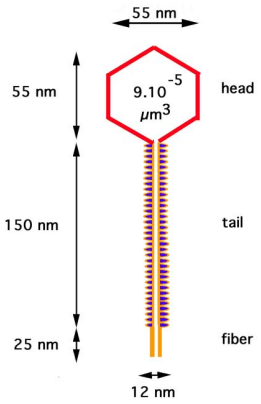
b.

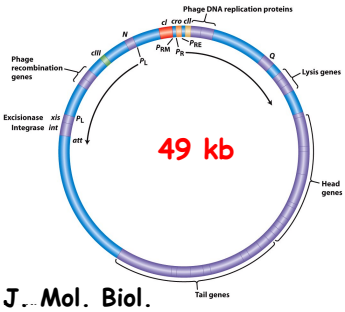
- Much More Efficient
- Can Use Less DNA
- Get Lots More Clones
- Need Lots of Clones For Large Genome

Structure of the λ Phage and Its Genome



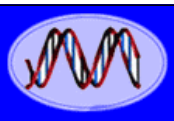






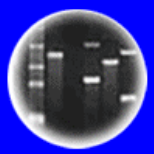
First Genome Sequence

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



DNA
Genetic Code of Life

Entire Genetic Code of a Bacteria

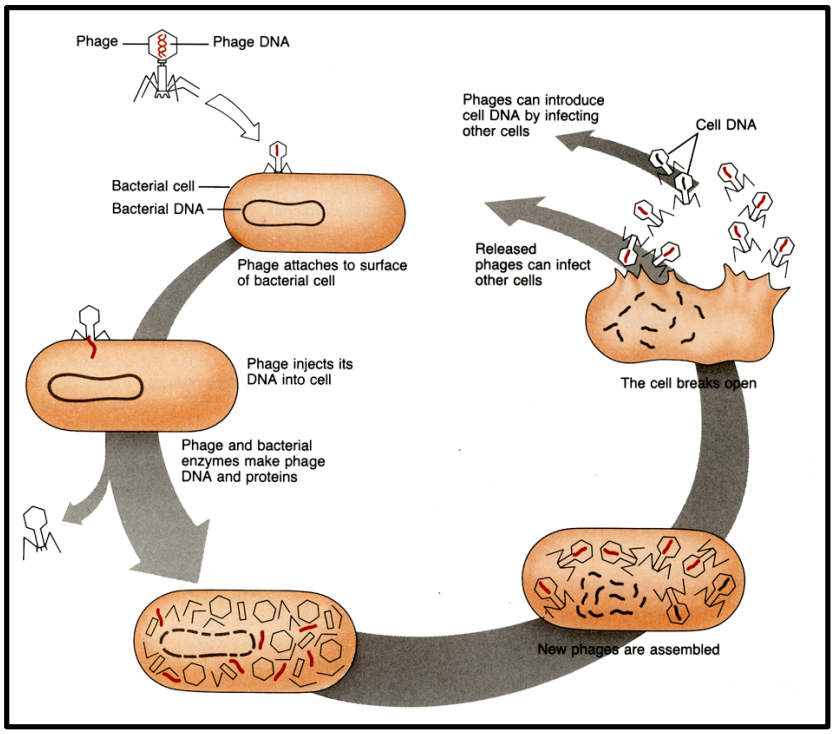


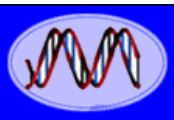
DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

Plants of Tomorrow

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





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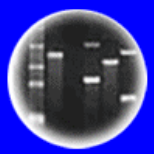
Entire Genetic Code of a Bacteria

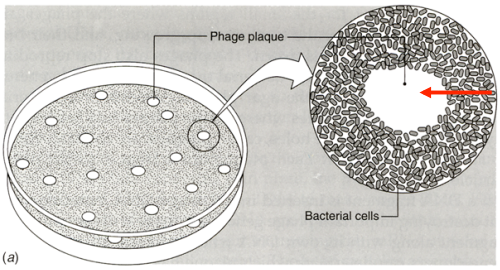


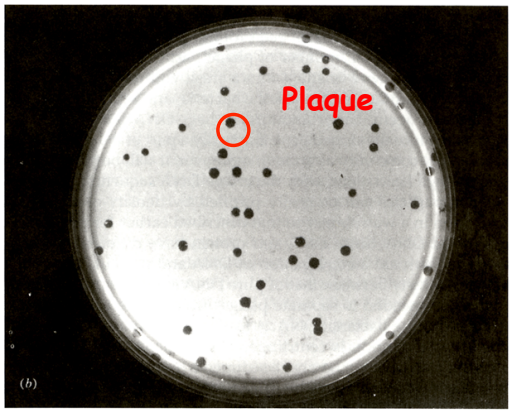
DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

Plants of Tomorrow

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

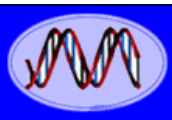
1. Long DNA Segments Can Be Cloned (~20kb) Need Fewer Clones For Whole Genome!
2. Can Clone DNA Segments In Viral Genome & Self-assemble With Viral Proteins Into Virus In A Test Tube!

∴ Make Recombinant Viruses In The Lab!

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

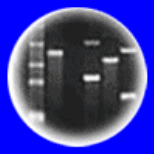
Much Higher Efficiency For Getting Recombinant DNA →Bacterial Cells Compared With Dna Transformation.

∴ Set More Clones Per Amount Of Recombinant DNA!



DNA
Genetic Code of Life

Entire Genetic Code of a Bacteria

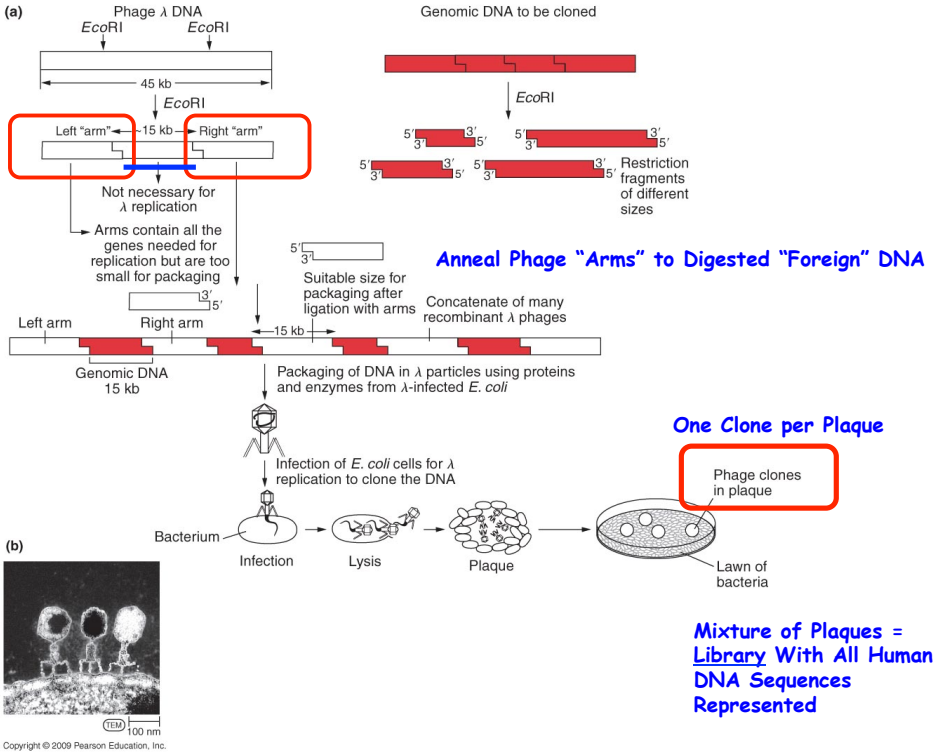


DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

Plants of Tomorrow

Using a Bacterial Virus To Clone the Human Genome



Anneal Phage "Arms" to Digested "Foreign" DNA

One Clone per Plaque

Mixture of Plaques = Library With All Human DNA Sequences Represented

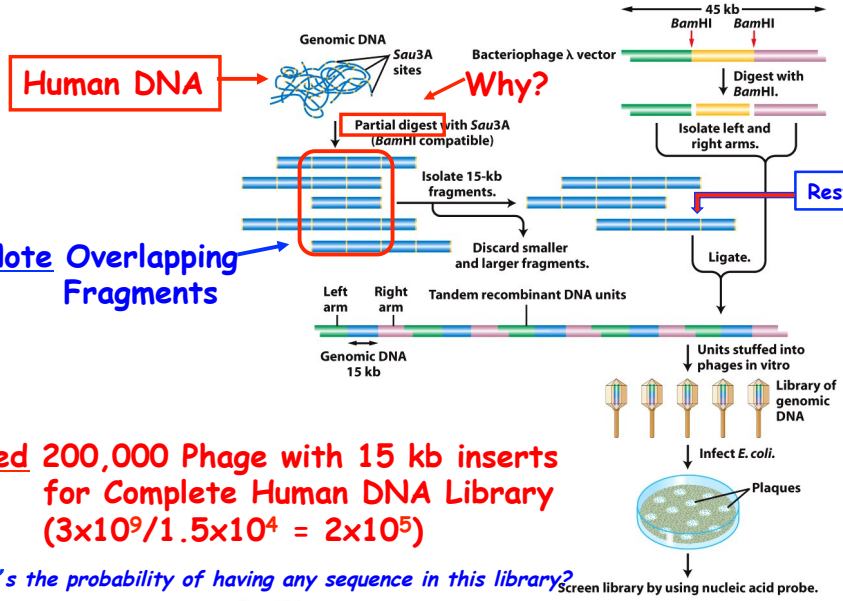
Cloning the Human Genome and Screening for the Factor VIII Gene

Human DNA

Why?

Note Overlapping Fragments

Restriction Cleavage Site



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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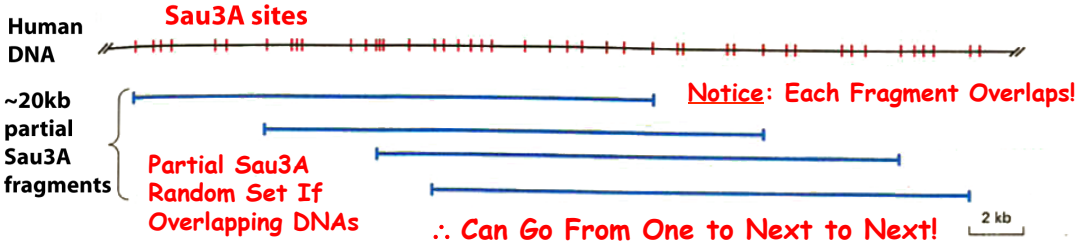
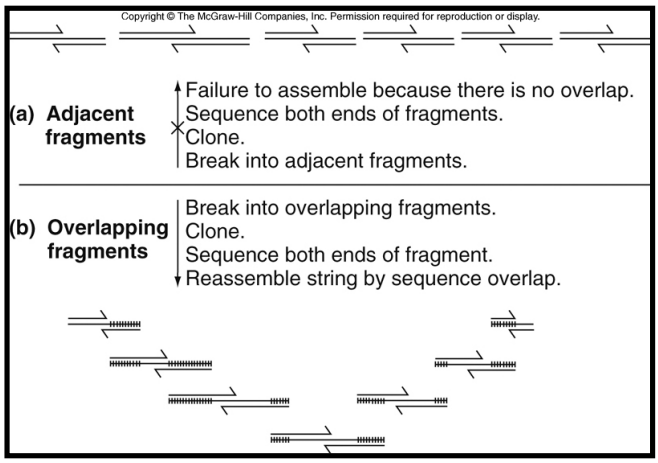
Why Partial Digestion? An Important Concept!

What is Complete & Partial Digestion?

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

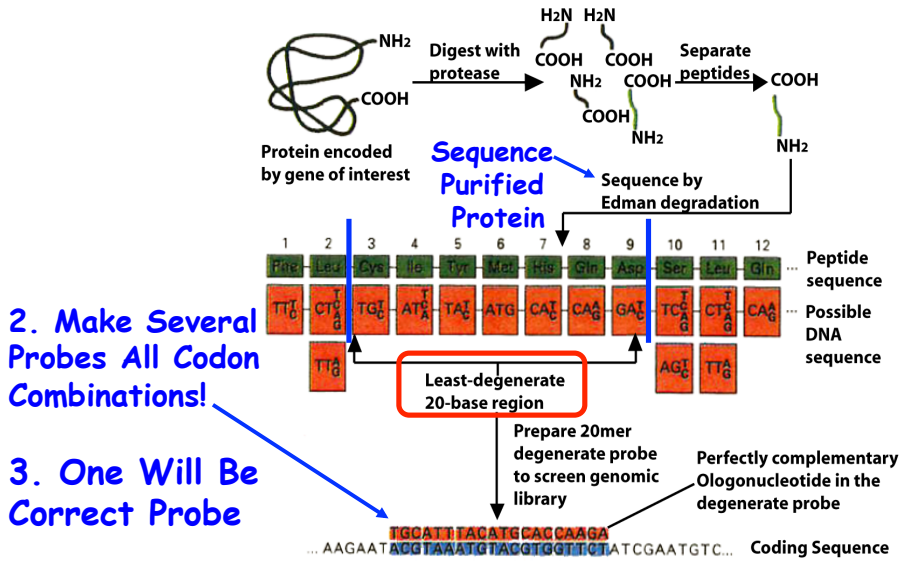
1st letter	Second Letter: U	Second Letter: C	Second Letter: A	Second Letter: G	3rd letter
U	UUU Phe UUC Phe UUA Leu UUG Leu	UCU Ser UCC Ser UCA Ser UCG Ser	UAU Tyr UAC Tyr UAA Stop UAG Stop	UGU Cys UGC Cys UGA Stop UGG Trp	U C A G
C	CUU Leu CUC Leu CUA Leu CUG Leu	CCU Pro CCC Pro CCA Pro CCG Pro	CAU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



2. Make Several Probes All Codon Combinations!

3. One Will Be Correct Probe

1. Use Genetic Code

How many Combinations of Synthetic Probes?

2x3x2x1x2x2x2=96

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

(b) Synthesizing DNA probes based on reverse translation

Protein sequence	N	Glu	Asp	Met	Trp	Tyr C
Degenerate coding sequences	5'	GAA GAG	GAT GAC	ATG	TGG	TAT 3' TAC
		②	②	①	①	②

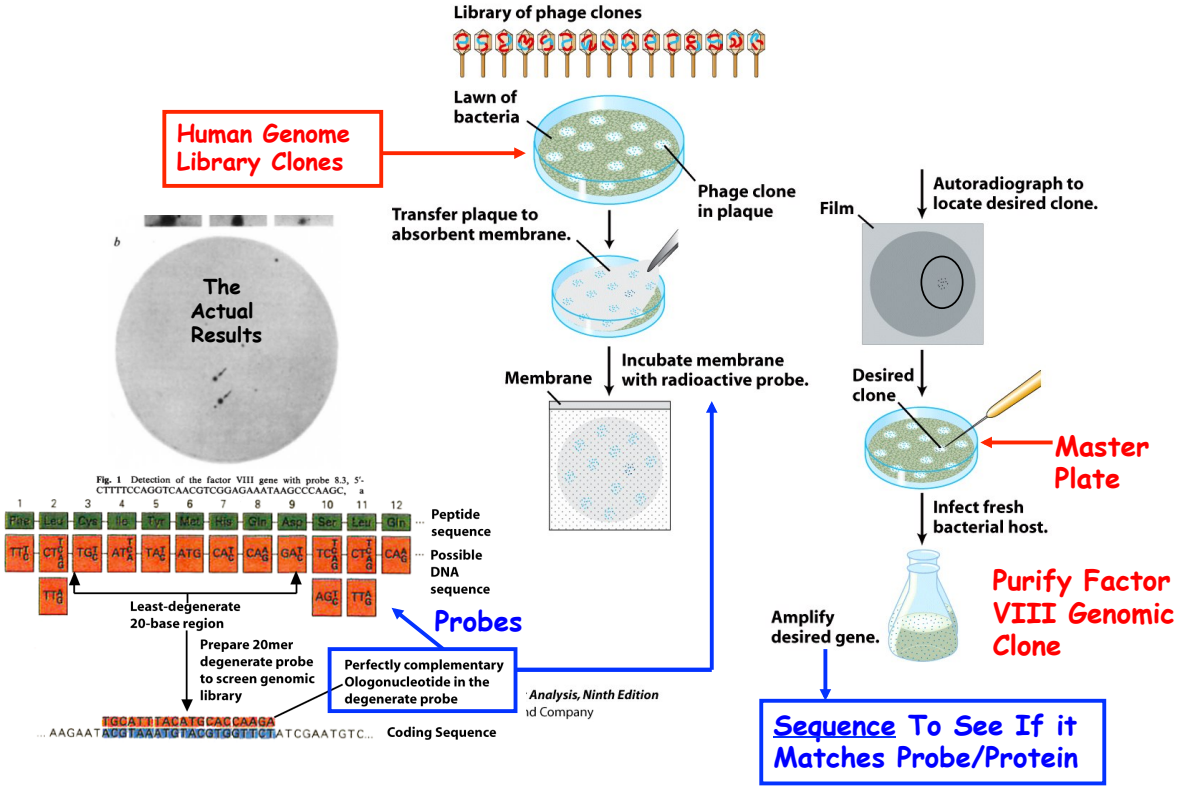
Sequences that must be present in the probe = 8 Codon Combinations

Mixed Probes (Contains All 8 Sequence Combinations)

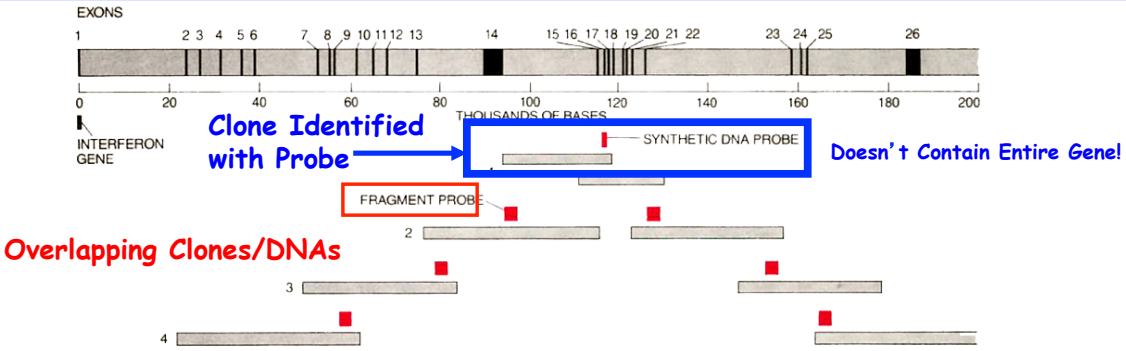
GAAGATATGTGGTAT
GAGGATATGTGGTAT
GAAGACATGTGGTAT
GAGGACATGTGGTAT
GAAGATATGTGGTAC
GAGGATATGTGGTAC
GAAGACATGTGGTAC
GAGGACATGTGGTAC

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

Finding The Factor VIII Gene Or Part of Gene!!



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



How Find Clones with Rest of Gene? Key Question !

Remember - the library contains overlapping DNA clones ∴ can use one part of first clone to re-screen library & "walk" to other gene regions- using restriction maps & sequencing (compare with protein sequence) as guides!

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

Step Three

Finding the Entire Factor VIII Gene? Walking & Sequencing

Walking Up and Down Genes and Chromosomes

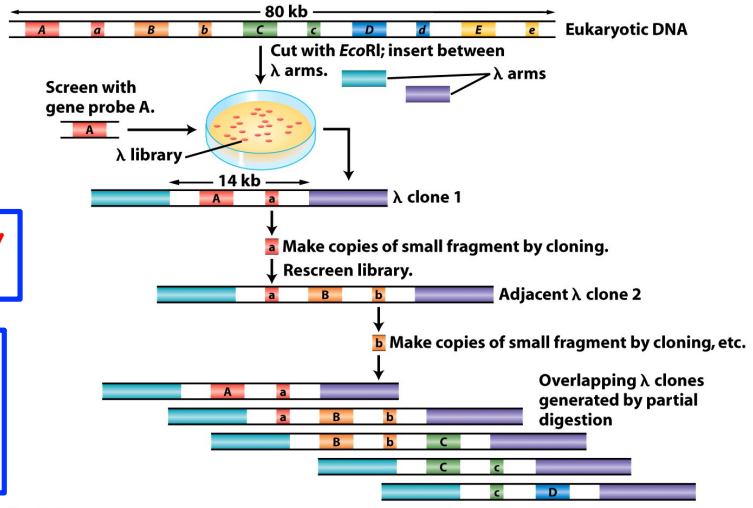


Figure 20-13
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Reiterative Library Screening Process

Find Overlapping Clones By Restriction Site Mapping

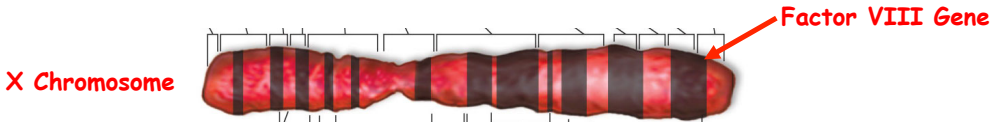
Basis of Genome Projects & Whole Genome Sequencing

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)]

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

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

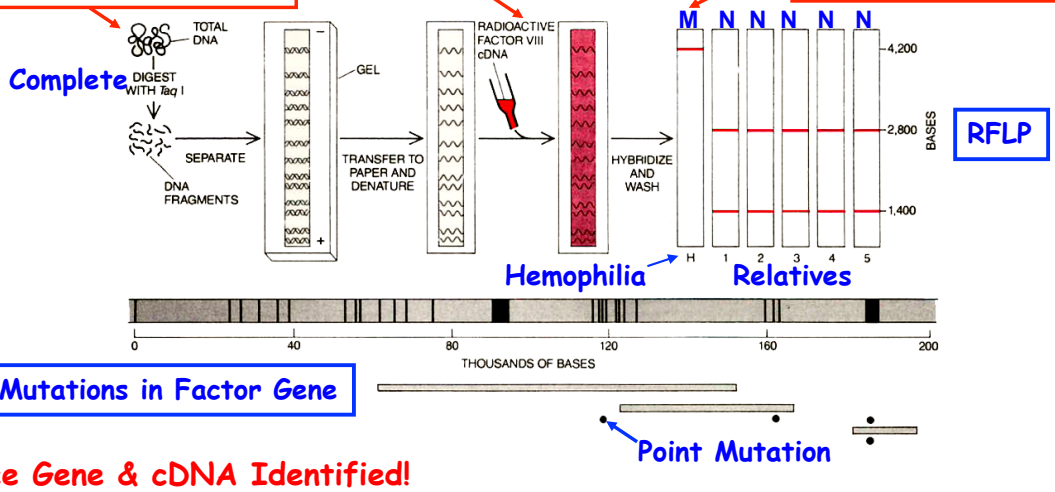
Need To Screen Across the Gene for Markers -- Family Specific

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

Once Gene & cDNA Identified!

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

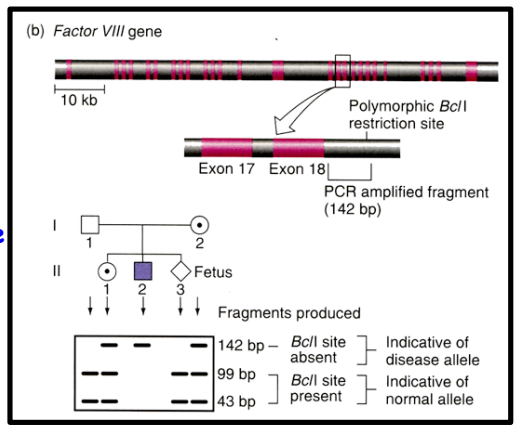
Mutations Arise Independently in Families

Using PCR and RFLPs (Markers) to Screen For 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

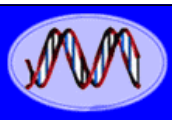
[–] = Disease allele



The 21st Century Approach!

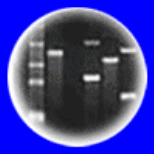
1. Sequence the Entire Gene & Find Mutation
2. Then Synthesize Primers to Test Family Members Using PCR

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



DNA
Genetic Code of Life

Entire Genetic Code of a Bacteria



DNA Fingerprinting

Cloning: Ethical Issues and Future Consequences

Plants of Tomorrow

Step Four

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

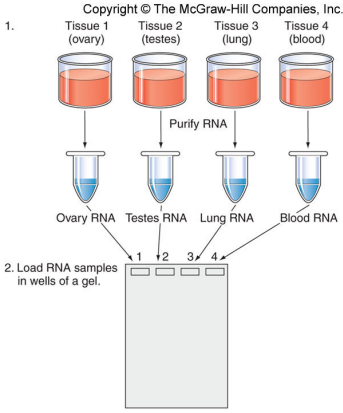
Recall: Eukaryotic Genes Provide Obstacles for Efficient Protein Production in Genetically Engineered Cells!

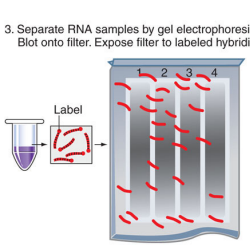
Introns! Switches!

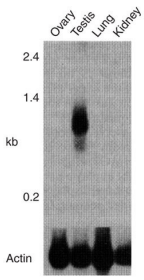
Making the Drug

Need cDNA Not Gene

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





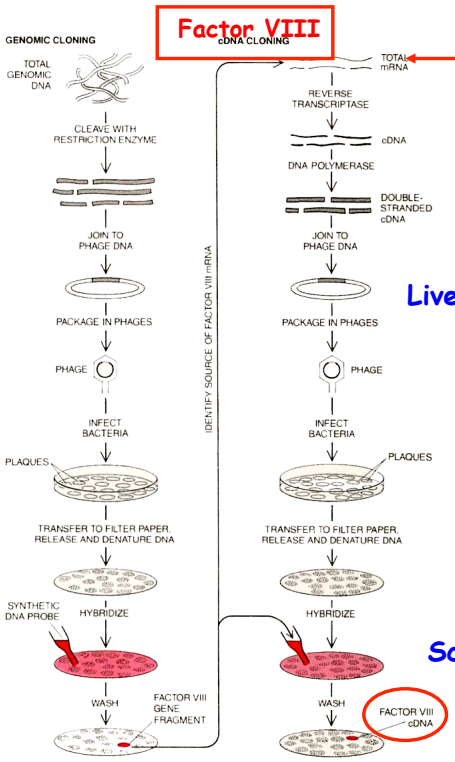


RNA Blot Is Like a DNA Blot Except That RNA is on Gel & Blotted

Factor VIII Gene Is Highly Active in Liver!

Could Also Use PCR (RT-PCR)

Using Factor VIII Gene Probe to Identify Factor VIII cDNA clone



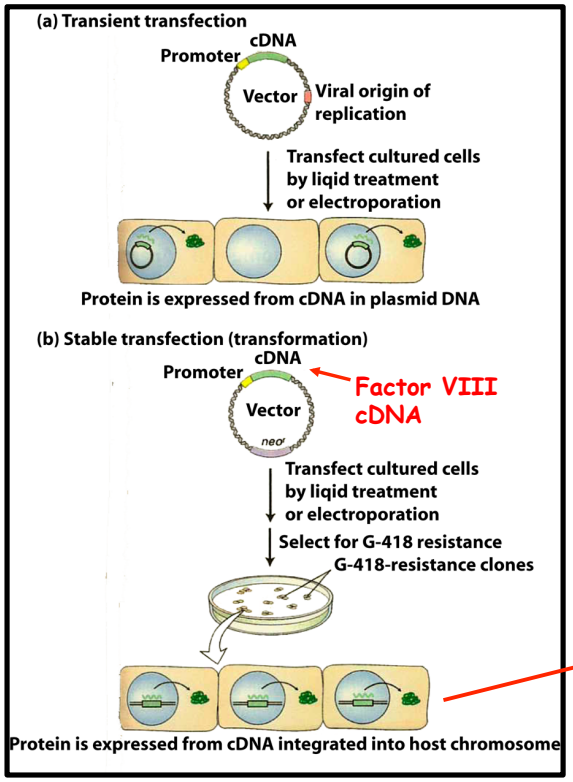
Liver mRNA

Liver cDNA Library

Factor VIII cDNA → Used to Make Drug !

Screen Liver cDNA Library

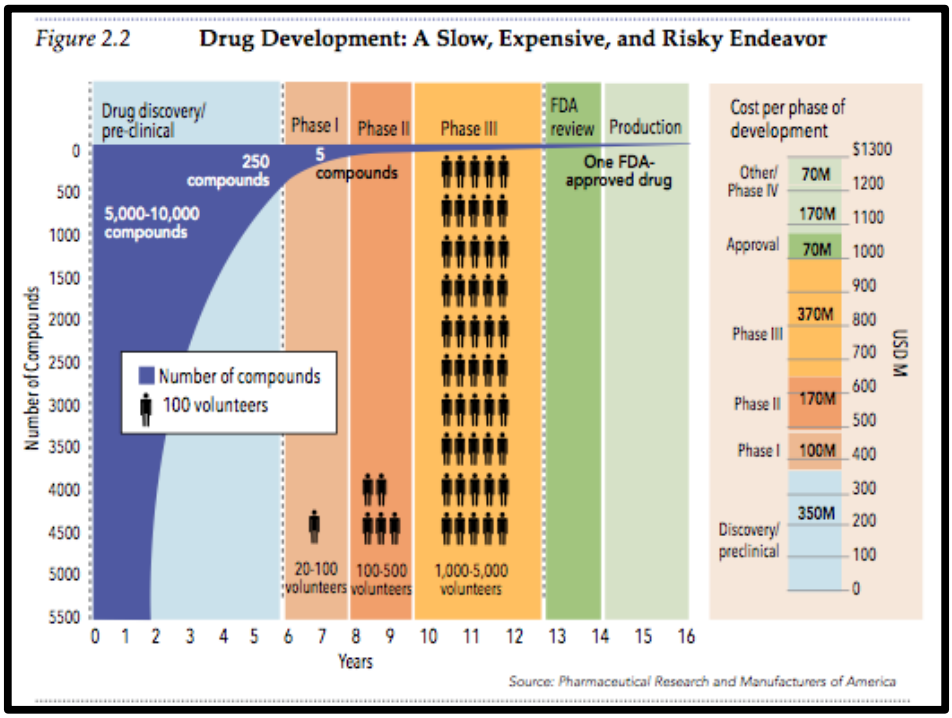
Engineer Factor VIII cDNA to Produce Protein in Host Cell & Synthesize Factor VIII in Mammalian Cells



Why Mammalian Cells?

Purify Factor VIII Protein!

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



A Long and Expensive Process!

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.

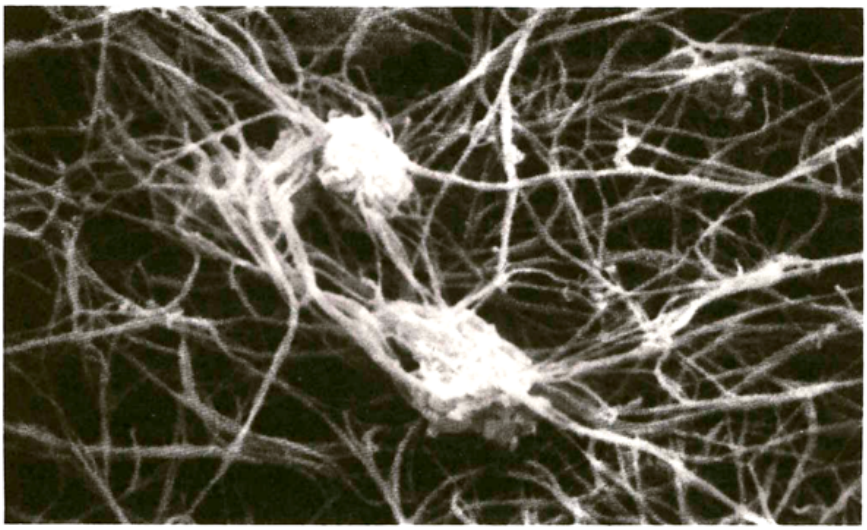
Prophylactic Treatment Costs $300,000/ Year!
Most Hemophiliacs Use "On Demand" or As Needed

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

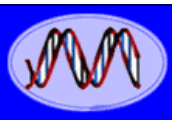
TIME Partners with CNN

Gene Therapy Shows Promise for Treating Hemophilia

By ALICE PARK Monday, December 12, 2011

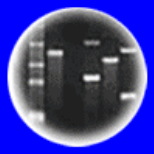
The First Ever In-Human Gene Editing Will Try and Combat Hemophilia

Factor IX – Hemoglobin B
FDA-Approved Clinical Trial
2016



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)