

Recombinant DNA Technology

Why Do Genetic Engineering?

1. Produce desired proteins *in vitro* for therapeutic use.
2. Have rice produce as much starch as a kernel of corn (*in vivo* production).
3. Gene therapy
4. Elucidate the function of BMP's

Steps in Genetic Engineering

1. Isolation of gene of interest
2. Isolation of plasmid DNA
3. Manipulation of DNA sequence
 - a. Cutting- Restriction enzymes
 - b. Splicing- DNA ligase
4. Transformation of bacteria
5. Selection of "correct" bacteria

Prokaryote Advantages

1. Grow fast
2. Manipulation easier
3. Eukaryotic technology still somewhat embryonic

Prokaryote Disadvantages

1. Can't splice out introns
2. Introns are needed for good expression
3. Size of DNA that can be put into bacteria is limited
4. Prokaryotes don't glycosylate proteins

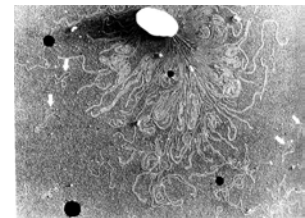
Plasmids

Plasmid- small, circular, extrachromosomal DNA which replicates independently of host chromosomal DNA.

Most (experimental) derived from a single clinical specimen in 1974

Low copy # vs. high copy number

Incompatible plasmids



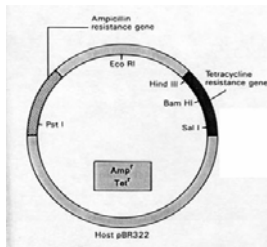
Isolation of Plasmid DNA

- Lysis by boiling
- Alkaline lysis
- Detergents or organic solvents

Isolation of Plasmid DNA Other Steps

- Centrifuge denatured proteins
- Precipitate nucleic acids with salt/EtOH
- Quantify

Plasmid map



- Ori
- antibiotic resistance gene(s)
- restriction sites

• Figure: Harpers Review of Biochemistry

Manipulation of DNA Sequence

Restriction enzymes

Restriction enzyme- an enzyme which cuts specific DNA sequences, endonuclease
“blunt end” vs. “sticky end”

Cleavage is restricted to specific, 4-6 bp sequences (foreign bacteria); always palindromic sequence

More than 800 are now known

Restriction Endonucleases

Type I- multisubunit, endonuclease and methylase activities, cleave at random up to 1000 bp from recognition sequence

Type II- cleave DNA within recognition sequence, require no ATP, most monomers

Type III- multisubunit, endonuclease and methylase about 25 bp from recognition sequence

Select Restriction Endonucleases

Table 29-2
Recognition Sequences for Some Type II Restriction Endonucleases

<i>Bam</i> HI	(5') G G A T C C (3') C C T A G G ↓	<i>Hind</i> III	(5') A A G C T T (3') T T C G A A ↓
<i>Cla</i> I	(5') A T C G A T (3') T A G C T A ↓	<i>Nco</i> I	(5') C C C G C C G C C (3') G C C G G G C C ↓
<i>Eco</i> RI	(5') G A A T T C (3') C T T A A G ↓	<i>Pst</i> I	(5') C T G C A G (3') G A C G T C ↓
<i>Eco</i> RV	(5') G A T A T C (3') C T A T A G ↓	<i>Pvu</i> II	(5') C A G C T G (3') G T C G A C ↓
<i>Nae</i> III	(5') G C C C (3') C C C G ↓	<i>Hin</i> III	(5') C A C N N N G T C (3') C T G N N N C A G ↓

Arrows indicate the phosphodiester bonds cleaved by each restriction endonuclease. Asterisks indicate bases that are methylated by the corresponding methylase (where known). N denotes any base. Note that the name of each enzyme consists of a three letter abbreviation of the bacterial species from which it is derived (e.g., *Bam* for *Bacillus amyloliquefaciens*, *Eco* for *Escherichia coli*). The Roman numerals included in the enzyme names (e.g., *Bam*HI) distinguish different restriction endonucleases isolated from the same bacterial species rather than the type of restriction enzyme.

Generating a Plasmid map

- restriction sites
- sizes when insert included

Cloning Vectors

1. Plasmids- 5,000 to 400,000 bp
useful for putting 0.01-10 kb in
2. Bacteriophages-virus that infects bacteria
useful for putting 10-20 kb in
3. Cosmids- artificially generated
useful for putting 20-50 kb in
4. YACs- yeast artificial chromosomes
new, 500 kb
5. One newer yet?

Other Important Vectors

1. Baculovirus- infects insect (*Sf9*, e.g.) cells
2. For plants, wound and infect with engineered *Agrobacterium tumefaciens*

Transformation of Bacteria

CaCl₂ Transformation

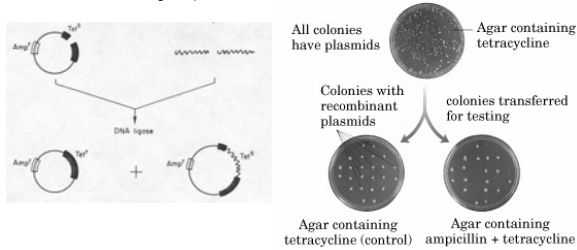
- Cells and DNA incubated together in CaCl₂ at 0°C, then heat shock at 42°C
- How this makes cells “competent” to take up DNA is not known
- Only a small percent of cells take up DNA- must select for them

Newer Methods of Transformation

- Lipofectin® and similar molecules
- Electroporation
- Microinjection

Selection of “Correct” Bacteria

Antibiotic Resistance Genes are a Part of Many (Constructed) Plasmids



- Follow with replicate plating of transformants on Amp and Tet

Blue-White Screening Promega Corp; Madison, WI

- pGEM-3Z, e.g.
 - *Amp^r*
 - *lacZ*
 - polycloning site in *lacZ* gene
 - T7 promoter one side, SP6 other

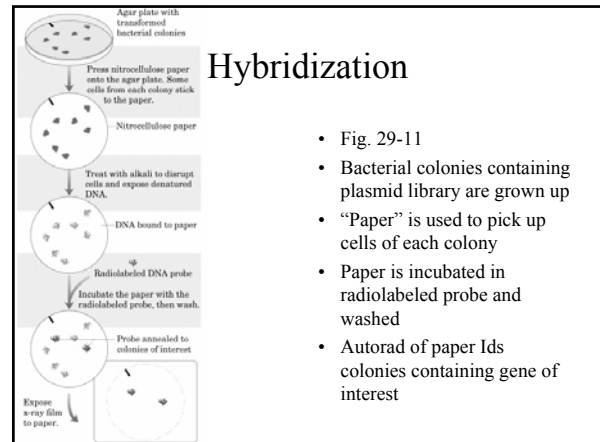
Isolation of Gene of Interest

Isolation of Gene of Interest Use of Antibodies

- Ab ppt protein
- Prot being synth on mRNA
- Generate cDNA
 - reverse transcriptase
 - DNA polymerase
- Must have protein in ~pure form

Isolation of Gene of Interest Genomic Library Screening

- Isolation of total genome
- Fragments and their sizes
- How many fragments to get entire genome can be calculated
- Fragments put into a vector
- Vectors are hybridized with a probe
- Don't need protein, but must know at least part of sequence

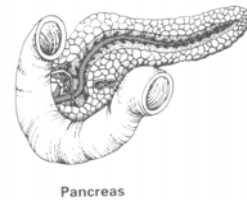


Isolation of Gene of Interest Polymerase Chain Reaction (PCR)

- Taq polymerase
- Equipment- thermocycler
- Procedure- Taq + template + primer
- Don't need protein, but must know at least part of sequence
- The real power here is ability to amplify DNA

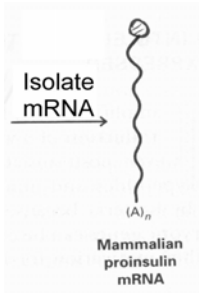
Summary

Figure: Stryer, Biochemistry



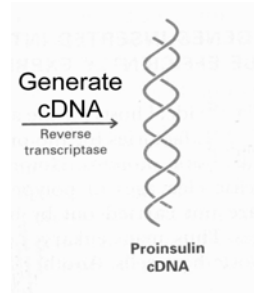
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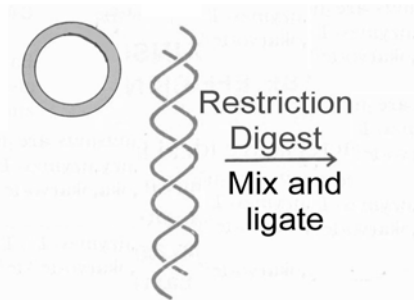
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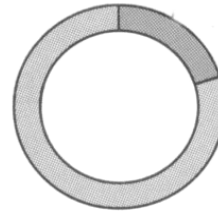
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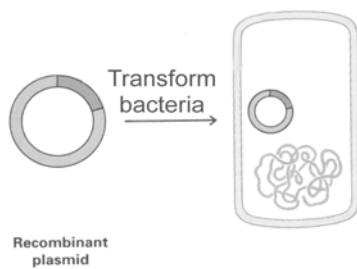
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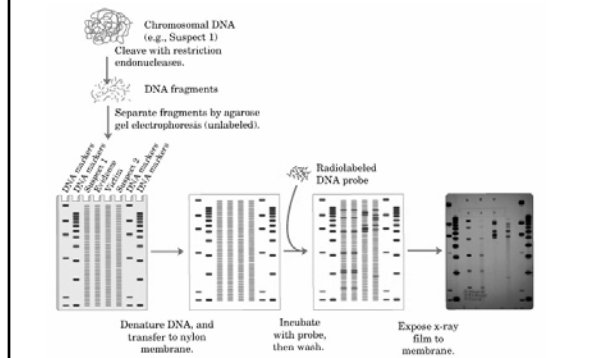
Polymorphisms

- Occur ~ once every 500 nt
- Usually in noncoding region
- If in coding region, leads to disease
- Used to ID victims and suspects and parents

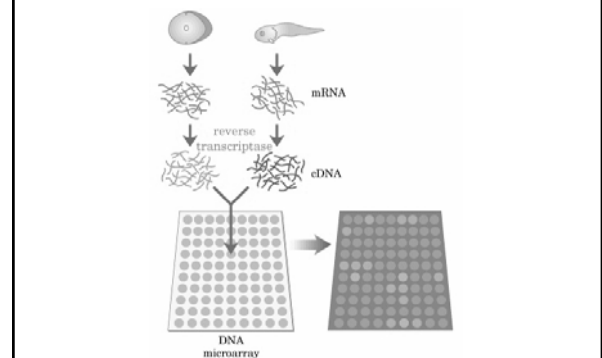
RFLP

- Restriction Fragment Length Polymorphism
- Same pattern which exists in >1% of the population
- Inherited
- Thousands known

Box 29-1



DNA Microarrays



Mammalian Expression Promoters

Promoter	Source	Advantage
CMV	Human cytomegalovirus immediate-early gene	Proven high-level expression in a variety of mammalian cell lines
EF-1 α	Human elongation factor 1 α subunit gene	Strong constitutive expression in mammalian cells; use in cell lines that down-regulate viral promoters
UbC	Human ubiquitin C gene	Efficient expression across a broad range of tissues and mammalian cell types
Sv40	Simian virus 40	Constitutive expression with higher expression levels in cells that express the large T antigen