



## Restriction endonucleases

- Cleave DNA in a sequence-specific manner
- Evolved as a defense mechanism against infection by foreign DNA
- Different restriction enzymes in different organisms
- Protect host DNA by modification-methylation
- Nomenclature: **EcoRI**

Genus
Species
Strain
Enzyme number

- Many restriction enzymes recognize "palindromic" sites

EcoRI cuts: 5'—GAATTC—3'  
3'—CTTAAG—5'

To yield:

5'—G—OH      and      5'—ATTC—3'  
3'—CTTAA—5'                      HO—G—5'

- EcoRI will not cut at this site if the Adenine is methylated, as a means of protecting its own DNA from being digested.

5'—GAA<sup>m</sup>TTC—3' ← will not be cut by EcoRI  
3'—CTTA<sup>m</sup>AG—5'

## Digestion of DNA

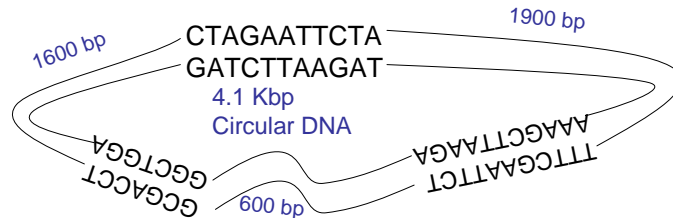
- Digestion of a particular DNA gives specific, "invariant" fragments of characteristic size
- Example: 3.2 Kbp DNA

700 bp
2000 bp
500 bp

5'-----ATGAATTCCA-----GCCGAATTCTTT-----3'  
3'-----TACTTAAGGT-----GCCGTTAAGAAA-----5'

↓  
**EcoRI**

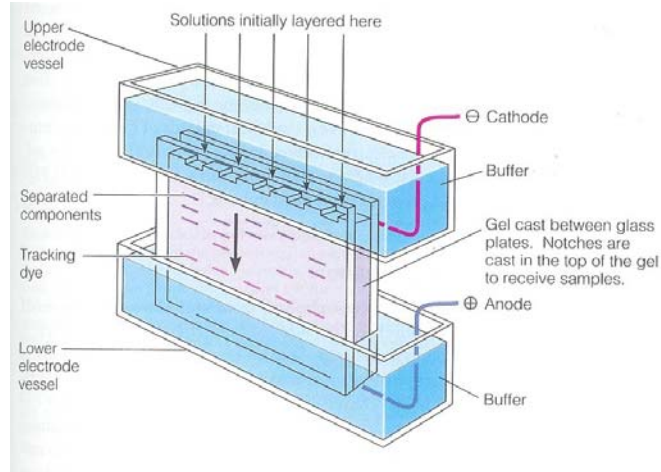
Restriction fragments of 2.0, 0.7, 0.5 Kbp



↓  
**EcoRI**

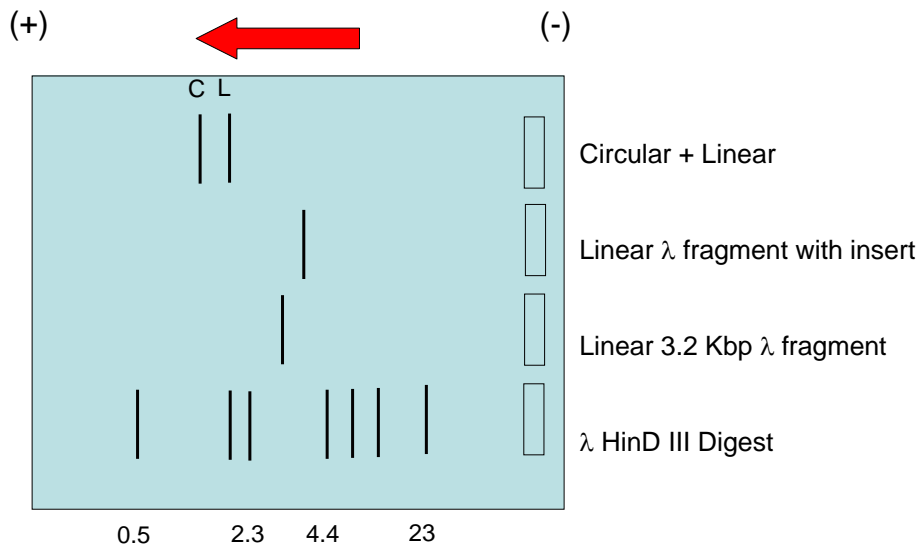
Restriction fragments of 1.9, 2.2 Kbp (Linear!)

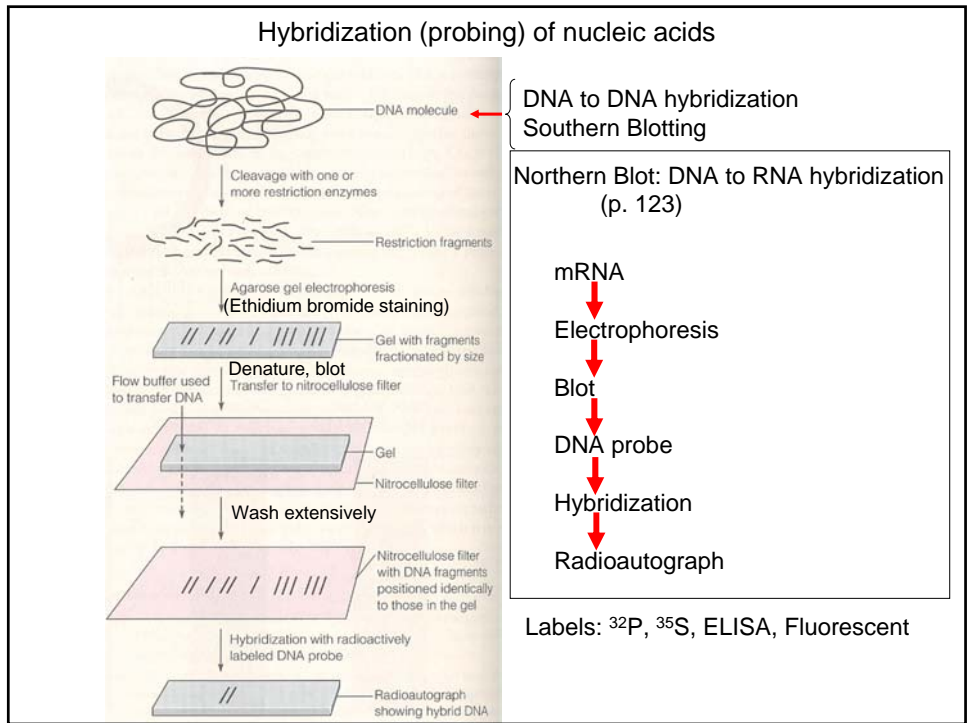
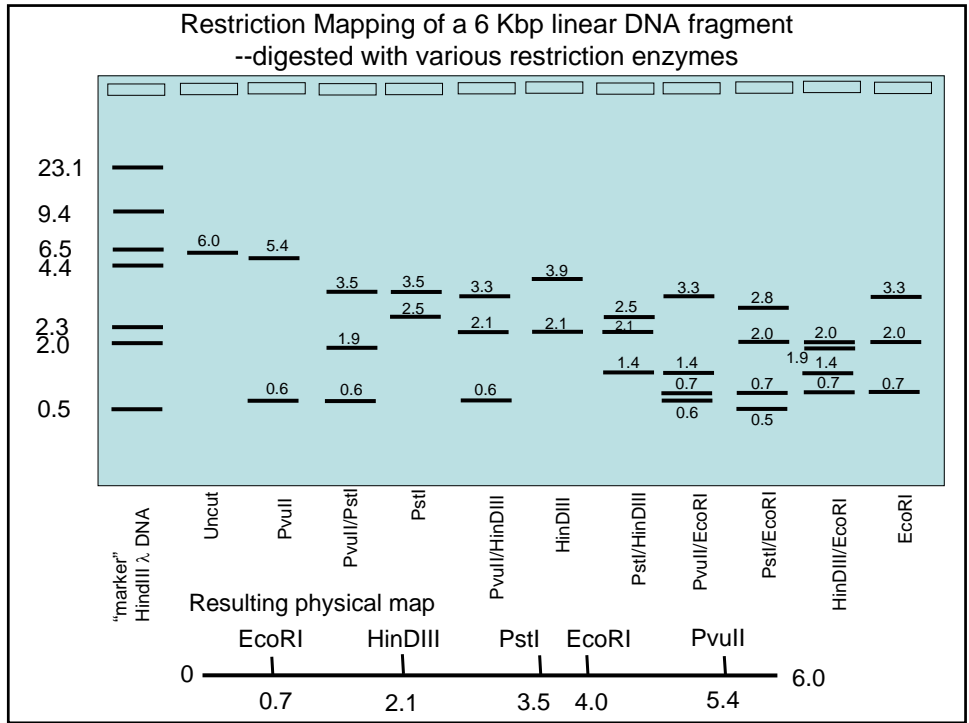
### Size-separation of DNA molecules and fragments by Electrophoresis

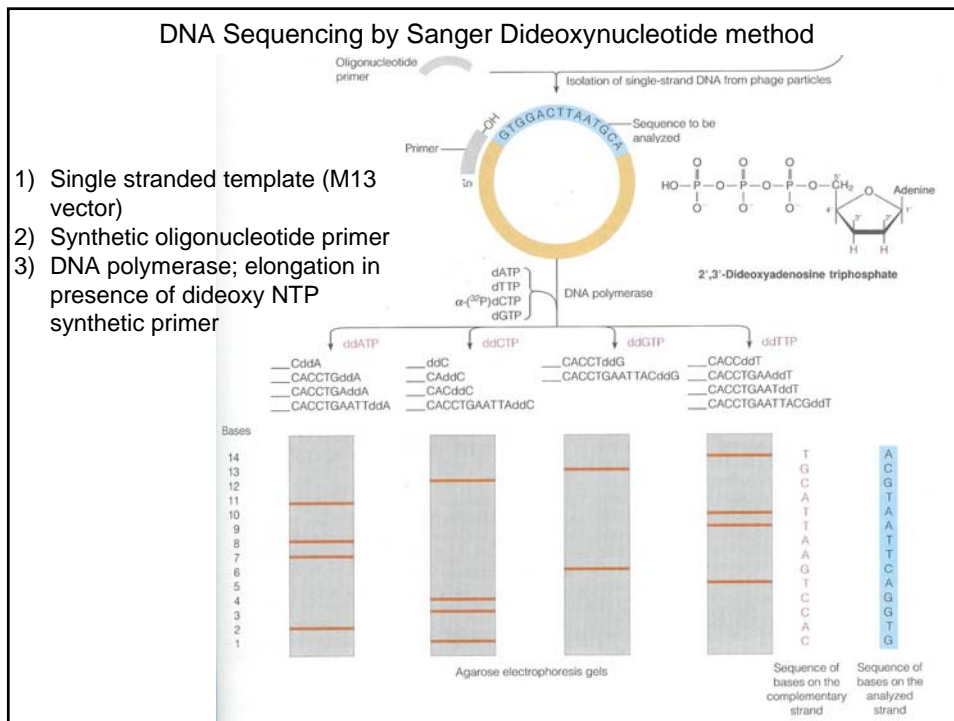
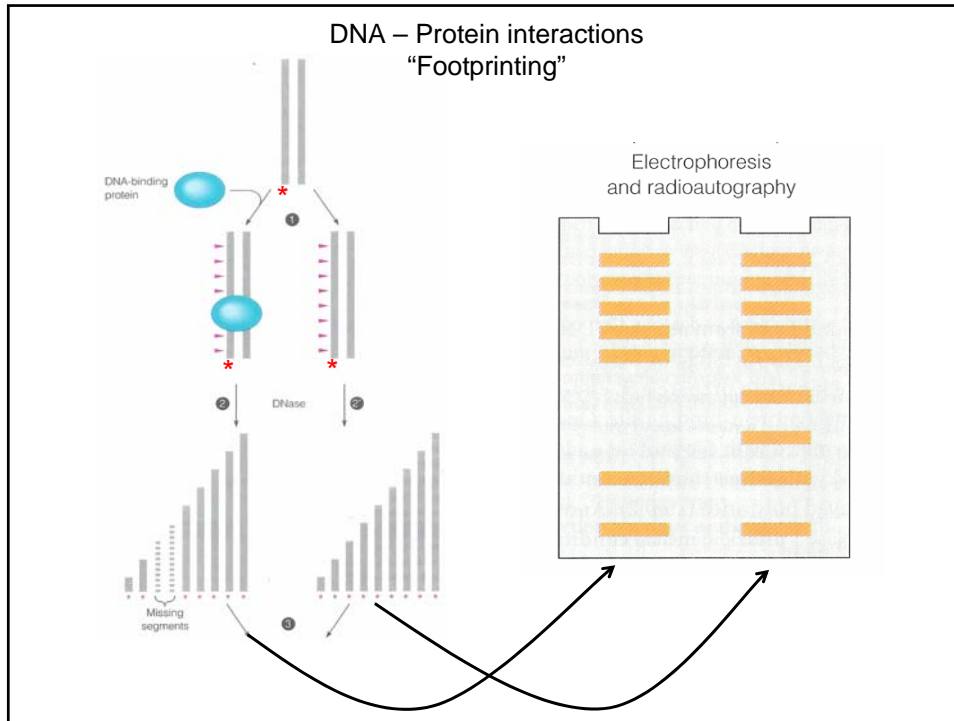


Rate of DNA MIGRATION is inversely proportional to length

- Size of DNA
- Concentration of Agarose
- Conformation of DNA
- Applied voltage







## Recombinant DNA and cloning

### A. Introduction

“Cloning” is a way to isolate a specific DNA fragment (a “gene”) and produce it in a pure form in large amounts. Cloning makes detailed analysis possible that would otherwise be difficult or impossible without the gene in pure form, separated from the rest of the DNA of the organism.

Cloning comprises techniques that provide a powerful approach for

1. Understanding eukaryotic genes
2. Providing large scale production of scarce proteins, e.g. peptide hormones, insulin
3. Controlled alteration of genetic composition of cells

### B. Cloning specific DNA fragments

1. Some definitions and concepts
  - a. Cloning vectors...usually plasmids, DNA molecules capable of autonomous replication (circular)
  - b. Foreign DNA...fragment to be studied, that is not originally part of the cloning vector
  - c. Recombinant DNA...DNA molecules constructed *in vitro*, consisting of DNA from 2 or more sources (i.e. cloning vector with foreign DNA inserted)

### 2. Steps in Cloning

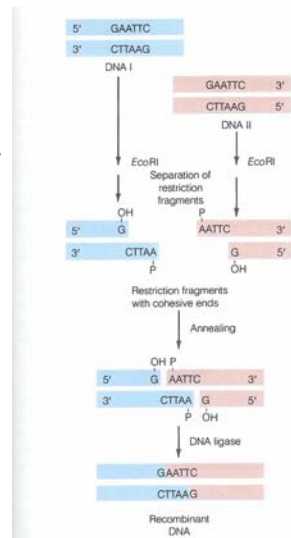
- a. Cut cloning vectors with a restriction endonuclease
- b. Cut out a piece of foreign DNA with the same (or compatible) restriction endonuclease
- c. Join the foreign DNA to the vector using DNA ligase
- d. Introduce the recombinant vector into cells (transfection, transformation) in which they can replicate
- e. Identify and isolate cells that contain the recombinant vector. From these, other cells (or clones) may be obtained.

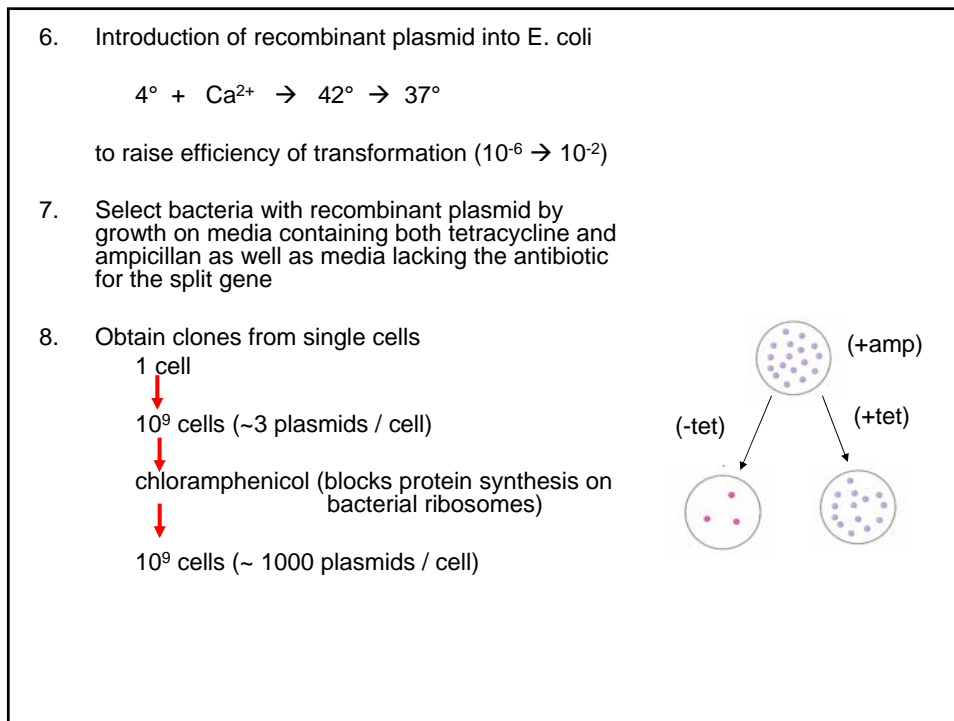
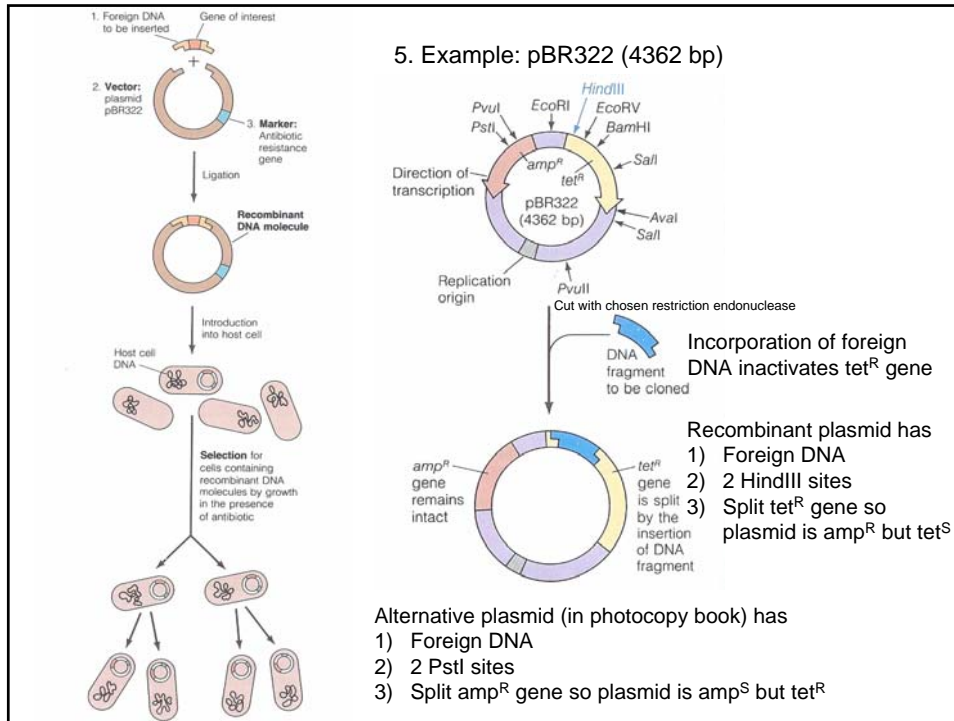
### 3. Cloning vectors

- a. Bacterial plasmids: pBR 322, p SP6
- b. Bacterial viruses:  $\lambda$  gt 11
- c. Animal viruses: SV40

### 4. Plasmids

- a. Small circular ds DNA molecules
- b. Contain genes for antibiotic resistance, to allow selection by growth on media containing (lacking) antibiotics
- c. Contain several restriction sites
- d. Can be replicated (amplified) in bacteria



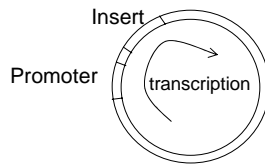


9. Isolation of cloned fragment
  - a. Concentrate bacteria with recombinant plasmid
  - b. Lyse
  - c. Differential precipitation of plasmid DNA
  - d. Cut out foreign DNA with Pst I or HindIII
  - e. Separate plasmid and foreign DNA on agarose gel



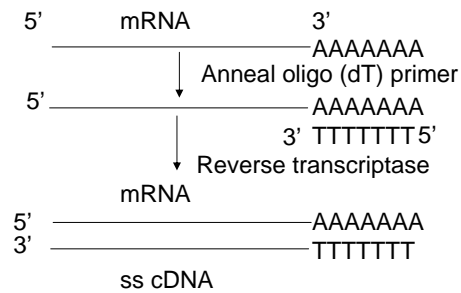
C. Expression of foreign genes in E. coli

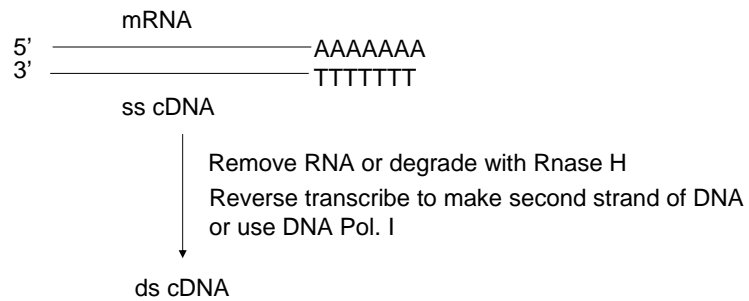
1. Expression vectors
  - Vectors with a regulated bacterial promotor (eg  $\beta$  Gal) upstream from insertion site
  - Can construct plasmids that contain sequences causing expressed proteins to be secreted (to minimize degradation)



2. Limitations of Eukaryotic gene expression in bacteria
  - a. No splicing. Genes with introns cannot be expressed properly. Can be circumvented by using cDNA
  - b. Bacteria cannot process of modify eukaryotic proteins (e.g. glycosylation, phosphorylation)
  - c. Codon usage varies
3. Examples of eukaryotic proteins produced in E. coli
  - a. Somatostatin
  - b. Human growth hormone
  - c. Insulin (now mostly produced in yeast cells)
  - d. Interferons

D. Construction of cDNA (complementary DNA) from mRNA





#### Other tricks

Can add tails (oligo C, G) with terminal transferase or add "linkers" with ligase to facilitate coupling with vector DNA

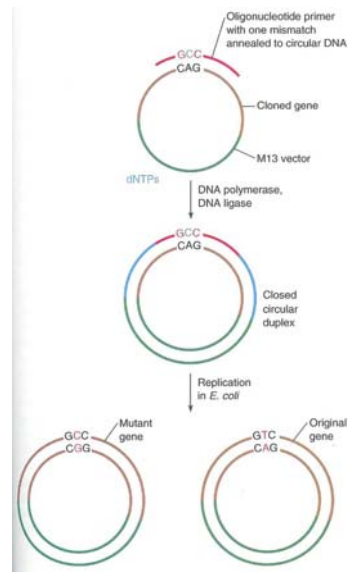
Foreign DNA can be inserted behind different promoters which are responsive to different regulatory signals

Site-directed mutagenesis:  
The ability to change individual codons or groups of codons in expressed coding sequences.

Primer contains one (or only a few) mismatched bases

Extend primer with DNA pol., ligate to generate ds DNA with one (or a few) mismatches

Replication of primer strand will introduce mutation at the site of the mismatch.  
Variations of this procedure are used to alter amino acids at active sites of enzymes (one at a time), to introduce new restriction enzyme cleavage sites and other directed, specific alterations



## Applications of Recombinant DNA technologies

- A. Construction of genetic "libraries" to assist in isolation / characterization of specific genes and gene products:
  - cDNA libraries / expression libraries
  - genomic DNA libraries
  - cosmids
  - YAC's (yeast artificial chromosomes)
  - Human Genome Project (and others.....)
- B. Production of scarce proteins using expression vectors
- C. Production of vaccines, hormones, blood clotting factors, etc.
- D. Diagnostics: antibody and oligonucleotide probes:
  - cancer cells, detection / imaging
  - bacterial and viral diseases (Legionnaires, AIDS)
  - Prenatal diagnoses
  - Forensics
- E. Therapeutics
  - Human insulin, Human growth hormone, interferons, growth factors, blood clotting factors, Plasminogen activator, tumor necrosis factor, novel recombinant antibodies, synthetic peptides as recombinant vaccines (Hep B, malaria, rabies, AIDS)
- F. Gene Transfer
  - Plants---increased yield, improved tolerance, herbicide res., disease res.
  - Transgenic animals---production of scarce proteins (eg in cow's milk), studies of eukaryotic gene regulation
  - Human gene therapy

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