Chapter 32: Genetic Engineering

- **Key Terms**
- **Cloning** describes propagation of a DNA sequence by incorporating it into a hybrid construct that can be replicated in a host cell.
- A **cloning vector** is a plasmid or phage that is used to "carry" inserted foreign DNA for the purposes of producing more material or a protein product.

32.2 Cloning Vectors Are Used to Amplify Donor DNA

- Fragments of DNA are generated for cloning by cleavage with restriction enzymes.
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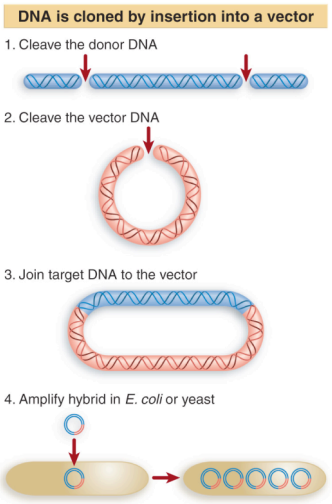
- A **cloning vector** is a plasmid or phage chromosome that is used to perpetuate a cloned DNA segment.
- Cloning vectors may be
  - bacterial plasmids,
  - phages,
  - and cosmids (combo phage and plasmid),
- Some cloning vectors have sequences allowing them to be propagated in more than one type of host cell.

A donor DNA is generated by cleavage by restriction enzymes so that its ends can be joined to the ends of a cloning vector cleaved by the same restriction enzyme.

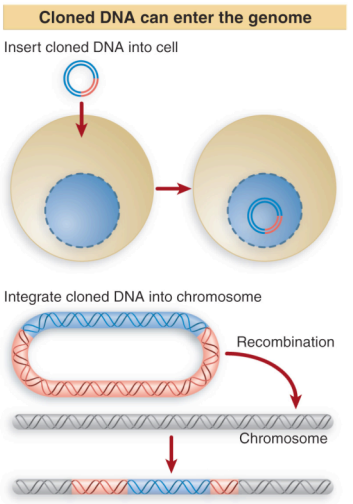
The hybrid molecule is then introduced into a bacterium or yeast in which it can replicate to produce many more copies.



Introducing new DNA into the genome requires cloning the donor sequence,

delivery of the cloned DNA into the cell,

and integration into the genome.



DNA can be released into target cells by methods that pass it across the membrane naturally,

such as a viral vector (in the same way as a viral infection)

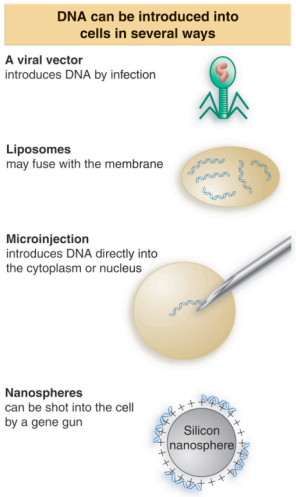
or by encapsulating it in a liposome or sugar moieties

(which fuse with the membrane).

Or it can be passed manually,

by microinjection,

or by coating it on the exterior of nanoparticles that are shot into the cell by a gene gun that punctures the membrane at very high velocity.



Each restriction enzyme cleaves a specific target sequence

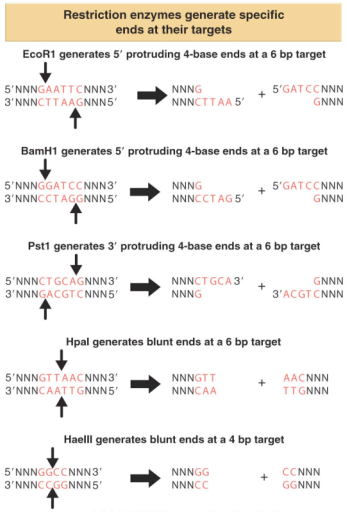
(usually 4-6 bp long).

The sites of cleavage on the two strands may generate:

5' protruding ends,

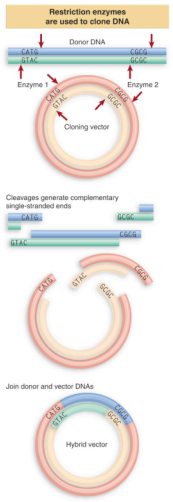
3' protruding ends,

or blunt ends.



Restriction enzymes can generate complementary single-stranded ends in donor and vector DNA by making staggered breaks in their target sequences.

Crosswise joining of the fragments generates a hybrid DNA molecule.

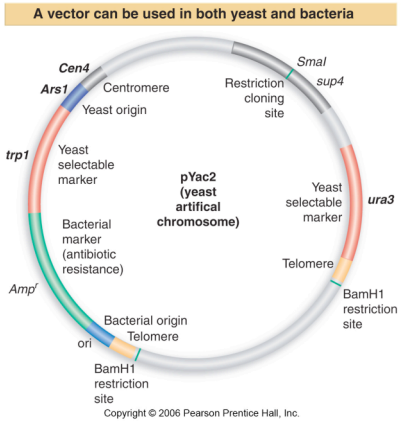


Cloning vectors may be based on plasmids or phages or may mimic eukaryotic chromosomes.

Several types of cloning vectors are available

Vector	Features	Isolation of DNA	DNA limit
Plasmid	High copy number	Physical	10 kb
Phage	Infects bacteria	Via phage packaging	20 kb
Cosmid	High copy number	Via phage packaging	48 kb
BAC	Based on F plasmid	Physical	300 kb
YAC	Origin + centromere + telomere	Physical	>1 Mb

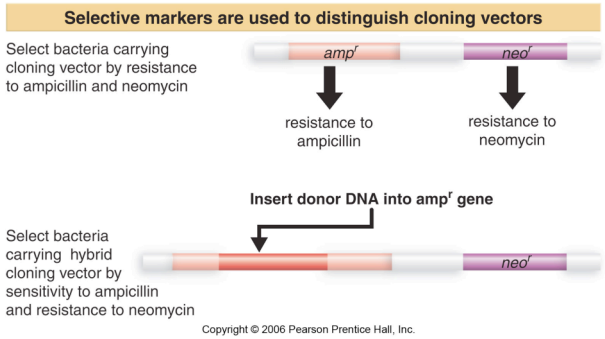
pYac2 is a cloning vector with features to allow replication and selection in both bacteria and yeast. Bacterial features include an origin of replication and antibiotic resistance gene. Yeast features include an origin, centromere, two selectable markers, and telomeres.



32.3 Cloning Vectors Can Be Specialized for Different Purposes

- Amplification of a cloned sequence requires a selective technique to distinguish hybrid vectors from the original vector
- Promoter activity can be assayed by using reporter genes.
- A **reporter gene** is a coding unit whose product is easily assayed
  - Usually an enzyme
  - such as chloramphenicol transacetylase, beta-galactosidase, luciferase
  - it may be connected to any promoter of interest so that expression of the gene can be used to assay promoter function.
- Exons can be identified by placing inserted DNA into an intron that is flanked by two exons.

Bacteria carrying a cloning vector can be selected by resistance to an antibiotic marker carried by the vector. Bacteria carrying a hybrid cloning vector with an insertion of donor DNA can be distinguished by loss of activity of the vector gene into which the insertion was made.



The CAT assay can be used to follow the activity of any eukaryotic promoter.

The promoter is linked to the gene for chloramphenicol transacetylase in a cloning vector.

Extracts are made from target cells carrying the vector.

The ability of the extracts to acetylate chloramphenicol is directly proportional to the units of the enzyme, which is determined by the efficiency of the promoter.

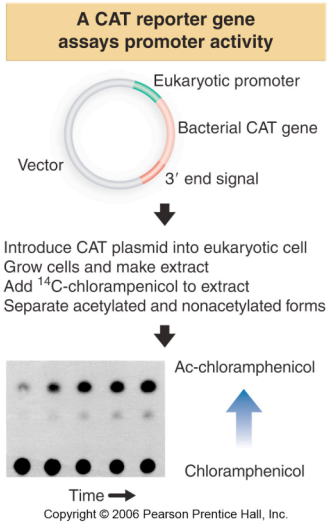
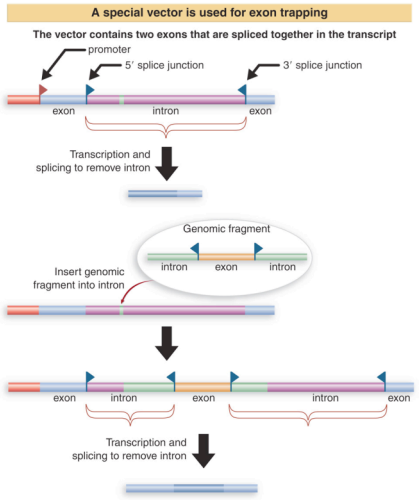


Figure: 32-11 A special vector is used for exon trapping

- A special splicing vector is used for exon trapping.
- If an exon is present in the genomic fragment, its sequence will be recovered in the cytoplasmic RNA,
- but if the genomic fragment consists solely of sequences from within the intron, splicing does not occur, and the mRNA is not exported to the cytoplasm.

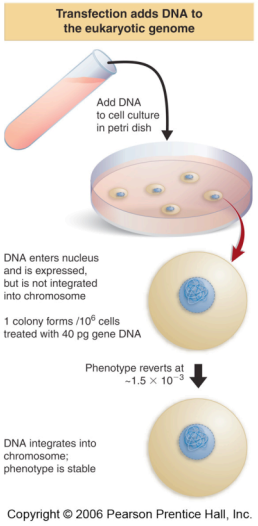


32.4 Transfection Introduces Exogenous DNA into Cells

- **Transfection** of eukaryotic cells is the acquisition of new genetic markers /genes by incorporation of added exogenous DNA
- **Transient transfectants** have foreign DNA in an unstable—i.e., extrachromosomal—form.
  - Remains in plasmid form
  - Does not integrate into the genome
- DNA that is transfected into a eukaryotic cell forms a large repeating unit of many head to tail tandem repeats
- **Stable transfectants** have foreign DNA that is integrated into a host chromosome
- Genes carried by the transiently or stably transfected DNA can be expressed.

DNA added to a cell culture can enter the cell and become expressed in the nucleus, initially as an unstable transfectant.

The cell becomes a stable transfectant when the DNA is integrated into a random chromosomal site.



32.5 Genes Can Be Injected into Animal Eggs

- **Transgenic** animals are created by introducing DNA prepared in test tubes into the germline.
  - The DNA may be inserted into the genome or exist in an extrachromosomal structure
- DNA that is injected into animal eggs can integrate into the genome
- Usually a large array of tandem repeats integrates at a single site.
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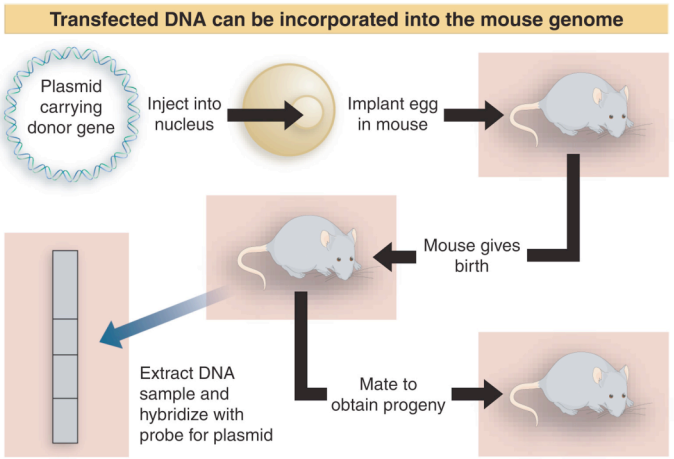
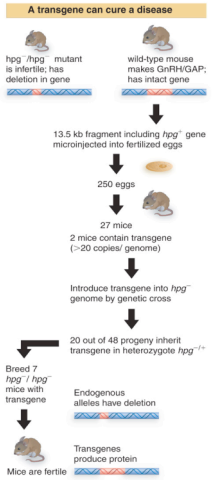
- Expression of the DNA is variable and may be affected by the site of integration and other epigenetic effects.



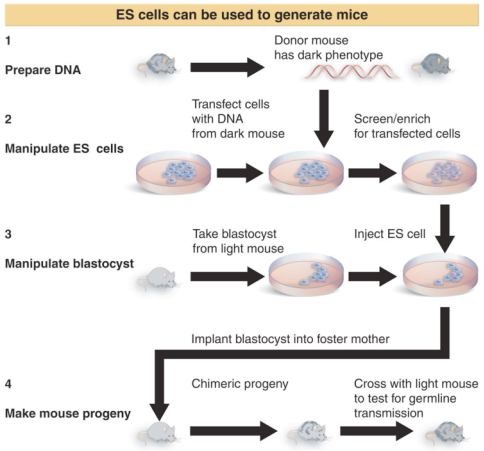
Hypogonadism can be averted in the progeny of *hpg* mice by introducing a transgene that has the wild-type sequence.



32.6 ES Cells Can Be Incorporated into Embryonic Mice

- ES (embryonic stem) cells that are injected into a mouse blastocyst generate descendant cells that become part of a chimeric adult mouse
- When the ES cells contribute to the germline, the next generation of mice may be derived from the ES cell.
- Genes can be added to the mouse germline by transfecting them into ES cells before the cells are added to the blastocyst.

ES cells can be used to generate mouse chimeras, which breed true for the transfected DNA when the ES cell contributes to the germline.

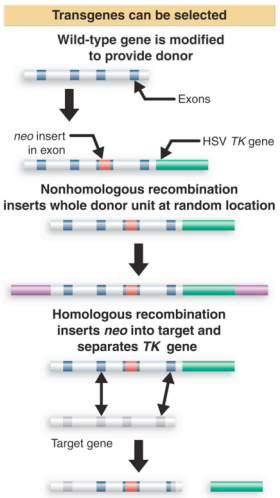


32.7 Gene Targeting Allows Genes to Be Replaced or Knocked Out

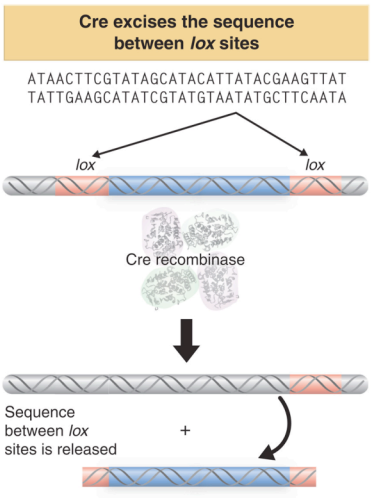
- An endogenous gene can be replaced by a transfected gene using homologous recombination.
- The occurrence of a homologous recombination can be detected by using two selectable markers,
  - one of which is incorporated with the integrated gene,
  - the other of which is lost when recombination occurs.
- A gene **knockout** is a process in which a gene function is eliminated, usually by
  - replacing most of the coding sequence with a selectable marker *in vitro*
  - and transferring the altered gene to the genome by homologous recombination.
- A gene **knock-in** is a process similar to a knockout, but more subtle mutations are made
  - such as nucleotide substitutions or the addition of epitope tags.
- The Cre/*lox* system is widely used to make inducible knockouts and knock-ins.

The Knockout technique

A transgene containing *neo* within an exon and *TK* downstream can be selected by resistance to G418 and loss of *TK* activity.



The Cre recombinase catalyzes a site-specific recombination between two identical *lox* sites, releasing the DNA between them.

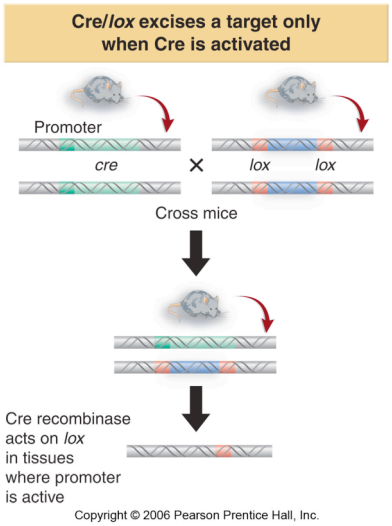


By placing the Cre recombinase under the control of a regulated promoter, it is possible to activate the excision system only in specific cells.

One mouse is created that has a promoter-*cre* construct, and another that has a target sequence flanked by *lox* sites.

The mice are crossed to generate progeny that have both constructs.

Then excision of the target sequence can be triggered by activating the promoter.



An endogenous gene is replaced in the same way as when a knockout is made but the *neo* gene is flanked by *lox* sites.

After the gene replacement has been made using the selective procedure, the neomycin gene can be removed by activating Cre, leaving an active insert.