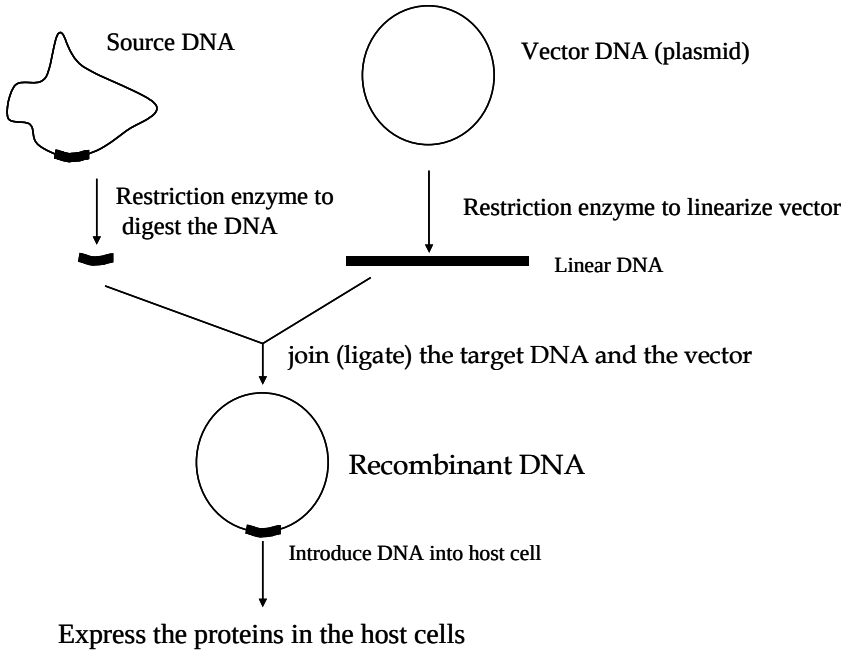
Chap 3 Recombinant DNA Technology

Introduction

- Core of contemporary biotechnology. In 2011, the sales of biologics have hit 200 billion USD worldwide and >75 billion in the US in 2014. The majority of biologics are recombinant proteins produced using recombinant DNA technology.
- Also named gene cloning, molecular cloning or genetic engineering.
- Transfer DNA (foreign DNA, target DNA, cloned DNA or insert DNA) from one organism to another.
- General procedure (Cohen et al. PNAS USA, 70: 3240-3244, 1973.



I. Restriction Endonuclease (restriction enzyme)

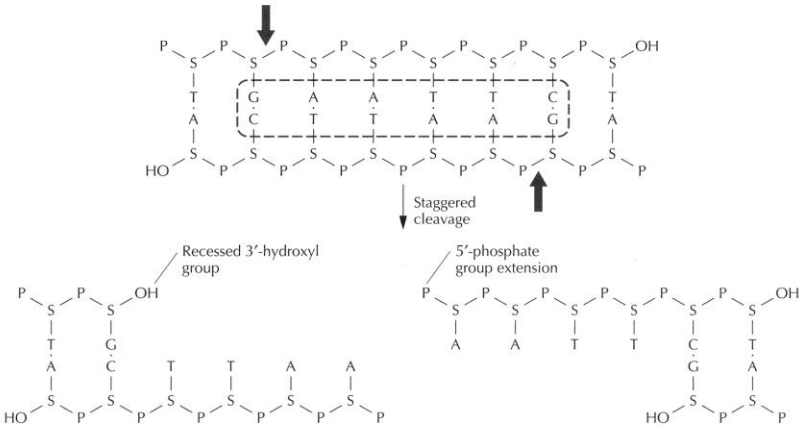
DNA molecule can be cut by:

1. passing DNA thru a small-bore needle to break DNA into 0.3-0.5 kb fragments➔random
2. restriction enzyme which recognizes DNA internally at specific bp sequences (usually 4-6 bp, palindromic, i.e. two strands are identical when read in either direction).

Examples of RE

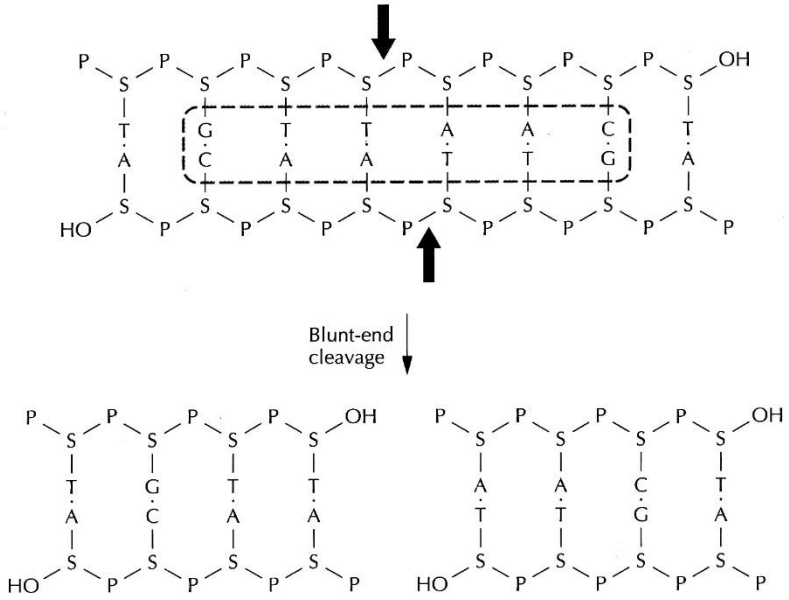
- *Eco*RI
  - Eco: *E. coli*, R: R13 strain, I: roman numeral to indicate the order of characterization of different enzymes. Overhang: the bases that hang out,
  - Recognizes GAATTC and cuts it into sticky ends

Figure 4.2 Symmetrical, staggered cleavage of a short fragment of DNA by the type II restriction endonuclease *Eco*RI. The bold arrows show the sites of cleavage in the DNA backbone. S, deoxyribose sugar; P, phosphate group; OH, hydroxyl group; A, adenine; T, thymine; C, cytosine; G, guanine. The *Eco*RI recognition sequence is highlighted by the dashed line.



- *Hin*d II: recognizes GTTAAC and cuts it into blunt ends

Figure 4.3 Blunt-end cleavage of a short fragment of DNA by the type II restriction endonuclease *Hin*dII. The bold arrows show the sites of cleavage in the DNA backbone. For abbreviations, see the legend to Fig. 4.2. The *Hin*dII recognition sequence is highlighted.



~ TABLE 15.1

Characteristics of Some Restriction Endonucleases

	Enzyme Name	Pronunciation	Organism in Which Enzyme is Found	Recognition Sequence and Position of Cut*	Number of Cleavage Sites in DNA from λ	Number of Cleavage Sites in DNA from pBR322
Enzymes with 6-bp Recognition Sequences	*Bam*HI	"bam-H-one"	*Bacillus amyloliquefaciens H*	5′ G↓G A T C C 3′ 3′ C C T A G↑G 5′	5	1
	*Bgl*II	"bagel-two"	*Bacillus globigi*	A↓G A T C T T C T A G↑A	5	0
	*Eco*RI	"echo-R-one"	*E. coli RY13*	G↓A A T T C C T T A A↑G	5	1
	*Hae*II	"hay-two"	*Haemophilus aegyptius*	R G C G C↓Y Y C G C G↑R	>30	11
	*Hin*dIII	"hin-D-three"	*Haemophilus influenzae* R_d	A↓A G C T T T T C G A↑A	6	1
	*Pst*I	"P-S-T-one"	*Providencia stuartii*	C T G C A↓G G A C G T↑C	18	1
	*Sal*I	"sal-one"	*Streptomyces albus G*	G↓T C G A C C A G C T↑G	2	1
	*Sma*I	"sma-one"	*Serratia marcescens*	C C C↓G G G G G G↑C C C	3	0
Enzymes with 4-bp Recognition Sequences	*Hae*III	"hay-three"	*Haemophilus egyptius*	G G↓C C C C↑G G	>50	22
	*Hha*I	"ha-ha-one"	*Haemophilus hemolyticus*	G C G↓C C G↑C G	>50	31
	*Hpa*II	"hepa-two"	*Haemophilus parainfluenzae*	C↓C G G G G↑C C	>50	26
	*Sau*3A	"sow-three-A"	*Staphylococcus aureus 3A*	↓G A T C C T A↑G	116	22
Enzymes with 8-bp Recognition Sequences	*Not*I	"not-one"	*Nocardia otitidis-caviarum*	G C↓G G C C G C C G C C G G C↑G	0	0

*In this column the two strands of DNA are shown with the sites of cleavage indicated by arrows. Since there is an axis of two-fold rotational symmetry in each recognition sequence, the DNA molecules resulting from the cleavage are symmetrical.

Key: R = purine; Y = pyrimidine.

- Note: RE are found primarily in bacteria to cut the bacteriophage[1] DNA as a defense system. The bacterial DNA is resistant because its DNA is chemically modified (methylation of cytosine to form 5 methylcytosine) to mask most of the recognition sites. (purine: A or G, pyrimidine: C or T)

II. DNA ligase

- Fragments cut by RE need to be joined to form a recombinant DNA (rDNA)
- DNA ligase (mainly from bacteriophage T4) catalyzes the formation of phosphodiester bonds at the ends of DNA➔ rDNA

[1] Bacteriophage: The virus that infects bacteria.

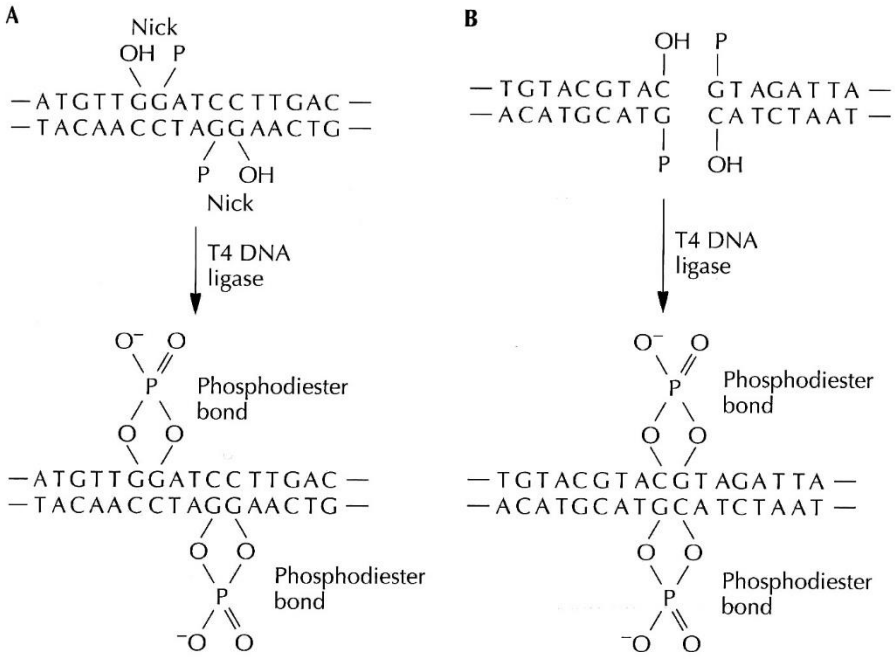


Figure 4.6 Mode of action of T4 DNA ligase. The enzyme T4 DNA ligase forms phosphodiester bonds by joining 5′-phosphate and 3′-hydroxyl groups at nicks in the backbone of double-stranded DNA. **A.** Ligation of sticky-ended DNA. **B.** Ligation of blunt-ended DNA. A, C, G, and T represent nucleotides.

- T4 DNA ligase is the only DNA ligase that efficiently joins blunt-end termini under normal conditions. Sticky ends ligations are usually carried out at 12-15°C. Blunt-end ligation is usually more difficult and is carried out at RT (room temperature) with 10-100X more enzyme than sticky end ligations. The ligase activity is strongly inhibited by [NaCl]>150 mM.

III. Cloning strategies

- In some cases, DNA needs to be modified for cloning. Modification can be achieved using enzymes.

Modifying enzyme	Function
T4 &T7 DNA pol	Removal of 3' protruding ends
T4 &T7 DNA pol Klenow fragment (C-terminal proportion (70%) of *E. coli* DNA pol I, possess the DNA pol activity and 3'->5' exonuclease activity but lacks 5'->3' exonuclease activity	Filling in 3' recessive ends
Terminal transferase	DNA-independent, add ≈10 nt to the 3' end in 30 min

Joining of incompatible ends [2]

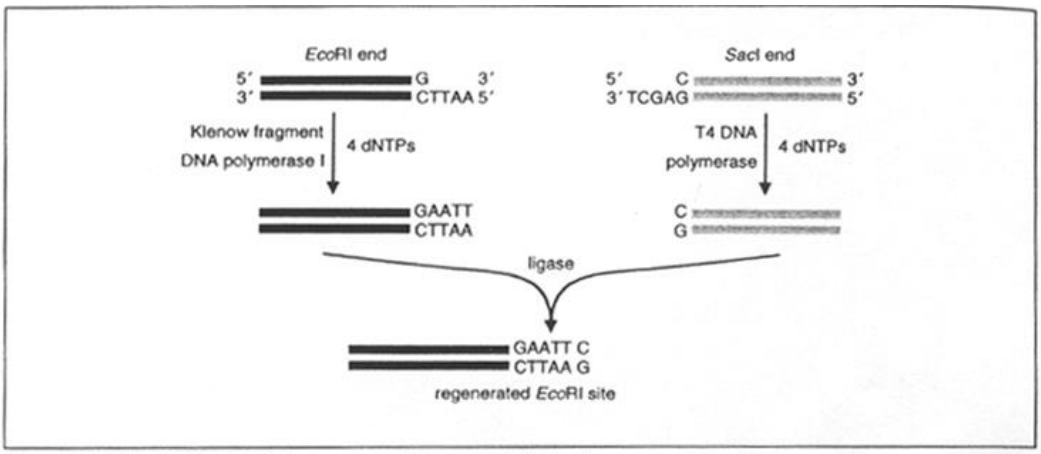


Figure 3.16.1 Joining DNA fragments with incompatible ends.

Joining of blunt ends

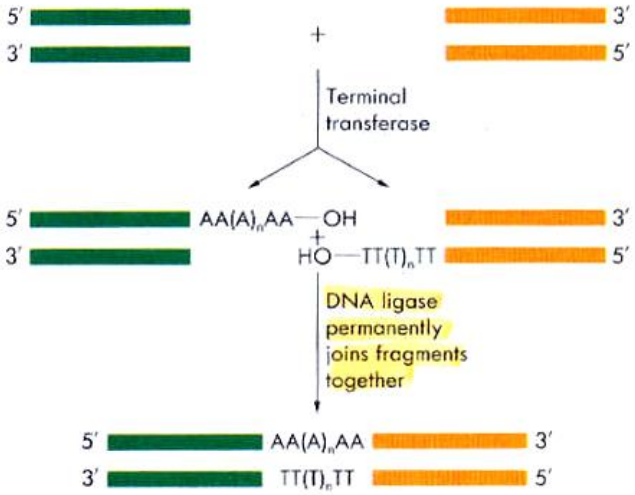
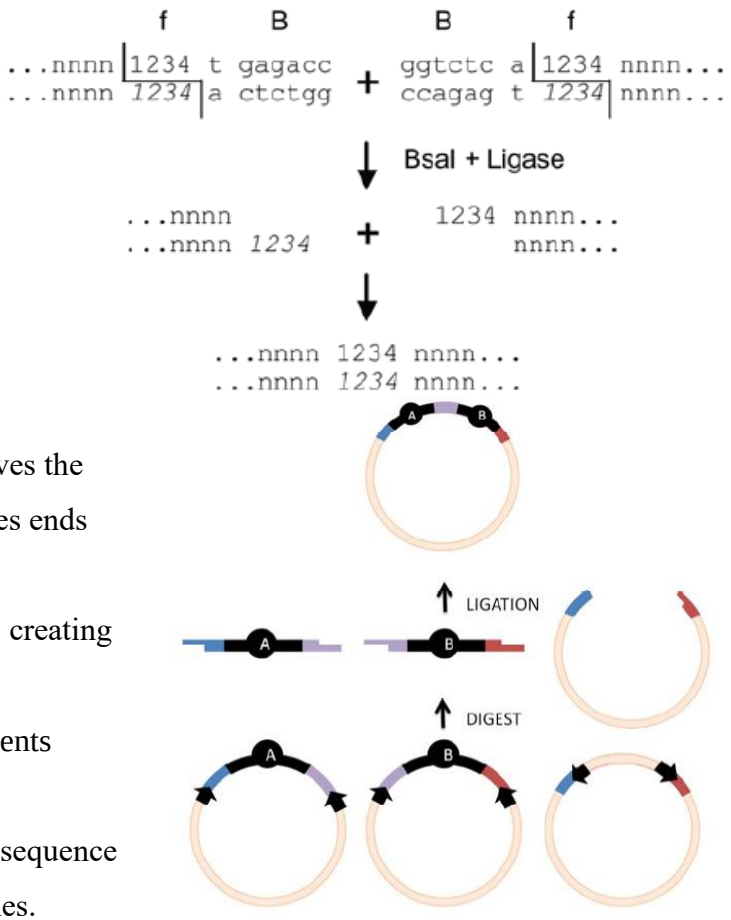


FIGURE 5-9
Two blunt-ended DNA fragments can be joined together by adding poly(dA) and poly(dT) tails to the ends of the fragments. The complementary tails will form base pairs, and enzymes can be used to fill in any single-stranded gaps and join the fragments permanently.

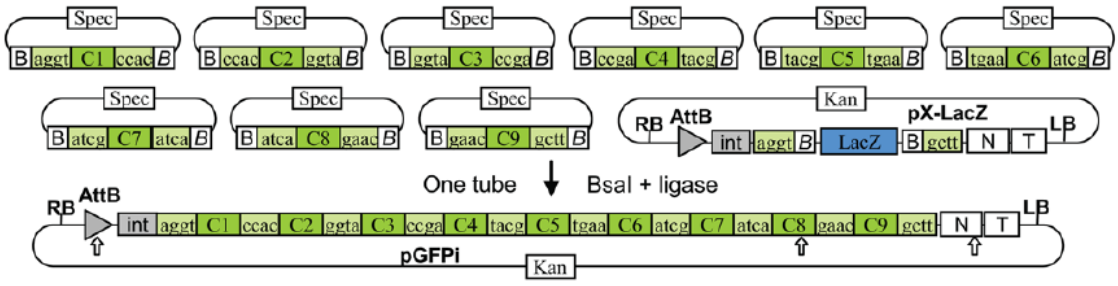
Golden Gate Shuffling

- Traditional cloning methods are inefficient and time consuming to join multiple fragments.
- Golden Gate Shuffling is a protocol to assemble separate DNA fragments together into a vector in one step and one tube.

- This cloning strategy is based on the ability of <u>type IIS restriction enzymes (e.g. BsaI) to cut outside their recognition site (e.g. t gagacc)</u>.
  - Two DNA ends terminated by the same 4 nucleotides (sequence f, composed of nucleotides 1234) can be synthesized, where sequences f are flanked by a BsaI recognition sequence, B.
  - The type IIs restriction enzyme removes the enzyme recognition sites and generates ends with complementary 4 nt overhangs.
  - These ends can be ligated seamlessly, creating a junction that lacks the original site.



- Ex: One-pot one-step assembly of 9 fragments
  - First select a number of 4 nucleotides 'recombination sites' on a nucleotide sequence alignment of several homologous genes.
  - The selection of these recombination sites defines modules that consist of a core sequence (C1-C9) flanked by two 4 nt sequences.
  - These 9 modules can be amplified by PCR with primers designed to add flanking BsaI sites on each side of the modules (the BsaI cleavage sites perfectly overlapping with the recombination sites) and cloned into 9 plasmids separately.
  - The recipient expression vector, pX-LacZ contains two BsaI sites compatible with the first (C1) and last (C9) modules.
  - Mix 9 module plasmids and 1 recipient plasmid into one tube. Add BsaI and ligase.



IV. Cloning Vectors

- To make the rDNA useful, one must have the gene of interest, the other fragment must enable the cellular maintenance of the rDNA=> plasmid is the most commonly used cloning vector.

Plasmids:

1. Self-replicating, ds, circular DNA in bacteria, independent of chromosomal DNA
2. Some encode resistance to antibiotics, others carry genes for the utilization of unusual metabolites.
3. Different plasmids can co-exist in cells, each may have different functions.
4. 1-500 kb, usually multiple copies exist.
5. Have the origin of replication (*ori*), for plasmid replication initiation.

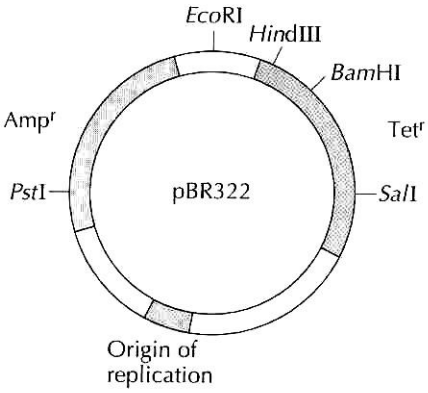


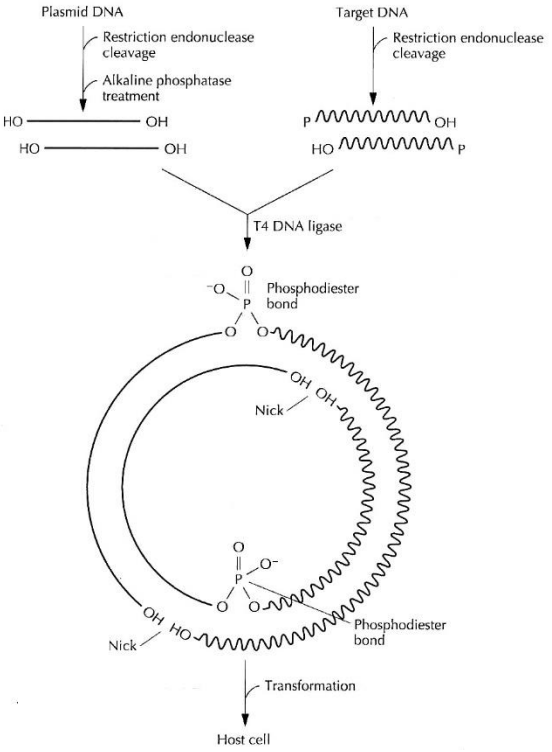
Figure 4.7 Genetic map of the plasmid cloning vector pBR322. Unique *Hin*dIII, *Sal*I, *Bam*HI, and *Pst*I recognition sites are present the genes for tetracycline resistance (Tet^r) and ampicillin resistance (Amp^r). The unique *Eco*RI site is just outside the tetracycline resistance gene. The origin of replication functions in the bacterium *E. coli*. The complete DNA sequence of pBR322 consists of 4,361 bp.

- Essential features of cloning vectors
  1. Origin of replication
  2. Small (<15 kb) for efficient transfer into *E. coli*.
  3. Multiple unique restriction sites[2] into which the foreign DNA can be inserted (multiple cloning site, MCS).
  4. Selectable markers for identifying cells harboring the rDNA, and whether the foreign DNA has been inserted.
- ex: pBR322 (p indicates plasmid)
  - Contains *Ori* and several unique restriction sites (*Hind*III, *BamH*I..)
  - Two antibiotics resistance genes against ampicillin (Amp^r) and tetracycline (Tet^r).

[2] Sequences that can be recognized by restriction enzyme

Cloning:

- For cloning, both plasmid and the target DNA are treated with the RE and ligated with T4 DNA ligase.
- The plasmid DNA may self-ligate after RE digestion. To minimize the self-ligation, the digested plasmid can be treated with alkaline phosphatase to remove the phosphate group.
- The two phosphodiester bonds are formed by T4 ligase and able to hold both molecules together despite the nicks.
- After transformation and the ensuing replication cycles, host cell ligase system produces new complete DNA w/o nicks.



V. Transferring genes into cells

- After construction, the rDNA is transferred into cells by one of the following methods:
  - Transformation: Transferring genes into prokaryotic cells. Expose bacterial cells to $CaCl_2$ or PEG (polyethylene glycol) to make cells competent (able to take up exogenous DNA)[3]. Mix the cells with the recombinant DNA and apply a heat shock (rapid increase of temperature from 37 to 42°C) (in the tube). The membrane can transiently open to uptake DNA.
  - Electroporation: apply a brief electric pulse to induce transient openings. Can be used to transfer large DNA into bacteria (See Appendix).
  - Transfection: Transferring genes into eukaryotic cells. rDNA is mixed with liposome (cationic phospholipid that can form micelle and fuse with membrane) so that rDNA is encompassed. The mixture is added to cells for gene delivery.

[3] In $CaCl_2$ method, the competency can be obtained by creating pores in bacterial cells by suspending them in a solution containing high concentration of calcium. DNA can then be forced into the cell by heat shock treatment at 42˚C for transformation.

- Gene gun: DNA is coated with gold particles and bombarded into cells (e.g. plant cells)
- Microinjection: used to introduce genes into single cells (e.g. eggs for the generation of transgenic animals). An extremely fine pipette is used to directly inject DNA into the nucleus of cells (e.g. fertilized egg or embryo) so DNA is integrated into the chromosome. The transfected egg is then implanted into an animal.

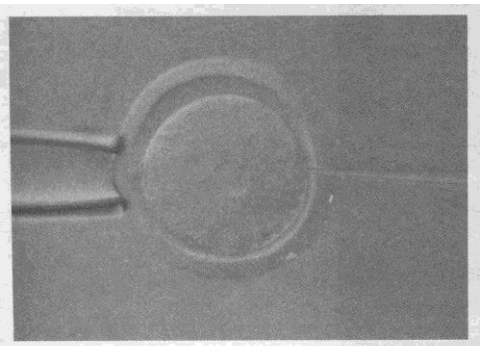


Figure 4.2 Microinjection of DNA into a pronucleus of a fertilized animal egg.

- Virus vectors: recombinant viruses are created and used to <u>infect</u> or <u>transduce</u> cells.

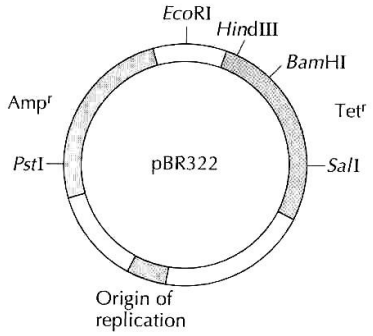
- Note:
  - The host cells (e.g. *E. coli* Top10) must lack the genes for RE used, otherwise the cloned gene would be cleaved.
  - Transformation efficiency is typically low (<0.1%).
  - Some cells are transformed by unwanted plasmids (the original plasmid that re-circularize), or non-plasmid DNA➔ in a population of cells, not all cells receive a recombinant plasmid➔ selection is needed.

VI. Selection

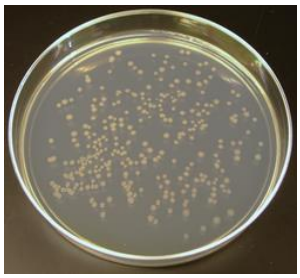
- Identify and select the cells containing the desired recombinant DNA.

Example: a gene cloned into the *Bam*HI site of pBR322

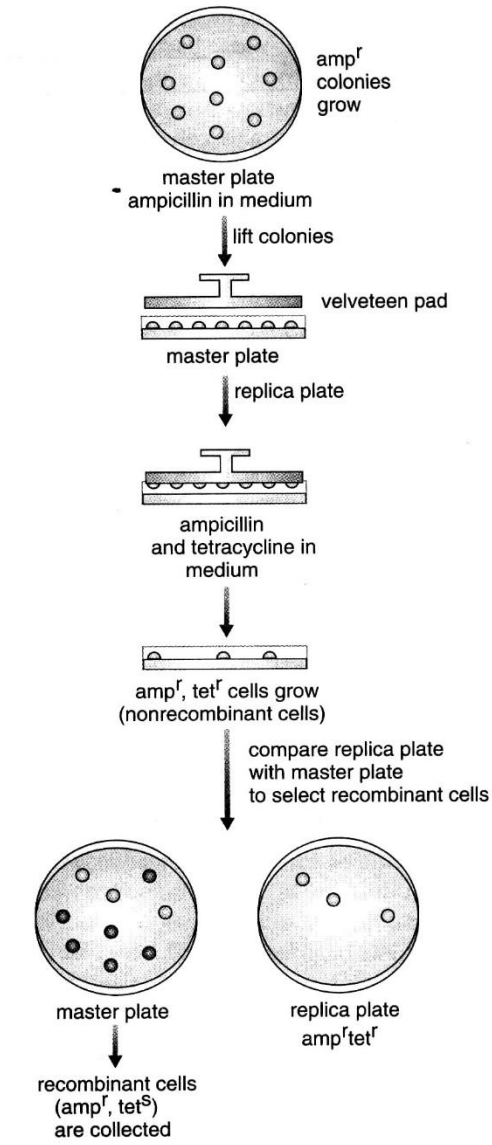
- If the foreign DNA is inserted at the *Bam*HI site➔the Tet^r gene is disrupted in the recombinant plasmid➔ the desired cell is <u>Amp^rTet^s</u> (resistant to Amp but sensitive to Tet); the cells with the re-circularized pBR322 are <u>Amp^rTet^r</u>.



- Replica plating:
  - Grow diluted cells on agar plate[4] with Amp. Amp^r cells can grow into colony[5] (in the master plate)



  - A sterile pad (e.g. nylon filter paper) is pressed against the master plate containing Amp, cells from the colony adhere to the pad.
  - The pad is then pressed against medium in a second agar plate (replica plate containing both Tet and Amp), transferring cells to the second plate. The locations of these cells are identical to the original colonies on the master plate.
  - Surviving colony: Amp^rTet^r
  - Dead colony: Amp^rTet^s
  - Compare the replica plate with the master plate. Pick the Amp^rTet^s colony (the cells with the r plasmids) on the master plate.



ıre 4.4 Selecting for recombinant cells after transformation.

Replica Plating.mp4

- Note:
  - pBR322 and replica plating are seldom used nowadays because screening is time-consuming. Replica plating may be used for counter selection.

[4] Agar plate: Culture plate filled with agar (solid medium for cell growth)

[5] Colony: a group of cells originating from the same single cell

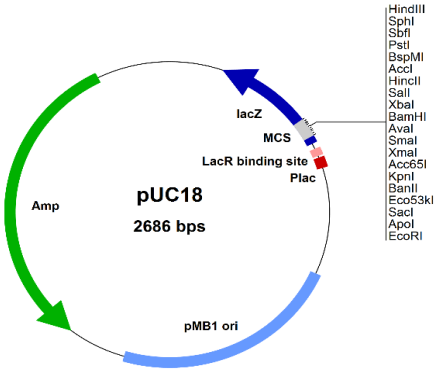
- New vectors normally contain reporter genes and multiple cloning sites.

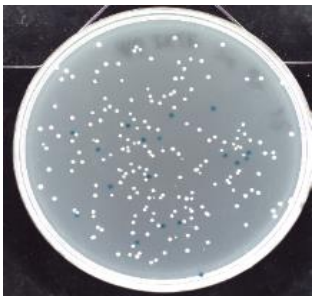
Improved vectors

- Multiple cloning sites (MCS): allow the choice of different restriction enzyme (containing many restriction recognition sites)
- Reporter genes:

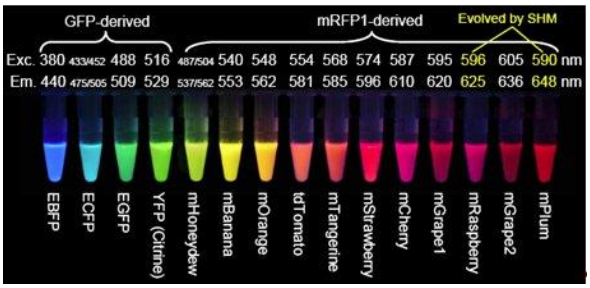
Examples of reporter proteins:

1. β-galactosidase: encoded by *LacZ* gene; breaks down X-gal (a lactose analogue) and produces blue color in the medium. In the pUC18 plasmid, if the foreign gene is cloned into the MCS and disrupt the *LacZ* gene, the colony will be white.





2. GFP (green fluorescent protein): can be excited at 395 nm and emit fluorescence at 510 nm and observed by fluorescence microscope and quantified by fluorimeter. There are many variants (GFPuv, EGFP, RFP, EYFP, mCherry[6], AmCyan1[7]).



3. Luciferase: catalyzes a bioluminescent reaction to generate light. The light intensity can be recorded and quantified. Firefly luciferase is often used.

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[6] mCherry is a red monomer which matures extremely rapidly, making it possible to see results very soon after activating transcription. Excitation maximum: 587 nm, emission maximum: 610 nm

[7] Living Colors AmCyan1 is a cyan fluorescent protein that was isolated from the coral reef organism *Anemonia majano*. Cyan fluorescent proteins such as AmCyan1 are ideal for simultaneous detection of two or more events in the same cell or cell population, because their excitation and emission spectra are distinct from other fluorescent proteins.

$$luciferin + ATP + O_2 \xrightarrow{firefly\ luciferase} oxyluciferin + AMP + PP_i + CO_2 + light$$

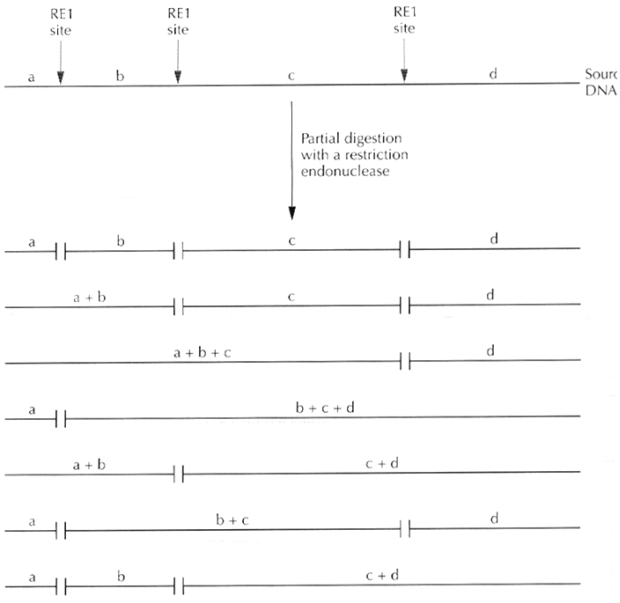
- Note: Other bacteria such as *Bacillus subtilis* or *Agrobacterium tumefaciens* (農桿菌, containing Ti plasmid commonly used for gene transfer into plant cells) can be used as host cells.

VII. Creating a Gene Library

- Objectives: e.g. (1) for genome sequencing; (2) isolation of genes that encode the proteins; (3) creating new protein variants
- (genomic) library: a collection of clones that contain every gene (in the genome)
  - in prokaryotes: coding regions are continuous
  - in eukaryotes: exons are separated by introns➔ different cloning strategies

Creating a prokaryotic gene library:

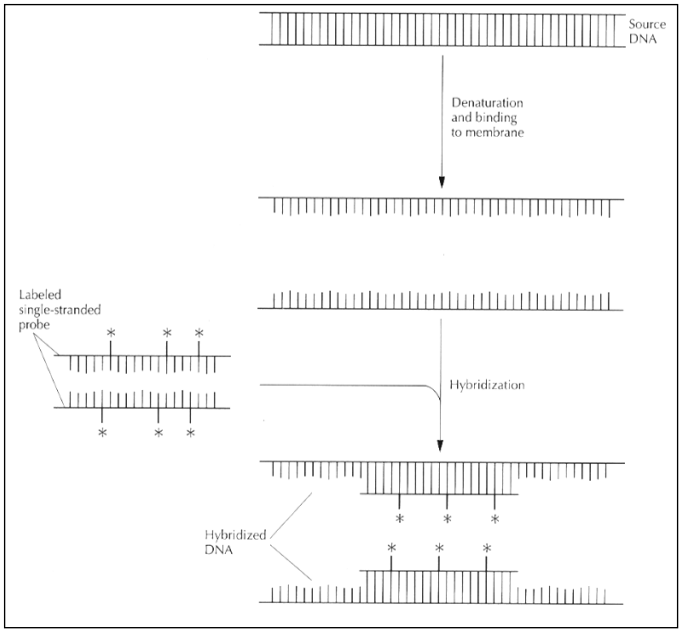
- Cut the complete genomic DNA with RE and insert each fragment into a vector.
- Transform the vectors to cells to form colonies.
- The specific colony (clone) that carries the target DNA is identified, isolated and characterized➔ the collection of the clones is called the "library".
- Partial digestion: vary the length of time and amount of RE for digestion to obtain all possible fragments.
  - A complete library theoretically contains all the genomic DNA of the source organism.
  - Problem: some fragments may be too large to be cloned➔incomplete library
  - Solution: form a library with another RE

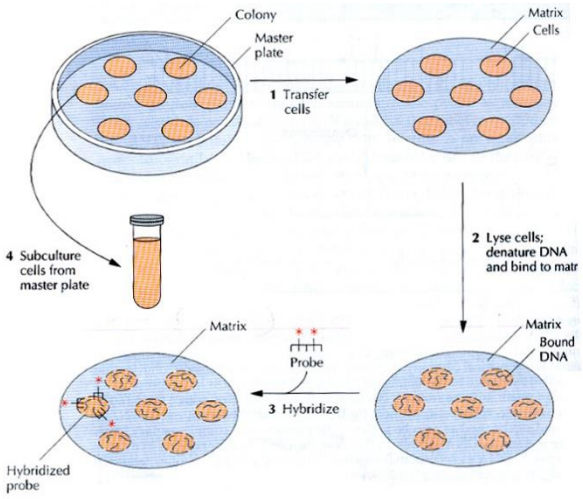


Screening of the clones

(1) by DNA hybridization:

- Depends on base pairing (hybridization) between the probes[8] and the target sequence.
- Denaturation: heat DNA to break the H-bond, so that the d.s. DNA becomes s.s.
- Add the probe for hybridization. The probe sequence can be deduced from known mRNA or protein sequence.





- The probes can be 100-1000 nt although larger or smaller probes may be used.
- Usually, stable base pairing requires a match of >80% within a segment of 50 bases.
- Because most genomic libraries are created from partial digestion, a number of clones may give a positive response➔further check (e.g. RE mapping, protein assay…) is needed.

Colony hybridization met

(2) by immunological assay

- Similar to DNA hybridization, but Ab replaces the DNA probe

[8] Probe: a short DNA that is designed to bind specific target DNA. The probe is labeled with a fluorescent or luminescent (e.g. digoxigenin) dye for facile detection

(3) by protein assay

- Suitable for enzymes (such as β-gal, amylase…)
- Add substrate into the medium
- Detect the right colony by color change (see reporter gene previously)

- Overall: very time-consuming➔ high-throughput screening.

Creating an eukaryotic cDNA (complementary DNA) library

- Start from mRNA (to avoid intron problems) that has a poly-A tail at 3'end

(1) Purification of mRNA

- Lyse the cells with lysis buffer to release the RNA sample. Load the samples containing mRNA to a column containing beads conjugated with oligo(dT) for purification. The resultant product is a pool of mRNAs (a mixture containing many different mRNAs).

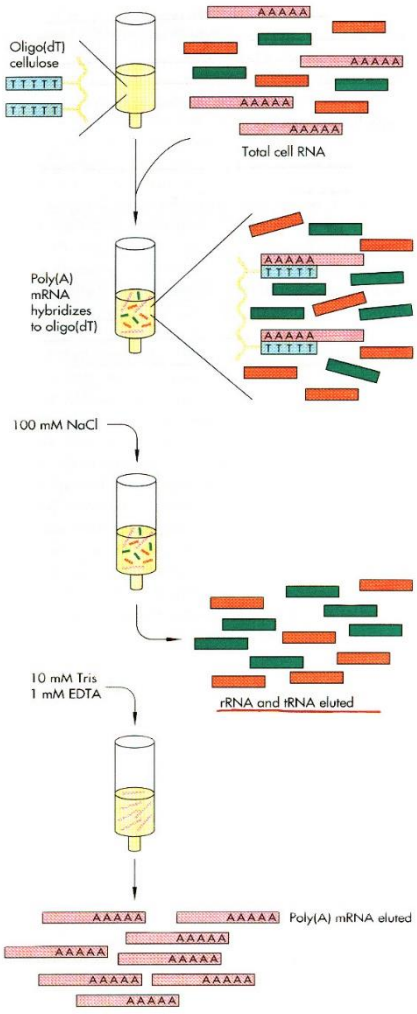
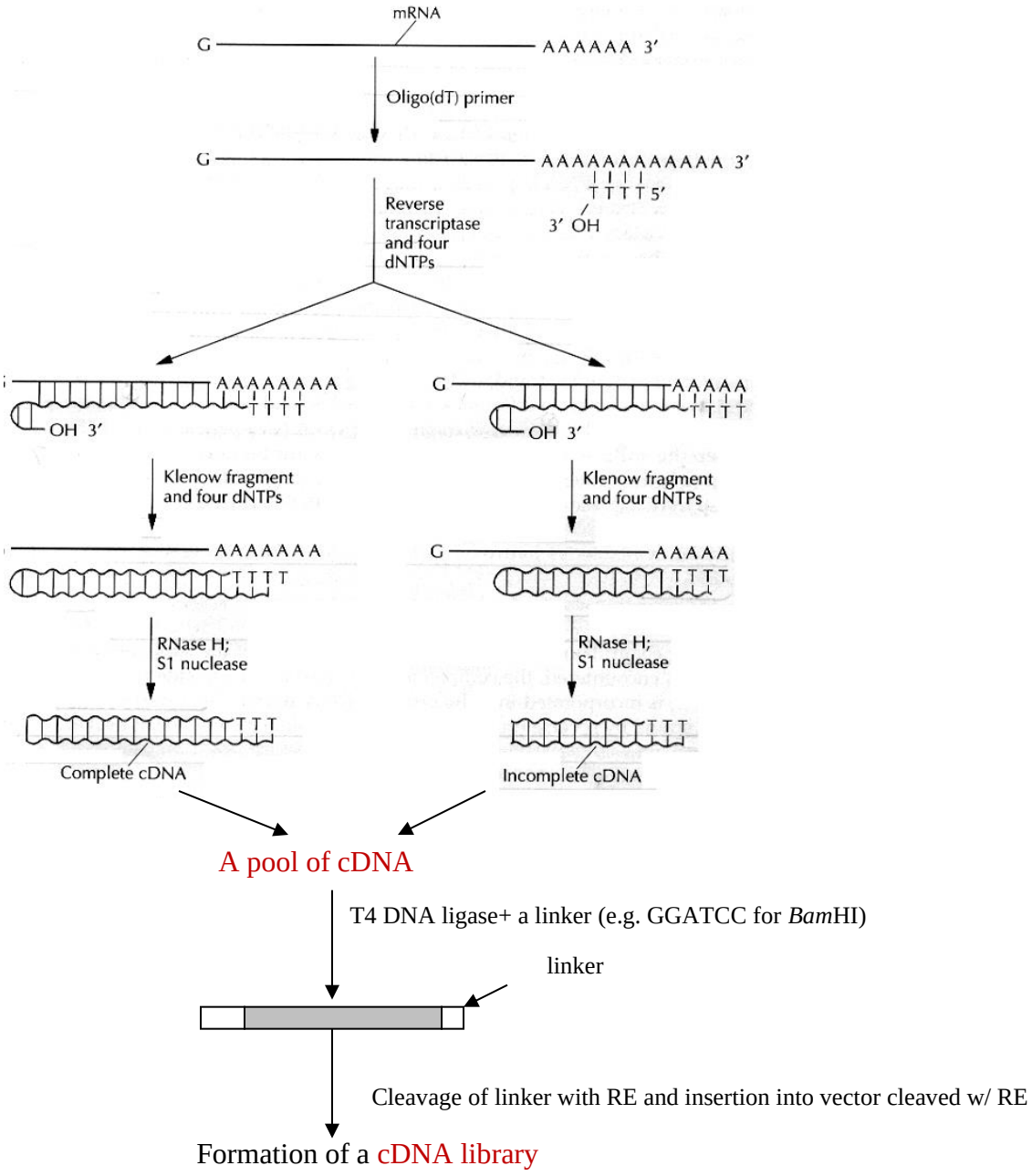


FIGURE 7-2
Isolation of poly(A) RNA. Most eukaryotic mRNAs carry a poly(A) tail, which can be used to purify the mRNA fraction from the bulk of cellular RNA. Cellular RNA is passed over a column consisting of an inert material, often cellulose or agarose, to which oligonucleotides consisting entirely of deoxythymidine (dT) residues have been attached. The poly(A) tails hybridize to this oligo(dT), causing the mRNA to stick to the column, while the rest of the RNA runs through. After extensive washing of the column to remove the last traces of contaminating material, the column is washed with a buffer of low ionic strength. Under these conditions the poly(A)·oligo(dT) hybrids dissociate, and the purified mRNA washes off the column.



(2) Converting mRNA to cDNA by reverse transcription

- First stage: Reverse transcription (RT synthesizes the 1st strand)
  - oligo(dT) base pairs with poly A tail and provides a 3' hydroxyl group to prime the synthesis.
  - RT (reverse transcriptase): from RNA viruses (e.g. murine leukemia virus)
  - RT is a multifunctional enzyme that possesses

(1) RNA-dependent DNA pol activity: uses RNA as the template and 4 dNTP to direct the synthesis of 1st-strand cDNA. RT synthesis of DNA usually is incomplete. The DNA strand usually turns back for a few nt to form a hairpin loop.

(2) RNAse H activity (degrades RNA in an RNA:DNA hybrid from either 5' or 3' terminus.

(3) DNA-dependent DNA pol activity (but very low efficiency)

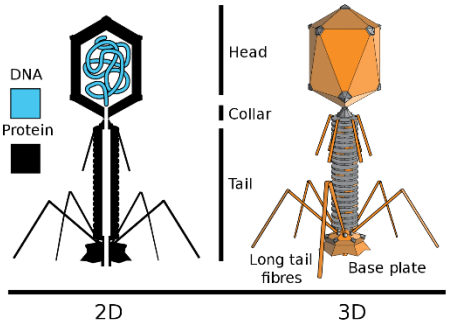
- 2nd stage: DNA amplification (can be done in another tube)
  - Klenow fragment (or Taq pol) uses the first DNA strand and 4 dNTP to synthesize the 2nd strand.
  - S1 nuclease opens the hairpin loop.
- 3rd stage: DNA ligase joins the fragments, cloning into vectors.
- Reverse transcription can be coupled to PCR (RT-PCR) in the second stage to amplify the cDNA. Reverse transcription occurs in a tube (60 min at 37°C) and generates the 1st strand cDNA, then we can take an aliquot to another tube for 2nd stage PCR.

VIII. Vectors for cloning large pieces of DNA

- Why these vectors?
  - A larger genomic library is likely to include all the genetic material
  - More likely that a particular gene is entirely in a single clone, important for the analysis of complex eukaryotic proteins.
  - But plasmid-based vectors can contain only up to 10-15 kb, otherwise it's difficult to deliver into cells.

(1) Bacteriophage λ vector (≈20 kb)

- A virus that infects bacteria. Phage λ DNA is packaged in the head and, after attachment, is injected into the cells.
- Causes lysogenic infection:
  - λ DNA integrates into the host chromosome at specific attachment sites (attP on the phage DNA and attB on the bacterial chromosome) and maintain as a benign guest.



- Under stressed conditions (nutritional or environmental)➔λDNA is excised and enters a lytic cycle.

Packaging of λ DNA into heads

- λ DNA is ds and linear, ≈ 50 kb with a 12 base single-stranded extension at the 5' end of each strand➔ cohesive ends (cos). Cos ends are complementary, after injection into *E. coli*, the cos ends join to form a circular molecule.
- The DNA replicates into a linear λ DNA consisting of contiguous lengths of 50 kb
- The volume of the head is sufficient for 50 kb,
  - >52 kb ➔can't fit into the head
  - <38 kb ➔ noninfectious particles
- An enzyme at the head opening recognizes cos sequence and cuts the DNA➔ each newly assembled phage has a DNA of ≈ 50 kb in length.

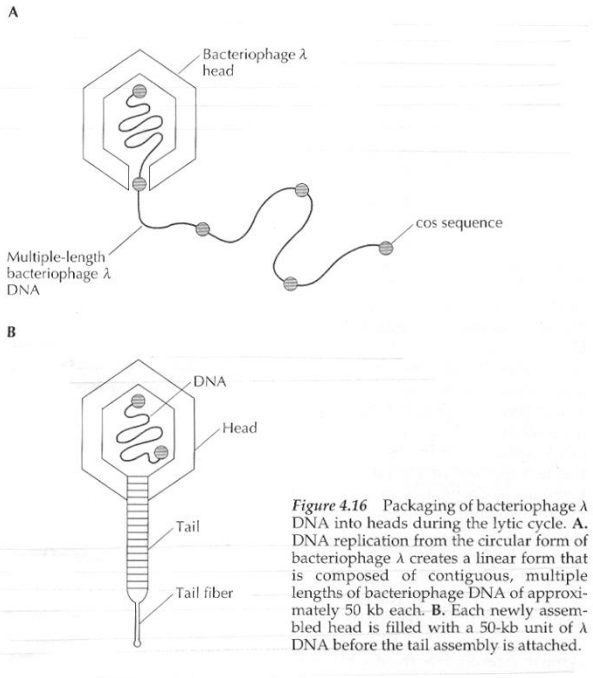
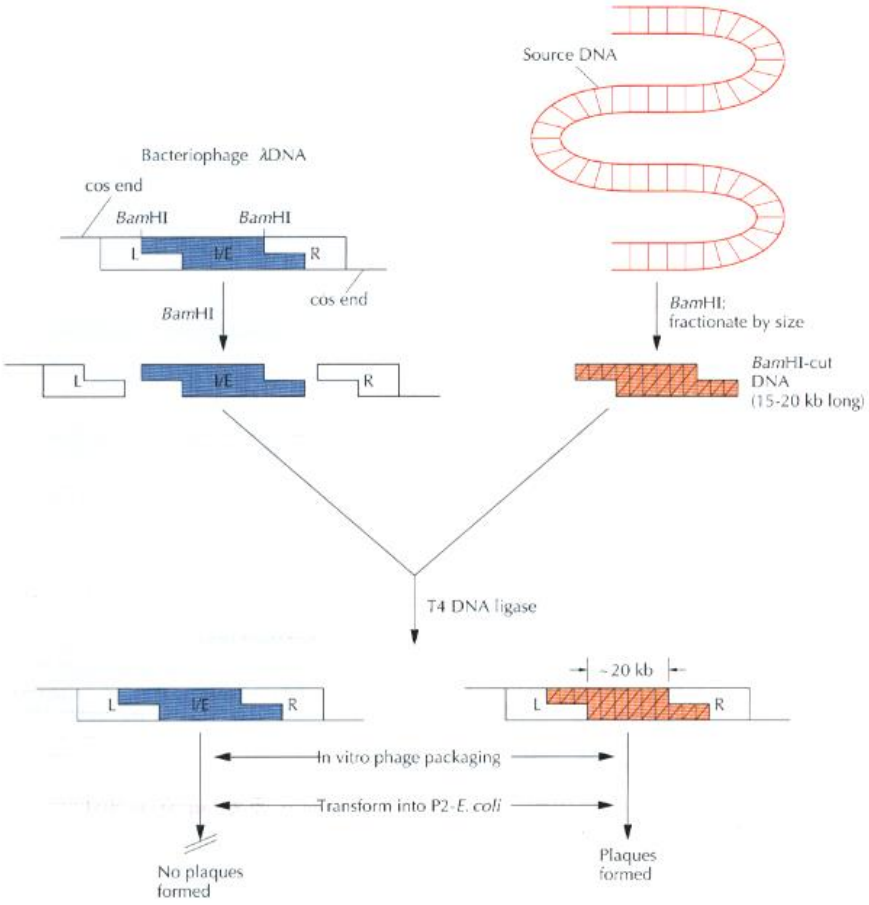


Figure 4.16 Packaging of bacteriophage λ DNA into heads during the lytic cycle. **A.** DNA replication from the circular form of bacteriophage λ creates a linear form that is composed of contiguous, multiple lengths of bacteriophage DNA of approximately 50 kb each. **B.** Each newly assembled head is filled with a 50-kb unit of λ DNA before the tail assembly is attached.

Phage λ cloning system

- λ genome can be cut w/ *BamH* I into 3 segments:
  - Right arm (R): for DNA replication and cell lysis
  - Left arm (L): for production of heads and tails
  - I/E: for integration and excision
- Cut the target and phage DNA with *BamH*I
- T4 DNA ligase to join the target DNA of 15-20 kb with L and R arms
- Add empty phage heads and tails (assembled from expressed proteins) ➔ 50 kb units of DNA are packaged *in vitro* (in the test tube) to form infectious phage particles (*in vitro* packaging). Other products from the ligation either too large or too small couldn't be packaged.

- Infect *E. coli* with the bacteriophage particles.
  - Recombinant phage λ undergoes lytic cycles in *E. coli*➔ **plaques** form (reconstituted phage λ won't form plaques.
  - Wild-type phage results in lysogenic infection➔ no plaques form.



- Screen the plaques by DNA probes (hybridization), immunological methods or DNA sequencing.

(2) Cosmid: Combines the features of phage λ and plasmid

(3) YAC : 100-1300 kb, unstable

(4) BAC : 100-400 kb, more stable, essential in Human Genome Project (HGP)

(5) HAC (Human artificial chromosome): >2000 kb, containing the telomere and human α satellite DNA (repetitive sequences in the centromere, the 170 kb momomer forms arrays of repeats of up to several Mb), mimicking human chromosomes and is used for gene expression (therapy).

Appendix

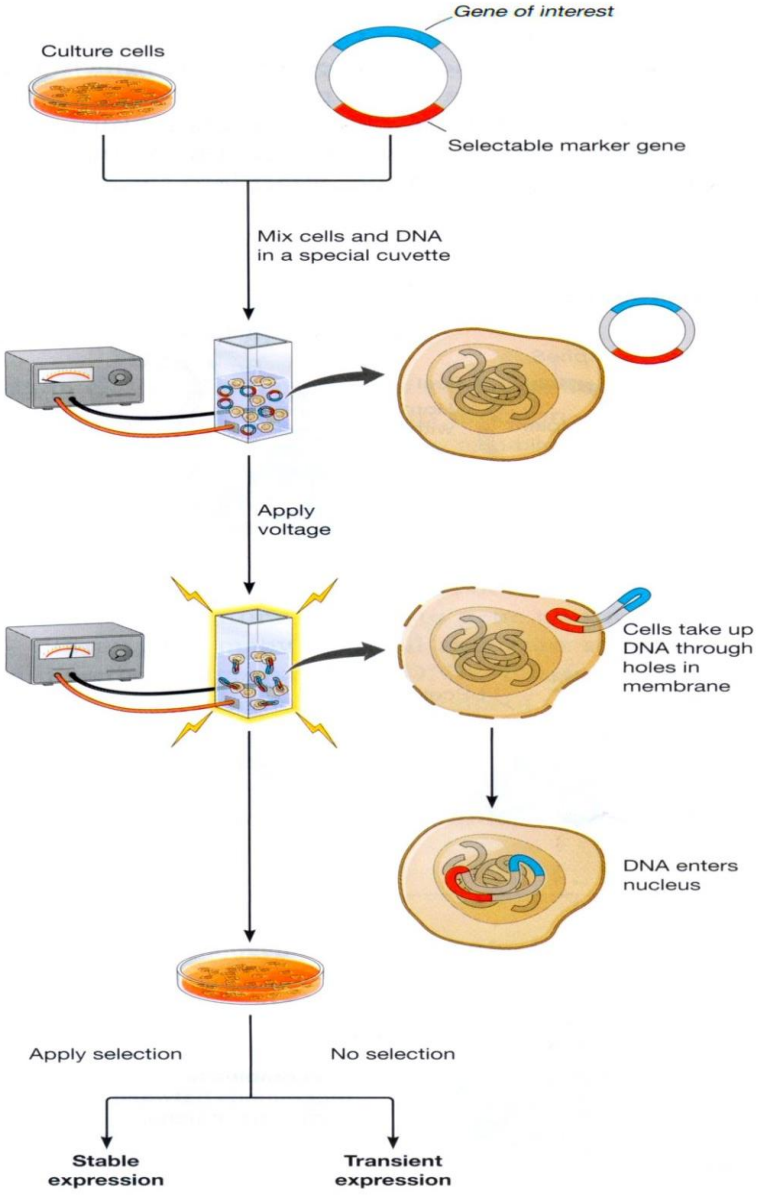


FIGURE 6-8

Gene transfer by electroporation. Cells are mixed with the DNA to be transfected and placed in a small chamber with electrodes connected to a specialized power supply. A brief electric pulse is discharged across the electrodes, which transiently opens holes in cell membranes. DNA enters the cells, which are removed and plated in fresh medium. The cultures can be harvested for experiments during the transient expression phase, or selection can be applied to isolate stably transfected clones.

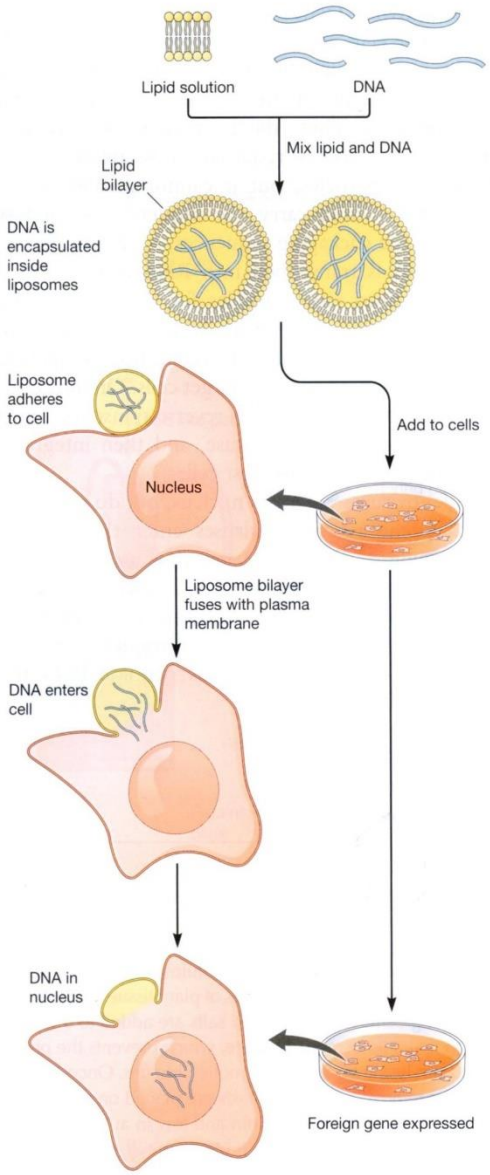


FIGURE 6-9

Transfection using liposomes (*lipofection*). DNA, which is negatively charged at near-neutral pH because of its phosphodiester backbone, is mixed with lipid molecules with positively charged (cationic) head groups. The lipid molecules form a bilayer around the DNA molecules, which creates liposomes that are mixed with cells. Most mammalian cells are negatively charged at their surface, so the positively charged liposomes interact with the cells. Cells take up the lipid–DNA complexes, and some of the transfected DNA enters the nucleus.

See [1]

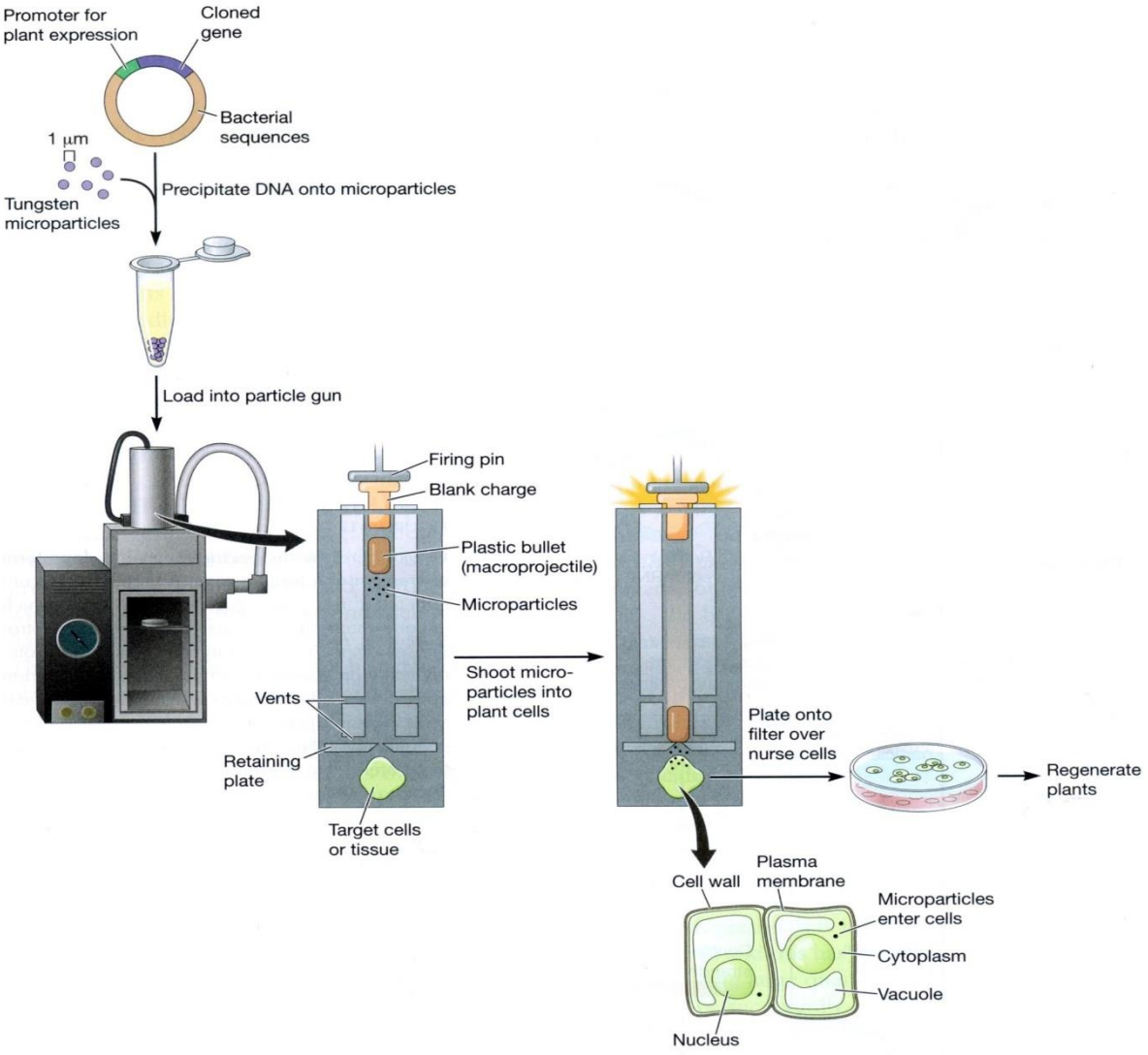


FIGURE 6-11

Direct transfer of DNA into plant cells by microprojectile bombardment ("shotgun"). A thick coat of DNA is deposited onto the surface of a 1-μm-diameter tungsten or gold particle by precipitation with calcium chloride. The beads are placed on the end of a plastic bullet (the "macroprojectile") in the barrel of a particle gun designed especially for this purpose. The target plant tissue or suspension cells are placed next to a small opening at the end of the barrel. The macroprojectile is propelled toward the cells by an explosive charge and, as it slams into the retaining plate, the particles it carries pass through the aperture and hit the cells. The barrel of the gun and the specimen chamber are usually evacuated, otherwise the air resistance slows the velocity of the microparticles. Plant cells can withstand a vacuum for as long as 2 min. Following bombardment, the cells are transferred to a cell culture plate and plants are regenerated as described in Fig. 6-10.

See [1]

References:

[1] Watson J, Myers RM, Caudy AA, Witkowski JA. Recombinant DNA: Genes and Genomes. New York: W.H. Freeman and Company, 2007.

[2] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, *et al.* Short protocols in molecular biology. . New York: John Wiley & Sons, 1999.