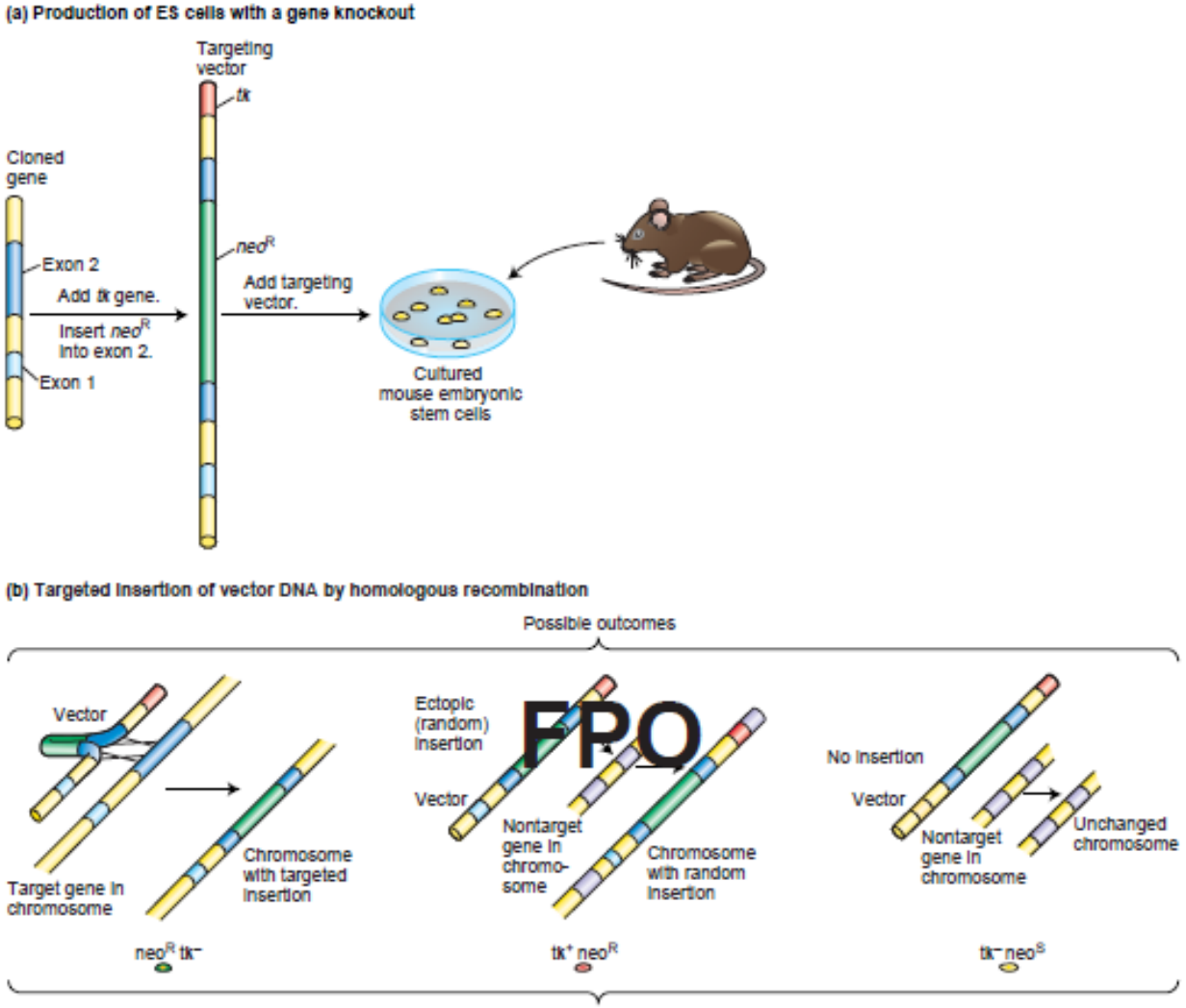

(a) Production of ES cells with a gene knockout
Targeting vector
tk
Cloned gene
Exon 2
Add tk gene.
Insert neoR into exon 2.
Exon 1
neoR
Add targeting vector.
Cultured mouse embryonic stem cells
(b) Targeted insertion of vector DNA by homologous recombination
Possible outcomes
Vector
Target gene in chromosome
Chromosome with targeted insertion
neoR tk−
Ectopic (random) insertion
Vector
Nontarget gene in chromo-some
Chromosome with random insertion
tk+ neoR
No insertion
Vector
Nontarget gene in chromosome
Unchanged chromosome
tk− neoS
(c) Selective cells with gene knockout
Neomycin analog
Ganciclovir
Add to median.
Kills neoS cells
Kills tk+ cells
Cell with no insertion
Cell with targeted insertion
Cell with random insertion
Cells carrying targeted mutation

The herpes simplex virus thymidine kinase (HSV-TK) converts ganciclovir (GCV) into a toxic product and allows selective elimination of TK+ cells in vitro and in vivo.

It is currently being used in clinical gene therapy trials as a therapeutic gene or as a safety marker.

Specifically, GCV is used to exert selective pressure on cells transfected with the "cell suicide gene" HSV1-tk.

Normally, eukaryotic cells can survive in the presence of the non-toxic prodrug GCV. However, upon expression of HSV1-tk, GCV is converted to GCV-monophosphate by HSV1-TK and further phosphorylated to the diphosphate and triphosphate forms by host kinases.

GCV-triphosphate, a lethal toxin, is incorporated into the DNA of replicating eukaryotic cells causing premature DNA chain termination and apoptosis

Collins and Hohn[2] developed a new type of plasmid vector which combines the high efficiency of transfection with packaged 'phage' particles and the advantage of using a plasmid vector to accept larger segments of foreign DNA.

This new type of vector differs from the usual plasmid vectors in the presence of a small piece of lambda DNA, the so-called cohesive end site.

Lambda DNA in the virus is linear with two single-stranded ends of twelve nucleotides in length.

Because these ends are complementary, they are called the cohesive ends or *cos* sites.

Such vectors are, therefore, designated as cosmids.

Cosmid hybrids can be packaged into lambda phage heads and this allows the cloning of the DNA fragments up to **35-45 kb**.

Plasmids have been constructed which contain a fragment of lambda DNA including the cos site

Plasmid vectors that can be packaged into lambda phage heads ("cosmids") have been constructed.

Cosmids accept larger inserts than lambda phage (30-45kb) and can be efficiently introduced into host bacteria through in vitro packaging.

In fact, only a small region in the proximity of the cos site is required for recognition by the packaging system (Hohn 1975).

Plasmids have been constructed which contain a fragment of a DNA including the cos site (Collins& Briining 1978, Collins & Hohn 1979, Wahl et al. 1987, Evans et al. 1989).

These plasmids have been termed **cosmids** and can be used as gene-cloning vectors in conjunction with the in vitro packaging system.

Packaging the cosmid recombinants into phage coats imposes a desirable selection upon their size.

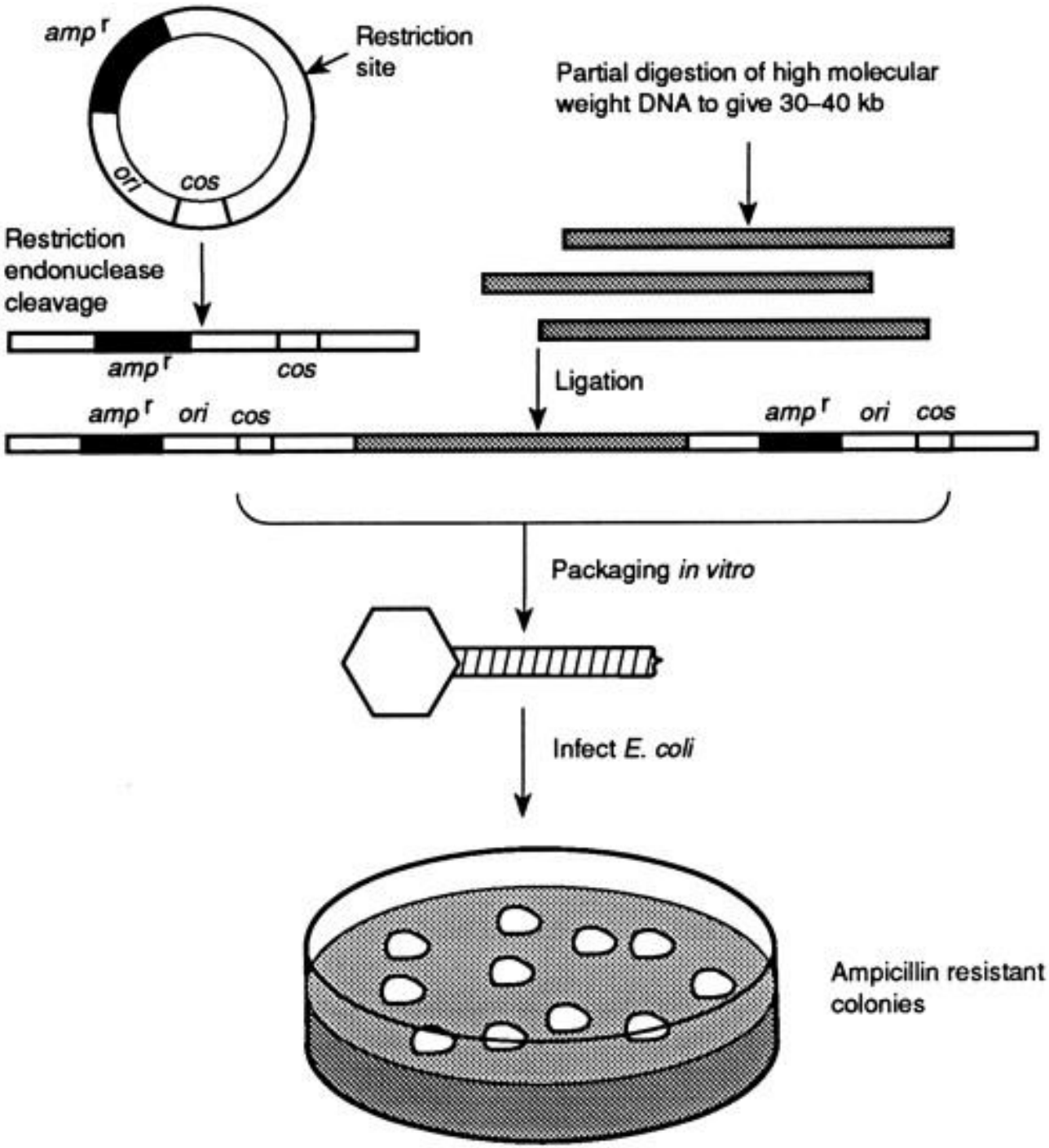
With a cosmid vector of 5 kb, we demand the insertion of **32-47 kb** of foreign DNA much more than a phage-lambda.vector can accommodate.

Cosmids provide an efficient means of cloning large pieces of foreign DNA.
Because of their capacity for large fragments of DNA, cosmids are particularly attractive vectors for constructing libraries of eukaryotlc genome fragments.

Note that, after packaging *in vitro, the particle is used to infect a suitable host.*

The recombinant cosmid DNAis injected and circularizes like phage DNA but replicates as a normal plasmid without the expression of any phage functions.

Transformed cells are selected on the basis of a vector drugresistance marker.


amp r
Restriction site
ori
cos
Partial digestion of high molecular weight DNA to give 30–40 kb
Restriction endonuclease cleavage
amp r
cos
Ligation
amp r ori cos
amp r ori cos
Packaging in vitro
Infect E. coli
Ampicillin resistant colonies

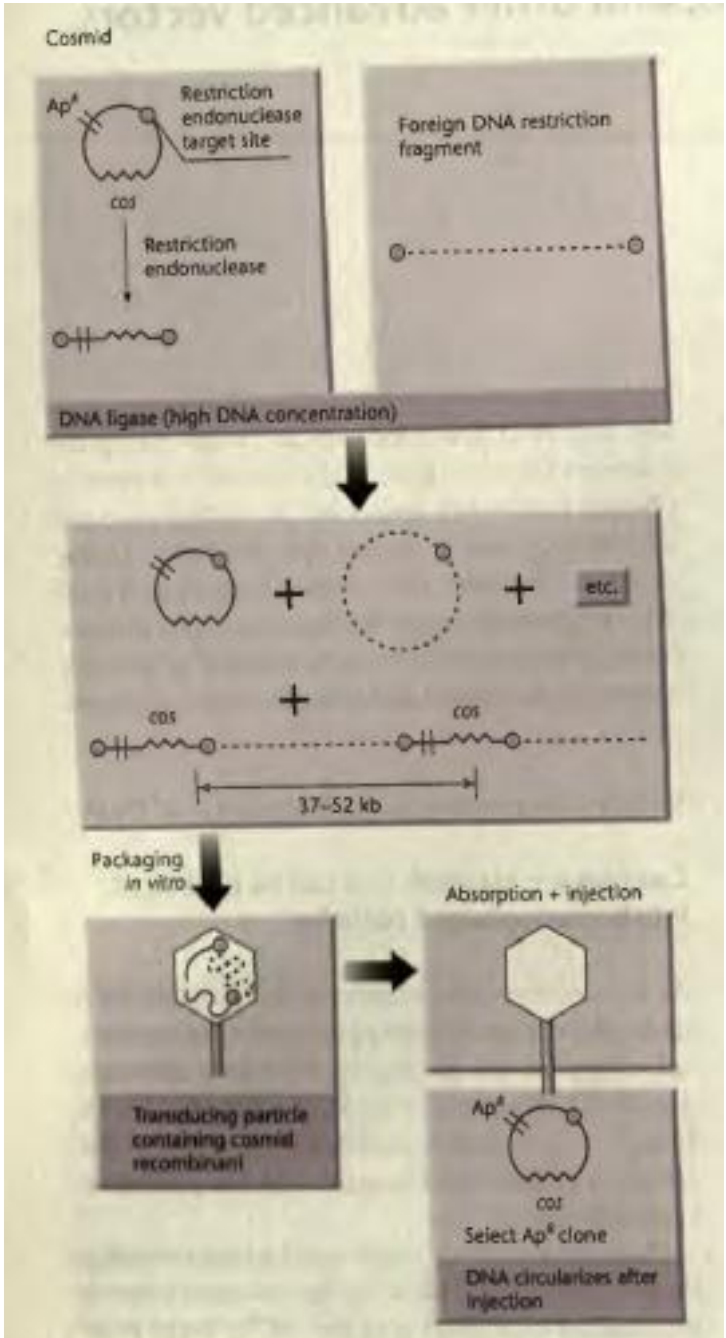
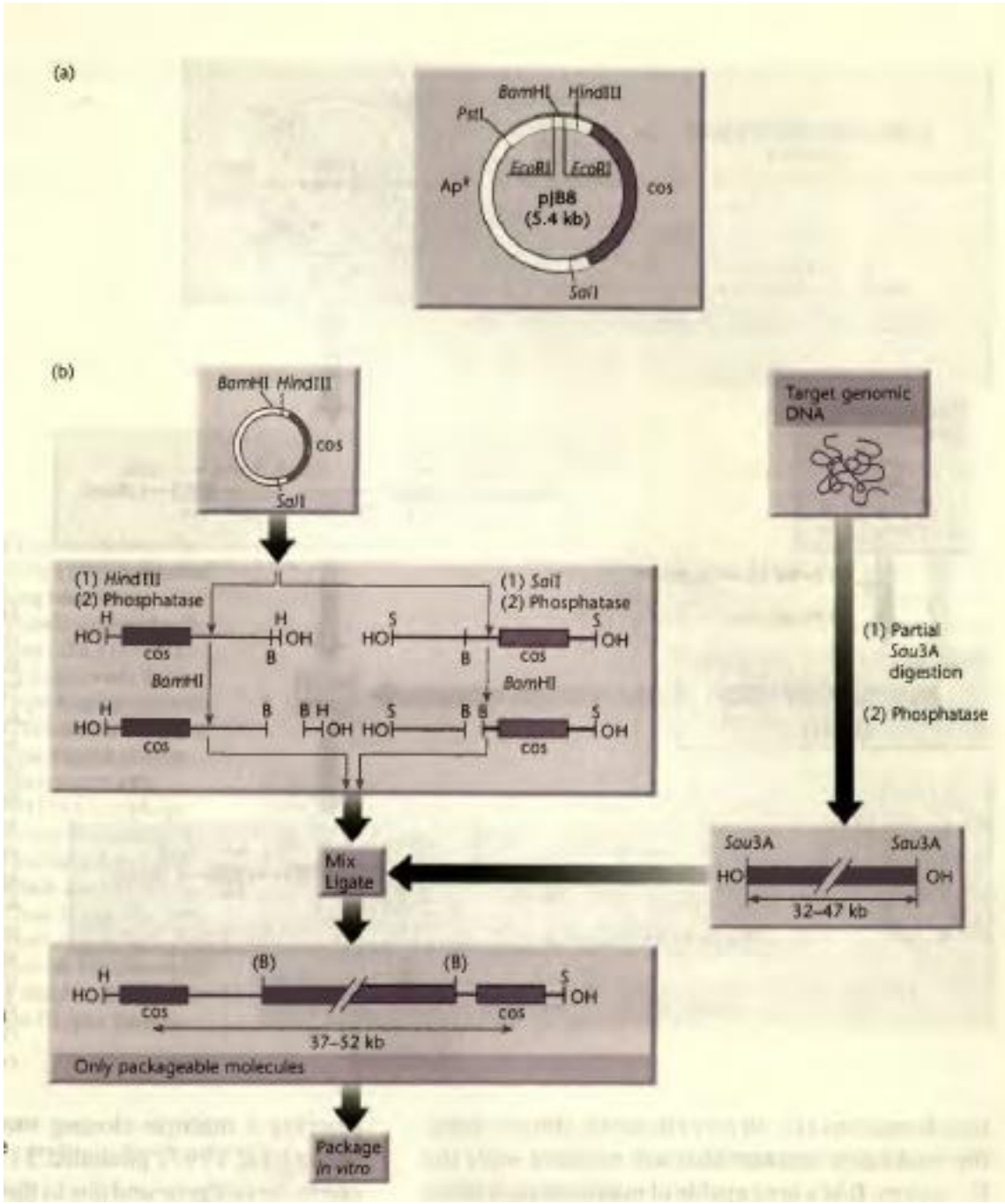


Fig. 5.1 Simple scheme for cloning in a cosmid vector. (See text for details.)



Lytic–Lysogenic options.

The process of a phage infecting a bacterium and producing progeny is referred to as a lytic infection.
Some phage, like T4, are only capable of lytic growth.

Some phage are also capable of maintaining their chromosome in a stable, silent state within the bacteria. This is called lysogeny.

Phage that are capable of both a lytic and lysogenic pathway are called temperate phage.

P1 and lambda are temperate phage.

M13 is unusual because phage continually exit from a bacterium without killing it. For this reason, M13 is not considered to have a true lysogenic state and is not a temperate phage.

When the bacterium contains a silent phage chromosome, it is referred to as a lysogen. The incorporated phage genome is referred to as a prophage.

P1 is a temperate bacteriophage (phage) that infects Escherichia coli and some other bacteria. A unique feature of phage P1 is that during lysogeny its genome is not incorporated into the bacterial chromosome, as is commonly observed during lysogeny of other bacteriophage.

When undergoing a lysogenic cycle, the phage genome exists as a plasmid in the bacterium,. P1 has an icosahedral “head” containing the DNA, attached to a contractile tail with six tail fibers.

At around **93Kbp** in length, the genome of the P1 phage is moderately large compared to the genomes of others, lambda (48Kbp), and Ff (6.4Kbp).

In the viral particle it is in the form of a linear double-stranded DNA molecule. Once inserted into the host, it circularizes and replicates as a plasmid.

- PI vector system which has a capacity for DNA fragments as large as 100 kb (Sternberg 1990, Pierce et al. 1992).

- Thus the capacity is about twice that of cosmid clones but less than that of yeast artificial chromosome (YAC) clones.

- The PI vector contains a packaging site (pac) which is necessary for in vitro packaging of recombinant molecules into phage particles.

- The vectors contain two loxP sites. These are the sites recognized by the phage recombinase, the product of the phage cre gene, and which lead to circularization of the packaged DNA after it has been injected into an E. coli host expressing the recombinase.

- Clones are maintained in E. coli as low-copy-number plasmids

Temperate phage, such as P1, have the ability to exist within the bacterial cell they infect in two different ways.

In **lysogeny**, P1 can exist within a bacterial cell as a circular DNA, in that it exists by replicating as if it were a plasmid and does not cause cell death.

Alternatively, in its **lytic** phase, P1 can promote cell lysis during growth, resulting in host cell death. During lysogeny, new phage particles are not produced. In contrast, during lytic growth many new phage particles are assembled and released from the cell.

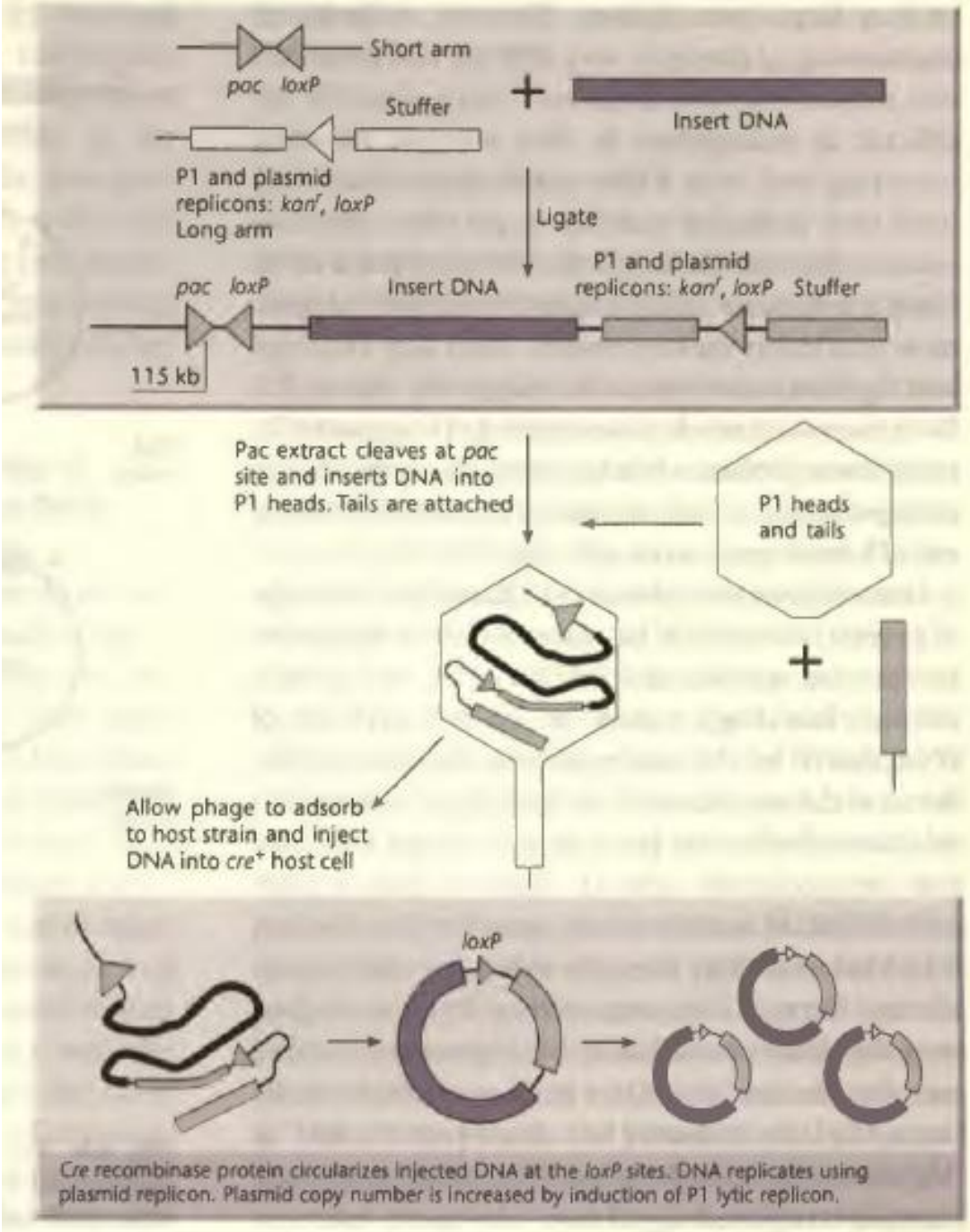


Fig. 5.4 The phage P1 vector system. The P1 vector Ad10 (Sternberg 1990) is digested to generate short and long vector arms. These are dephosphorylated to prevent self-ligation. Size-selected insert DNA (85–100 kb) is ligated with vector arms, ready for a two-stage processing by packaging extracts. First, the recombinant DNA is cleaved at the *pac* site by pacase in the packaging extract. Then the pacase works in concert with head/tail extract to insert DNA into phage heads, *pac* site first, cleaving off a headful of DNA at 115 kb. Heads and tails then unite. The resulting phage particle can inject recombinant DNA into host *E. coli*. The host is *cre*$^+$. The *cre* recombinase acts on *lox*P sites to produce a circular plasmid. The plasmid is maintained at low copy number, but can be amplified by inducing the P1 lytic operon.

Bacterial Artificial Chromosomes (BACs)

BAC (bacterial artificial chromosome) vectors, derived from the F plasmid, can carry inserts ranging from **150 to 300 kb**.

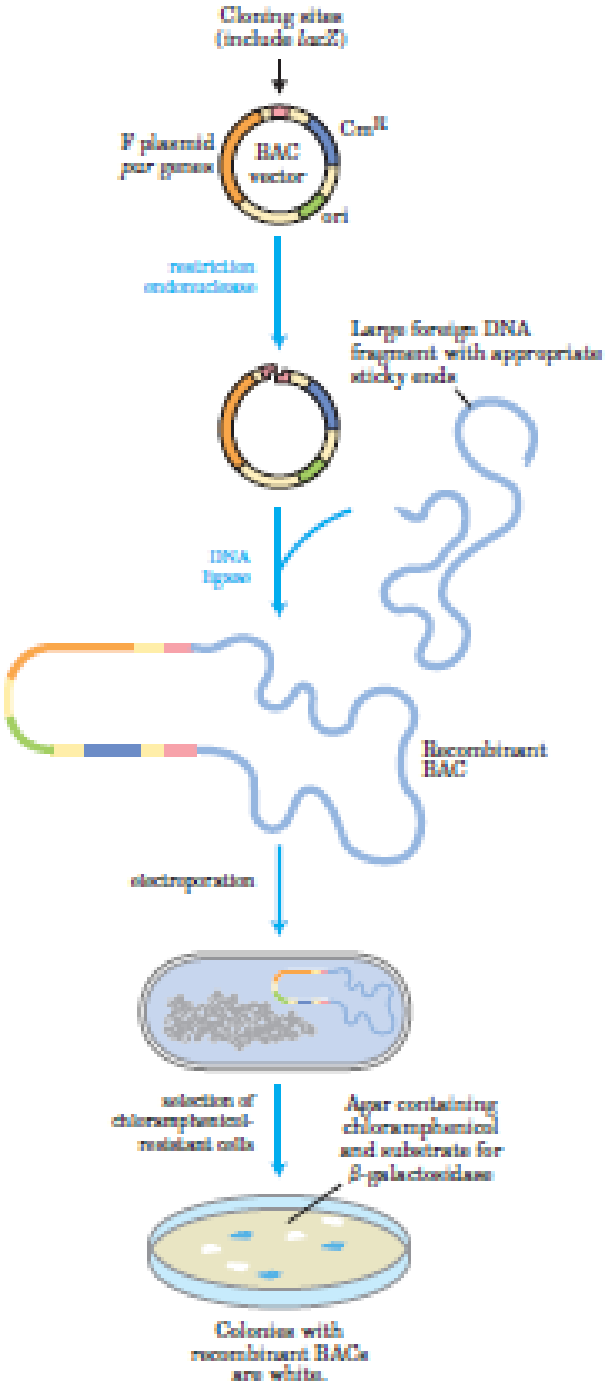
Bacterial artificial chromosomes are simply plasmids designed for the cloning of very long segments (typically 100,000 to 300,000 bp).

They generally include selectable markers such as resistance to the antibiotic chloramphenicol (CmR), as well as a very stable origin of replication (ori) that maintains the plasmid at one or two copies per cell.

The large circular DNAs are then introduced into host bacteria by electroporation.

These procedures use host bacteria with mutations that compromise the structure of their cell wall, permitting the uptake of the large DNA molecules.

Finally, inserts **larger than 300 kb** require a eukaryotic vector system called **YACs** (**yeast artificial chromosomes,**



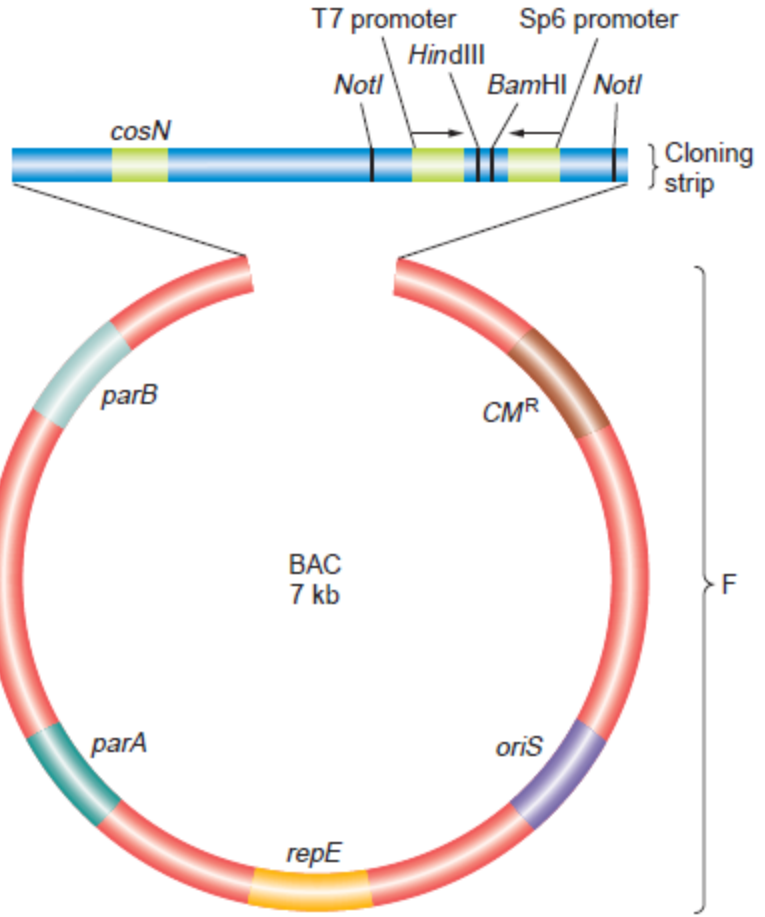


Figure 11-9 Structure of a bacterial artificial chromosome (BAC), used for cloning large fragments of donor DNA. CM^R is a selectable marker for chloramphenicol resistance. *oriS*, *repE*, *parA*, and *parB* are F genes for replication and regulation of copy number. *cosN* is the *cos* site from λ phage. *Hin*dIII and *Bam*HI are cloning sites at which donor DNA is inserted. The two promoters are for transcribing the inserted fragment. The *Not*I sites are used for cutting out the inserted fragment.

Table 5.1 Maximum DNA insert possible with different cloning vectors. YACs are discussed on p. 213.

Vector	Host	Insert size
λ phage	*E. coli*	5–25 kb
λ cosmids	*E. coli*	35–45 kb
P1 phage	*E. coli*	70–100 kb
PACs	*E. coli*	100–300 kb
BACs	*E. coli*	≤300 kb
YACs	*Saccharomyces cerevisiae*	200–2000 kb