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BT 207

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Vectors - 2

Cloning vectors

Allowing the exogenous DNA to be inserted, stored, and manipulated mainly at DNA level.

1. **Plasmid vectors**
2. **Bacteriophage vectors**
3. **Cosmids**
4. **Fosmids**
5. **BACs & YACs**
6. **MACs**

Cosmid vectors

A cosmid is a plasmid that contains phage sequences that allow the vector to be packaged and transmitted to bacteria like a phage vector.

A hybrid plasmid that contains a Lambda phage **cos sequence**. Cosmids (**cos sites + plasmid = cosmids**)

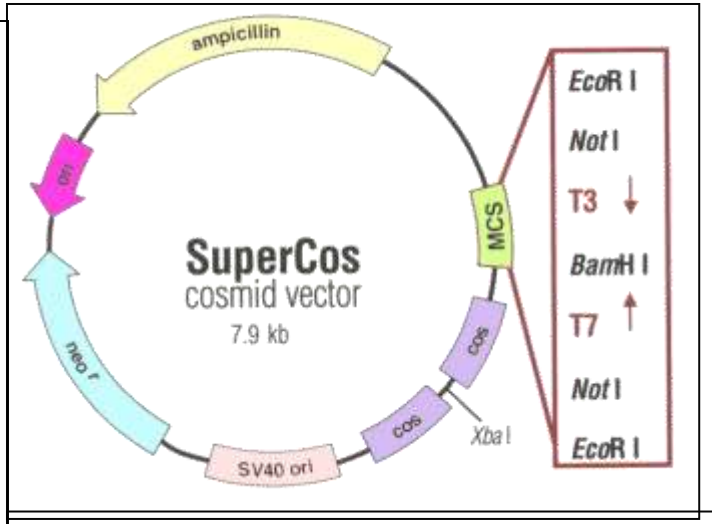
Cos sequences are ~200 base pairs long and essential for packaging.

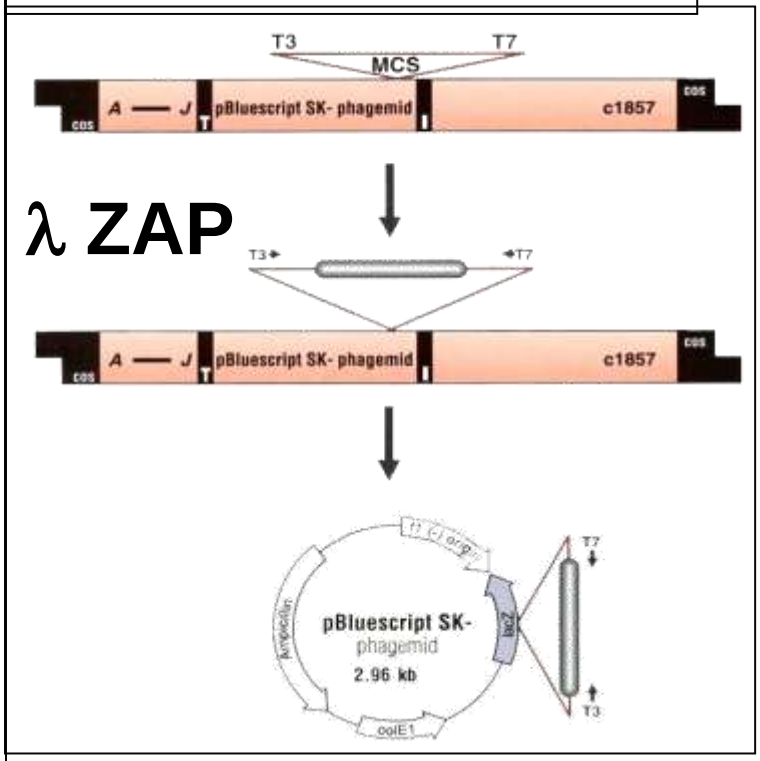
Combine the properties of plasmid vectors with the useful properties of the *cos* site

Advantages:
Useful for cloning very large DNA fragments (32 - 47 kbp)

Inherent size selection for large inserts

Disadvantages:
Not easy to handle large plasmids (~ 50 kbp)



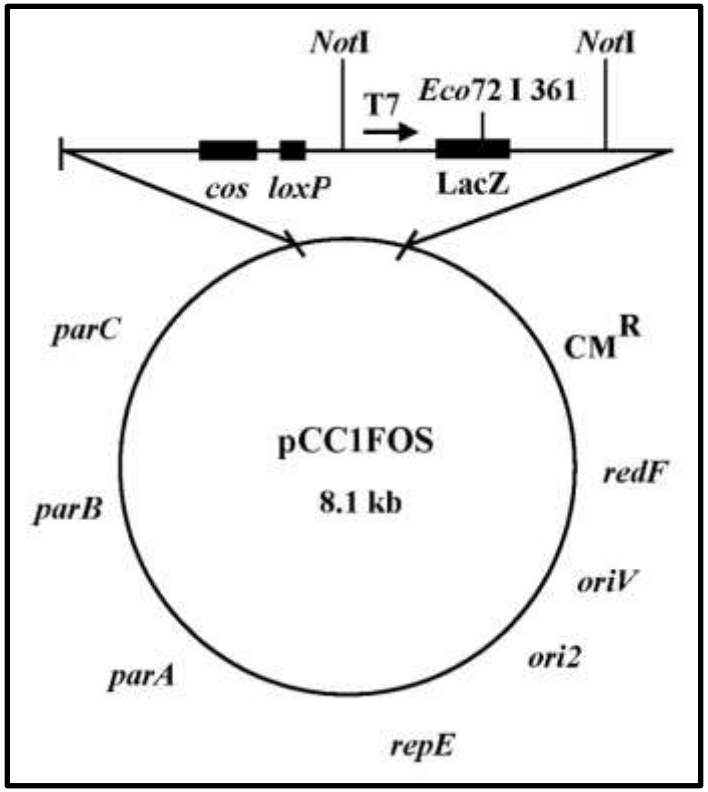


- **Fosmids** are similar to cosmids but are based on the bacterial F-plasmid.
- The F plasmid or F-factor is a large, 100-kbp, circular conjugative plasmid of *Escherichia coli* and was originally described as a vector for horizontal gene transfer and gene recombination
- The cloning vector is limited, as a host (usually *E. coli*) can only contain one fosmid molecule.
- Fosmids can hold DNA inserts of up to 40 kb in size.
- A fosmid library is prepared by extracting the genomic DNA from the target organism and cloning it into the fosmid vector.
- The ligation mix is then packaged into phage particles and the DNA is transfected into the bacterial host.
- Bacterial clones propagate the fosmid library.
- The low copy number offers higher stability than vectors with relatively higher copy numbers, including cosmids.
- Fosmids may be useful for constructing stable libraries from complex genomes.
- Fosmids have high structural stability and have been found to maintain human DNA effectively even after 100 generations of bacterial growth.

Fosmids contain several functional elements:
OriT (Origin of Transfer): The sequence which marks the starting point of conjugative transfer.

OriV (Origin of Replication): The sequence starting with which the plasmid-DNA will be replicated in the recipient cell.

tra-region (transfer genes): Genes coding the F-Pilus and DNA transfer process.



DNA libraries using Fosmids

- The first step in sequencing entire genomes is cloning the genome into manageable units of some 50-200 kilobases in length.
- It is ideal to use a fosmid library because of its stability and limitation of one plasmid per cell.
- By limiting the number of plasmids in the cells the potential for recombination is decreased, thus preserving the genome insert.

Plasmid, Cosmid, Fosmid

BACs : Bacterial Artificial Chromosomes

Bacterial Artificial Chromosomes (BAC) have been developed to hold much larger pieces of DNA than a plasmid can.

Originally created from par of an unusual plasmid present in some bacteria called the F' plasmid.

The F' plasmid allows bacteria to give its genome to another bacteria.

F' has origin of replication and bacteria have a way to control how F' is copied.

In 1992, Hiroaki Shizuya took the parts of F' that were important, cleaned it up, and turned it into a vector.

Advantages:

Useful for cloning extremely large DNA fragments (> 300 kbp)

This is very important for genome sequencing projects

Disadvantages:

Not easy to handle extremely large DNA molecules

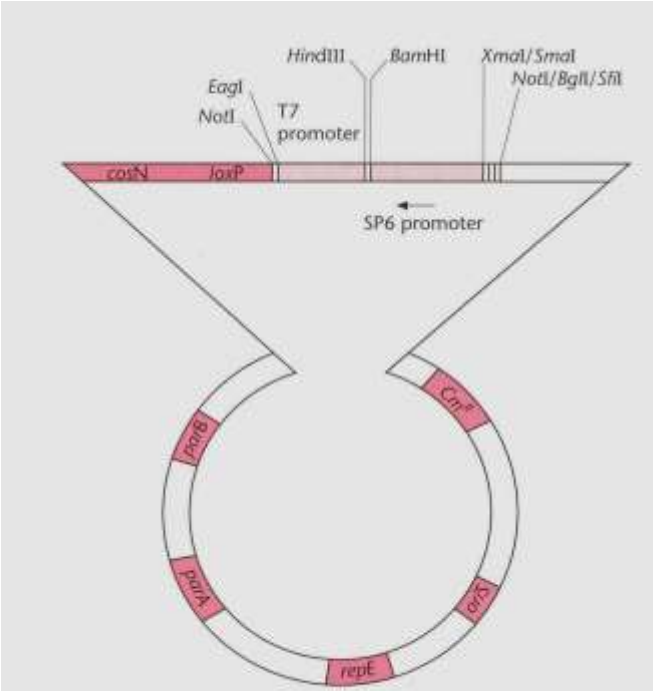
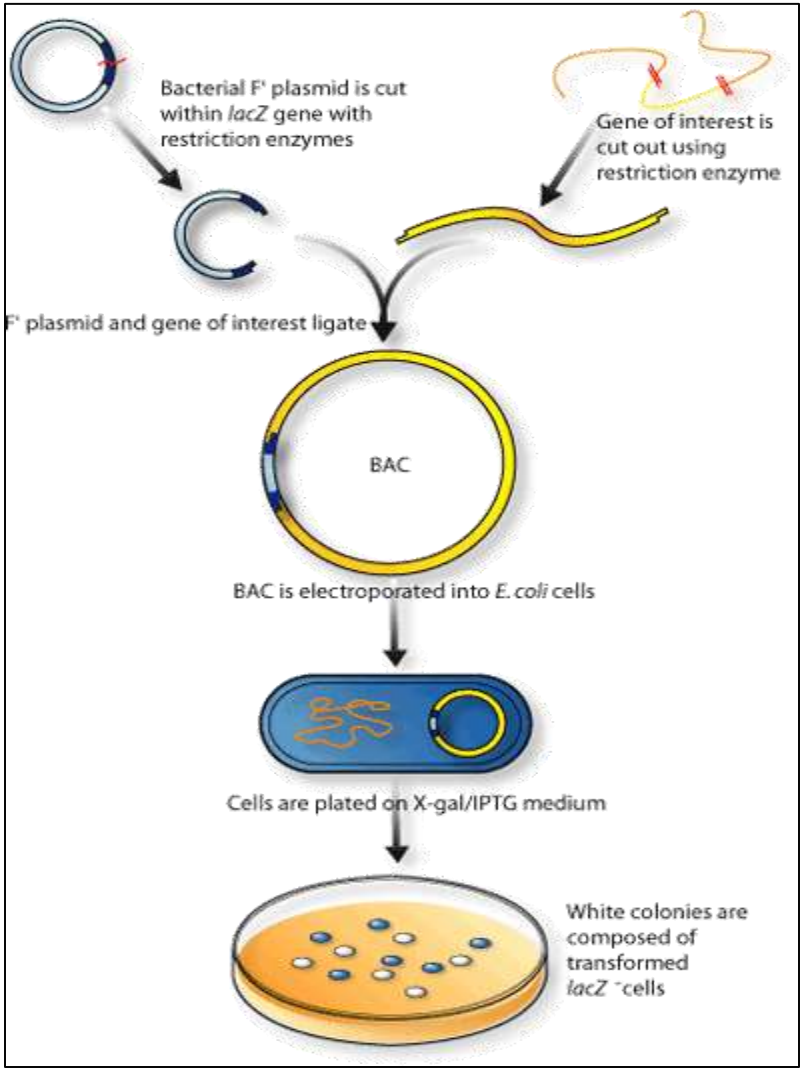
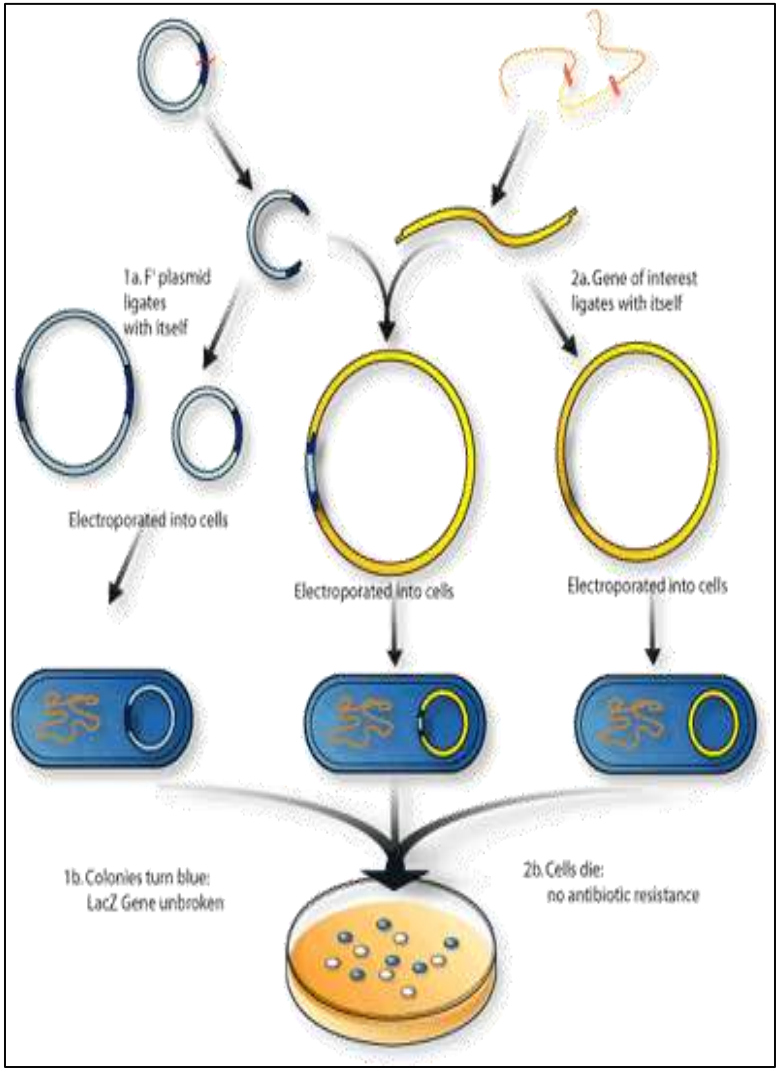


Fig. 5.5 Structure of a BAC vector derived from a mini-F plasmid. The *ori*S and *rep*E genes mediate the unidirectional

- *ori*S and *ori*E mediate replication
- *par*A and *par*B maintain single copy number and proper plasmid partitioning
- Chloramphenicol^{R} marker



Transforming a bacterium using a BAC vector

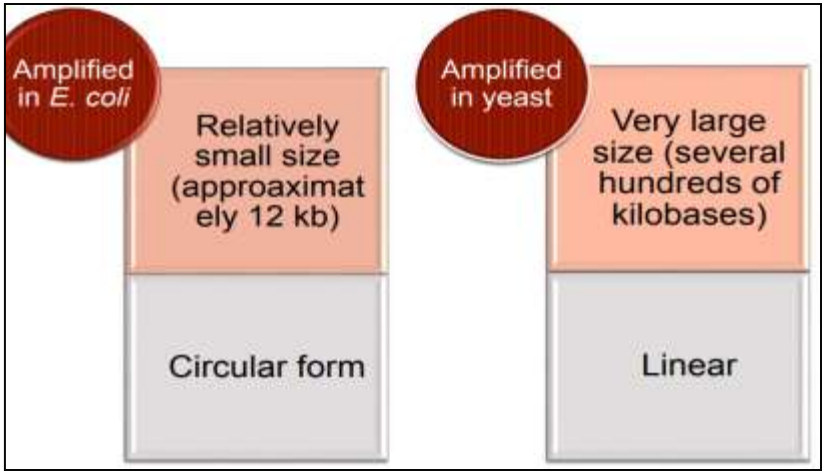


Selecting for transformed bacteria

Yeast artificial chromosomes (YACs)

Yeast artificial chromosome (YAC) is a human-engineered DNA molecule used to clone DNA sequences in yeast cells

YACs are plasmid shuttle vectors capable of replicating and being selected in common bacterial hosts such as *Escherichia coli*, as well as in the budding yeast *Saccharomyces cerevisiae*.



YEAST ARTIFICIAL CHROMOSOMES

- YAC is an artificially constructed chromosome that contains a
  - ❑ Centromere
  - ❑ Telomeres
  - ❑ Autonomous replicating sequence (ARS) element required for replication and preservation of YAC in yeast cells
- ARS elements are thought to act as replication origins

❖First described in 1983 by Murray and Szostak

Purpose:

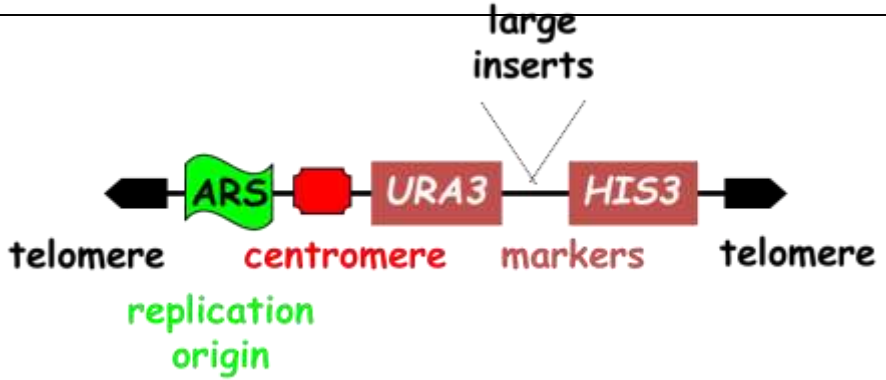
- Cloning vehicles that propogate in eukaryotic cell hosts as eukaryotic Chromosomes
- Clone **very** large inserts of DNA: 100 kb - 10 Mb

Features:

- YAC cloning vehicles are plasmids
- Final chimeric DNA is a linear DNA molecule with telomeric ends: **Artificial Chromosome**

Yeast Artificial Chromosomes (YACs)

- Genetically engineered yeast mini chromosomes.
- Capable of carrying inserts of 200 - 2000 kbp in yeast
- Contain a yeast origin of replication, yeast centromere, two yeast telomeres, a selectable marker, and a polycloning site.
- Marker - URA3 encodes Orotidine 5'-phosphate decarboxylase **(ODCase)**, which is an enzyme that catalyzes one reaction in the synthesis of pyrimidine ribonucleotides (a component of RNA). Loss of ODCase activity by mutation leads to a lack of cell growth unless uracil or uridine is added to the media. The presence of the URA3 gene in yeast restores ODCase activity, facilitating growth on media not supplemented with uracil or uridine, thereby allowing selection for yeast carrying the gene.
- **HIS3** gene - *Saccharomyces cerevisiae* - encodes Imidazoleglycerol-phosphate dehydratase which catalyses the sixth step in histidine biosynthesis.

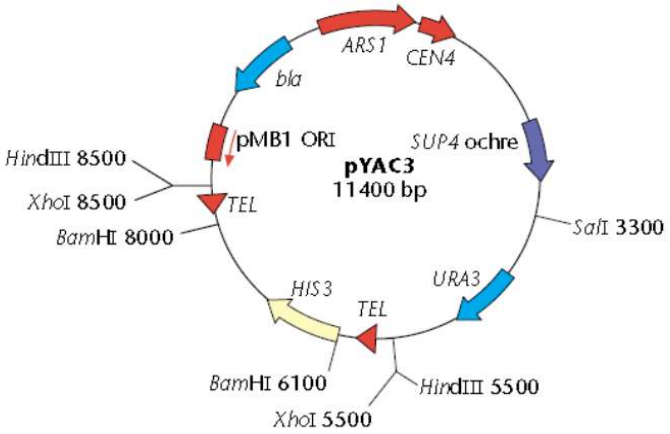


YAC cloning vector

Yeast Artificial Chromosomes (pYACs) Plasmids

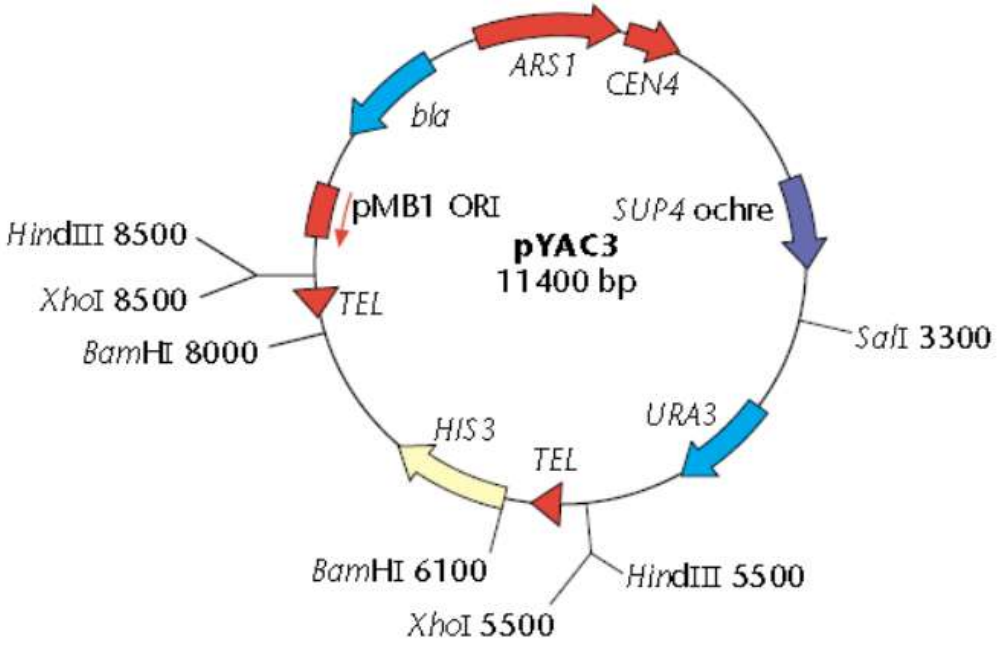
- Many different yeast artificial chromosomes plasmids exist, such as pYAC3 and pYAC4 plasmids.
- The basic structural features of YACs were developed from the yeast centromere shuttle-plasmids YCp series.
- These are composed of:
  - double-stranded circular DNA sequences carrying the b-lactamase gene bla and the bacterial pMB1 origin of replication
- Include yeast ARS1 with its associated CEN4 DNA sequence, as well as the URA3 selectable marker.

Circular map of plasmid vector pYAC3



- Yeast HIS3 is flanked by a telomere-like DNA sequence that are adjacent to two recognition sites for the BamHI restriction enzyme.
- Most of these YACs also contain the cloning site in the middle of the SUP4 suppressor of an ochre allele of a tyrosine transfer RNA (tRNA) gene.

Circular map of plasmid vector pYAC3



CONSTRUCTION OF YAC

- A YAC is built using an initial circular plasmid

typically broken into two linear molecules using restriction enzymes

- DNA ligase is then used to ligate a sequence or gene of interest between the two linear molecules
- forming a single large linear piece of DNA

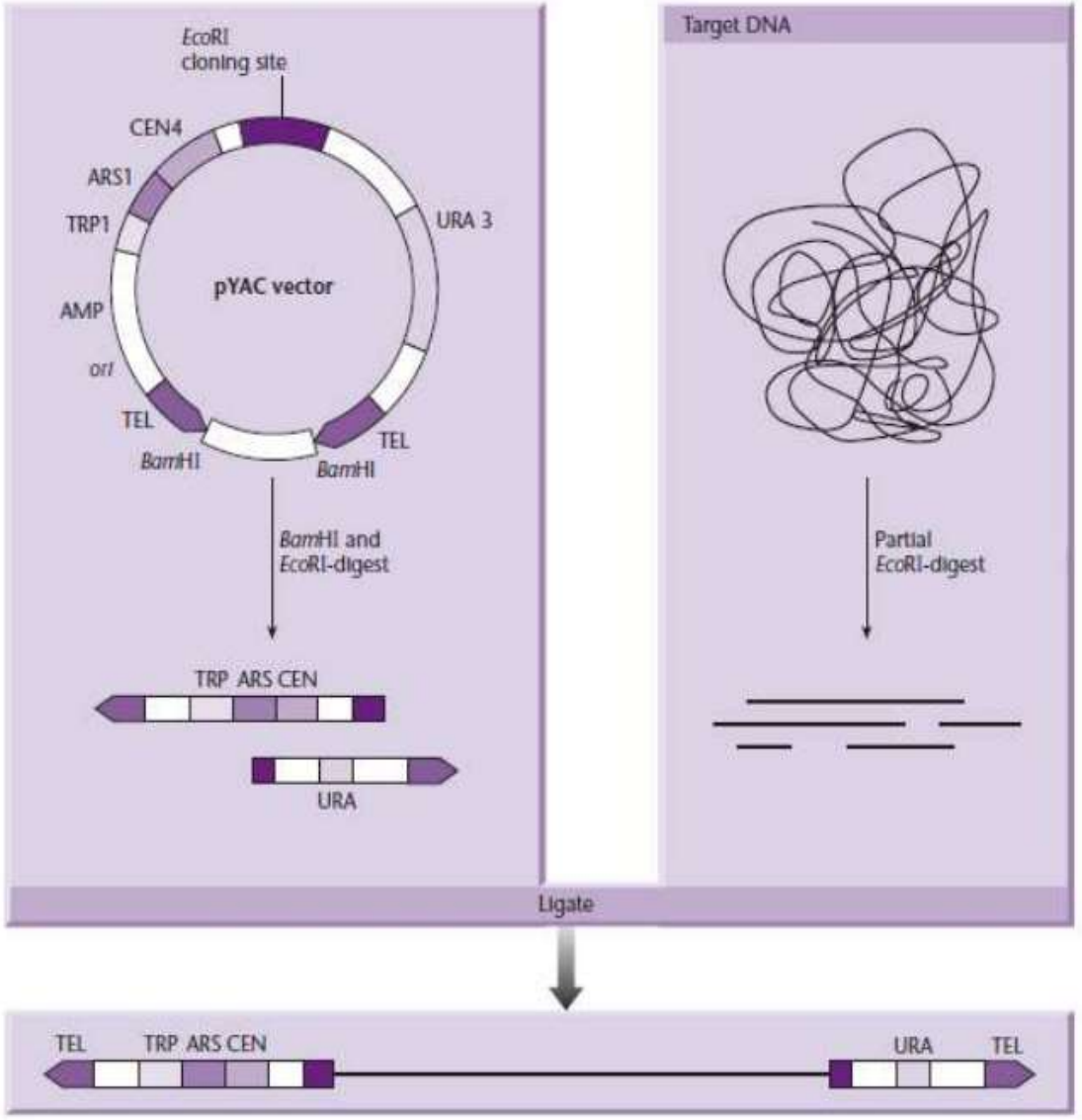


Fig. 11.3
Construction of a yeast artificial chromosome containing large pieces of cloned DNA. Key regions of the pYAC vector are as follows: TEL, yeast telomeres; ARS1, autonomously replicating sequence; CEN4, centromere from chromosome 4; *URA3* and *TRP1*, yeast marker genes; Amp, ampicillin-resistance determinant of pBR322; *ori*, origin of replication of pBR322.

- This inserted gene compensates for a mutation in the yeast host cell that causes the accumulation of red pigment
- The host cells are normally red, and those transformed with YAC only, will form colourless colonies
- Cloning of a foreign DNA fragment into the YAC causes insertional inactivation, restoring the red colour
- Therefore the colonies that contain the foreign DNA fragment are red. The yeast artificial chromosome (YAC) vector is capable of carrying a large DNA fragment (up to 2 Mb)
- Transformation efficiency is very low.

Homologous Recombination

- In recombinationally-targeted YAC cloning, YACs are assembled *in vivo, by* recombination, and not by ligation *in vitro*
- Recombination takes place between a target segment of the exogenous DNA, and the YAC vector that contains sequences homologous to these targets

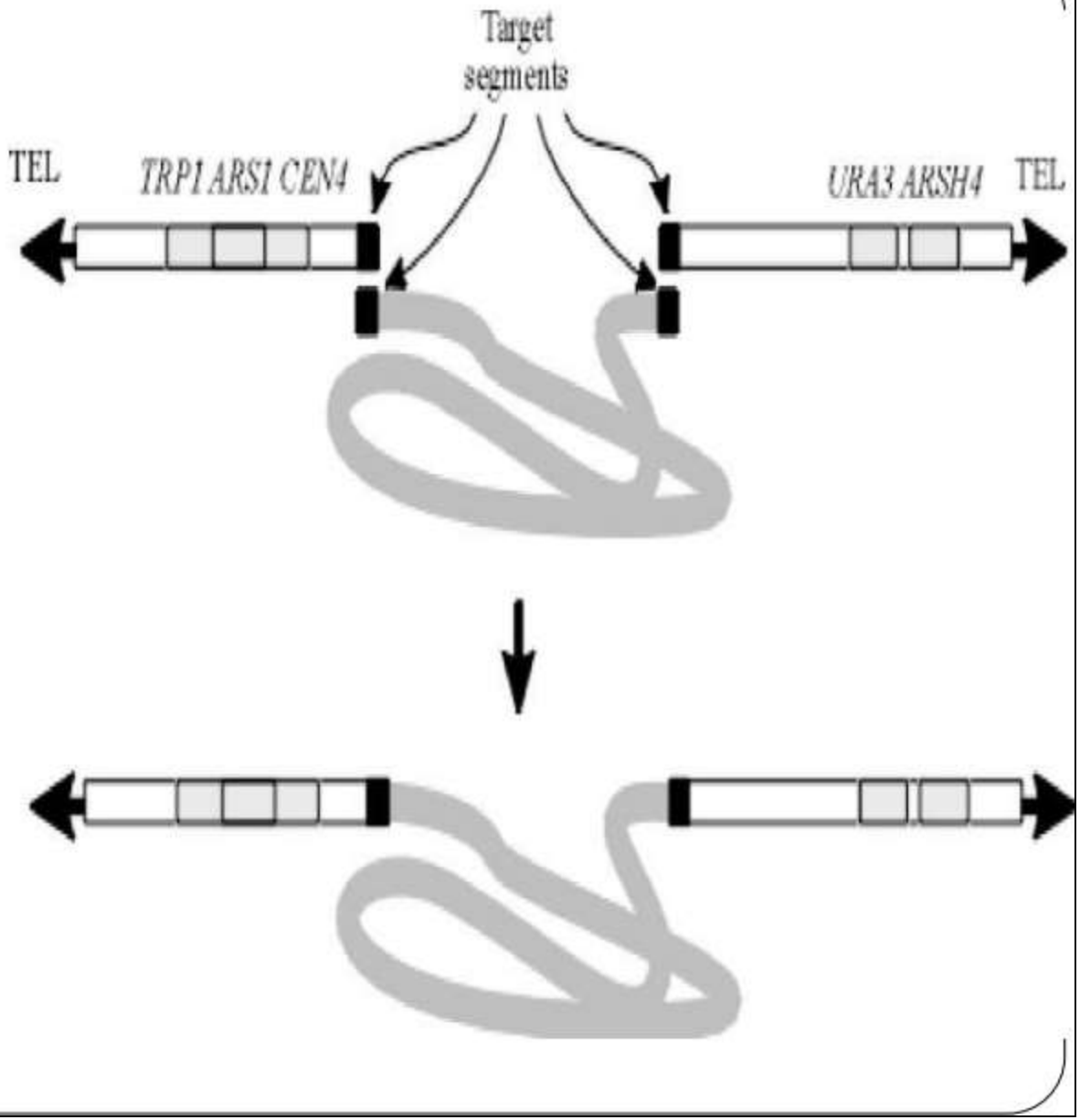
- Firstly two YAC vectors arms and the exogenous segment(flanked by desired sequences) are transformed into the yeast cell
- Then followed by recombination
- Results in formation of desired stable YACs.

- How to deliver the recombinant DNA in host?
- Electroporation
- How to screen positive colonies?

Marker –

- URA3 gene
- HIS3 gene
- Positive selection in media lack of uracil or uridine/Histidine

Figure.Recombinational targeted cloning with YAC vectors. A yeast strain is transformed with a mixture of the two YAC vector arms and large fragments of DNA. Recombination in vivo results in the formation of a specific YAC clone. The two YAC vector arms are derived from linearized plasmids that contain targeting segments that are homologous to the termini of the DNA segment that is to be cloned.



Application of YACs – Generating whole DNA libraries of the genomes of higher organisms

P1 derieved artificial chromosome - PAC

- The P1-derived artificial chromosome (PAC) are DNA constructs that are derived from the DNA of P1 bacteriophage.
- They can carry large amounts (about 100-300 kilobases) of other sequences for a variety of bioengineering purposes.

PAC vectors

- Phage artificial chromosome or P1-derived artificial chromosome (PAC) is a form of chromosome derived through biological manipulation and it originates from a 'phage' instead of a 'plasmid'.
- Genomic libraries constructed in the original Bacterial Artificial Chromosome (BAC) and P1 Artificial Chromosome (PAC) cloning systems were very useful for completion of the Human Genome Project.
- Libraries constructed in these vector systems were used to generate physical maps of all twenty three-chromosome pairs and served as the templates for DNA sequencing.
- PACs can accommodate larger inserts of DNA than a plasmid or many other types of vectors. Sometimes, the number of inserts can be as high as 300 kilobase pairs.

PACs and BACs

- PACs - P1-derived Artificial Chromosomes
- *E. coli* bacteriophage P1 is similar to phage lambda in that it can exist in *E. coli* in a prophage state.
- Exists in the *E. coli* cell as a plasmid, NOT integrated into the *E. coli* chromosome.
- P1 cloning vehicles have been constructed that permit cloning of large DNA fragments- few hundred kb of DNA
- Cloning and propogation of the chimeric DNA as a P1 plasmid inside *E. coli* cells

- BACs - Bacterial Artificial Chromosomes
- These chimeric DNA molecules use a naturally-occurring low-copy number bacterial plasmid origin of replication, such as that of F-plasmid in *E. coli*.
- Can be cloned as a plasmid in a bacterial host, and its natural stability generally permits cloning of large pieces of insert DNA, i.e. up to a few hundred kb of DNA.

Construction of PACs through electroporation:

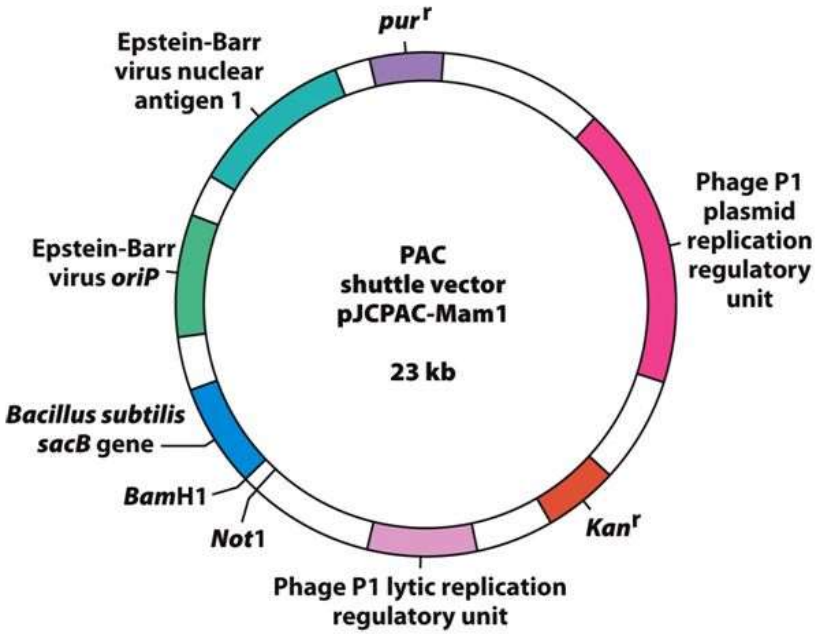
- During the construction of PACs, P1 phage containing cells will undergo a process known as 'electroporation', which will increase the permeability of the cell membrane and allow DNA material to enter the cell and couple with the existing DNA.

- This process will give rise to PACs and from there onwards, the PACs can replicate within the cell through 'lysogeny', without destructing the cell or incorporating into rest of the chromosomes.

- The PAC vector pJCPAC-Mam1 is a 23 kilobase (kb) shuttle vector that is highly versatile and useful for functional studies in human tissue culture cell lines.

- pJCPAC-Mam1 contains the P1 single copy replicon for low copy expression and a multi-copy lytic replicon under the control of the lac repressor for high copy expression in *Escherichia coli*, wild type and mutant loxP sites for generation of bidirectional nested deletions in any clone of interest and the Epstein Barr Virus (EBV) latent replication origin oriP, the Epstein Barr Nuclear Antigen 1 (EBNA1) gene, and a puromycin-resistance gene for propagation in mammalian cells.

The PAC Mammalian Shuttle Vector pJCPAC-Mam1



Uses of PACs:

- PACs are in high demand when it comes to cloning important biomedical sequences, which are essential for many scientific functions.
- One of its main uses is the genome analysis and map based cloning of complex plants and animals, which requires isolation of large pieces of DNA rather than smaller segments.
- Although there are other forms of artificial chromosomes which can accommodate more base pairs than PACs, relative user friendliness of these vectors makes it a popular choice among many biomedical researchers.

Mammalian Artificial Chromosomes Vector

- Chromosomes in eukaryotes have evolved as vehicles for **nuclear genes** and have developed specialized nucleoprotein structures for this purpose, some of them are centromeres, telomeres and origin of replication.

- Alphoid arrays are found at all human centromeres and consist of a 171 bp monomer organized in higher order repeats encompassing 0.5-5 Mb.

- They have been considered the best candidate for the specific DNA requirement for centromere function.

- Mammalian Artificial Chromosomes formation was observed following transfection of a 100 kb yeast artificial chromosome (YAC) containing alphoid sequence from chromosome 21 with uniform higher order repeats and frequent CENP-B boxes, a conserved motif binding the CENP-B protein.

- The YAC had been retrofitted with terminal human telomere sequence, but no other human DNA was included.

- This YAC construct generated cell lines containing Mammalian Artificial Chromosomes at frequencies ranging from 10 to 100% cells.

Human artificial chromosomes (HACs) and
Mouse artificial chromosomes (MACs)

- Human artificial chromosomes (HACs) have been generated mainly by either a 'top-down approach' (engineered creation) or a 'bottom-up approach' (de novo creation).

- HACs with acceptor sites exhibit several characteristics required by an ideal gene delivery vector, including stable episomal maintenance and capacity to carry large genomic loci plus their regulatory elements, thus allowing the physiological regulation of the introduced gene in a manner similar to that of native chromosomes.

- Mouse artificial chromosomes (MACs) with acceptor sites were also created from a native mouse chromosome. The microcell-mediated chromosome transfer (MMCT) technique for manipulating HACs and MACs in donor cells in order to deliver them to recipient cells is required for each approach.

Construction methods HAC

● There are currently two accepted models for the creation of human artificial chromosome vectors.
➔ Top-down approach (engineered chromosome)
➔ Bottom up approach (de novo artificial chromosome)
● The generated HACs are 1–10 Mb in size, consisting of multiple copies of rearranged input DNA molecules.

In **engineered chromosome** by a top-down approach, minichromosomes or chromosomes derived from endogenous chromosomes generated by natural fragmentation of chromosomes, telomere-directed chromosome breakage, or radiation-induced chromosome breakage, containing an endogenous functional centromere. The chromosomes can then be transferred into other cell lines by microcell-mediated chromosome transfer (MMCT).

In **de novo artificial chromosome** by a bottom-up approach, exogenous chromosomes can be circular or linear, created de novo from cloned chromosomal components, either naturally occurring or synthetic high-order -satellite DNA arrays introduced on α bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC), or p1-derived artificial chromosome (PAC) vectors, which have a functional centromere and autonomously replicate and segregate.

Human artificial chromosome

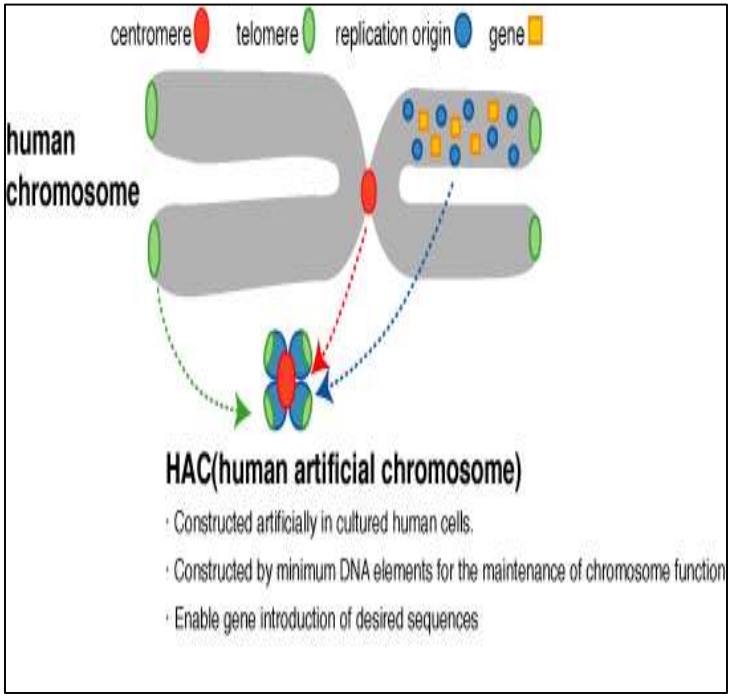
- A human artificial chromosome (HAC) is a microchromosome that can act as a new chromosome in a population of human cells.

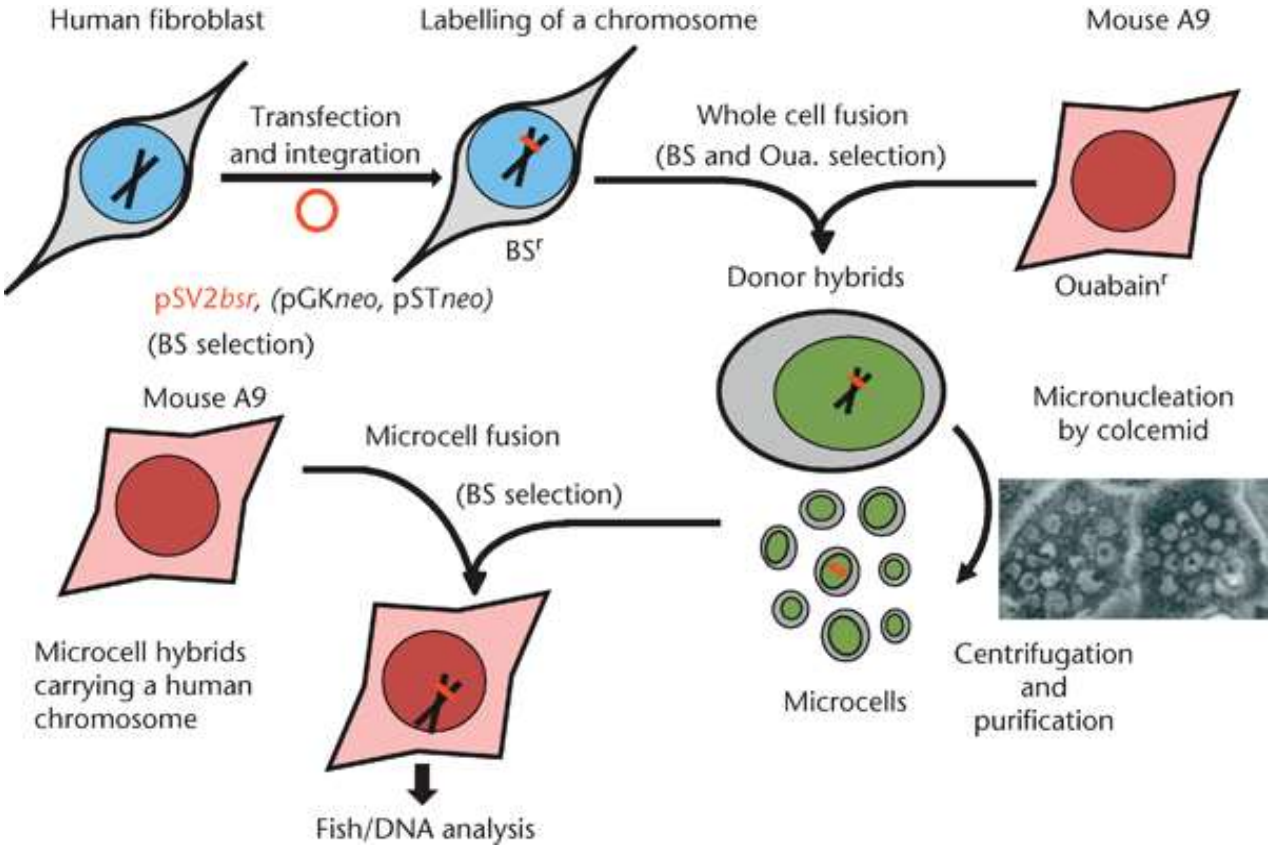
- That is, instead of 46 chromosomes, the cell could have 47 with the 47th being very small, roughly 6-10 megabases (Mb) in size instead of 50-250 Mb for natural chromosomes, and able to carry new genes introduced by human researchers.

- Ideally, researchers could integrate different genes that perform a variety of functions, including disease defense.

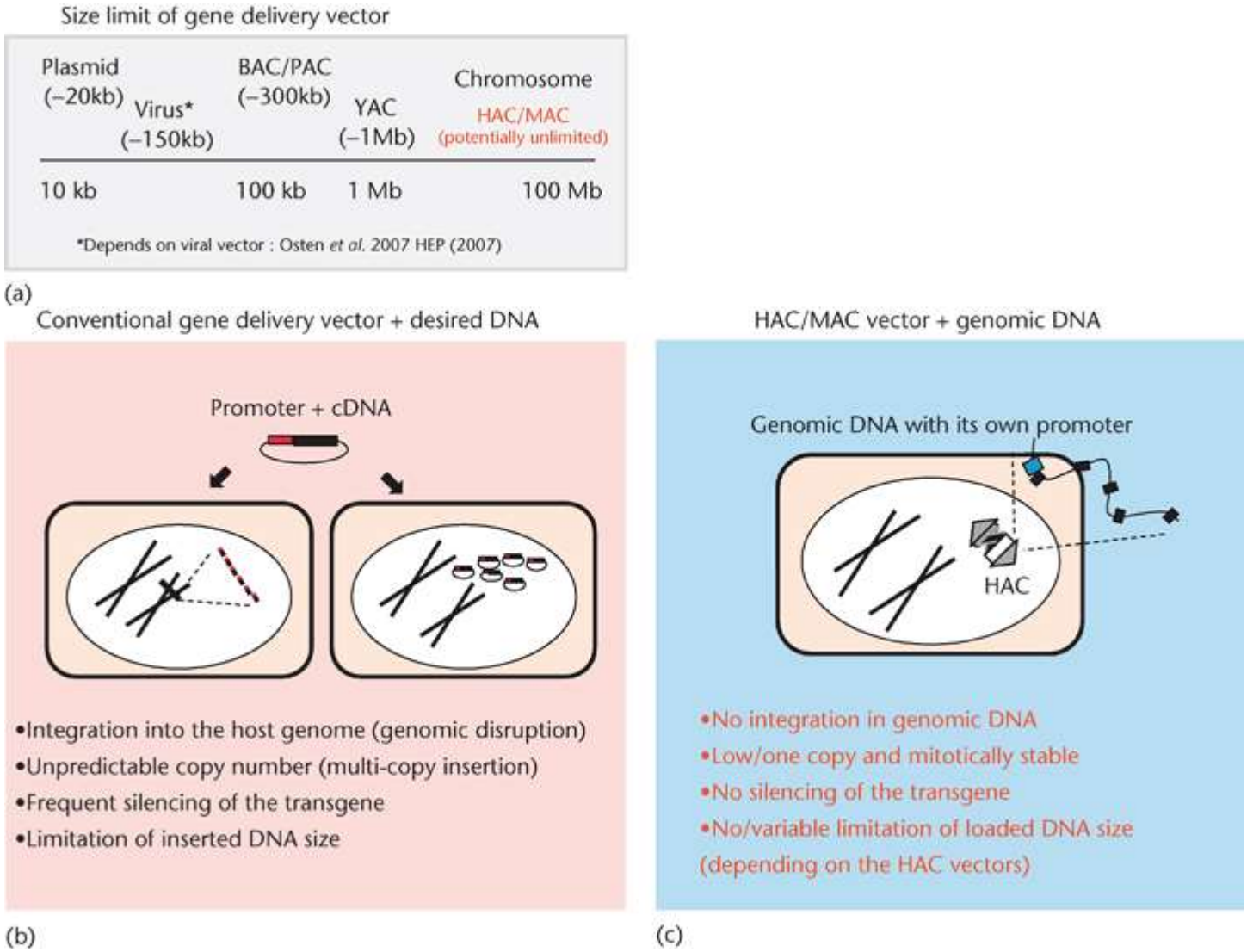
- A human artificial chromosome (HAC) is a mini-chromosome that is constructed artificially in human cells. Using its own self-replicating and segregating systems, a HAC can behave as a stable chromosome that is independent from the chromosomes of host cells.

- The essential elements for chromosome maintenance and transmission are the following three regions:
  (1) the “replication origin,” from which the duplication of DNA begins,
  (2) the “centromere,” which functions in proper chromosome segregation during cell division, and
  (3) the “telomere,” which protects the ends of linear chromosomes.

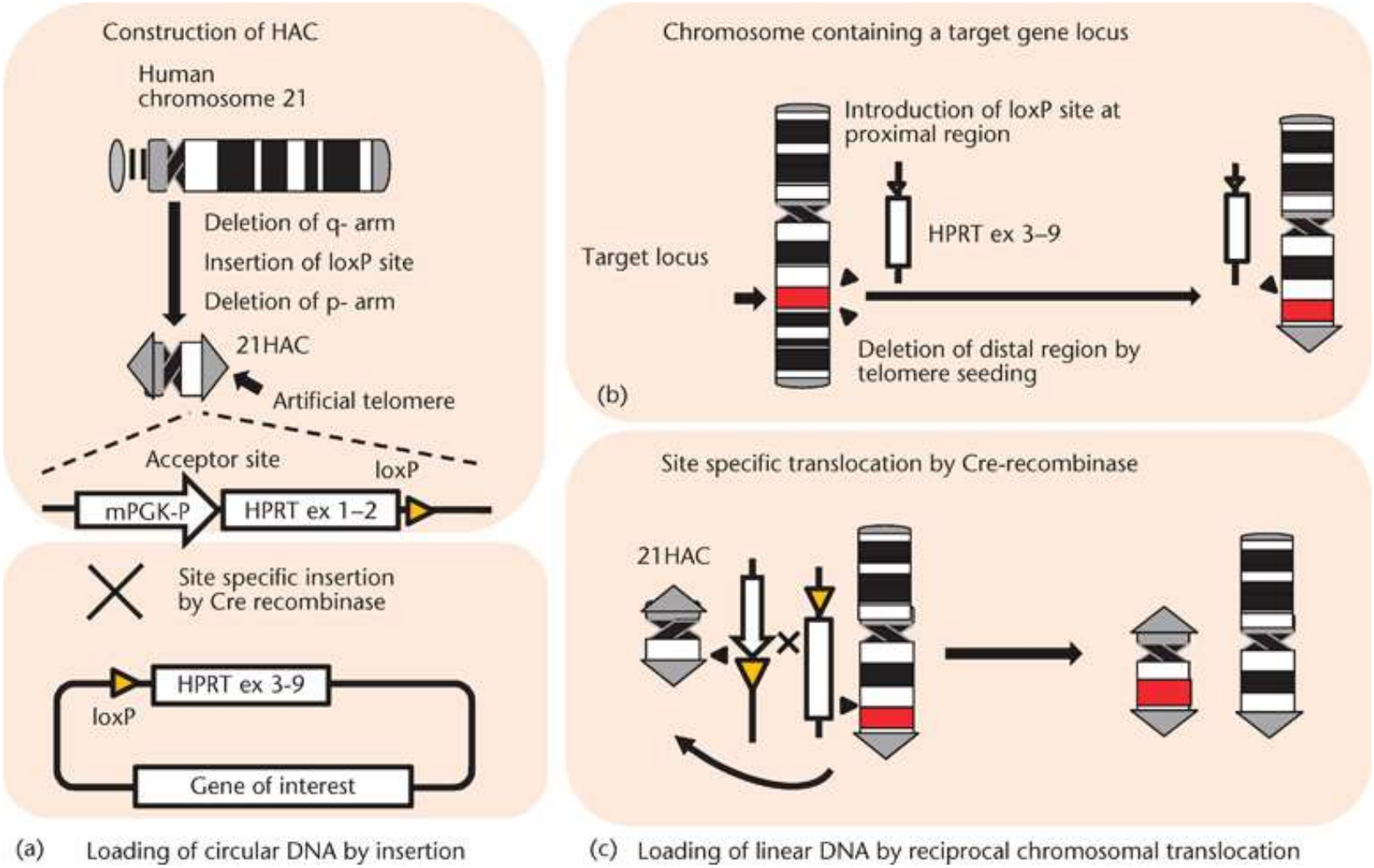




Construction of mouse A9 hybrid cells carrying a single human chromosome by MMCT. The first step involves marking the human chromosome in the fibroblasts with a selection marker and fusing the fibroblasts with mouse A9 cells. The second step is the introduction of the marked human chromosome from the donor hybrid to the recipient A9 cells. The procedure can be divided into several parts: micronucleation of the donor hybrids by colcemid treatment, enucleation in the presence of cytochalasin B, purification of the microcells, fusion with the recipient A9 cells, drug selection of the microcell hybrids, identification of the transferred human chromosome by fluorescence *in situ* hybridisation and DNA analyses



Potential characteristics of HACs. (a) Size limits for gene delivery vectors. The maximum deliverable DNA size in each vector is described. HAC vectors as well as chromosomes can carry DNA fragments larger than 1 Mb size. The size limits depend on each vector. (b, c) Limitations and consequences of gene delivery with conventional vectors such as viruses or plasmids and with HACs.



Two types of gene loading to HAC. (a) Construction of a HAC vector from human chromosome 21 using the top-down approach. The 21HAC is equipped with a loxP site for loading the gene of interest. A site-specific recombination event mediated by Cre recombinase is selected by reconstruction of the functional HPRT gene, which confers HAT resistance. (b) The gene of interest, isolated in a circular vector, is introduced into the HAC by site-specific insertion. (c) A megabase size gene locus, which is above the capacity of circular cloning vectors, is introduced into the HAC by site-specific reciprocal chromosome translocation.

Advantage of HAC

- Alternative methods of creating transgenes, such as utilizing YACs and BACs, lead to unpredictable problems.

The genetic material introduced by these vectors not only leads to different expression levels, but the inserts also disrupt the original genome.

HACs differ in this regard, as they are entirely separate chromosomes. This separation from existing genetic material assumes that no insertional mutants would arise. This stability and accuracy makes HACs preferable to other methods such as viral vectors, YACs and BACs.

- HACs allow for delivery of more DNA (including promoters and copy-number variation) than is possible with viral vectors.

Applications of HAC

- HACs are useful in expression studies as gene transfer vectors, as a tool for elucidating human chromosome function, and as a method for actively annotating the human genome.
- HACs have been used to create transgenic animals for use as animal models of human disease and for production of therapeutic products.
- HAC can carry genes to be introduced into the cells in gene therapy.

Future applications of HAC and MAC vectors