Yeast Artificial Chromosomes

Remigiusz Arnak, *International Centre for Genetic Engineering and Biotechnology, Trieste, Italy*

Carlo V Bruschi, *International Centre for Genetic Engineering and Biotechnology, Trieste, Italy*

Valentina Tosato, *International Centre for Genetic Engineering and Biotechnology, Trieste, Italy*

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Advanced article

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Yeast artificial chromosomes (YACs) are shuttle-vectors that can be amplified in bacteria and employed for the cloning and manipulation of large deoxyribonucleic acid (DNA) inserts (up to 3 Mb pairs) in the yeast *Saccharomyces cerevisiae*. Artificial chromosomes can be conveniently built and modified in yeast cells using *in vivo* homologous recombination, a novel process known as 'recombineering'. The capacity of YACs to accommodate large DNA fragments is exploited to clone clusters of genes surrounded by their native DNA context, where regulatory elements are located. This is important for biotechnology, when YACs are used for engineering genetic determinants of new biochemical pathways for production of secondary metabolites and for heterologous protein expression. YACs can be retrofitted with the appropriate selectable markers and transmitted to cells of different organisms allowing the generation of transgenic animals. Finally, YACs are largely employed in the production of full-scale genomic libraries, for mapping and functional analysis purposes.

Introduction

Yeast artificial chromosomes (YACs) are plasmid shuttle-vectors able to accommodate large fragments of deoxyribonucleic acid (DNA) (up to 3 Mb). This is much more than capacities offered by regular plasmids (around 10 kb), λ bacteriophage (20 kb) or cosmids (45 kb) (Sambrook and Russell, 2001). YACs are capable of replicating and being selected in common bacterial hosts such as *Escherichia coli*, as well as in the budding yeast *Saccharomyces cerevisiae*. They are of relatively small size (approximately 12 kb) and of circular form when they are amplified or manipulated in *E. coli*, but are linearised when introduced as cloning vectors in yeast. The latter process involves cleavage at strategically located sites, in two (DNA) arms. These are subsequently ligated to appropriate DNA inserts before transformation into recipient yeast cells converted to spheroplasts, where telomere (TEL) sequences are added by the cell's telomerase apparatus. In this linear form, these specialised vectors contain all three *cis*-acting structural elements essential for behaving like natural yeast chromosomes: an autonomously replicating sequence (ARS) necessary for replication; a centromere (CEN) for segregation at cell division; and two TELs for maintenance and end protection. In addition, their capacity to accept large DNA inserts enables them to reach the minimum size (150 kb) necessary for chromosome-like mitotic stability and for fidelity of transmission in yeast cells. YACs have several advantages over other large capacity vectors like bacterial artificial chromosomes (BACs) or P1-derived artificial chromosome (PACs). These include accommodation of DNA segments thousands of kilobases in size and stable maintenance of cloned eukaryotic DNA due to its compatibility with the yeast replication machinery. Moreover, they are amenable to large-scale plasmid amplification in *E. coli* and to creation of site-specific genetic changes within the exogenous DNA sequences by using the faithful and efficient yeast mechanism of homologous recombination. Recently, YACs have become instrumental for the study of complex genome organisation as well as for its manipulation in higher eukaryotes, for industrial production of complex macromolecules as well as for the

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construction of artificial microorganisms. **See also**: Centromeres; Homologous Genetic Recombination in Eukaryotes; Restriction Enzymes; Telomeres; Yeasts

Overview of Yeast Artificial Chromosomes Plasmids (pYACs)

The basic development of the YACs started in 1981 together with the characterisation of the yeast DNA origin of replication (ARS), centromeric sequence (CEN) required for chromosome transmission during cell division and telomeric sequence responsible for stability (TEL). By placing these three *cis*-acting DNA elements together on a plasmid, the first stably maintained minichromosomes were constructed (Hsiao and Carbon, 1981). In 1983, a 55 kb long DNA molecule containing yeast chromosome elements was constructed, becoming the first artificial chromosome ever generated (Murray and Szostak, 1983). In 1987 Burke *et al*. (1987) constructed pYAC3 (**Figure 1**) and pYAC4 vectors and used them for cloning a several hundred kilobase pairs-long DNA fragment. These two constructs soon became basic vectors used as scaffolds for constructing many other YACs. Indeed, many present YAC vectors are refined descendants of these two original plasmids. The basic structural features of pYAC vectors were developed from the yeast CEN shuttle-plasmids YCp series. These are composed of double-stranded circular DNA sequences carrying the β-lactamase gene *bla* and the bacterial pMB1 origin of replication, conferring resistance to ampicillin and the ability to replicate in bacteria, respectively. They also include yeast ARS1 with its associated CEN4 DNA sequence, as well as the *ura3* selectable marker. On this basic scaffold plasmid the yeast *his3* was cloned, flanked by a TEL-like DNA sequence derived from the termini of the *Tetrahymena pyriformis* macronuclear ribosomal DNA, which allows the formation of functional TELs in yeast, which are adjacent to two recognition sites for the *Bam*HI restriction enzyme. Most of these YACs also contain a cloning site in the middle of the *sup4* suppressor of an *ochre* allele of a tyrosine transfer ribonucleic acid (tRNA) gene; this enables restoration of the normal white colour phenotype in otherwise red *ade1* and/or *ade2* nonsense mutants (Fisher, 1969). Accordingly, in the insertional inactivation cloning process, the *sup4* gene is disrupted by the DNA insert, thus removing the suppression of the *ade* mutations and allowing their phenotypic expression as red colour. Usually, for library construction, the vector pYAC-RC is utilised (Marchuk and Collins, 1988). This shuttle plasmid is a derivative of pYAC3 and it was constructed by removal of *Cla*I and *Sal*I sites in the parental vector followed by the insertion of a polylinker into the unique *Sna*BI site within the *sup4* intron. This insertion does not disrupt the function of *sup4* allowing the red/white visual selection of recombinant molecules. It has been used for generating libraries from complex genomes (such as *Caenorhabditis elegans* and *Hordeum vulgare*) fragmented via restriction endonucleases that cut infrequently (Kleine *et al*., 1997). **See also**: Artificial Chromosomes; Bacterial Antibiotic Resistance; Genetic Engineering: Reporter Genes; Prokaryotic Replication Origins: Structure and Function in the Initiation of DNA Replication

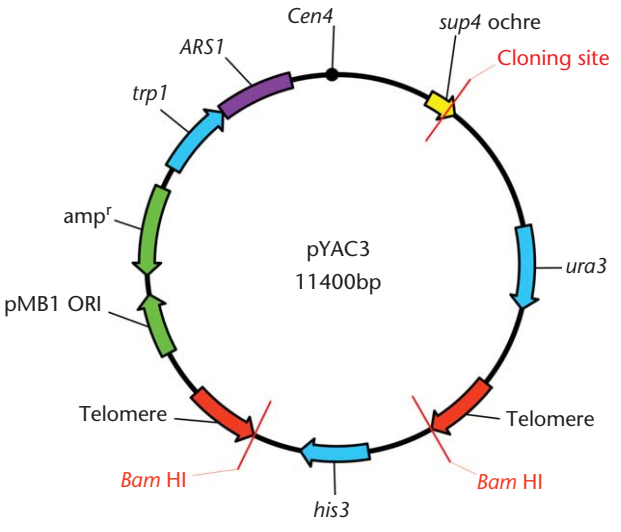

Figure 1 Circular map of plasmid vector pYAC3. The picture shows yeast auxotrophic markers (blue), elements responsible for propagation and selection in bacteria (green), chromosomal structural elements (red and violet) and strategic restriction sites.

Construction of YACs

After plasmid DNA purification, two distinct digestions are performed: the first with *Bam*HI that cuts twice adjacent to the two telomeric DNA sequences flanking the *his3* gene, which therefore is deleted from the plasmid and subsequently lost (**Figure 2a**). This digestion generates a long linear fragment carrying telomeric sequences at each end. The loss of the *his3* gene is used as negative selective marker to counterselect uncut pYAC molecules. The second digestion consists of the opening of the multiple cloning site within the *sup4* gene (**Figure 2a**). As a result of this second digestion, two linear fragments are produced as left and right arms of the future linear YAC (**Figure 2b**). The selective markers are thus separated: *trp1* adjacent to *ARS1* and *CEN4* on the left arm and *ura3* on the right arm. Large DNA fragments with ends compatible to the cloning site used, obtained from the desired genome source by digestion with the appropriate restriction endonuclease, are ligated with phosphatase-treated YAC arms, to create a single yeast-transforming DNA molecule (**Figure 2c**). Primary transformants can be selected for complementation of the *ura3* mutation in the host, and successively for complementation of the host *trp1* mutation, thereby ensuring the presence of both chromosomal arms. Transformant colonies containing the exogenous DNA insert within the *sup4* gene are detected by their red colour, due to

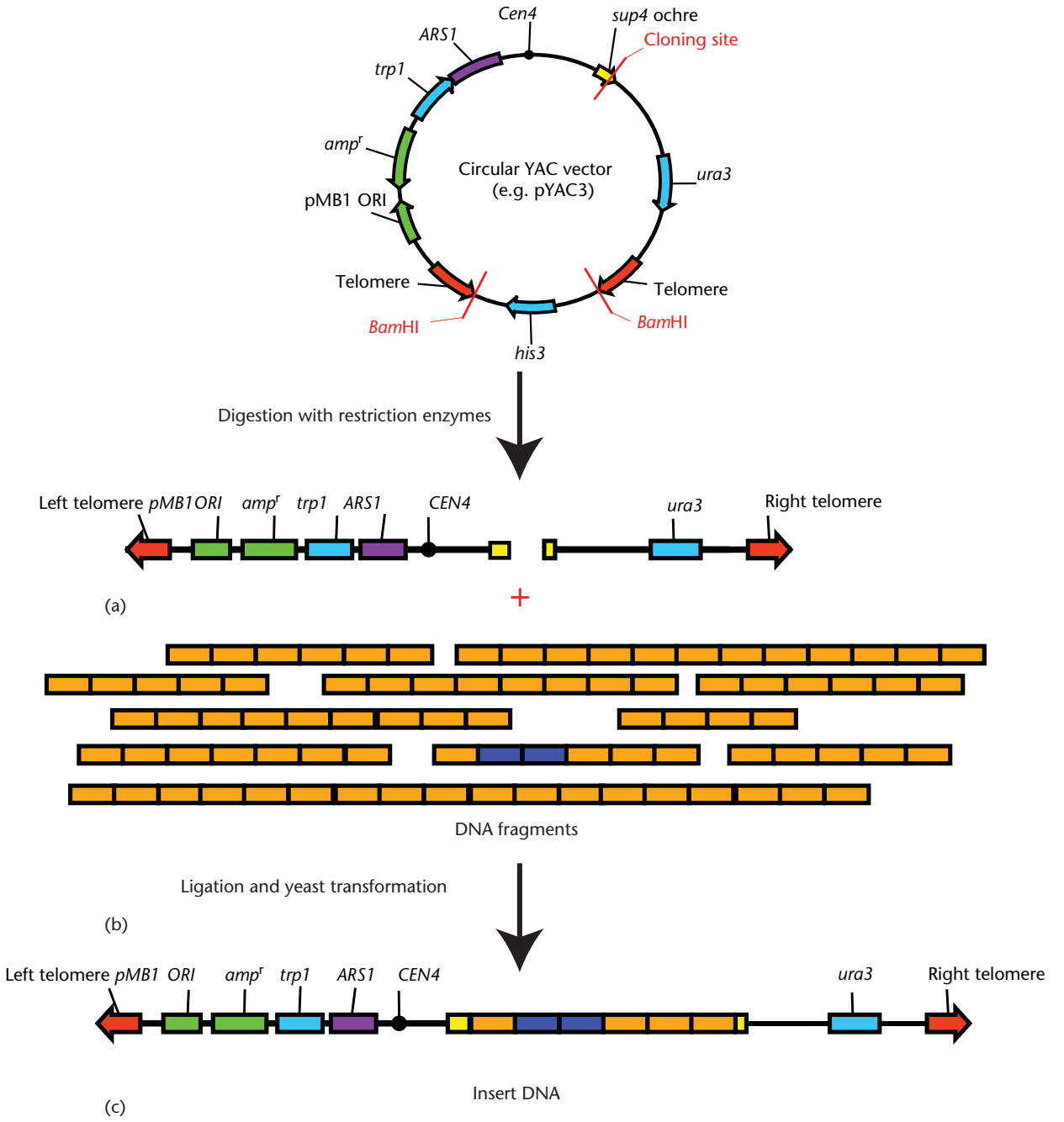

Figure 2 Construction of the YAC. (a) A circular YAC vector able to replicate and be selected in *E. coli*, due to the presence of the bacterial *ori* origin of replication and the *bla* gene for ampicillin resistance (marked in green) and be propagated in yeast cells as a linear molecule containing all necessary chromosomal elements: yeast centromere *CEN4* (black dot), autonomously replicating sequence *ARS1* (violet) and two *Tetrahymena* telomeric sequences *TEL* (red arrows) functional in yeast after linearisation with the *Bam*HI restriction endonuclease. The yeast *sup4* gene (yellow) contains a cloning site and is used as a colour marker for selection of YACs containing exogenous insert DNA. (b) DNA fragments with ends compatible to the YAC cloning site are prepared from source DNA. After double digestion of the YAC vector, the markers used to select for transformants are separated on two chromosomal arms: *trp1* on the left and *ura3* on the right arm (blue boxes). (c) Chromosomal arms ligated with exogenous DNA are selected after transformation of an appropriate yeast strain (*ura3*, *trp1* and *ade2*). Adapted from Burke *et al.* (1987).

the inactivation of the suppressor activity and the consequent accumulation of a red metabolic precursor in *ade* host cells. After the *in vitro* construction of the new YAC, one of the main problems remains its large-scale amplification. Recently (Noskov *et al.*, 2011), detailed protocols for isolation and purification of large circular YACs have been described. According to these methods, large circular YACs up to 600 kb in size can be easily

prepared in microgram quantities. The high quality of this DNA allows successive restriction digestions as well as easier transfection of mammalian cells. Alternatively, YACs can be constructed by means of homologous recombination in the process called transformation associated recombination (TAR). Further modifications of the artificial chromosomes by the *in vivo* homologous recombination system within yeast cells can be carried out by recombinagenic targeting (see below). **See also**: Chromosomes: Methods for Preparation; Plasmids

Biological Features of YACs

The stability of YAC vectors in yeast per se is similar to that of natural chromosomes (in the order of $10^{-5}/10^{-6}$) provided that all three structural elements (ARS, CEN and TEL) are present and functional and, in addition, that the minimal required size is reached by the insertion of enough exogenous DNA. However, the genetic and biochemical background of the host cell also plays an important role in determining the stability of YACs. Indeed, several mutations are known to affect YAC stability and segregation together with natural chromosomes. For example, alterations in the expressions of genes such as *bub1*, *bub2* and *bub3*, *mad1*, *mad2* and *mad3*, *cdc6*, *cdc20*, *pds1* and others lead to chromosome losses, including YACs. In particular, two genes, *zds1* and *zds2*, encode proteins that alter short YAC (10–15 kb) stability by interaction with the Sir-peptides family. SIR2 and SIR4 proteins may protect YACs from the meiotic recombination machinery enhancing their stability. Another important consideration is that faithful and efficient duplication of YACs is guaranteed only if other DNA sequences functionally competing with ARS do not exist on the construct. This point is particularly relevant when unknown DNA inserts are cloned in the YAC vector, as is the case for genomic libraries, in which there could be cryptic or otherwise unknown ARS-like sequences able to interfere with the ARS function. **See also**: Gene Expression in Yeast

Modifying YACs by Homologous Recombination

Depending on the experimental systems and the yeast strains, different selectable markers and restriction sites are appropriate on the YAC vectors. These can be constructed *in vitro* by standard techniques and then used for subcloning DNA fragments. Additionally, existing YAC clones can be modified by homologous recombination in yeast, a process called 'retrofitting' (**Figure 3**). Accordingly, genetic markers can be modified by simply transforming YAC-containing yeast cells with a disruption cassette carrying the desired genetic marker flanked by short DNA sequences homologous to one of the markers present on the artificial chromosome. The same result can be obtained by using one arm of the artificial chromosome carrying the new genetic marker as the transforming molecule. As shown in **Figure 3a**, the introduction of the yeast *lys2* gene, together with the mammalian selectable marker *neo* encoding resistance to neomycin, into the *ura3* gene on the right arm of a YAC is achieved by exploiting homologous recombination techniques. *neo* is the usual marker gene for subsequent selection of mammalian cells after YAC transfection (see below). Moreover, disruption of this marker provides a selectable replacement marker for introducing specific genetic changes within the exogenous DNA insert (**Figure 3**). The application of this simple technique allows, for example, the identification of tumour-suppressor genes in mammalians (Barton *et al.*, 1990). **See also**: DNA Replication: Prokaryotes and Yeast; Homologous Genetic Recombination in Eukaryotes

A yeast integrative plasmid (YIp) carrying the mutated copy of an exogenous DNA segment and the functional copy of a previously disrupted marker can be used for this purpose. After plasmid integration, the target DNA is duplicated in tandem via homologous recombination, generating a YAC with one wild type and one mutant copy of insert DNA ('pop-in'). At this stage, YACs with only the mutated copy of the marker can be obtained after spontaneous recombination within duplicated DNA regions ('pop-out') by growing the transformed cells in the absence of selective pressure for several generations (**Figure 3b**). However, the frequency is not very high in this case (from 10^{-4} to 10^{-5} per generation) and there is approximately the same probability for both the mutated and wild type copy of the marker to remain within the cell on the YAC vector. In the latter case it is necessary to perform additional molecular tests of the obtained pop-outs.

TAR Technology

The isolation of genomic DNA as circular or linear YACs is possible by using TAR (Larionov *et al.*, 1996). Initially, TAR cloning was based on recombination between one repeated region within transformed genomic DNA fragments (such as an Alu or LINE element) and one repeat of the same sequence on both free ends of a co-transformed linearised plasmid that also contains a yeast CEN and a TEL. The TAR approach eliminates the step of usually difficult and problematic ligations and substitutes it with a recombination event. Another advantage of TAR is no need for the compatibility of restriction digestion sites. In TAR, yeast cells are co-transformed with the two arms of an artificial chromosome together with an exogenous DNA fragment. Chromosome arms are separated by enzymatic digestion and each of the two has been provided with a short region of sequence homology (usually 400–500 bp) called anchor (also hook), ligated at its end. It is also feasible to isolate a gene with vectors containing a repeat hook of approximately only 60 bp. Anchors are homologous to the sequences flanking the exogenous DNA fragment carrying the genetic determinant(s) of interest. The

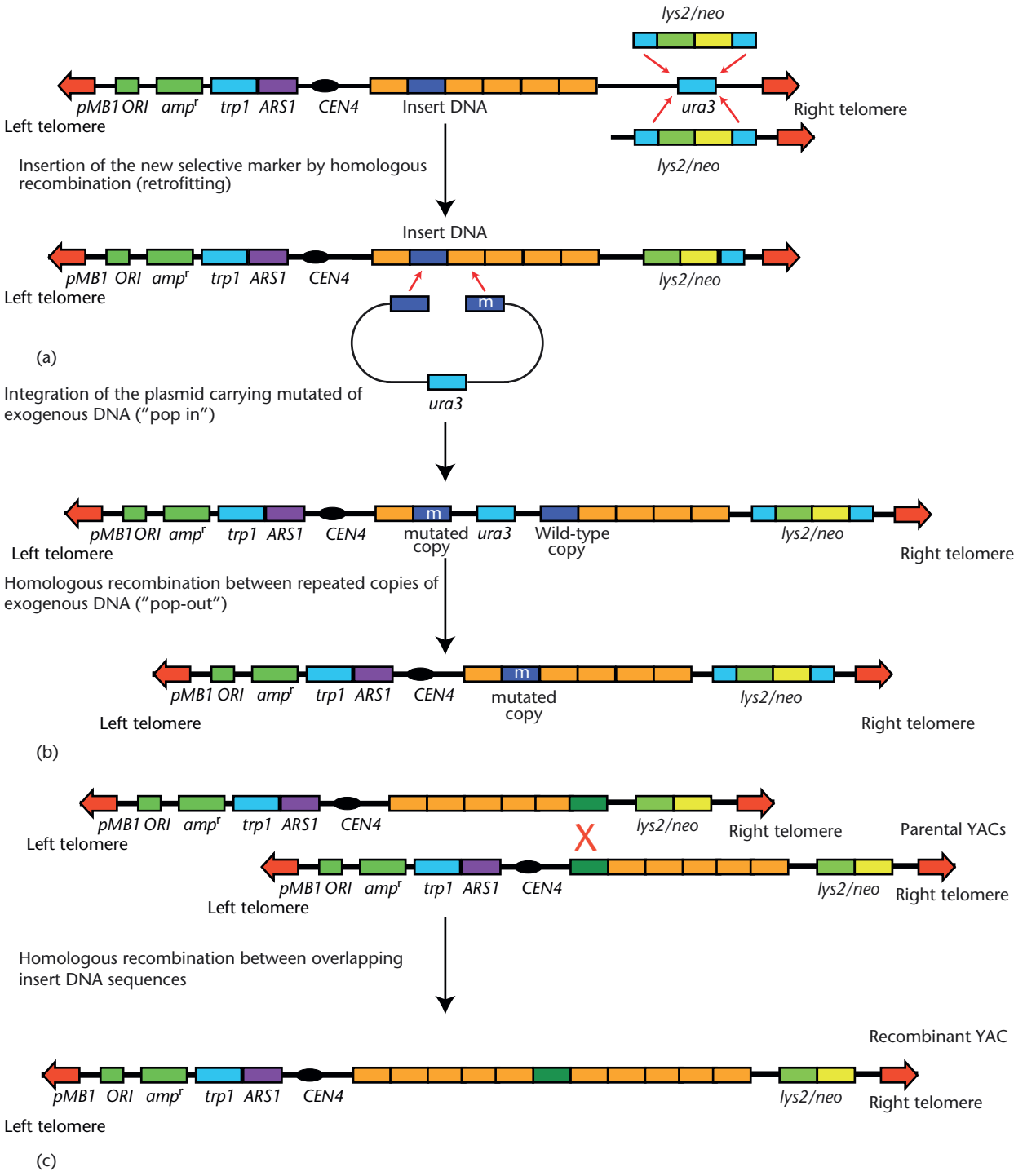

Figure 3 YAC modification by homologous recombination. (a) New selective markers *lys2* (green box) and *neo* (yellow box) are introduced into the *ura3* gene present on YAC by one-step disruption: transformation with disruption cassette (top) or modified right YAC arm (bottom). (b) Inactivation of the *ura3* gene allows subsequent modification of the insert DNA by using linearised yeast integrative plasmid containing a functional copy of the *ura3* gene and a mutagenised copy of the exogenous DNA fragment. After plasmid integration (pop-in), two copies of target DNA are present (wild-type and mutated). A YAC containing only the mutated copy of the exogenous DNA is obtained after homologous recombination and loss of the integrative plasmid carrying the wild-type equivalent (pop-out). (c) Reconstruction of two smaller overlapping YACs into a larger recombinant YAC by recombination between homologous regions of the insert DNA (dark green boxes).

homologous recombination between the anchors and the fragment produces a linear artificial chromosome harbouring the chosen DNA fragment. The TAR approach can be used also to selectively clone large DNA fragments from within YACs into smaller vectors that subsequently can be transformed into bacterial cells for easy and large-scale propagation of plasmid DNA. This possibility removes the problematic isolation of YACs, as they are often in the size of native yeast chromosomes. For this purpose, the pClasper vector was developed and is often used in functional analysis of large genomic regions. Inserts in the pClasper are less prone to chimerism, as they are in the circular form and are far easier to be isolated (Bradshaw *et al*., 1995). Recently, the pClasper-based assay was used to generate mouse models of human diseases in order to study gene function in lymphangiogenesis and lymphoid organ development during ontogeny and chronic inflammation (Bentley *et al*., 2010). The great power of recombinagenic targeting is that using the specific anchors on TAR cloning vectors it is possible to isolate almost any desired piece of DNA from a chosen genome. One of the important uses of this technique is the isolation of human genomic DNA fragments otherwise poorly clonable in BACs or PACs and covering large gaps in the human genomic DNA sequence (Leem *et al*., 2004). Thanks to this strategy, the characterisation of highly repetitive DNA fragments (as human CENs and kinetoplastidae-family variant surface glycoproteins) was possible. In fact, another adaptation of TAR, using a pEB2 vector, was performed, in order to isolate the VAR genes of *Plasmodium falciparum*, responsible for the chronic infection by the parasite (Gaida *et al*., 2011). TAR was used for cloning any genomic fragment up to 250 kb as circular YAC with an efficiency from 1% to 5% (Kouprina and Larionov, 2008).

Use of YAC

YAC vectors were initially created for the cloning of large exogenous DNA segments in *S. cerevisiae* but soon became chromosomal-like platforms for a variety of *in vivo* experiments. The spectrum of YAC applications is very wide, from generating DNA libraries of the entire genomes of higher organisms to identifying essential mammalian chromosomal sequences necessary for the future construction of specialised mammalian artificial chromosomes (MACs). The availability of YAC libraries has greatly advanced the analysis of genomes previously cloned in cosmid vectors. For example, YAC clones have been used as hybridisation probes for the screening of complementary DNA (cDNA) libraries, thus simplifying the characterisation of unidentified genes. Today, the use of artificial chromosomes allows positional cloning of disease genes and preparation of DNA probes for diagnostic purposes. YACs can be employed for gene therapy in mammals since they are starting points in creating human artificial chromosomes (HACs). HACs can accommodate large genes together with their context DNA and thanks to their stable episomal maintenance are of great value for gene therapy, especially in curing diseases caused by recessive mutations (Kazuki *et al*., 2011; Perez-Luz and Diaz-Nido, 2010). One of the most important uses of YACs is the exploitation of their behaviour as endogenous chromosomes to explore genomic instability. YACs have been used to develop assays in budding yeast to identify mutants with enhanced rates of gross chromosomal rearrangements (GCRs). Among these mutants, which are defective in chromosome integrity, either DNA replication initiation and elongation proteins or a mitotic condensation peptide, were recognised. Moreover, specific YACs containing the breakpoint cluster region from the highly unstable human MLL (myeloid/lymphoid or mixed-lineage leukaemia) gene were created ad hoc to investigate the frequency of chromosomal translocations (Tennyson *et al*., 2002). Another major application of YACs is in the study of regulation of gene expression by *cis*-acting, controlling DNA elements, that are present either upstream or downstream of large eukaryotic genes, after the transfer of these YACs from yeast to mammalian cells. Recent technological developments allow the transfer of YACs into mouse embryonic stem (ES) cells and the subsequent generation of transgenic mice. Investigators have begun to employ these artificial chromosomes for the *in vivo* study of multigenic loci in mammalian cells. YACs are not useful tools only in biomedicine, but they represent a fundamental instrument for metabolic compounds production on industrial scale. Organic synthetic chemistry to produce several macromolecules used as drugs is expensive and time consuming. The natural microbial fermentation by microorganisms to generate the same molecules is often the best and first choice. For example, the fermentative production of flavonoids was achieved in yeast. Flavonoids are the most common group of polyphenolic compounds in the human diet and are known for their strong antioxidant activity in the prevention of cancer and cardiovascular diseases. Recently, the entire flavonoid pathway was cloned in YACs to generate a small library for yeast transformation (Naesby *et al*., 2009). For this work, genes codifying for the enzymes of the flavonoid pathway were selected from plants, *Aspergillus* and *Rhodosporium* and cloned into vectors based on pYAC4. The confirmation of the entire functional pathway was performed with the detection of kaempferol whereas the clones with incomplete pathway were detected by the accumulation of intermediate such as naringenin and they were therefore discarded. These kind of YAC-based technologies allow the industrial production of compounds, some of which otherwise not amenable to organic synthesis, and indicate that YACs are suitable for targeted reconstitution of a particular heterologous pathway in yeast. Finally, the newest and original utilisation of YACs is to generate a 'synthetic yeast' (Baker, 2011). Jeff Boeke (John Hopkins University, Baltimore, USA) is building artificial yeast chromosomes, each about the same size as *Mycoplasma mycoides* genome. This approach can be used to answer a

wide variety of questions about fundamental properties of chromosomes, genome organisation, RNA splicing, structure and evolution. Many answers will be provided scrambling this new synthetic genome via specific recombination among the YACs. **See also**: Artificial Chromosomes

YAC Genomic Libraries

BACs and the PACs can accommodate inserts of exogenous DNA up to 300 kb. Although this represents an average insert size close to the one obtained after YAC preparation *in vitro*, it is possible to construct YACs with megabase-long inserts using the precise homologous recombination process of yeast. The prerequisite for this technique is the isolation of YAC clones containing neighbouring genomic DNA inserts. Initial screening for specific sequences usually results in several clones, which most probably contain overlapping inserts. Following insert fingerprinting by conventional Southern hybridisation or polymerase chain reaction (PCR)-based methods, using tandemly repeated sequences as markers, it is possible to obtain detailed physical maps allowing an accurate alignment of two inserts by comparing their individual restriction patterns. Further neighbouring DNA inserts can be found by chromosome 'walking' techniques that require novel sequence information to design DNA probes at the ends of the existing contigs. One of these techniques is screening by hybridisation using contig ends as probes; another is screening by PCR techniques that allow the selective amplification of sequences composed of two neighbouring contigs. More recently, the simultaneous alignment among YAC, BAC and PAC libraries via fingerprinting comparison, provided an integrative approach to obtain with high accuracy the physical map of large genomes (Kleine *et al*., 1997; Krzywinski *et al*., 2004). Manual screening by conventional filter hybridisation of YAC libraries is still used (Sanchez and Lanzer, 2006a) even if, nowadays, robotic systems for YAC libraries analysis are replacing the traditional techniques. In the last years, efficient targeted library construction using TAR were built (Del Portillo *et al*., 2001; Gaida *et al*., 2011). These and other works suggest that YACs are presently more used to generate ad hoc libraries of gene families rather than to build complete genome maps as it was done in the past. Many of these gene families belong to parasites, eukaryotic microorganisms deeply studied for their ability to evade the human immune system response. Detailed protocols have been developed to specifically target genomic regions, such as the *var* of *P. falciparum* and the *vir* of *Plasmodium vivax*, which are responsible for antigenic variation in pathogenic hosts and nonmodel organisms (Sanchez and Lanzer, 2006b). **See also**: Genome Mapping; Polymerase Chain Reaction (PCR); Southern Blotting and Related DNA Detection Techniques

Once clones with overlapping inserts are found, a YAC with a continuous contig can be obtained at high frequency by inducing meiosis in diploid yeast cells formed after mating two haploid strains containing one each of the two YACs (Silverman *et al*., 1990). This allows meiotic recombination to occur between the two homologous overlapping sequences of the inserts, thus generating a recombinant YAC harbouring both inserts and joined at the point of crossing-over. To prevent the generation of mitotically unstable dicentric or acentric YACs, the insert DNA must be cloned in the same 5′ to 3′ vector orientation. After meiotic division and sporulation, spores containing a single recombinant YAC created by the precise physical exchange of homologous insert DNA between two parental YACs are frequently found among tetrads (**Figure 2c**). Following several cycles of recombination between overlapping sequences it is possible, therefore, to reconstruct and maintain in a single YAC vector the original DNA sequence of a eukaryotic genome fragment more than 2 Mb in size (Den Dunnen *et al*., 1992). So far, in library construction, chromosome and YAC splitting techniques were routinely used but, more recently, protocols based on calcium alginate bioactive beads were developed (Kawakami *et al*., 2006) bypassing the problem of degradation of large molecule DNA by promoting their compaction with polyamines.

Production of Transgenic Animals with YACs

Transgenic animals become recently an invaluable tool in studying the role of particular genes and its variants for health maintenance and development of diseases. Using artificial chromosomes it is possible to re-create an entire gene that includes all the exons, introns and regulatory elements necessary for correct expression, regardless of the size of these mammalian elements that may exceed 200 kb, rendering PACs and BACs counterproductive. The only vectors suitable for these purposes are YACs, which can accommodate DNA fragments large enough to cover the whole genomic *loci* and its regulatory elements. The first transgenic mouse was produced in 1980 (Gordon *et al*., 1980) and since that time, many other transgenic species were created including invertebrates (fruit fly, nematode), fishes (zebra fish, carp), various amphibians (like *Xenopus laevis*), chickens, rats, rabbits, dogs, sheep, cattle and pigs. The evolutionarily closest transgenic human relative generated up to now is the primate marmoset *Callithrix jacchus*. Owing to being closely related to human, it is currently the best model for studying human diseases and possible therapies (Sasaki *et al*., 2009). The transgenic technology is not only used for scientific purposes, but it has also many practical applications. Transgenic cows are resistant to bovine spongiform encephalopathy (BSE) due to lack of the prion receptor, transgenic rabbits, goats, pigs and chickens are used for recombinant protein production (like human erythropoietin or von Willebrand factor).

Moreover, transgenically engineered pigs are used as organ donors for xenotransplantations.

The first stage in the generation of these transgenic animals is preparation of YACs carrying the required genes. YACs carrying an insert of interest, and retrofitted with an appropriate mammalian selective marker, can be transferred to mammalian cells by using several different techniques. Purified vector DNA can be introduced by pronuclear microinjection or by lipofection with YACs previously embedded in a lipid solution. The disadvantage here includes physical damage to the large DNA molecules during the purification process and, consequently, a low yield of intact integrated YAC copies and partial transgene integration. To overcome these drawbacks, sophisticated injection methods have been developed (Takahashi and Ueda, 2010), like the intracytoplasmic sperm injection (ICSI) method that was developed to enhance the efficiency of the pronuclear microinjection standard method. Founders with germline transmission of an intact and functional transgene of 250 kb were produced and 35% of live transgenic animals were recovered. In this field of application, the efficiency of DNA transgenesis achieved is often 10 times greater than that usually obtained by standard microinjection, thus enabling to use a reduced number of individuals in animal experimentation (Moreira *et al*., 2006, 2007). Another approach, the fusion of yeast spheroplasts containing YACs with mammalian cells, eliminates the problem of *in vitro* YACs disintegration by virtue of the absence of physical DNA isolation. With this method intact YAC molecules can be transferred into the mammalian cell nucleus. However, in addition to the desired YAC vector, a substantial portion of the yeast genome can also be transferred, although this does not seem to affect either the YAC stability or the ability of the cell to differentiate (Jakobovits *et al*., 1993; Pavan *et al*., 1990). Furthermore, it has been found that mouse ES cells carrying YACs transferred by this technique are able to repopulate mouse tissues and retain the function of human DNA sequences in the developed chimaeric mice. **See also**: Transgenic Mice Production

YACs in the Construction of MACs

YACs were developed following the identification and isolation of the DNA sequence elements essential for chromosome replication and maintenance in yeast cells. Using the same strategy, YACs are being used for isolation of functionally analogous mammalian DNA sequences in order to develop MACs. Mammalian TELs can be isolated from DNA fragments by complementation, using modified YAC vectors containing only one *Tetrahymena* TEL, a yeast CEN and a replication origin (Cross *et al*., 1989). After ligation of the modified vector with the insert DNA, only YACs containing the second arm with mammalian telomeric sequences can transform yeast cells, forming linear minichromosomes. Mammalian CENs and origins of replication are complex genetic elements that cannot be easily defined by sequence, but can only be selected by function. Fortunately, almost all large segments of mammalian DNA are capable of autonomous replication, thus diminishing the need to isolate replication origins. However, short mammalian origins (from 448 to 4300 nt) have been characterised and cloned into YACs promoting the development of HACs. The main difficulty in constructing MACs by using YACs is the isolation and maintenance of the mammalian CEN due to its large size and high instability in the yeast cell (Neil *et al*., 1990). First attempts in construction of α-satellite-based (Grimes and Cooke, 1998) and *Epstein-Barr virus* (*EBv*)-based MACs (Tolmachova *et al*., 1999) showed that the use of the YACs is a promising technique for construction of stable MACs. Human CENs from several chromosomes have been successfully isolated on various YAC vectors utilising the TAR cloning technique, confirming YACs as good starting point for 'bottom up' assembly of MACs (mammalian DNA already cloned into YACs is used to assemble MAC, in facts it is *de novo* assembly of the chromosome) (Kouprina *et al*., 2003). Fully developed MACs using YAC technology were recently reported for pigs as swine artificial chromosomes (SACs) representing novel vectors for swine transgenesis (Poggiali *et al*., 2002). As further evolution technology, a SAC was transferred from swine cells into sheep cells remaining stable and functional up to 52 generations (Cavaliere *et al*., 2009). The experiment resulted in the formation of a *de novo* MAC whose size was determined by atomic force microscopy. The generation of shuttle MACs based on the analysis of centromeric sequence similarities among mammalian species is therefore possible. Many are the applications of MAC vectors. Among them, the significant MAC formation assay has been used to confirm that the recognition sequence of CEN protein B is necessary for *de novo* CEN assembly in humans. **See also**: Centromeres; *Epstein–Barr Virus*; Telomeres

YACs in Synthetic Biology

The capacity of YACs to accommodate large DNA fragments is of extreme use for synthetic biology in the production of secondary metabolites. These are the big group of substances used widely in every branch of modern industry with strong propensity towards pharmaceutics. Some of them are synthesised within modern organic chemistry laboratories but often their synthesis is not possible or not economically efficient. The same problem concerns the isolation of secondary metabolites from living organisms (often the compound is produced very quickly), from specific tissues or under particular conditions that make it very difficult to obtain the product, or that make it very expensive or both. These problems can be efficiently solved by the use of heterologous systems (like bacteria or yeast) for the expression of the desired components. The situation gets more complicated if the compounds are not native for the host organisms and their synthesis requires

complex, multistep biochemical pathways carried out by a large functional groups of enzymes. For this purpose, special expression-enhanced yeast artificial chromosomes are used (eYACs). Biochemical pathway engineering can be done in two different ways. In the direct approach, the final compound and all enzymes involved in its synthesis are known. This approach is based on the direct cloning of target genes into YAC even though they might be derived from many different organisms. Recently, a different strategy was developed, allowing production of compounds never seen before in nature. Instead of directly cloning the desired genes and shaping the final result, the mixture of mRNA from different species and environment are transcribed into cDNAs. Then, individual cDNAs are integrated into a yeast expression cassette and used for random assembly of expression-enhanced artificial chromosomes. In such a way different enzymes are expressed in different configurations creating novel pathways not occurring naturally and consequently producing compounds with unknown features, impossible to produce by synthetic chemistry. This approach opens the completely new, wide and of almost unlimited possibility field of secondary metabolites with not only pharmaceutical potential (Naesby *et al*., 2009). Other systems also exist, allowing expression in mammalian cells without the need of integration of any foreign DNA into the genome (e.g. artificial chromosome engineering system (ACEs)).

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