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*review*

# Mammalian Genome Recombineering: Yeast, Still a Helper Microorganism of Choice?

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**Summary**

Functional studies of complex mammalian genomes have been revolutionized by the development of the recombineering methodology. Recombineering represents the sum of *in vivo* recombinant DNA techniques used for the production and manipulation of targeting vectors by the process of homologous recombination within the host microorganism. Although this methodology had initially been developed in yeast, the term recombineering was coined after successful introduction of similar techniques in bacterial cells. Since then, due to simplicity of amplification, manipulation and purification of mammalian targeting vectors, *Escherichia coli* has become the dominant helper microorganism in functional genomics studies. However, some types of experiments in functional genomics still employ yeast as a unique host for the manipulation of megabase-sized mammalian genomic regions.

*Key words*: yeast, mammalian genome, homologous recombination, recombineering

## Introduction

During the last few years, draft sequences of many mammalian genomes, including the human, have been released and several other sequencing projects are underway. Completion of draft sequences and functional studies of individual components of the sequenced genomes are the next big challenges. Our understanding of newly sequenced regions in mammalian genomes initially comes from alignment with sequences of a known function from less complex eukaryotic, or even prokaryotic, genomes. However, a large number of mammalian coding regions cannot be easily related to the sequences of known genes. Therefore, the real function of many unrelated mammalian DNA sequences has to be revealed from the results of genetic studies in model organisms. The mouse is probably the best mammalian model organism for this type of studies, due to the established sophisticated transgenic systems based on murine embryonic stem (MES) cells and gene targeting (GT) methodology that allow the introduction of precise genetic modifications into the mouse genome (*1–4*). A major limitation of the standard GT methodology in MES cells is the requirement for complex DNA constructs for targeting and selection. The production of these constructs, using common recombinant DNA techniques, involves two major steps: isolation of the desired genomic segment to be modified, following time-consuming construction and screening of a genomic library, and subsequent introduction of a desired genetic modification within the isolated genomic segment using restriction enzymes as a major tool (Fig. 1a). This approach is limited by the requirement for a specific cleavage site in both cloning vector and genomic DNA, leaving little possibility to design the desired targeting vector. Additionally, the gene targeting frequency and the size of the genome modification are directly influenced by the low accommodation capacity of the conventionally used cloning vectors. Due to these methodological problems, the production of GT vectors has represented, for a long time, a major bottleneck for many functional genomics experiments.

The development of new techniques that make use of homologous recombination (HR) to construct targeting vectors (**recombi**nation-mediated genetic engi**neering**

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termed **recombineering**, Fig. 1b) has overcome many limiting steps of the techniques previously employed. The yeast *Saccharomyces cerevisiae*, with impressively high frequency of homologous recombination, is the microorganism of choice for the production of recombinant targeting constructs using the recombineering approach (*5,6*). Due to the inherent yeast recombination machinery, it is possible to clone any desired piece of genomic DNA, using a technique known as transformation-associated recombination (TAR) cloning. This cloning technique significantly reduces the time and effort needed for the isolation of a genomic segment from genomic libraries. Once isolated, it is possible to introduce virtually any genetic change and selective marker within a future targeting construct in yeast, using PCR amplified cassettes sharing very short homology with the genomic segment. Another and perhaps the most significant advancement that yeast recombineering can offer is the accommodation of large genomic segments in yeast artificial chromosomes (YACs).

Although the use of YAC-based vectors enabled numerous functional studies of complex megabase-sized mammalian genomic sequences (*7*), difficulties in purification, frequent chimaerism of genomic segments and

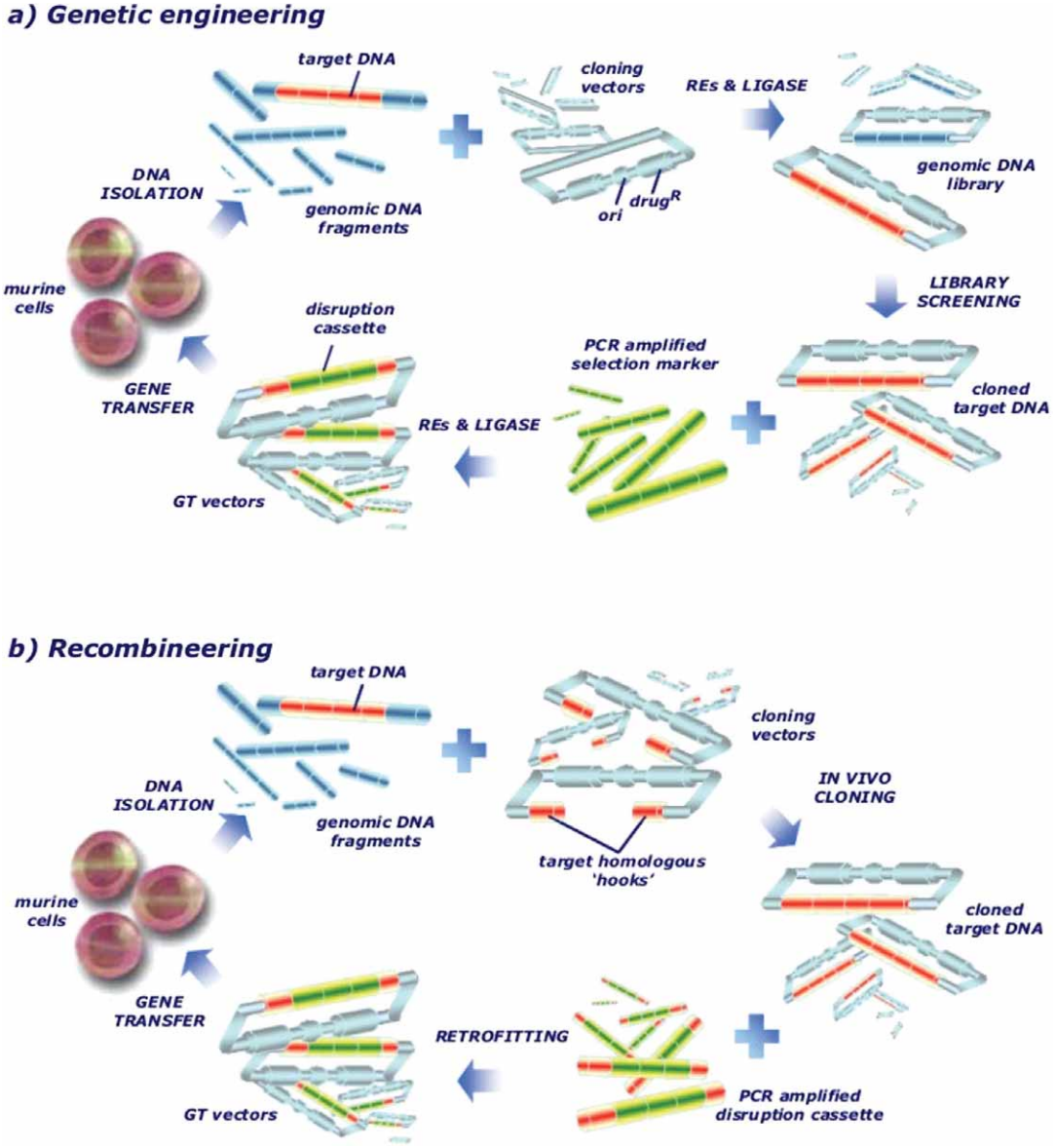


**Fig. 1.** Comparison of recombinant DNA techniques *in vitro* (genetic engineering) and *in vivo* (recombineering) for the production of murine gene targeting (GT) vectors: a) conventional genetic engineering technology involves time-consuming preparation and screening of the genomic library, resulting in isolation of the target DNA segment, followed by its modification using restriction enzymes (RE) and ligases in order to obtain the final targeting construct. Both the flanking regions of the genomic segment and the position of the selective marker are strictly defined by the position of the appropriate RE sites within the genomic DNA; b) recombineering methodology allows quick one-step isolation of the desired genomic DNA segment relying on recombination between short homologies present in the cloning vector and genomic DNA. The same principle is subsequently used for the insertion of a selective marker, resulting in a targeting vector carrying a genomic segment modified at virtually any position ($drug^R$ – selective marker comprising the resistance to certain antibiotic; *ori* – origin of replication)

their low stability made handling of YAC vectors quite complicated. Construction of equivalent vectors, bacterial artificial chromosomes (BACs) and P1 artificial chromosomes (PACs) in the bacterium *Escherichia coli*, solved the problems encountered previously with YACs. However, due to the low efficiency of HR in the bacterial host, modification of these vectors involved mostly standard recombinant DNA techniques. Recently, recombineering techniques have also been developed in *E. coli* using phage-encoded, instead of endogenous recombination proteins, making the bacterial cell as competent in HR as yeast and the microorganism of choice for the construction of targeting vectors. In this review, the advantages and disadvantages of recombineering in both microorganisms will be compared, with major focus on the yeast *S. cerevisiae* and its ability to still be considered a valuable tool for mammalian genome engineering (for more on the bacterial recombineering topic see reviews *8*,*9*).

## Basic Recombination Studies in Yeast as a Starting Tool for Recombineering

Fundamentals of cloning techniques based on homologous recombination were established in the early 1980s by Orr-Weaver *et al.* (*10*) while studying recombination using transformation in yeast as a model system. Previous experiments evidenced that, when the transforming DNA was a non-replicative hybrid plasmid, transformation could occur only by plasmid integration into yeast chromosomes *via* homologous recombination (*11*,*12*). Whereas intact circular plasmids display low frequency of integration by a single reciprocal crossover, plasmids linearized within a region of homology to yeast chromosomal DNA are integrated into the homologous chromosomal site at much higher frequencies. More surprisingly, plasmids containing a double-strand gap within sequences homologous to the yeast genome integrate with high efficiency by crossover. During the process of integration, the double-strand gap is repaired *via* homologous recombination using chromosomal information as a template. The final structure is identical to that obtained from the integration of a circular DNA molecule (*10*).

A step further was achieved using plasmids containing a yeast origin of replication (also referred to as autonomously replicating sequence, or ARS) that can produce transformants both with and without integrating into chromosomal DNA, thus rendering both crossover and non-crossover products. When the transforming DNA is a gapped replicative plasmid, approximately equal numbers of integrated and non-integrated plasmids are recovered, following the correct repair of a double-strand gap. Since recombinational repair of a double-strand gap involves transfer of genetic information from one DNA molecule to another, non-crossover events result in non-reciprocal transfer or conversion of chromosomal information onto extra-chromosomal plasmids (*13*–*15*).

These observations subsequently led to the development of a one-step gene disruption methodology in yeast by Rothstein in 1983 (*16*). Complete deletion of a corresponding chromosomal gene (null mutation) is performed by gene replacement, such that a marker gene replaces the deleted sequence. Originally, DNA fragments flanking the gene of interest are cloned on both sides of a yeast marker gene and, upon transformation, homologous recombination between the flanking regions results in deletion of the gene of interest and simultaneous integration of the marker gene. The method has undergone many improvements since. A breakthrough was accomplished when Baudin *et al.* (*17*) demonstrated that a PCR-amplified marker gene, flanked by short stretches of genomic sequence, was sufficient to target homologous integration. The minimum amount of homology required was shown to be 30 bp on each side of a selectable marker (*18*). These findings resulted in the development of a PCR-mediated technique for gene disruption in *S. cerevisiae*, facilitated by the design of high fidelity DNA polymerases (*19*). Constructs for gene manipulation, thus, can be generated rapidly and accurately by PCR, without previous time-consuming construction of plasmid clones containing the gene of interest (*17*,*20*). The system was further improved by using a heterologous marker gene in order to avoid problems of gene conversion associated with the use of yeast endogenous marker genes and recipient yeast strains not completely devoid of the marker gene (*21*). The most extensively used heterologous marker is the dominant resistance module *kanMX*. It contains the *kanr* open reading frame of the *E. coli* transposon *Tn*903 (*22*), fused to the *TEF* promotor and terminator sequences from the filamentous fungus *Ashbya gossypii* (*23*), and permits efficient selection of transformants resistant against the antibiotic geneticin (G418) (*24*,*25*). Since the number of marker genes is limited, marker rescue is an essential requirement in the construction of yeast strains with multiple gene disruptions. A high occurrence of mitotic recombination between non-tandem direct repeats in yeast was used to develop various marker recycling procedures, all based on homologous or site-specific recombination between homologous sequences flanking the marker gene, such as *Salmonella hisG* (*26*), *S. cerevisiae FRT* (*27*) and *loxP* (target sites for Cre recombinase of phage P1) (*28*,*29*). After gene disruption, recombination between the two repeats results in marker removal, leaving behind a single repeat at the deleted gene locus. Alternatively, marker recycling procedures based on religation of DNA ends, such as I-*Sce*I-induced popping-out (*30*), have been developed. Besides considerably higher efficiency compared to other marker recycling approaches, another advantage of the I-*Sce*I pop-out system is the substantially shorter sequence (50 bp) that is left behind after popping-out, which contrary to recombinase-induced popping-out does not contain an active I-*Sce*I site. However, genomic copies of a repeat, as well as any other heterologous sequence, can cause increased mistargeting of constructs containing the same sequence, or unexpected chromosomal rearrangements *via* intra- or interchromosomal recombination. Hence, much effort has been made to accomplish marker removal, such that no heterologous material is retained. A novel PCR-mediated gene deletion and marker recycling system, designated seamless gene deletion, has recently been introduced for use in *S. cerevisiae* (*31*). A 40-bp sequence, derived from a region adjacent to the targeted locus, is

placed in an integrating construct to generate direct repeats on both sides of the targeted gene. Thus, after integration and subsequent excision of the marker gene, both *via* homologous recombination, no foreign or additional sequences are left in the genome. Another possible approach for marker recycling is a two-step cloning-free process based on transformation of yeast by oligonucleotides, referred to as *delitto perfetto* (idiom used to represent 'perfect deletion'). The first step involves integration of a counterselectable reporter (CORE) cassette, followed by a transformation step with specifically designed oligonucleotides that eliminate the CORE cassette. This strategy generates products having only the desired modification, such as single or multiple base change, an insertion, a small or large deletion, or even random mutations, whereas foreign DNA disappears without a trace (*32*).

## *In vivo* Plasmid Vector Construction in Yeast

Homologous recombination between overlapping DNA fragments in yeast has not only been used for the introduction of various modifications into genomic DNA, but it has also been a very valuable tool in plasmid DNA manipulation. It all began with the pioneering work of several research groups in the late 1980s, who demonstrated that a double-strand DNA break in a vector could be repaired by co-transformation with a linear DNA fragment containing sequences that flank the break (*33*,*34*). This finding served as the foundation for a wide variety of yeast-based DNA cloning and manipulation methods. Ma *et al.* (*35*) developed a convenient and simple method for the construction of new plasmids of desired structure. In order to obtain a recombinant circular plasmid, they used yeast co-transformation with a linearized plasmid and a DNA restriction fragment of appropriate homology to serve as a substrate for recombinational repair. Conveniently, this procedure does not require homology of the free ends of the involved DNA molecules for efficient recombination between internal homologous regions. The method has been particularly useful for the incorporation or removal of selectable markers, centromeres and replication elements, and has served for the construction of an extended series of yeast centromere, episomal and replicating (YCp, YEp, and YRp) plasmids. Analogous to improvements in the gene disruption methodology, the technique for plasmid construction *via* homologous recombination in yeast was further simplified by the use of PCR-amplified oligonuceotides with 30–40 bp of homology to a linearized yeast plasmid. During co-transformation into yeast, homologous recombination occurs at a position directed by the PCR oligonucleotide, rendering the desired plasmid construct (*36*).

Thus, a decade after the first manipulations of endogenous and/or exogenous DNA *via* homologous recombination in yeast were performed, virtually any modification of genomic DNA and plasmid vectors could easily and accurately be accomplished by means of an available technique (Fig. 2).

## Transformation-Associated Recombination (TAR) Cloning

At about the same time, pursuing the requirements of functional genomics, two research groups were investigating the possibility of isolating large chromosomal regions directly from genomic DNA, another implementation of recombinational cloning in *S. cerevisiae* (*37*,*38*). Previously, several vectors for cloning large DNA fragments had been designed (plasmids, phages and cosmids), with Yeast Artificial Chromosomes (YACs) (*39*,*40*) being an ultimate vehicle of choice for cloning large fragments of DNA. Results of Ketner *et al.* (*41*), who managed to recover the adenovirus genome as a YAC, suggested that it was possible to recover even single copy genes by recombinational targeting. Following that finding, two groups in collaboration, led by Michael A. Resnick and Vladimir Larionov, developed a new technology, transformation-associated recombination (TAR) cloning (Fig. 3), employing a process similar to that of gap repair during homologous recombination (*38*). TAR cloning enables selective, accurate and quick isolation of entire genes or large chromosomal regions from complex

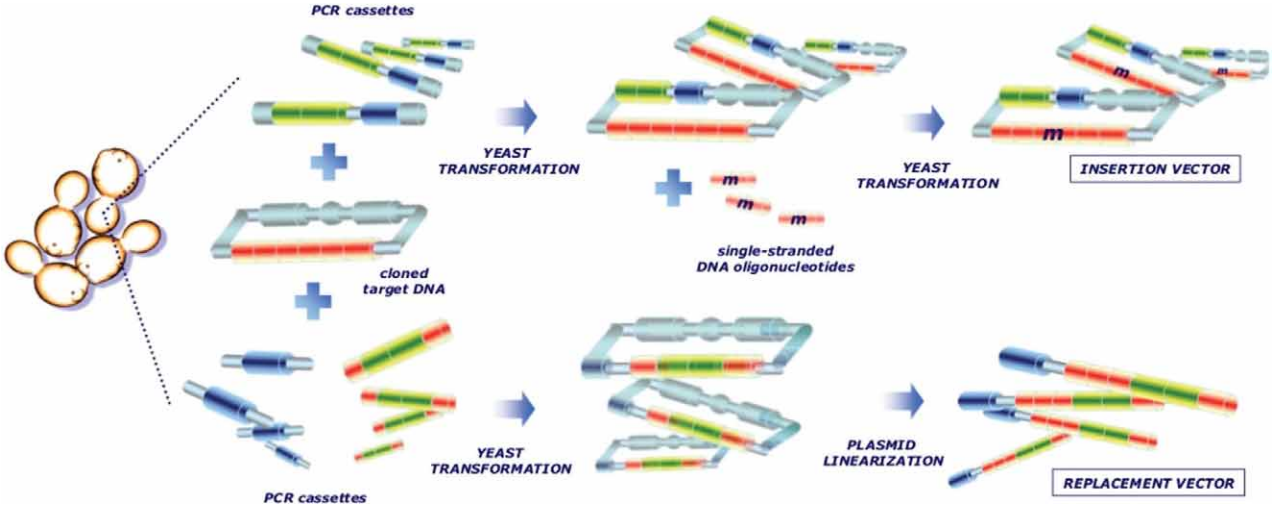


**Fig. 2**. Production of mammalian targeting vectors in yeast using homologous recombination

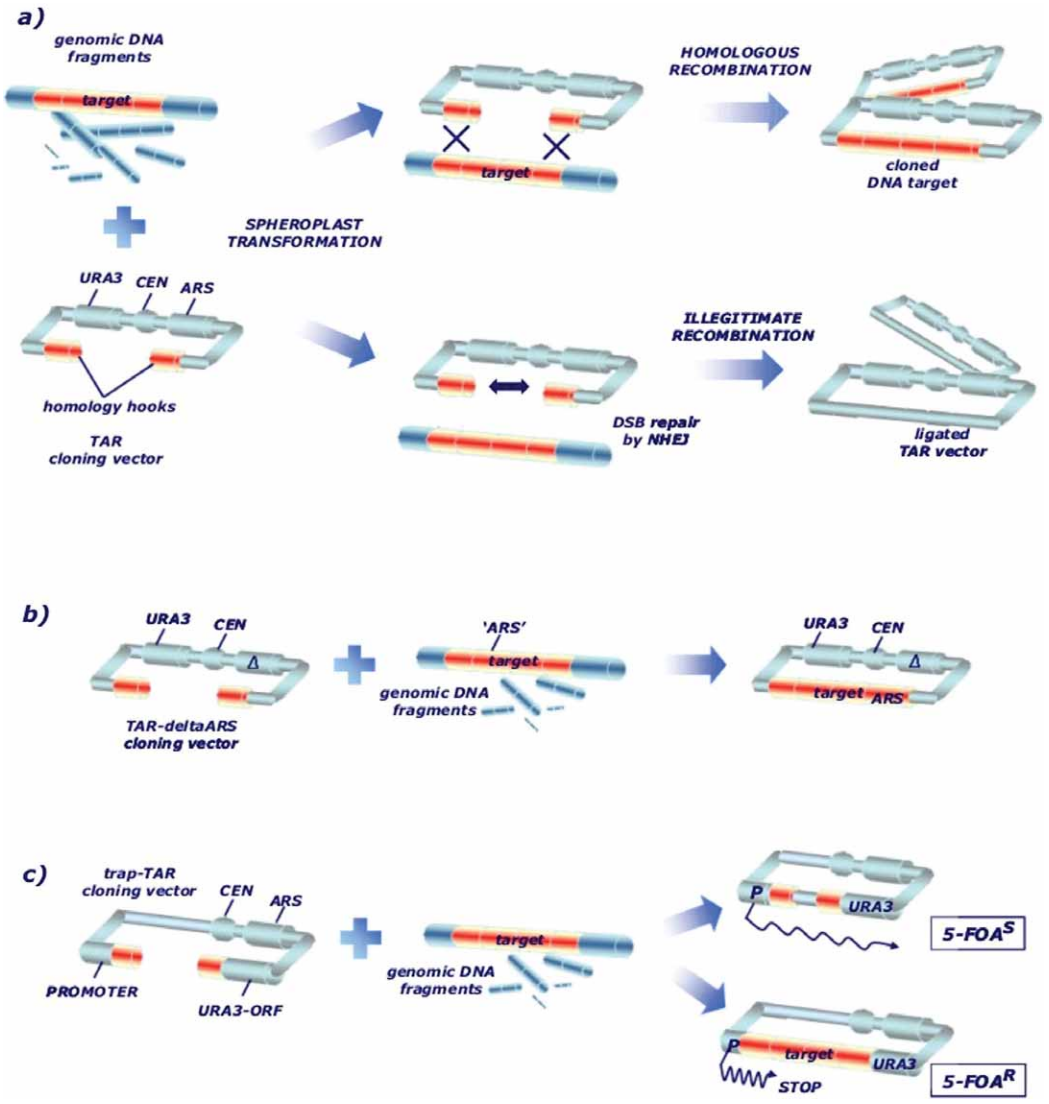


**Fig. 3.** Transformation-associated recombination (TAR) cloning: a) co-transformation of yeast spheroplasts with genomic DNA and a TAR cloning vector, containing a yeast selective marker (URA3), a centromere (CEN) and an autonomously replicating sequence (ARS), can result in two types of recombinant products: a vector containing the targeting sequence cloned, as a consequence of the DSB repair by HR, or a ligated vector after NHEJ. In order to increase the frequency of the HR event, two approaches have been developed: b) by using TAR vectors without the ARS which give transformants only in case of an HR event with a mammalian genomic segment containing an ARS-like DNA sequence, or c) by positioning the hooks between upstream activation sequences and the ORF of a negative selection marker, such as URA3, resulting in positive TAR clones only after insertion of a genomic segment (the NHEJ event would result in the 5FOA sensitive clones)

genomes without the need for previous construction of a genomic library. Thus, it minimizes standard *in vitro* manipulation of cellular DNA prior to cloning, which can lead to breaks and nicks, contributing to the assembly of aberrant DNA molecules (*42*,*43*). The technique is based on *in vivo* homologous recombination, during yeast spheroplast co-transformation, between genomic DNA and a linearized TAR vector. The vector contains two targeting 'hooks', homologous to 5′- and 3′-sequences flanking the region of interest, which can be as small as 60 bp (*44*) (selectivity of cloning decreases significantly when the homology is shorter), a yeast centromere and a selectable marker. Recombination between the linearized vector and genomic DNA fragments results in formation of circular yeast artificial chromosomes (YACs) that can replicate and segregate in yeast. Equivalently, the use of two linearized vectors, one containing only a telomere and the other a centromere and a telomere, results in the assembly of a linear YAC. Besides the difference in the rate of the desired recombinational event, in favour of the linearized vector (circular TAR cloning is a bimolecular event, whereas in linear TAR cloning the reaction is trimolecular), circular YACs have several advantages over their linear counterparts. Most prominent is the simplicity of isolation, as they can be separated from linear yeast chromosomes by standard alkaline lysis techniques (*45*), or simply by pulsed-field gel electrophoresis (PFGE), since large open-circular molecules are trapped in the starting well. Circular YACs are more resistant to shear stress than linear YACs, and can be easily manipulated. Furthermore, they can be easily modified by homologous recombination into BACs and transferred into *E. coli* cells, simplifying DNA isolation for further physical and functional analyses. Moreover,

it has been shown that circular YACs exhibit structural and segregational stability comparable to that of linear YAC molecules (*46*). The quality of genomic DNA is critical for the efficiency of the method. Preparation of high molecular mass DNA in agarose plugs is an option, as it prevents genomic DNA from shearing (90 % of genomic DNA consists of fragments greater than 1 Mb), but at the same time agarose fragments inhibit spheroplast transformation. Therefore, DNA preparation in aqueous solution is preferable. Also, the yield of the transformants increases with decreasing size of genomic DNA fragments, possibly due to more efficient penetration of smaller fragments into the spheroplasts, with an optimal range between 300 and 500 kb for cloning fragments greater than 200 kb (*47*). So far, DNA fragments up to 600 kb have been non-selectively isolated in yeast using common genomic repeats (such as LINEs and SINEs) as targeting hooks (*38,46*), whereas under optimized conditions, specific chromosomal segments up to 250 kb can be isolated with high selectivity (*48*).

### *Improvements of the TAR cloning methodology*

The TAR cloning methodology has undergone many improvements over the last few years, and has become a very powerful tool in the post-genomic era. The initial low efficiency of transformation was significantly increased using the highly transformable *S. cerevisiae* strain VL6-48N, generated especially for the purpose of TAR cloning (*49*). A distinctive feature of this strain is the deletions of various endogenous genes coding for yeast auxotrophic markers, which eliminate the occurrence of false positive transformants due to spontaneous mutations or homologous recombination between the plasmid bearing the marker gene and its equivalent genomic locus. Thus, vectors with multiple yeast genetic markers can be efficiently used.

Even so, one of the major technical problems was the low frequency (~0.5 %) at which the desired region was obtained, especially in the case of single-copy gene isolation (*50,51*). The finding that TAR vectors recombine preferentially with homologous sequences at the ends of the targeted genomic fragment was critical for the improvement of TAR cloning protocols (*44*). The frequency of a desired event increases approximately 20 times by introducing double-strand breaks (DSB) near the targeted regions. This can be achieved either by treating genomic DNA with a specific endonuclease prior to transformation, or by introducing specific DSBs *via* RecA-assisted restriction endonuclease (RARE) cleavage, a technique that allows selective cleavage of a DNA molecule at a single, predetermined restriction site (*52,53*). Limitations for wide applicability of the original method were posed by two imperatives in TAR vector construction – two specific targeting sequences that function as 'hooks' and lack of an ARS element (Fig. 2b).

Although quite a few draft sequences have been released until today, in many cases there is only limited sequence information available, such as a 3′-end specific sequence or a sequence tagged sites (STS). STSs are short (200 to 500 bp) DNA sequences at 3′-ends of most human genes with a single occurrence in the human genome, and serve as landmarks in genome mapping. Approximately one STS has been identified per 100 kb of the human genome. Based on this fact, a modified version of the TAR cloning method, radial TAR cloning (*54*), has been developed using a vector with a single specific gene sequence as one of the hooks, while the other hook is a common repeated sequence such as *Alu* and *B1* for human and mouse DNA, respectively. The repeated elements enable isolation of a set of nested overlapping fragments that extend from the specific hook to different homologous upstream or downstream positions. In contrast to TAR cloning with two specific hooks, radial TAR cloning can produce chimaeras (*55*). This cloning artefact occurs due to the presence of multiple homologies for the common repeat hook in the genomic DNA, but does not represent a hindrance, since chimaeric clones can be identified by sequencing the ends of the cloned inserts and thus efficiently eliminated. The use of vectors lacking an ARS element allows screening of TAR cloning events against a high vector background (clones without an insert), but at the same time limits the TAR cloning methodology to isolation of DNA fragments containing at least one ARS or ARS-like sequence. ARS-like elements are short AT-rich sequences (~50 bp) containing a non-conserved 17-bp core consensus sequence (*56*). Although sequences that function as ARSs in yeast are abundant in all eukaryotic genomes and occur on the average every 20 to 40 kb (*57*), in chromosomal regions with multiple repetitive elements, such as heterochromatin blocks, telomeres and the centromere, ARS frequency might be reduced. To clone specifically the regions lacking ARS-like elements, a modified TAR cloning method has been developed using a TAR vector with an inserted ARS, and URA3 as a counter-selectable marker (*58*, Fig. 3c). The hooks are placed between the promoter derived from the *Schizosaccharomyces pombe ADH1* gene and the open reading frame of the *URA3* gene. The used promoter exhibits specific spacing requirements for its function such that the permissive distance between the TATA box and the transcription initiation site is at most 130 bp, which correlates with two hooks 60 bp in size. In this way, insertion of any sequence between the hooks results in the initiation of transcription at an alternative site, and thereby inactivation of *URA3* expression which confers resistance to 5-fluoroortic acid. Thus, negative genetic selection allows screening of TAR cloning events against high background vector recircularization by the process of end-joining. Circularization of linear plasmid DNA by ligation was observed already in early transformation experiments in yeast (*11*), implicating the existence of an additional repair pathway for DNA double-strand breaks, independent of recombination. Today it is known that non-homologous end-joining (NHEJ) is a critical mechanism for DNA repair and has been highly conserved throughout eukaryotic evolution (*59*). Disruption of the yeast genes *YKU70*, *YKU80* or *LIG4*, coding for major components of NHEJ – Yku70p, Yku80p and Lig4p, respectively, drastically reduces repair of plasmids bearing 5′- or 3′- loose ends, while residual repair in these strains is inaccurate (*60,61*). Thus, one way of avoiding high background, when using TAR vectors containing ARS, could be the use of mutant yeast strains with defective NHEJ.

### *YAC stability and propagation in yeast*

Once the desired YAC clone has been obtained, there is still the issue of stability of such large fragments

of heterologous DNA in *S. cerevisiae*. There is evidence that YAC clones containing mammalian DNA may be unstable and undergo deletions or rearrangements during mitotic propagation in yeast. Such events are most likely promoted by homologous recombination (HR) between large amounts of repeated sequences within mammalian DNA during the cloning process and/or mitotic propagation (*62–64*). Genes specifically associated with HR in mitotic cells include *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59* and *RPA* (*65–68*). Therefore, since the early 1990s, several groups have been investigating the integrity of YACs in yeast strains proficient or deficient in DNA repair/recombination. However, the results of those studies are not consistent, as different groups report contrary data. There is evidence, based on several independent studies, that the frequency of transformation-associated alterations and mitotic instability of YACs, containing heterologuous DNA, is significantly reduced in *rad52* mutant strains (*69,70*). Yet, Kohno *et al.* (*71*) report even greater stability of a number of loci in *rad51rad52* double mutants in comparison with *rad51* and *rad52* single mutants. However, mutations in *RAD52*, initially characterized as causing a defect in the recombinational repair of DSBs (*72*), also result in a variety of defects, including strong reduction of various types of induced and spontaneous recombination (*13,73, 74*). These pleiotropic effects account for a reduced growth rate and transformability of *rad52* mutants compared to wild type (WT) strains, as well as an inability to manipulate YACs *in vivo* due to the lost recombination function of the host and somewhat elevated rates of chromosome loss and mutation. Moreover, as the *RAD52* gene product is essential for meiotic recombination, the homozygous *rad52* null mutant is able to go through sporulation, but inviable spores are produced (*74*). Since manipulation of yeast often involves mating and sporulation (meiosis) as a method of altering the genetic background, introduction of a *rad52* null mutation into YAC hosts might preclude the use of standard genetic techniques for manipulation of YAC clones. Based on these observations, a system that accomplishes YAC stabilization and provides an opportunity for additional YAC modification by recombination *in vivo* has been developed. Transfer of YACs from WT strains into strains with a conditional *RAD52* gene is performed by *kar1* mating (*75*). This technique takes advantage of the properties of *kar1* mutant strains – if one of the mating yeast cells carries a mutation in the *KAR1* gene, nuclear fusion fails (*76*). Most of the daughter cells formed by budding from the heterokaryon (zygote containing two nonfused parental nuclei) receive one of the haploid chromosomal sets, generating haploid cells with a mixed cytoplasm. However, transfer of natural yeast chromosomes, as well as YACs, from one haploid nucleus to another during mitosis has been demonstrated to occur at low frequency (*77*). Thus, *kar1* mating eliminates the need for crosses and sporulation or, alternatively, for the isolation and retrotransformation of YACs into new hosts.

Alternatively, Le and Dobson (*78*) suggested that yeast strains with mutations in *RAD54*, one of the members of the *RAD52* epistasis group, might represent improved YAC cloning hosts. Indeed, there is evidence that for some sequences, with respect to faithful maintenance of the DNA, a *rad54* strain might represent a better cloning host than a *rad52* strain. In an attempt to develop a system for stabilization of YAC clones, a novel *rad54-3* strain with a temperature-sensitive (ts) conditional mutation for double-strand break repair has been developed. Ts mutants have several unique advantages over other inducible systems, including fast temporal response, high reversibility, and the applicability to any developmental stage of an organism. The temperature-sensitive *rad54-3* allele blocks mitotic recombination between tandemly repeated sequences and significantly stabilizes YAC clones. In addition, yeast carrying the *rad54-3* mutation has growth and transformation rates comparable with WT strains, and more importantly, can undergo normal meiosis after mating with a *rad54* null. The original YAC, present in the *rad54-3* strain used in these crosses, can be recovered afterwards, unaltered in most products of the cross. Thus, the *rad54-3* mutant is unique in allowing all standard genetic manipulations, previously implemented in *RAD+* yeast hosts, with the advantage of a reduced level of homologous recombination, which makes it an improved YAC cloning host. In addition, it may allow cloning of regions that have been unclonable in other systems, since *rad54-3* mutants can maintain more stably tandem arrays of DNA repeats.

### *YAC modifications and delivery into mammalian cells*

Like any other technology, TAR cloning has some limitations that have to be taken into account. Still, it has become a very valuable tool, enabling, with comparable accuracy to PCR methods, the rescue of large chromosomal regions in yeast as YACs within only 2 weeks. Furthermore, known mutations and new modifications, including point mutations, deletions and insertions, can easily be introduced into the cloned DNA fragments hundreds of kilobases in size (Fig. 4). Precise alterations in both the coding and regulatory regions are essential to understand the role of specific sequences in gene function. The modified DNA can then be tested functionally in mammalian cells. To investigate the expression and fate of YACs, it is necessary to establish them stably in recipient cells. In order to promote their stable retention in mammalian cells by selection, modification of YACs by incorporation of appropriate markers, a process called 'retrofitting', is indispensable. Standard techniques of gene disruption and insertion allow the design of YACs with specific markers by recombinant DNA manipulation. Over the years, series of different plasmid vectors for efficient YAC 'retrofitting', containing various marker gene combinations, have been designed. Yeast integration vectors with dominant selectable marker genes allow targeted integration into left (centromeric) and right (non-centromeric) YAC arms, as well as into mammalian-derived inserted DNA, and provide a simple system for YAC retrofitting. Among the first markers used for selection in mammalian cells were the herpes simplex virus thymidine kinase (TK) gene (*79,80*) and the mammalian hypoxanthine-guanine phosphoribosyltransferase (HPRT) (*81,82*), strictly to be used in cell lines deicient in the respective genes and cultured in hypoxanthine/aminopterin/thymidine (HAT) medium (*83*). A more widely used selectable marker is the bacterial re-

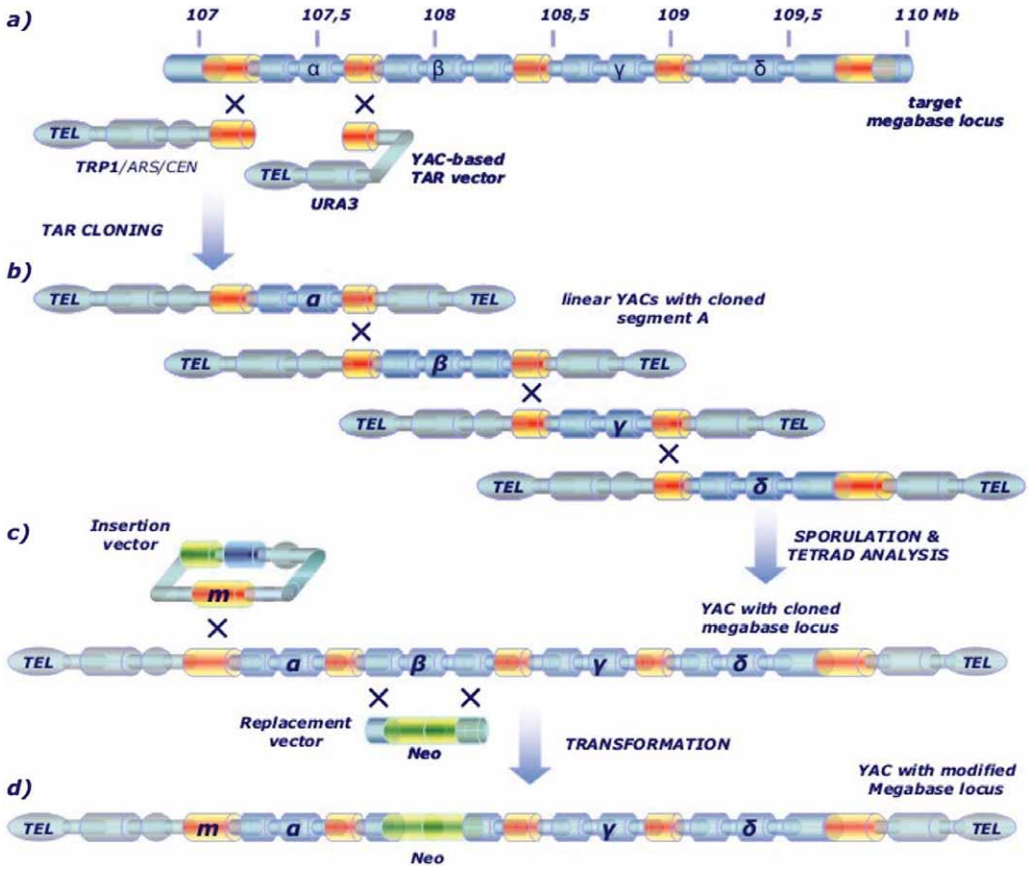


**Fig. 4**. YAC recombineering in yeast: TAR cloning of several mammalian genomic segments (α, β, γ, and δ), which can be assembled into a single YAC vector, after several meiotic recombination rounds, due to the overlapping homologous regions (a,b), and modified by introduction of site-specific mutations or a marker insertion at a desired site by transformation of a YAC-carrying strain with adequate insertion or replacement vectors (c,d)

sistance gene of transposon Tn5, encoding the enzyme neomycin phosphotransferase II (neo), which gives resistance to various aminoglycoside antibiotics, including neomycin, kanamycin, paromomycin and geneticin (G418), and permits selection of stable YAC clones when introduced into mammalian cells (*84,85*).

At the very beginning of the 1990s, a number of different approaches were developed for an efficient introduction of YACs carrying large fragments of DNA into mammalian somatic cell lines, including calcium phosphate coprecipitation (*86*), electroporation (*87*), lipofection (*88*), spheroplast fusion (*89*), and microinjection (*90*). The results of several groups suggest that virtually any gene could be transferred into mammalian cells *via* YACs and expressed at a level comparable to the corresponding endogenous gene (*88,91,92*). However, it was becoming increasingly obvious that, when compared to somatic cell cultures, transgenic mice were a substantially better system for analysing gene function and regulation *in vivo*. In 1993, within a week of one another, three groups working independently reported their pioneer work on generation of transgenic mice with YACs. Remarkably, three different techniques were described for the delivery of YAC DNA into mammalian cells: pronuclear microinjection of gel-purified YAC DNA (*93*), lipofection of YAC DNA into embryonic stem (ES) cells (*94*), and yeast spheroplast fusion with ES cells (*95*).

Although introduced more than a decade ago, the delivery of YACs into the germline cells by conventional, pronuclear (PN) microinjection, while feasible, remains often troublesome. It is likely to correlate with the purity and integrity of YAC DNA isolates, as well as the limited number of molecules that are injected due to physical constraints of extremely large DNA molecules. Most importantly, even though standard YAC DNA isolation techniques, appropriately modified for high molecular mass DNA, allow efficient isolation of intact DNA molecules, there is a high possibility of subsequent mechanical shearing during handling in the PN microinjection procedure. To overcome this problem, a similar approach, using the intracytoplasmic sperm injection (ICSI) technique (*96*), has recently been employed to introduce large DNA constructs into mouse germline cells (*97*). It has been shown that this method is definitely more efficient in producing stable incorporation into the host genome and correct phenotypic expression of large DNA constructs. Even so, the specialized equipment and expertise required for microinjection techniques are often replaced by mass delivery techniques, such as lipofection of ES cells.

Introduction of YACs into the germline of mice *via* ES cells (*94,95*), with subsequent generation of chimaeric mice, albeit more difficult and time-consuming than the use of germline cells themselves, offers the possibility for

structural and functional characterization of YAC transgenes prior to the generation of transgenic mice (*98,99*). However, variability in transfer efficiency and high frequency of deletions and rearrangements in inserted YAC DNA have been the major obstacles in using lipofection as a technique of choice. Along with appropriate modifications of the procedure, the low percentage of clones containing intact DNA after lipofection in initial experiments was significantly increased by the use of agents such as polyethyleneimine (PEI). PEI neutralizes the negative charge of DNA giving rise to more compact molecules, and thus protects YAC DNA from breakage during isolation (*100,101*). In addition, it is capable of destabilizing the endosome at low pH, thereby protecting YAC DNA from degradation upon entering the host cell, resulting in 100 % of clones containing intact DNA among positive transfectants (*101*).

Alternatively, yeast spheroplast fusion with ES cells provides unlimited possibilities for introduction of chromosomal fragments several megabases in size into mammalian cells, and results in high percentage of ES cells containing intact integrated YAC DNA. A major advantage of this approach is the possibility to bypass the laborious procedure of isolation of large quantities of purified high molecular mass DNA. Yeast spheroplast fusion with ES cells was so far successfully employed to generate mice without apparent adverse effects, making this approach a preferable choice for the generation of transgenic animals. However, a substantial disadvantage of this method is the effective co-transfer and subsequent co-integration of variable and uncontrolled amounts of the yeast genome into mammalian cells (*95,98,99*), with the possibility of interference with complex expression patterns of higher eukaryotes, which still remains to be elucidated. When successfully transferred into mammalian cells, the stability of YAC DNA is usually maintained by integration into the chromosomal DNA, rather than by extrachromosomal propagation (*85,89*).

Before carrying out subsequent experiments, it is crucial to evaluate the integrity, organization and copy number of YAC transgenes inserted into the host genome. The presence and integrity of YAC DNA in the mammalian genome are initially assessed by an extensive PCR screen with a set of primer pairs specific for both left and right YAC-vector arms, as well as internal sequences (if no sequence data is available, STS sequence information is used). Further on, detailed genome mapping is performed by digestion of total DNA with rare cutting restriction enzymes, followed by pulsed-field gel electrophoresis (PFGE) and subsequent Southern blot analysis. A more sophisticated and less laborious approach for mapping integrated YAC DNA is provided by high resolution chromatin/DNA fiber fluorescence *in situ* hybridization (fiber-FISH) (*102,103*), a powerful, direct and sensitive technique with a wide resolution range. Fiber-FISH enables visualization of labelled DNA sequences, as little as 1 kb apart, on decondensed ('naked') DNA fibers. This method has not only been useful for determining the size and structure of foreign insertions in the mammalian genome, but also for determining the impact of integration on the high-order structure of the host chromosome. It has been shown that most stably transfected cell lines carry single or few (<5) copies of intact integrated YAC transgenes, while the presence of multiple copies, albeit possible, is uncommon.

### *TAR cloning applications*

All these findings contributed to the development of a wide variety of applications for the TAR cloning methodology. Over the years, TAR cloning has been used to construct both random and specific DNA libraries, as well as to isolate unique regions and full-size genes from genomes of humans, primates, mouse and other organisms. The methodology greatly simplifies the assembly of chromosome- and subchromosome-specific libraries (*38,46,104*), substituting labour-intensive and time-consuming methods, such as chromosome sorting by flow cytometry prior to cloning, or random cloning of genomic DNA from hybrid cell lines. The construction of such libraries is a valuable tool both for the recovery of disease genes identified by the radiation hybrid technique (*105,106*), as well as sequencing of new genomes. Another contribution of the TAR cloning methodology in sequencing projects is the assistance in the recovery of gaps on existing chromosomal sequences (*55,107*). Most of the draft genome sequences available at the present moment consist of stretches of contigs separated by gaps or regions in which the exact sequences are not known (*108*). Those gaps most probably contain chromosomal regions that are either toxic, unstable, or not efficiently clonable in *E. coli* libraries used for DNA sequencing. Approximately 6 % of such human DNA sequences have been efficiently cloned and propagated in yeast using TAR generated YACs (*107*).

Yet, one of the most outstanding contributions of the TAR cloning methodology, with respect to the needs of modern functional genomics, is the capacity to specifically isolate complex genomic loci, containing full-size single-copy genes with all endogenous intronic sequences and regulatory regions. TAR cloning is also highly efficient in the isolation of gene homologues, synthenic regions and entire gene families (*109,110*) since up to 15 % of sequence divergence does not prevent recombination in yeast (*111*). Physical analysis of the so far isolated genomic loci, including human HPRT (60 kb), hTERT (60 kb), ASPM (70 kb), SPANX-C (83 kb), BRCA1 (84 kb), BRCA2 (90 kb), PTEN (120 kb) and KA11 (200 kb), among others, has demonstrated high fidelity with respect to the DNA sequence, which is of exceptional importance for structural and functional analysis.

Still, the problem of full length gene delivery into human cells remains. Many types of viral and episomal shuttle vectors, which do not cause insertional mutagenesis or silencing in host chromosomes, have been developed for gene delivery and expression in mammalian cells. However, viral vector systems are considerably limited by possible silencing of transgene expression, significant cytopathology at high multiplicities of infection and the risk to trigger a severe immune response in the host. Thus, such systems are only partially satisfactory both for gene therapy and animal biotechnology. At present, the most promising vector system, with a potential therapeutic application, involves mammalian artificial chromosomes (MACs). So far several MACs have been designed. Human artificial chromosomes (HACs) can accommodate large regions of mammalian DNA, they

are stably maintained at low copy number in the host nucleus, and do not contain any viral genes that could elicit an immunogenic response. In addition to providing stability, the chromatin context of HACs ensures authentic regulation of expression patterns of mammalian genes. Several studies have so far demonstrated the efficiency of HACs as a delivery vehicle (*112*,*113*). The strategies for the design of HACs represent a classical case of learning by doing, and can be generally divided in two groups: 'top down' and 'bottom up'. The 'top down' approach is based on truncation of a human chromosome using targeting vectors containing telomeric sequences in order to obtain a substantially smaller, so called, minichromosome (*114–116*), whereas in the 'bottom up' strategy the cloned chromosomal elements are assembled *in vitro* into a unit that can initiate *de novo* formation of a HAC in human cells (*117–119*). The second approach, although more attractive because it is not restricted to a specific cell line with a truncated chromosome, is still to be optimized. Progress in the development of this approach in the last decade has been, in part, hindered by insufficient understanding of the complex structure of mammalian chromosomes and structural requirements for *de novo* HAC formation. One of the greatest obstacles has been the inability to clone and produce large, stable fragments of highly repetitive human centromeric DNA, comprised primarily of alphoid satellite DNA with repeating units 171 bp in length organized into higher order repeats that vary from 0.2 to 5 Mb in length (*63*). The difficulty in propagating alpha satellite DNA is largely due to the tendency of tandemly repetitive DNA to recombine into smaller arrays. To overcome this problem, centromeric regions were isolated with high selectivity using the TAR cloning methodology (*48*). Subsequent characterization of individual clones elucidated several elements required for *de novo* assembly of centromeres, but major progress had not been made until a novel strategy for rapid construction of synthetic alphoid DNA arrays with a predetermined structure was developed (*120*). The method includes concatamerization of DNA into short repeats (using RCA or directional *in vitro* ligation), followed by the assembly of short repeats into long arrays by *in vivo* homologous recombination in yeast (TAR cloning). The 'bottom up' approach was also employed for construction of the first swine artificial chromosome (SAC) (*121*), which is expected to be of great use in the production of transgenic pigs for xenotransplantation.

Thus, in the future, the use of TAR cloning could facilitate the construction of the first generation of MACs with predetermined structure, which will unquestionably lead to the development of artificial chromosome-based gene applications for biotechnological purposes and biomedicine.

## Helper Microorganism of Choice in Recombineering: Bacteria or Yeast?

Almost all previously described *in vivo* recombinant DNA techniques have also become possible in the bacteria *E. coli* by the introduction of phage-encoded enzymes for homologous recombination instead of inappropriate endogenous equivalents (*8*,*9*, Fig. 5). Previously, the main barrier for the development of an efficient *in vivo* recombinant DNA methodology in bacteria was the exonucleolitic activity of the RecBCD enzymatic complex which degrades any linear DNA molecule introduced into the bacterial cell. Initial attempts to resolve this problem by introducing *recBC* mutations into the *sbcD* and *sbcC* mutant background, or by incorporating Chi sites at the very ends of the transforming linear DNA

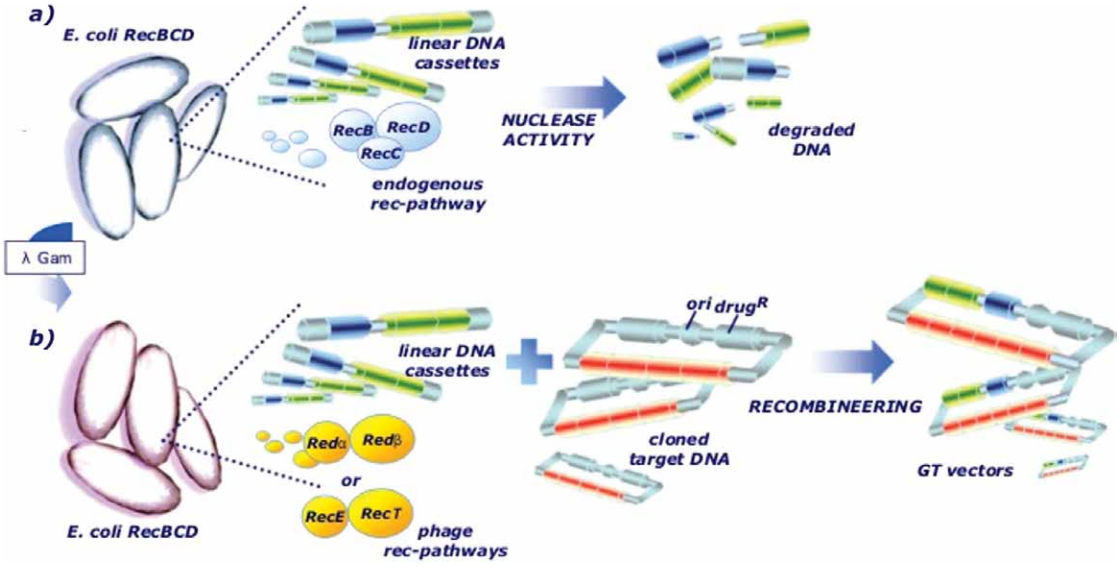


**Fig. 5.** Modifying the recombination machinery of *E. coli* in order to enable recombineering: a) the exonucleolitic activity of the RecBCD enzymatic complex degrades any linear dsDNA cassette introduced into the wild type bacterial cell and blocks its possibility to be used for modification of cloned target DNA; b) expression of the bacteriophage λ Gam protein inhibits the RecBCD, thereby protecting the linear dsDNA cassette from degradation. A simultaneously expressed pair of phage proteins Redα/Redβ or RecE/RecT process and anneal correctly the linear dsDNA cassette with homologous regions within the cloned DNA target, thereby enabling the production of the desired gene targeting vectors

molecule that attenuates RecBCD activity, were eventually abandoned due to their limited applicability. Another option for manipulation of genomic segments in bacteria was the use of circular DNA constructs that are stable in RecBCD wild type strains, with the necessity to provide RecA function which is essential for the integration of circular DNA molecules by homologous recombination. As most bacterial strains usually used for the preparation of BAC or PAC genomic libraries are RecA--deficient, this was enabled by the introduction of a plasmid molecule encoding the wild type *recA* gene. The complicated preparation of circular targeting constructs with relatively large DNA segments homologous to the genomic DNA on BACs, and increased BAC instability during RecA protein expression, were the main reasons for abandoning the latter approach. Problems with bacterial endogenous recombination proteins were successfully resolved by simultaneous expression of three phage proteins: the constitutive expression of the bacteriophage λ Gam protein that inhibits RecBCD, and the conditional expression of two other pairs of proteins which provide proper processing and pairing of linear dsDNA constructs with the genomic target: RecE and RecT proteins from the cryptic RAC prophage, or Redα and Redβ proteins from the bacteriophage λ. The RecE and Redα exonucleases process the ends of linear dsDNA fragments, preparing in this way the ss tails to be annealed by the action of the RecT and Redβ protein, respectively. This phage-encoded recombination activity could be simply introduced into any recA deficient *E. coli* strain with a BAC clone containing the desired target DNA by the transformation with plasmids bearing λ Gam and RecET or Redα,β-encoding genes. Simplicity of this approach made bacterial recombineering a powerful platform for high-throughput production of targeting vectors and therefore a new standard for functional genomics studies *(122,123)*.

Usual comparisons of *in vivo* recombinant DNA techniques with BACs in *E. coli* and YACs in *S. cerevisiae* demonstrate a substantial advantage of bacterial recombineering (Table 1). However, a more detailed insight makes this advantage less prominent. A standard statement, regarding the non-natural linkage of genomic segments cloned into these artificial chromosomes, is that YACs are often chimaeric, whereas chimaerism is very rare in BACs. The main reason for a low frequency of chimaerism in BACs lays in the standardized use of RecA-defficient bacterial strains for the preparation of genomic libraries. Several studies have shown that YAC clones can also display low frequency of chimaerism comparable to BACs, if appropriate recombination yeast mutants are used for genomic DNA cloning (*69*,*78*). The same principle can be applied to the comparison of genomic DNA stability: unwanted rearrangements are not frequent in BACs due to the absence of RecA activity, whereas the potent yeast recombination machinery frequently generates deletions or translocations in YACs. Again, if the conditional *rad52* or *rad54* yeast mutant strains are used for YAC propagation, genomic DNA repeats present in the cloned segment can be stably maintained. Purification of high quantities of intact genomic DNA required is the main advantage of BACs. Purification of sufficient quantities of genomic DNA in YACs is laborious, and usually requires YACs to be transferred to *E. coli* for subsequent manipulation. This can be partially overcome by using spheroplast fusion as the method of choice for YAC delivery into the mammalian cell, with the risk of concomitant transfer and integration of the yeast genomic DNA.

Similar to TAR cloning in yeast, the newly developed *E. coli* recombination systems also allow subcloning of desired genomic fragments by the process of gap repair (*124*). This is achieved by transformation of the appropriate bacterial BAC host strain with a linear plasmid molecule, amplified by PCR using chimaeric primers (primers carrying sequences homologous to the target DNA on their 5'-ends). However, there are significant differences in approaches for selective cloning of genomic segments by gap repair between the yeast and bacterial systems. Firstly, the standard yeast TAR cloning technique uses co-transforming mammalian DNA for cloning of the desired genomic segment, whilst this is not the case in the described bacterial approach. It would be interesting to see if the co-transforming mammalian DNA could also be used in bacteria. Secondly, the genomic segments subcloned by bacterial gap repair, so far, are quite short: up to 80 kb compared to more than 600 kb obtained by yeast TAR cloning (*38*). Most probably, this is affected by the difference in the accommodation capacity of bacterial and yeast cloning vectors and represents the main advantage of YACs, as they can accommodate megabase-size genomic inserts, whereas BACs can carry inserts up to 300 kb or less.

Table 1. BAC recombineering in *E. coli vs.* YAC recombineering in *S. cerevisiae*, adapted from (*8*)

| BACs/YACs | *Escherichia coli* | *Saccharomyces cerevisiae* |
|---|---|---|
| DNA insert chimaerism | 'rare' chimaeric BAC clones (recA$^-$) | 'frequent' chimaeric YAC clones (*rad52*, *rad54*) (*69*,*78*) |
| DNA stability (directed and inverted repeats) | 'stable' DNA repeats (recA$^-$) | 'unstable' DNA repeats (*rad52*, *rad54*) (*69*,*78*) |
| DNA isolation | **easy to purify** intact BACs (electroporation) | difficult to purify intact YACs (spheroplast fusion) (*85*) |
| Subcloning by gap repair/TAR cloning | up to 80 kb (*124*) | from 70 up to >600 kb (*38*) |
| DNA accomodation | 200–300 kb | **megabase size** inserts (2.4 Mb) (*40*,*125*) |

## Conclusions

According to the facts described above, both BAC recombineering in *E. coli* and YAC recombineering in *S. cerevisiae* could be considered an equally powerful tool for manipulation of mammalian genomes. The choice between the two microorganisms depends exclusively on the purpose of the genetic manipulation to be performed. Simplicity of preparation and manipulation of the standard targeting vectors is, in large part, the main reason to choose bacterial recombineering for the setup of systematic gene disruption projects. However, recombineering in yeast is still a better choice for the study of many complex mammalian genes, as their megabase size can only be accommodated in YACs.

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