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Review

Size matters: use of YACs, BACs and PACs in transgenic animals

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Abstract

In 1993, several groups, working independently, reported the successful generation of transgenic mice with yeast artificial chromosomes (YACs) using standard techniques. The transfer of these large fragments of cloned genomic DNA correlated with optimal expression levels of the transgenes, irrespective of their location in the host genome. Thereafter, other groups confirmed the advantages of YAC transgenesis and position-independent and copy number-dependent transgene expression were demonstrated in most cases. The transfer of YACs to the germ line of mice has become popular in many transgenic facilities to guarantee faithful expression of transgenes. This technique was rapidly exported to livestock and soon transgenic rabbits, pigs and other mammals were produced with YACs. Transgenic animals were also produced with bacterial or P1-derived artificial chromosomes (BACs/PACs) with similar success. The use of YACs, BACs and PACs in transgenesis has allowed the discovery of new genes by complementation of mutations, the identification of key regulatory sequences within genomic loci that are crucial for the proper expression of genes and the design of improved animal models of human genetic diseases. Transgenesis with artificial chromosomes has proven useful in a variety of biological, medical and biotechnological applications and is considered a major breakthrough in the generation of transgenic animals. In this report, we will review the recent history of YAC/BAC/PAC-transgenic animals indicating their benefits and the potential problems associated with them. In this new era of genomics, the generation and analysis of transgenic animals carrying artificial chromosome-type transgenes will be fundamental to functionally identify and understand the role of new genes, included within large pieces of genomes, by direct complementation of mutations or by observation of their phenotypic consequences.

Overcoming position effects in transgenic animals

The generation of transgenic animals is a routine method in many laboratories worldwide. Transgenesis is commonly applied to study gene function in development and disease, to devise new animal models of human genetic diseases or to produce recombinant proteins in fluids, mostly milk, of transgenic animals. However, there is still a major limitation in the method, namely, the uncertainty about the expression of each transgene. This is mainly caused by the stochastic event of transgene integration within the host genome and the nature of the transgenic constructs. It is accepted that host sequences surrounding the place of transgene integration can modify the expected expression pattern, potentially causing it to be ectopic, weak or even undetectable. This is currently interpreted as the result of chromosomal position effects (Wilson et al., 1990; Sippel et al., 1997) In addition, the limited knowledge of regulatory sequences for most genes favour the use of partial, often uncharacterised, genomic fragments that frequently function poorly in gene transfer experiments (Palmiter & Brinster, 1986).

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A number of strategies have been proposed to overcome these position effects and thus increase the probability of optimal expression for transgenes integrated at random locations. The incorporation of homologous intronic sequences in transgenic constructs were among the first suggestions recognised to improve expression of transgenes (Brinster et al., 1988; Whitelaw et al., 1991). Heterologous introns have also been routinely used in transgenic experiments and shown to improve expression in some cases (Choi et al., 1991; Palmiter et al., 1991). The inclusion of specific sequences such as scaffold/matrix-attachment regions (S/MARs) (McKnight et al., 1992; but see Gutierrez-Adan & Pintado, 2000), locus control regions (Grosveld et al., 1987; Ganss et al. 1994; Montoliu et al. 1996; most recently reviewed in Li et al., 1999) and insulators (Chung et al., 1993; Taboit- Dameron et al., 1999) have been reported to ameliorate transgenic expression. The adjacent co-integration of an abundantly expressed transgene has also been shown to rescue cDNA-type constructs present as a second neighbour transgene (Clark, 1997).

At best, targeting the site of integration by homologous recombination in ES cells (i.e., knock-in) would virtually solve any chromosomal position effects since the transgenic construct would then be controlled by all regulatory sequences present in the chosen endogenous locus. Alternatively, particular locations in the host genome can be selected according to their capacity to allow adequate expression patterns of experimental transgenes (Wallace et al., 2000). This strategy has been recently applied in transgenic sheep obtained by nuclear transfer from cultured somatic cells and served to identify the ovine α1(I) procollagen (*COL1A1*) locus as a permissive candidate for the insertion of therapeutically useful transgenes to be expressed in the milk at optimal levels (McCreath et al., 2000). Figure 1 summarises the above-mentioned strategies devised to overcome position effects in transgenic animals.

Position effects support the notion that genes are organised on chromosomes as contiguous but independent units referred to as expression domains (Elgin, 1990; Laemmli et al., 1992; Dillon & Grosveld, 1994). These expression domains are believed to remain insulated from neighbouring sequences and thought to include all regulatory elements that are necessary for correct gene expression. Thus, it is not surprising that standard transgenic constructs lacking most or some of these crucial regulatory sequences might display position effects when integrated randomly within the host genome. Most of the strategies devised to overcome such position effects have reported the progressive addition of regulatory elements, as a successful approach to improve the expression of transgenic constructs in a significant manner. Figure 2 shows a graphic representation of an expression domain along with the expected performance of different versions of a corresponding transgenic construct, progressively including more regulatory elements. Theoretically, the inclusion of all regulatory elements that are associated with a given expression domain in a transgenic construct would guarantee optimal expression levels in transgenic animals regardless of position of integration. Such conditions are present in artificial-

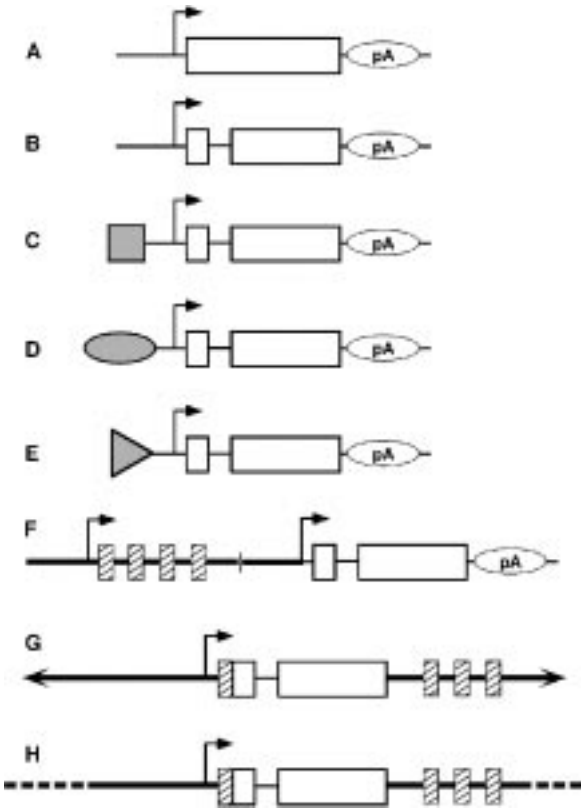

Figure 1. Diverse strategies devised to overcome position effects in transgenic animals (A) basic cDNA-type transgenic construct; (B) addition of intronic (homologous and heterologous) sequences; (C) addition of S/MAR sequences; (D) addition of a locus control region; (E) addition of insulating sequences; (F) transgene rescue by co-integration of an abundantly expressed transgene (stippled boxes); (G) use of genomic sequences contained within an artificial chromosome-type of transgenic constructs; and (H) gene targeting, knock-in. Symbols used: white rectangles (coding region of a gene), grey rectangle (S/MARs), grey oval (Locus Control Region), grey triangle (insulator), stippled box (coding region/exons of another gene, used to drive the expression of the transgene), white oval with pA (polyadenylation signal).

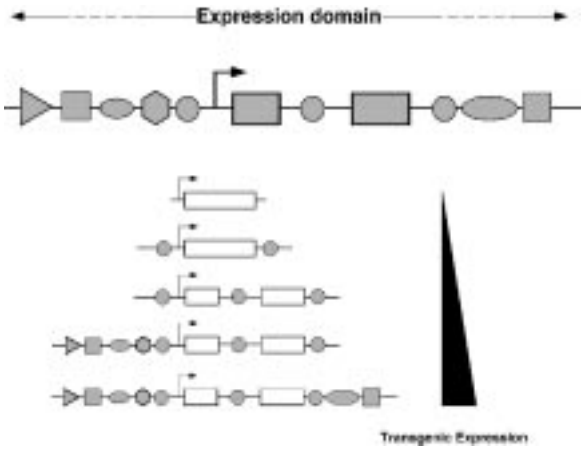

Figure 2. The expression performance of transgenic constructs normally depends on the presence of sufficient regulatory elements that identify an expression domain. The progressive addition of these elements usually correlates with an improved expression of transgenes. Symbols used are as in Figure 1 with the addition of: grey circles (enhancers), grey hexagon (repressor).

chromosome type vectors (YACs, BACs and PACs), due to their large cloning capacity. In this review we will discuss the use of these vectors in animal transgenesis.

The benefits and applications of using yeast artificial chromosomes (YACs) for animal transgenesis have been previously reviewed (Montoliu et al., 1993, 1994; Forget, 1993; Jakobovits, 1994; Lamb & Gearhart, 1995; Peterson, 1997a; Peterson et al., 1997b; Umland et al., 1997; Huxley 1998; Camper & Saunders 2000). The use of YACs in transgenesis is likely to ensure position-independent, copy-number dependent and optimal levels of expression of the transgenes, provided all regulatory sequences needed for the establishment and maintenance of the expression domain are located within the YAC. Thus, YAC transgenes can circumvent most position effects observed with standard constructs, being the recommended choice when regulatory sequences of a gene are not known (Lamb et al., 1993; Schedl et al., 1993b; Strauss et al., 1993; Hodgson et al., 1996; Ainscough et al., 1997; Fujiwara et al., 1997; Hiemisch et al., 1997; Porcu et al., 1997; Zweigerdt et al., 1997; Peterson et al., 1998; Li et al., 2000).

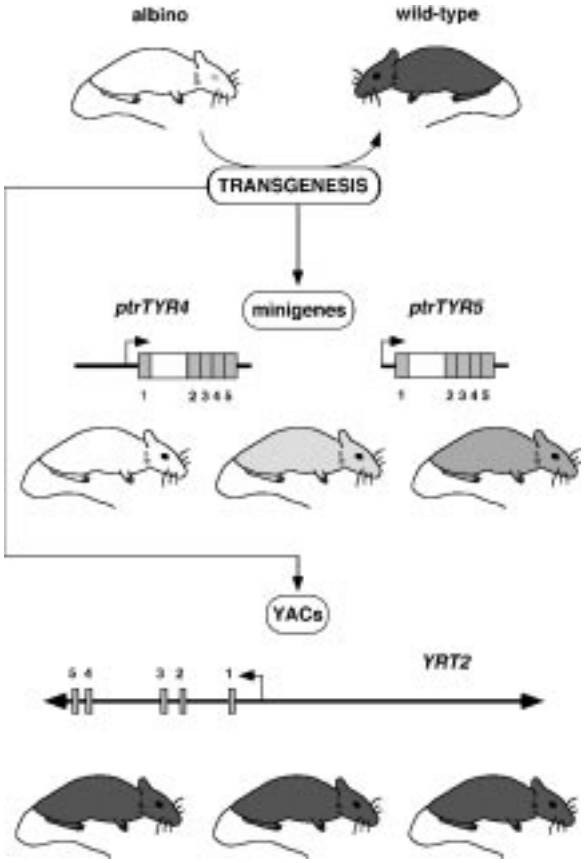

Figure 3. YACs versus standard constructs in transgenic mice, an example: rescue of the albino phenotype by introduction of functional tyrosinase constructs into mice. Standard minigene-type of constructs (ptrTyr4, 5.5 kb and ptrTyr5, 280 bp of tyrosinase promoter and upstream regulatory sequences, as reported in Beermann et al. (1990) and Klüppel et al. (1991), respectively) result in mice with variable expression of transgenes, showing pigmentation phenotypes weaker than that of wild-type animals. In contrast, YAC-type of constructs (i.e. YRT2, 250 kb encompassing the whole mouse tyrosinase locus, as reported in Schedl et al. (1993b)) produce transgenic mice indistinguishable of wild-type animals, without pigmentation variability and showing position-independent and copy number-dependent transgene expression.

Transgenic animals generated with YACs

YACs are eukaryotic cloning vectors capable of the stable maintainance of genomic fragments of DNA larger than 1 Mb (Burke et al., 1987). YACs are linear DNA molecules and are generated from vectors (such us pYAC4, Kuhn & Ludwig, 1994) that provide all functional elements for their maintenance in yeast cells as artificial chromosomes (Figure 4, Green et al., 1999). Their vast cloning capacity, compared to standard cloning vectors (plasmids, phages and cosmids), made them very attractive for gene transfer experiments. Several groups tested this hypothesis and evaluated their suitability for transgenesis in mice. Thus, in 1993, a number of independent teams succeeded in generating the first transgenic mice with YACs.

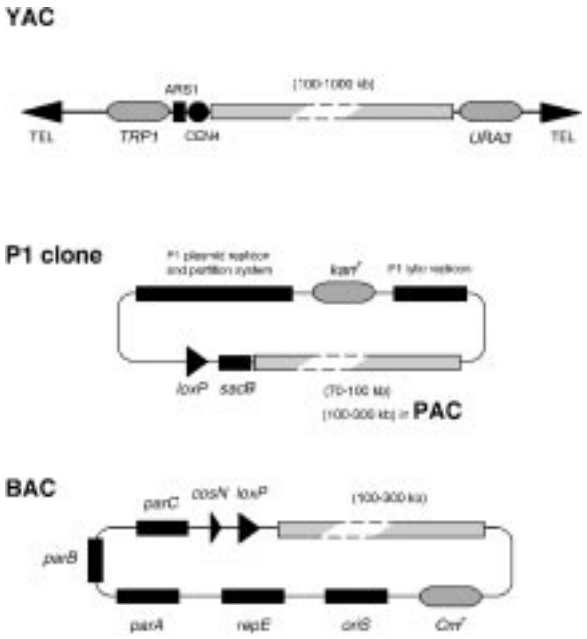

Figure 4. Schematic representation of a YAC, a P1-clone, a PAC and a BAC. Functional elements in each vector are shown in black. Selectable marker genes are shown in grey. Heterologous insert DNA is shown as a stippled discontinuous box. YAC, P1-clone, PAC and BAC modules shown here are derived from vector pYAC4 (Kuhn & Ludwig, 1994; Genbank U01086), pAd10SacBII (Pierce et al., 1992; Genbank U09128), pCYPAC2 (Ioannou et al., 1994, sequence derived from Genbank U09128, map available in http://www.chori.org/bacpac/) and pBeloBAC11 (Research Genetics, Genbank U51113, map available in http://www.tree.caltech.edu), respectively. P1-clones and PACs share vector sequences and only differ by their respective upper size limit on insert length, as indicated. Abbreviations: TEL, *Tetrahymena* telomere-derived sequence; CEN4, yeast *CEN4* centromere; ARS1, yeast autonomous replicating sequence 1; *TRP1*, yeast *TRP1* gene; *URA3*, yeast *URA3* gene; *loxP*, *loxP* site recognised by the Cre-recombinase protein; *kan*^{r}, kanamycine-resistance gene; *sacB*, bacterial gene used in positive selection for cloned inserts; *cosN*, *cosN* site from bacteriophage λ which may be cleaved by bacteriophage λ terminase; *oriS*, *repE*, *parA*, *parB* and *parC*, genes derived from the F factor of *Escherichia coli* needed for the autonomous replication, copy-number control and partitioning of the BAC; *Cm*^{r}, chloramphenicol-resistance gene . Not drawn to scale.

Three groups reported their pioneer work in two journals within a week of one another (Jakobovits et al., 1993; Schedl et al., 1993b; Strauss et al., 1993). Remarkably, three different techniques were described to deliver YAC DNA to the germline of mice: pronuclear microinjection of gel-purified YAC DNA (Schedl et al., 1993b), lipofection of YAC DNA into ES cells (Strauss et al., 1993) and yeast spheroblast fusion with ES cells (Jakobovits et al., 1993). Two of these YAC transgenes, the mouse tyrosinase and the collagen (*COL1A1*) genes, demonstrated transgenic expression at levels comparable to the corresponding endogenous genes (Schedl et al., 1993b; Strauss et al., 1993). Furthermore, analysis of several independent transgenic mouse lines carrying multiple copies of the YAC tyrosinase transgene permitted to prove position-independent and copy-number dependent expression (Schedl et al., 1993b). This study showed the faithful rescue of the albino phenotype of recipient animals by a 250 kb YAC tyrosinase transgene, as compared to the variability in pigmentation levels obtained with previous standard (and much smaller) tyrosinase constructs (Beermann et al., 1990; Tanaka et al., 1990; Kluppel et al., 1991). The comparison between standard (plasmid) and YAC tyrosinase transgenic mice is presented in Figure 3 and illustrates the generally good performance of YAC transgenes in gene transfer experiments observed in this particular and most other examples. Faithful expression was demonstrated with a 680 kb Myf-5 YAC transgene (Zweigerdt et al., 1997), whereas previous attempts made with standard Myf-5 transgenes driven by 5.5-kb 5′-upstream sequences failed to recapitulate the precise developmental expression pattern (Patapoutian et al., 1993). Similarly, a 130 kb YAC transgene containing both the *Igf2* and *H19* genes, was shown to display genomic imprinting effects according to their endogenous counterparts (Ainscough et al., 1997), in contrast with the previously described imprinting of mini-H19 transgenes, which only occured at multi-copy loci, inconsistently and prone to genetic background effects (Bartolomei et al., 1993; Pfeifer et al., 1996; Elson & Bartolomei, 1997). A number of independent experiments further confirmed the high perfomance of YAC constructs and their potential to overcome position effects in transgenic mice, according to the prediction of the model (Lamb et al., 1993; Strauss et al., 1993; Hodgson et al., 1996; Montoliu et al., 1996; Fujiwara et al., 1997; Hiemisch et al., 1997; Porcu et al., 1997; Peterson et al., 1998; Li et al., 2000). In most of these cases YAC transgene expression was found comparable to that of endogenous levels and largely determined by transgene copy-number. In addition, position-independent expression has been reported also in YAC constructs stably transfected in cells (i.e., Asselbergs et al., 1998; Vassilopoulos et al., 1999).

Some β-globin YAC transgenes, containing the β-globin LCR, have been reported to display uniform expression but position effect variegation in mice (Alami et al., 2000). However, these data are in good agreement with previous experiments carried out

in the endogenous mouse β-globin locus, suggesting the existence of unknown regulatory sequences that may compensate for LCR function when this specific sequence is removed from its endogenous normal context (Epner et al., 1998; Bender et al., 2000). Thus, the suboptimal performance occasionally observed with some YAC transgenes, such as the partial rescue of *GATA-3* mutant mice by YAC transgenes (Lakshmanan et al., 1998), is normally explained by the absence of additional regulatory elements that are required for correct expression pattern of the gene (Lakshmanan et al., 1999).

The use of YACs has been fundamental in molecular complementation of mutations, allowing the identification of new genes by transgene rescue of mutant phenotypes (i.e. Morgan et al., 1998; Majumder et al., 1998; Slee et al., 1999). Other applications have been explored with this new technique. The transfer of large genomic units enabled the production of human immunoglobulin light and heavy chains in transgenic mice (Davies et al., 1993; Choi et al., 1993; Zou et al., 1996) and, eventually, the generation of transgenic mice producing an almost complete repertoire of human antibodies in their sera from genomic unrearranged immunoglobulin loci cloned into YACs (Green et al., 1994; Fishwild et al., 1996; Mendez et al., 1997).

The overexpression of genes associated with human disease in mice via YAC transgenesis has also been investigated. For example, YACs containing the *APP* gene (>400 kb), encoding the amyloid precursor protein that accumulates abnormally in Alzheimer and other neurodegenerative diseases have been transferred to the germ line of mice aiming to produce animal models that were useful to study these human pathological conditions (Lamb et al., 1993; Pearson & Choi, 1993). However, the limited overexpression level achieved (two-fold) did not reproduce all the features of Alzheimer's disease (Murai et al., 1998) and triggered the generation of new YAC transgenic mice carrying mutant versions of the *APP* gene, and/or new candidate genes also associated with Alzheimer's disease (i.e. *apolipoprotein E4*, *presenilin-1* in Loring et al., 1996; Lamb et al., 1997, 1999).

YAC transgenic mice have also proven essential in the discovery of candidate genes responsible for the complex abnormal phenotype found in Down's syndrome patients. A panel of YAC transgenic mice was generated covering the human chromosome 21q22.2, a contiguous 2 Mb area known as the Down's syndrome region (Smith et al., 1995). A functional screening of these transgenic mice led to the discovery of a gene (*minibrain*) implicated in learning defects associated with Down's syndrome (Smith et al., 1997a).

A number of informative and advantageous transgenic mice have been generated with YAC-based β-globin locus (i.e. Peterson et al., 1993; Gaensler et al., 1993; Peterson et al., 1996; Liu et al., 1997; Calzolari et al., 1999). Proper developmental expression patterns were demonstrated for the human β-globin transgenes, compared to the endogenous murine copies. In Table 1 these and other YAC transgenic animals are summarised in a comprehensive manner, emphasising the most relevant features of each set of experiments.

The generation of transgenic animals with YACs has been extended to other mammals with comparable success. To date, transgenic pigs (Yannoutsos et al., 1995), rabbits (Brem et al., 1996; Rouy et al., 1998), and rats (Fujiwara et al., 1997, 1999b) have been generated with YAC transgenes. In livestock, the benefits of YAC transgenesis are fundamentally focused in two fields: xenotransplantation and the efficient production of recombinant proteins of interest in the milk of transgenic animals.

Technical considerations for the generation of YAC transgenic animals

A number of different methods have been devised to produce transgenic animals with YACs both using pronuclear microinjection and transfection into ES cells (Schedl et al., 1993a; Jakobovits et al., 1993, 1999; Strauss et al., 1993; Choi et al., 1993; Gaensler et al., 1993; Huxley 1998; Peterson, 1999). A protocol suitable for the preparation of YAC DNA for pronuclear microinjection can be found at the following WEB page: http://www.cnb.uam.es/~montoliu/prot.html.

Most of the present experiments make use of standard pronuclear injection with the appropriate technical considerations for DNA molecules of this large size. Early reported methods were based on the modification of YAC vector arms to allow amplification of YACs inside the yeast cells in order to increase the recovery of YAC DNA molecules (Schedl et al., 1993a, 1996a). Soon it was obvious that the amplification step was not essential for adequate purification of YAC DNA and in subsequent updated methods this step was omitted (Peterson, 1997a; Hiemisch et al., 1998). Furthermore, the amplification step in yeast cells required the presence of *HSV thymidine kinase* gene in one of YAC-vector arms which has been reported to impair

Table 1. Transgenic animals generated with YACs

Transgene	Size (kb)	Animal	Aim	Human disease/condition	Reference
Mouse	35, 100, 250	Mouse	Rescue of albino phenotype	Albinism	(Schedl et al., 1993b)
Tyrosinase		Rabbit	Identification of regulatory sequences		(Montoliu et al., 1996) (Brem et al., 1996)
Human *HPRT* locus	670	Mouse	Molecular complementation of mutation		(Jakobovits et al., 1993)
Mouse *α(I)collagen*	150	Mouse	Molecular complementation of mutation		(Strauss et al., 1993)
Human *Ig light chain*	300, 1300	Mouse	Production of human antibodies in mice		(Davies et al., 1993)
Human	85	Mouse	Production of human antibodies		(Choi et al., 1993)
Ig heavy chain			in mice		(Zou et al., 1996)
Human	220, 170	Mouse	Production of human antibodies		(Green et al., 1994)
Ig heavy and κ light chain	800, 1020		in mice		(Fishwild et al., 1996) (Mendez et al., 1997)
Human	400, 650	Mouse	Overexpression	Alzheimer	(Lamb et al., 1993, 1997)
APP			Model of human disease	Down syndrome	(Pearson and Choi, 1993)
Human β-globin locus	248, 150	Mouse	Analysis of regulatory sequences	Thalassemias	(Gaensler et al., 1993) (Peterson et al., 1993, 1995, 1996, 1998) (Bungert et al., 1995, 1999) (Liu et al., 1997) (Navas et al., 1998) (Calzolari et al., 1999) (Tanimoto et al., 1999)
Human	270, 370	Mouse	Pattern of expression and	Atherosclerosis	(Frazer et al., 1995)
Apolipoprotein (a)		Rabbit	regulation		(Rouy et al., 1998) (Acquati et al., 1999)
Human	108	Mouse	Analysis of structure		(McCormick et al., 1995, 1997a)
Apolipoprotein B		Rabbit			
Human Chromosome 21 region 21q22.2	430–1100	Mouse	Mouse model of human disease	Down Syndrome	(Smith et al., 1995, 1997)
Human *PMP22*	560	Mouse	Mouse model of human disease	Charcot-Marie-Tooth disease	(Huxley et al., 1996)
Human *PAX6*	420	Mouse	Mouse model for human disease	Aniridia	(Schedl et al., 1996b)
Human	420	Mouse	Xenotransplantation		(Yannoutsos et al., 1995, 1996)
MCP, CD59, CD46		Pig			(Langford et al., 1996)
Human *CFTR*	320	Mouse	Mouse model for human disease	Cystic fibrosis	(Manson et al., 1997)
Human *Hnf3γ-lacZ*	170	Mouse	Analysis of regulatory sequences		(Hiemisch et al., 1997)
Mouse *H19, Igf2*	130	Mouse	Genomic imprinting		(Ainscough et al., 1997)
Human *β(S)-globin*	240	Mouse	Mouse model for human disease	Sickle cell disease	(Chang et al., 1998)

Table 1. (*continued*)

Transgene	Size (kb)	Animal	Aim	Human disease/condition	Reference
Mouse *GATA-2*	120, 200 250	Mouse	Pattern of expression and regulation		(Zhou et al., 1998)
Mouse *GATA-3-lacZ*	120, 540 625	Mouse	Pattern of expression and regulation Identification of regulatory sequences		(Lakshmanan et al., 1998, 1999)
Human *WT1-lacZ*	280, 470	Mouse	Pattern of expression and regulation	Nephroblastomas	(Moore et al., 1998)
Human *SOX9-lacZ*	350	Mouse	Mouse model for human disease	Campomelic dysplasia	(Wunderle et al., 1998)
Human *Androgen Receptor*- CAG	450	Mouse	Mouse model for human disease	X-linked spinal and bulbar muscular atrophy	(La Spada et al., 1998)
Mouse *Inversin*	450	Mouse	Molecular complementation of mutation		(Morgan et al., 1998)
Mouse *downless*	200	Mouse	Mouse model for human disease	Autosomal hypohidrotic sctodermal dysplasia	(Majumder et al., 1998)
Human Asthma QTL	400	Mouse	Study of quantitative trait loci	Asthma	(Symula et al., 1999)
Human *IgH/c-myc*	240	Mouse	Mouse model for human disease	Burkitt's lymphoma	(Butzler et al., 1997) (Palomo et al., 1999)
Human *presenilin-1* (PS-1)	1000	Mouse	Mouse model for human disease	Alzheimer Down Syndrome	(Lamb et al., 1999)
Human *DAZ*	225	Mouse	Molecular complementation of mutation	Spermatogenic defects	(Slee et al., 1999)
Mouse *Xist*/Xic	430, 350 450	Mouse	X inactivation		(Heard et al., 1996) (Lee et al., 1999) (Matsuura et al., 1996)
Human *XIST*/XIC	320, 460, 480	Mouse	X inactivation		(Migeon et al., 1999) (Heard et al., 1999a,b)
Human *α-lactalbumin*	210	Rat	Expression in mammary gland (hGH)		(Fujiwara et al., 1997, 1999a)
Human *Huntingtin*	350, 600	Mouse	Mouse model of human disease	Huntington disease	(Hodgson et al., 1996, 1999)
Mouse *Myf-5*	680	Mouse	Analysis of regulatory sequences		(Zweigerdt et al., 1997)
Human *Macrophage scavenger receptor* (MSR class A)	180	Mouse	Analysis of gene function	Atherosclerosis	(de Winther et al., 1999)
Mouse Olfactory receptors	300	Mouse	Allelic inactivation		(Ebrahimi et al., 2000)
Human chromosome 5 5q31 cluster region	350–500	Mouse	Overexpression, discovery of new genes, genomic organisation		(Frazer et al., 1997)
Human *MJD1-CAG*	250	Mouse	Mouse model of human disease	Spinocerebellar ataxia 3 (Machado-Joseph disease)	(Cemal et al., 1999)

germ-line transmission of YAC transgenes in males (Fujiwara et al., 1997).

Regarding the handling and microinjection of YAC DNA, the presence of ionic strength (100 mM sodium chloride) has been reported to be required to stabilise YAC DNA molecules in solution. Further, the addition of polyamines is recommended. These compacting agents promote the formation of YAC DNA-polyamine complexes and prevent shearing upon handling and microinjection of YACs (Montoliu et al., 1995; but see Bauchwitz & Constantini, 1998). Different strategies have been suggested to concentrate YAC DNA for microinjection including a second standard gel electrophoresis after preliminary isolation on PFGE (Schedl et al., 1993a); dialysis in sucrose (Gaensler et al., 1993) and the use of specific spin filtration units (Peterson et al., 1993). The use of two gels followed by an agarase treatment is preferred in some laboratories over the filtration alternative. The later, although easier and faster, requires special care to avoid potential breakage of YAC DNA molecules during the more extensive pipetting steps. Apart from the above mentioned protocols, suitable for the pronuclear microinjection of YACs, adapted versions have been developed for the efficient transfection of YACs into somatic (Compton et al., 1999) and mouse ES cells (Lee & Jaenisch, 1996; Bauchwitz & Constantini, 1998).

YACs are normally isolated from the rest of endogenous yeast chromosomes by preparative PFGE techniques (i.e. Schedl et al., 1993a). However, YACs are generally of the same size range as the endogenous yeast chromosomes. Thus, the isolation of YAC DNA by electrophoretic techniques can be impaired by the presence of comigrating or closely migrating endogenous yeast chromosomes. Some studies have shown that the cointegration of contaminant yeast endogenous chromosomes does not seem to have an overt effect in the expression of YAC transgenes (Jakobovits et al., 1993; Green et al., 1994; Mendez et al., 1997). However, it is always preferable to microinject YAC DNA samples free of contaminating yeast endogenous chromosomes. This can be easily achieved by mobilising the YAC to alternate yeast hosts with defined karyotypic alterations (Hamer et al., 1995). This new set of hosts, called yeast window strains, have been engineered using recombination-mediated chromosome fragmentation. Each strain has defined alterations in its karyotype, which provide an electrophoretic interval devoid of yeast endogenous chromosomes, thus allowing the isolation of relatively pure YAC DNA regardless of YAC size (Hamer et al. 1995). All of the yeast window strains carry the *kar1-Δ15* mutation, thereby allowing the efficient transfer of a YAC from its original host into an appropriately selected yeast window strain using the *kar1*-transfer standard procedure (Spencer et al., 1994).

The overall efficiency of transgenesis with YACs (measured as the number of transgenic positive individuals found among newborns obtained) is comparable to that of standard DNA constructs although in some cases low efficiencies (<5%) are observed, most likely due to co-purified contaminants present in crude YAC DNA preparations. Although the usual number of YAC DNA molecules microinjected is much smaller than with standard plasmids (Brinster et al., 1985; Palmiter & Brinster, 1986), due to the bigger size of YAC transgenes, this does not seem to have an effect in transgenic efficiencies (Schedl et al., 1992, 1993b; Brem et al., 1996; Peterson, 1997a). The presence of vector sequences (10–15 kb long) found at either end of YACs does not appear to alter or prevent expression patterns of the borne transgenes (Schedl et al., 1992, 1993; Montoliu et al., 1996). Usually, 5–20% of newborn animals are found to be DNA-positive for the injected YAC transgene but only a variable proportion of them (20–70%) retains the entire YAC integrated in the host genome. Therefore, it is crucial to evaluate (i.e., by PCR) the integrity of YAC transgenes by analysing the presence of left and right YAC-vector arms, along with exhaustive Southern analysis with a set of internal probes, before subsequent experiments are actually carried out with selected founder animals (i.e. Smith et al., 1995; Montoliu et al., 1996; Brem et al., 1996; Peterson, 1997; Fujiwara et al., 1997). Additional methods that can be applied to assess YAC DNA integrity within the host genome include *recA*-assisted restriction endonuclease (RARE) analysis (Gnirke et al., 1993), restriction enzyme analysis with rare cutters (i.e. *SfiI*, *PpoI*; Peterson et al., 1998) and fiber fluorescence *in situ* hybridisation (FISH) (Rosenberg et al., 1995), a sophisticated method that uses stretched chromatin preparations to evaluate the integrity, organisation and copy number of integrated YAC transgenic sequences by FISH (Rosenberg et al., 1996).

Most transgenic animals generated with YACs carry single or few (<5) copies of transgenes integrated, in agreement with the limited number of DNA molecules that are microinjected. The presence of multiple copies (>5) is uncommon, but has been

reported (i.e. Schedl et al., 1993b, 1996b; Smith et al., 1995; Ainscough et al., 1997; Moore et al., 1998).

Alternatively, the introduction of YACs into the germ-line of mice can be achieved via ES cells with the subsequent generation of chimaeric mice (Strauss et al., 1993; Choi et al., 1993; Jakobovits et al., 1993; Green et al., 1994; Mendez et al, 1997). This approach, although more difficult and time consuming allows the functional and structural characterisation of YAC-transgenes prior to the generation of transgenic mice (Green et al., 1994; Mendez et al., 1997). ES cell clones can be screened for the presence of both YAC-vector arms and different copy-number integration events, single and multicopy arrays, can be selected for further analysis in transgenic mice (i.e. Heard et al., 1999a,b).

Besides these advantages, some inherent problems should be taken into account before using ES-cell based approaches for the transfer of YACs into the germ-line of mice. First, YAC-positively transfected ES cell clones are selected for G418 resistance (Strauss et al., 1993; Choi et al., 1993) or for complementation of hypoxanthine phosphoribosyltransferase (HPRT)-deficient ES cell lines in hypoxanthine–aminopterin–thymidine (HAT) medium (Jakobovits et al. 1993; Green et al., 1994; Mendez et al., 1997). These selection procedures require the use of drug-selectable marker genes that are either targeted into a YAC vector arm (*neomycin resistance* gene in Strauss et al., 1993; HPRT gene in Green et al., 1994; Mendez et al., 1997) or co-transfected in a separate plasmid (*neomycin resistance* gene in Choi et al., 1993). The selection procedure biases YAC integration into 'open' or 'active' chromatin, permissive for marker gene expression. Thus, in these cases, position effects on YAC transgene expression would not be observed, or be effectively masked, by pre-selection for integration into euchromatic regions. Further, the co-integration of YAC transgenes along with drug-selectable marker genes might interfere with the normal expression pattern of the YAC-borne gene, as it has been reported in standard gene targeting approaches (Fiering et al., 1995).

Second, the fusion of yeast spheroblasts carrying YACs with HPRT-deficient ES cell lines results in the effective transfer and co-integration of variable and uncontrolled amounts of the remaining yeast genome into the ES cell genome (Jakobovits et al., 1993; Green et al., 1994, Mendez et al., 1997). In some ES cell clones, even the entire 12 Mb yeast genome was deduced to be present by fingerprint analysis (Jakobovits et al, 1993). Surprisingly, adverse effects were not observed in transgenic mice derived from ES cell clones fused with yeast spheroblasts. However, it seems very unlikely to anticipate that none of the 6,000 genes known to be present in the yeast genome will not interfere, in some manner, with the complex gene expression programme of a mammalian cell. Therefore, the isolation of YAC DNA from yeast endogenous chromosomes prior to any gene transfer strategy should be, in our opinion, the recommended choice.

Transgenic animals generated with BACs/PACs

YACs are unique as vectors due to their huge cloning capacity and their unlimited potential for targeted modifications of incorporated genomic inserts (Schlessinger, 1990; Green et al., 1999). But, the routine work and handling of YACs requires specific skills and new expertise, which may not be present in all molecular biology laboratories. More importantly, several disadvantages are associated with YACs, including insert chimaerism (can be $>50\%$ of clones in a YAC library), insert instability, rearrangements and potential contamination with endogenous yeast chromosomes that can make difficult their efficient purification for microinjection or transfection into ES cells (Monaco & Larin, 1994; Green et al., 1999). To overcome these problems several other artificial chromosome-type vectors have been developed and have become popular. These include bacteriophage P1 clones (Sternberg et al., 1990, 1999; Pierce et al., 1992), bacterial artificial chromosomes (BACs) (Shizuya et al., 1992) and P1 bacteriophage-derived artificial chromosomes (PACs) (Ioannou et al., 1994). Figure 4 shows the basic modules from all artificial chromosomes-type vectors discussed in this review.

The bacteriophage P1 cloning system can efficiently accommodate 70–100 kb heterologous DNA inserts in *E. coli* (Sternberg 1999). P1 clones are usually derived from pAd10*sacB*II-type of vectors (Pierce et al., 1992). P1 clones are normally obtained by ligating genomic pieces of DNA with vector arms thereby generating a linear DNA molecule that is further processed and packaged in viral particles. Bacteriophage P1 particles are subsequently used to infect appropriate hosts where P1 clones are circularized by its *loxP*/Cre-recombinase system. Thereafter, P1 clones are subsequently maintained as single-copy circular plasmids. Copy-numbers, and hence DNA yield, can

be increased 10–30-fold before the DNA is isolated by regulating the activity of the bacteriophage P1 lytic replicon with IPTG. However, amplification of cloned DNA that contains repetitive sequences can lead to rearrangements (Birren et al., 1999). Upper limit size for inserts in P1 clones is fixed by the packaging capacity of bacteriophage particles (around 110 kb, including vector arms) (Sternberg 1999). PACs are very similar in structure to P1 clones (Figure 4, Ioannou et al., 1994; Birren et al, 1999). The main difference between PACs and P1 clones is that PACs lack the fixed upper size limit on insert length because PAC ligation mixes are transformed into their bacterial hosts by electroporation, whereas the generation of P1 clones involves the *in vitro* packaging step in bacteriophage particles (Sternberg, 1999; Birren et al., 1999). Thus, recombinant PACs achieve the same size range of inserts (100–300 kb) as do BACs (Ioannou et al., 1994; Monaco & Larin, 1994; Birren et al., 1999).

BACs, similar to PACs, are also circular plasmid DNA molecules that are hosted in *E. coli*. BACs can accommodate genomic inserts up to 300 kb and are derived from the F factor of *E. coli* (Shizuya et al., 1992). BAC vectors, such as pBeloBAC11, carry all sequences needed for autonomous replication, copy-number control and partitioning of the plasmid (Figure 4, Birren et al., 1999). In contrast to P1 clones and PACs, BACs are maintained as low-copy replicons and, correspondingly, yield lower quantities of DNA. For most applications, BACs and PACs are largely interchangeable and able to propagate large DNA inserts stably. Most protocols can be successfully applied to both types of clones, with the exception of antibiotic selection (kanamycin for PACs, chloramphenicol for BACs). Opposite to YACs, inserts cloned and maintained in BACs and PACs show low frequency (<5%) of chimaerism and much higher stability (Monaco & Larin, 1994; Birren et al., 1999). These new vectors have been used to generate genomic libraries that have been instrumental for most genome sequencing projects.

P1 bacteriophage clones were initially used to generate transgenic mice covering the human *apolipoprotein-B* gene (Linton et al., 1993), and the human chromosome 21q22.2, in combination with YACs, to isolate candidate genes associated with Down's syndrome (Smith et al., 1995). Furthermore, P1 clones and PACs have been successfully used to generate transgenic mice (McCormick et al., 1997b; Goodart et al., 1999; Chiu et al., 2000; Duff et al., 2000) and zebrafish (Jessen et al., 1999) in order to study long-range genomic interactions with the help of reporter genes.

The use of BACs in transgenic experiments was first reported in 1997 (Yang et al., 1997). The authors describe a simple method to modify a BAC that was then transferred to the germ-line of mice. Since then, a number of reports using BACs for transgenesis have been published along with pioneer revisions in this subject (Dewar et al. 1997; Nielsen et al., 1999; Heintz, 2000; Camper & Saunders, 2000). Table 2 summarises the reported BAC and PAC transgenes to date.

Similar to the work previously undertaken with YACs, BACs have been applied in a wide variety of studies including: molecular complementation of mutations (Antoch et al., 1997; Probst et al., 1998), *in vivo* studies of gene function (Yu et al., 1999; Zuo et al., 1999), analysis of gene dosage (Antoch et al., 1997; Yang et al., 1999), and the identification and analysis of regulatory sequences found at long distances (Nielsen et al., 1997, 1998; Kaufman et al., 1999). Further, BACs have been evaluated for their potential to improve mammary gland transgenesis and for the production of recombinant proteins in the milk of transgenic animals (Stinnakre et al., 1999; Zuelke, 1998).

Technical considerations for the generation of BAC transgenic animals

Several methods have been devised to purify BAC DNA for mammalian transgenesis (Yang et al., 1997; Chrast et al., 1999). A protocol suitable for the preparation of BAC DNA for pronuclear microinjection can be found at the following WEB page: http://www.med.umich.edu/tamc/BACDNA.html. BACs have been microinjected in three different forms: circular supercoiled plasmid, linearised DNA and purified insert. It is possible to obtain transgenic animals with undigested BACs that carry essentially intact insert DNA (Antoch et al., 1997; Duff et al., 2000), but there is always a risk of obtaining undesirable DNA molecules generated by random linearisation within constructs prior to the integration. Therefore, the preferred methods have been to microinject linearised BAC clones (Antoch et al., 1997; Probst et al., 1998; Jessen et al., 1998) or, better, the isolation of genomic inserts by PFGE after suitable enzymatic release (normally *Not*I) from vector sequences (Yang et al., 1997; Kaufman et al., 1999; Stinnakre et al., 1999). In some

Table 2. Transgenic animals generated with BACs/PACs

Transgene	Vector	Size (kb)	Animal	Aim	Human disease/condition	Reference
Human chromosome 21 region 21q22.2	P1 clone	70–100	Mouse	Animal model for human disease	Down's syndrome	(Smith et al., 1995)
Human *apolipoprotein B*	PAC	80	Mouse Rabbit	Overexpression	Lipoprotein metabolism	(Linton et al., 1993) (Fan et al., 1994)
Human *apolipoprotein E/CI/CIV/CII*	PAC	70	Mouse	Overexpression	Lipoprotein metabolism	(Allan et al., 1995)
Mouse *apolipoprotein B*	PAC	80	Mouse	Overexpression	Lipoprotein metabolism	(McCormick et al., 1997a,b)
Mouse *apolipoprotein B*	BAC	145 , 207	Mouse	Overexpression	Lipoprotein metabolism	(Nielsen et al., 1997)
Mouse *Zipro1* (RU49-*IRES-lacZ*)	BAC	169	Mouse	Expression pattern gene dosage analysis		(Yang et al., 1997, 1999)
Mouse *clock*	BAC	140	Mouse	Molecular complementation of mutation	Circadian rhytmns	(Antoch et al., 1997)
Zebrafish *GATA-2/GFP*	BAC	70, 80	Zebrafish	Expression pattern		(Jessen et al., 1998)
Mouse *RAG1-YFP* Mouse *RAG2-GFP*	BAC	50, 180	Mouse	Expression pattern		(Yu et al., 1999)
Goat *α-lactalbumin*	BAC	160	Mouse	Overexpression Copy-number dependence		(Stinnakre et al. 1999)
Mouse *α9acetilcholine receptor*	BAC	140	Mouse	Expression pattern		(Zuo et al., 1999)
Zebrafish (*rag 1-GFP*)	PAC	125	Zebrafish	Identification of regulatory sequences		(Jessen et al., 1999)
Zebrafish (*Hoxa-11b-lacZ*	PAC	100	Mouse	Identification of regulatory sequences		(Chiu et al., 2000)
Human *Tau*	PAC	200–250	Mouse	Overexpression	Neurodegenerative disease Alzheimer's disease	(Duff et al., 2000)
Human β-globin locus	BAC	100	Mouse	Identification of regulatory sequences	Thalassemias	(Kaufman et al., 1999)
Mouse *OCTN2* (carnitine transport)	BAC	131–204	Mouse	Molecular complementation of targeted mouse mutations (large deletions)	Triglyceride metabolism	(Zhu et al. 2000)
Human *cholesterol 7α-hydroxylase* (CYP7A1)	PAC	>150	Mouse	Identification of regulatory sequences		(Goodart et al., 1999)

Table 3. YAC versus BAC/PAC transgenesis

	YACs	BACs/PACs
Host cell	*Saccharomyces cerevisiae*	*Escherichia coli*
Type of DNA molecule	Linear chromosome	Circular plasmid
Insert size	Up to 1–2 Mb	Up to 300 kb
Protocols for handling and isolation of DNA	Difficult	Easy
DNA yield	Low	Medium (BACs) High (PACs)
Resistance to shearing	Low (fragile)	High (in supercoiled form)
Direct sequencing possible	No	Yes
Selection markers in host cell	Complementation of auxotrophic mutants resistance to drugs	Resistance to antibiotics *kan*^{r} (PACs) *Cm*^{r} (BACs)
Insert chimaerism	High (>50% clones)	Very low (<5%)
Insert rearrangements	Yes	Very rare
Modification capabilities	Plenty Reproducible protocols	Few (increasing) Protocols in evolution
Protocols for Mutagenesis	Easy	Difficult

cases, such as generating transgenic mice with BACs carrying mouse DNA inserts, it is helpful to microinject linearised BAC molecules with vector sequences attached that can serve later as tags (polymorphisms) to identify the presence of transgenes. Alternately, the excision of genomic inserts from BACs based on pBeloBAC11 vector derivatives by *Not*I digestion provides a few hundred vector base pairs at both ends (384 bp and 247 bp surrounding the *Hin*dIII cloning site) that can subsequently be used as tags or for PCR analysis. Similar to YACs, the presence of vector sequences in the microinjected BAC constructs does not seem to have an overt effect on the expression profiles of BAC transgenes, provided the size of the cloned genomic insert can accomodate most of the locus regulatory sequences (Kaufman et al., 1999).

When unique sites are not available the *recA*-assisted restriction endonuclease (RARE) technique may be employed to generate unique sites suitable for BAC insert excision (Boren et al., 1996; Nielsen et al., 1998; Nielsen et al., 1999). Alternatively, the presence of a unique *loxP* site in BAC/PAC-derived clones has been used for the effective Cre recombinase-mediated linearisation of transgenes before microinjection (Mullins et al., 1997).

BAC DNA can be efficiently stabilised in microinjection buffer by the addition of salt (usually sodium chloride) and polyamines, as reported for YACs (Schedl et al., 1993a), although polyamines can be excluded without obvious effects, presumably due to the smaller size of BACs (Kaufman et al., 1999; Yang et al., 1997).

BAC transgenic animals have also been prepared via the ES cell route by co-transfection of BAC transgenes along with a selectable marker, followed by the production of chimaeric mice that are bred to create transgenic lines (Kaufman et al., 1999).

Transgenic animals carrying either BAC or PAC transgenes have been generated with comparable efficiencies to that of standard constructs (5–20% of newborn animals). As with YACs, most of BAC transgenic animals carry a limited number of integrated transgene copies (<5), but up to 13 copies of a BAC transgene have been reported (Nielsen et al., 1997). There are not many studies addressing copy number-dependent expression of BAC transgenes. Nevertheless, position-independent and copy-number-related expression has been shown in goat α-lactalbumin BAC transgenes in mice (Stinnakre et al., 1999).

Unfortunately, not all BAC/PAC transgenes integrate in the host genome as intact DNA molecules. Again, similar to YACs, rearrangements and insertion of fragmented transgenes can occur with BACs (Antoch et al., 1997; Kaufman et al., 1999) suggesting that rearrangement appears to be primarily related to trans-

gene size, irrespective of YAC or BAC origin (Kaufman et al., 1999). Therefore, detailed Southern/PCR analysis of integrated BAC transgenes is recommended before further experiments are undertaken with selected transgenic lines.

In conclusion, BAC/PAC constructs closely mimic the optimal performance in transgenesis achieved with YACs (McCormick & Nielsen, 1998; Huxley, 1998; Kaufman et al., 1999; Heintz, 2000; Camper & Saunders, 2000). The choice of vectors should be based primarily on the technical skills of the laboratory and the expected size of the expression domain to be analysed in transgenic experiments. Relatively small genes (<100 kb) can be analysed with BACs/PACs, whereas bigger loci require the use of YACs. A comparison of YAC versus BAC/PAC transgenesis is presented in Table 3, indicating the benefits and problems associated with both systems.

Modification of YAC/BAC/PAC transgenes

BACs and PACs are more convenient to propagate and purify than YACs because they do not require specific methods other than adaptations of existing protocols, commonly applied to routine work with plasmids in bacterial cells (Sternberg, 1999; Birren et al., 1999). In contrast, YACs require unique and more tedious methods along with the need to become familiar with yeast cells, features which might have prevented their rapid dissemination and implementation in some laboratories (Green et al., 1999).

In spite of such apparent disadvantages, YACs have documented advantages over BACs with regard to their modification capabilities and the ease with which these are achieved. The yeast system offers an unlimited variety of modifications that can be introduced in YACs using standard protocols that exploit the efficient yeast endogenous homologous recombination system (Schlessinger, 1990; Monaco & Larin, 1994; Peterson, 1997b; Green et al., 1999).

In bacteria, a number of innovative methods have been developed recently to retrofit specific markers in BACs and PACs (Mejia & Monaco, 1997) and, remarkably, for easier mutagenesis of BACs and PACs (Yang et al., 1997; Zhang et al., 1998; Nielsen et al., 1998; Jessen et al., 1998; Chiu et al., 2000; Yu et al., 2000), opening the possibility of extending the range of BAC/PAC transgenic approaches to a similar degree of complexity as that of YAC transgenics. In this section we will discuss the modification potential of YACs compared to that of BACs and PACs.

Fragmentation vectors provide new telomeres along with selectable markers and have been used in YACs for a variety of purposes. These YAC vectors have been utilized to reduce the size of YAC constructs at precise locations, thereby generating a nested set of deletion derivatives (Montoliu et al., 1996; Wutz et al., 1997), to add selectable markers such as a neomycine-resistance gene (Lamb et al., 1993) or to combine size reduction with the addition of new features such as an amplification system (Schedl et al., 1993a; Fujiwara et al., 1997) or a reporter *lacZ* gene (Heard et al., 1996). Modified YACs are produced by targeted disruption of the original construct by homologous recombination driven in yeast cells. In some cases, homologous target sequences might not be known or available, but fragmentation can still be performed, at random locations, via repetitive sequence elements (i.e., B1 elements in the mouse genome; Zweigerdt et al., 1997; Lakshmanan et al., 1998). Following this approach, a YAC deletion series can be easily generated covering hundreds of kilobases in a single yeast transformation experiment, thus permitting the functional identification of distal regulatory elements (Zhou et al., 1998) or functional domains (Lee et al., 1999). With transgene DNA of human origin, an equivalent strategy can be devised using YAC fragmentation vectors that include *Alu* repeat sequences (Wunderle et al., 1998; Fujiwara et al., 1999b).

Modifications (i.e., deletions) at internal YAC sequences can also be performed by substituting the targeted sequence with a yeast selectable marker surrounded by neighbouring homologous sequences (Montoliu et al., 1996). Such YAC replacement vectors can also be used for the targeted insertion of heterologous sequences (Fujiwara et al., 1999a). However, these replacement type vectors leave behind the yeast selectable marker within the body of the YAC insert, which might interfere later with proper transgene expression. A cleaner alternative is to use the '*pop-in/pop-out*' method in yeast cells, which uses an integrative type of plasmid vectors called YIP (yeast integrative plasmid). Two rounds of homologous recombination are required to substitute the original sequence of a YAC with the desired mutation, without retaining the selectable marker. This elegant and powerful approach has been used in a variety of cases: to introduce point mutations at precise locations within a YAC (Duff et al., 1994; McCormick et al., 1995. 1997a; Lamb et al., 1997), to reproduce

trinucleotide repeat characteristics of a set of human diseases (Hodgson et al., 1999; La Spada et al., 1998; Cemal et al., 1999), to generate minor deletions at regulatory sequences of the YAC-borne gene (Giraldo et al., 1999), and to introduce a reporter gene (i.e., *lacZ*) by transcriptional fusion with the targeted gene (Hiemisch et al., 1997; Ainscough et al., 1997) or via IRES-facilitated transcription (Vassaux & Huxley, 1997).

Studies of human β-globin locus gene regulation during development have benefitted largely from the introduction of precise mutations by homologous recombination techniques in yeast cells and the analysis of modified YAC transgenes in mice (Peterson et al., 1998). Some of the engineered modifications (i.e., amino-acid exchange, 5′ breakpoints, internal deletions) correspond to equivalent defects found in patients affected by diverse forms of β-thalassemia syndromes and related human diseases (Peterson et al., 1995; Calzolari et al., 1999). Moreover, a large number of new mutations have been generated in human β-globin YAC transgenes to address the role of specific sequences (i.e. LCR) in the regulation of gene expression during development. These modifications include deletion of regulatory sequences (Peterson et al., 1996; Liu et al., 1997; Navas et al., 1998; Calzolari et al., 1999), inter-replacement of regulatory elements (Bungert et al., 1995) and alterations in the gene order or orientation of key regulatory sequences (Tanimoto et al., 1999).

The unlimited potential of YAC modifications is best illustrated by the generation of transgenic mice producing human antibodies (Mendez et al., 1997). In this report, the authors built megabase-sized YACs containing large contiguous genomic fragments corresponding to unrearranged human heavy and light immunoglubulin genes using homologous recombination in yeast. Final YAC constructs were obtained by step-wise fusion, via recombination, of smaller and partially overlapping YACs (Mendez et al., 1997).

BACs have been more difficult to modify than YACs, although it is likely that the progressive development of new techniques in this rapidly evolving field will see greater ease of modification. The main reason for difficulties in modifying BACs and PACs is that bacterial host cells are *recA* deficient, to prevent undesired recombination and rearrangements and to favour insert stability. Thus, several recombination pathways in bacteria have been explored that are normally absent in BAC host cells. Their utilization requires either the transfer of the BAC to a suitable bacterial strain or the exposure of the BAC-containing cell to the specific recombination machinery. Often these systems are not understood well and are prone to producing unexpected alterations that might give rise to the generation of erroneously modified BAC/PAC clones. Detailed structural analysis of resulting BAC clones is strongly recommended for the following methods.

The first describing a BAC modification system by homologous recombination in bacterial cells was that of Yang et al., (1997). Their approach is analogous to the yeast '*pop-in/pop-out*' strategy but is more complex, and has been used to target the incorporation of an IRES-*lacZ* reporter gene at precise internal locations within a BAC transgene. The expected homologous recombination event occurred at frequencies lower than those found in equivalent experiments with YAC constructs (Yang et al., 1997). This modification approach, based on the transient expression of *recA* protein, has been reproduced by several independent groups with BACs and P1 clones (Yu et al., 1999; Zuo et al., 1999; Payne et al., 1999). A simpler alternative was developed that uses the capacity of two properly oriented short DNA sequences (Chi-sites) to trigger the transfer of a DNA fragment located between them to homologous DNA by means of the recBCD pathway (Jessen et al., 1998). The authors showed that most BAC modified clones incorporated the desired homologous recombination event (a transcriptional fusion of a *lacZ* reporter gene) in the generation of transgenic zebrafish (Jessen et al., 1998). The same team reproduced chi-stimulated homologous recombination using a PAC clone with similar success (Jessen et al., 1999). Further experiments are necessary before the potential of this promising technique can be established.

A third modification system has been developed based on the *recE* and *recT* recombination pathway (Zhang et al., 1998). This approach, known as 'ET-cloning' uses homologous recombination driven by short sequences common to the BAC/PAC and the targeting vector. The authors showed the efficient modification of a P1 clone by targeting the insertion of an antibiotic resistance gene (Zhang et al., 1998). An updated version of the 'ET-cloning' method specially suited for the modification of BACs was recently reported, based on the functional counterparts of *recE* and *recT* proteins of bacteriophage λ (Muyrers et al., 1999). The combination of homologous recombination techniques coupled with the use of *FRT*/FLP and *loxP*/CRE site-specific recombination systems allows

the excision of the selectable marker employed to detect the very rare homologous recombination event (Buchholz et al., 1996; Zhang et al., 1998; Muyers et al., 1999). Despite the elegance and potential of the 'ET-cloning' method it has proven difficult to master in a number of laboratories that failed to obtain the desired BAC modification at the expected frequencies that were reported by the original authors. A possible solution was provided by placing all recombinogenic proteins under the control of a tightly regulated and inducible promoter, diminishing the risk of overexpression and the appearance of unwanted rearrangements (Narayanan et al., 1999). Using this modified protocol, called 'GET recombination', the authors could efficiently target the integration of a selectable marker (Narayanan et al., 1999), and the insertion of an EGFP reporter cassette within a 200 kb BAC carrying the human β-globin locus (Orford et al., 2000). The targeted insertion of the EGFP cassette also involved a series of deletions within the β-globin locus (up to 44 kb). Comparable results have been obtained with a BAC containing the mouse *whey-acidic protein* (*WAP*) gene that has been modified by the targeted insertion of a reporter secreted alkaline phosphatase gene (SEAP, Clontech) using 'GET recombination' (Aguirre & Montoliu, unpublished). The unlimited potential of the 'GET recombination' technique has been demonstrated by a recently developed method that allows the introduction of point mutations without leaving behind any operational sequences (Nefedov et al., 2000), analogous to the '*pop-in/pop-out*' strategies applied in YACs and BACs. In this case, the tetracycline resistance gene (TetR) has been used for both positive and negative selection in two consecutive rounds of homologous recombination. Using this technique, one of the most common β-thalassaemia mutations has been introduced into the intact β-globin locus present in a BAC (Nefedov et al., 2000).

Another modification system has been proposed recently for the targeted mutagenesis of BAC/PAC clones, based on the recombinogenic function provided by a defective λ prophage (Yu et al., 2000). Other mutagenesis methods, not using homologous recombination techniques, have been evaluated including the use of RARE cleavage in order to generate 5′ and 3′ BAC/PAC deletion derivatives (Nielsen et al., 1998), and the use of random-insertion mutagenesis using a transposon-mediated system (Brune et al., 1999).

Finally, YAC-shuttle vectors have been applied to convert P1 clones and PACs into YACs, to facilitate further modification using the yeast homologous recombination system (Chiu et al., 2000; Poorkaj et al., 2000). Conversely, methods have been established to transform standard linear YACs (up to 250 kb) into circular YACs that can also be propagated in *E. coli* as BACs, thereby facilitating sequencing and functional analysis of genomic regions (Cocchia et al., 2000).

Perspectives

Despite the differences between YAC and BAC/PAC transgenesis approaches, both are associated with optimal performance in transgenic experiments. The size of their genomic inserts, ranging from less than 100 kb to more than 1 Mb, normally guarantees the inclusion of most regulatory sequences that are relevant for the faithful regulation of a gene. Therefore, artificial chromosome-type transgenes are usually expressed in appropriate spatial- and temporal-specific manners.

At present, YACs are much easier to modify than BACs/PACs, though more difficult to handle. The ability to easily retrofit BAC/PAC clones is a great challenge for the immediate future. Nonetheless, due to the relevance of BACs and PACs in genome research and functional genomics, it is likely that this situation will change, once established and reproducible protocols are disseminated within the scientific community. Artificial chromosome transgenesis has been fundamental for the isolation of candidate genes by complementation of mutations or alterations in the phenotype, and the generation of improved animal models of human genetic diseases. Large contiguous chromosomal fragments can be functionally scanned by transgenic approaches using a set of overlapping YAC/BAC/PAC clones spanning the region (i.e. Smith & Rubin, 1997; Frazer et al., 1997; Zhu et al., 2000). The identification of genes associated with known quantitative trait loci (QTL) has also been shown to benefit from artificial chromosome transgenesis (Symula et al., 1999). These techniques are expected to have a major impact on the analysis of gene expression and function in systems with a high degree of complexity, such as the mammalian central nervous system, in which standard loss-of-function approaches usually do not provide a clue to understand the role of a gene. In this respect, gene dosage experiments produced by increasing overexpression of transgenes within YAC/BAC/PAC clones will be instrumental for the correct understanding of gene function (Heintz, 2000).

Finally, the benefits of artificial chromosome transgenesis will be exported to biotechnological applications, such as the production of pharmaceutical or nutriceutical proteins in the mammary gland of transgenic animals (Zuelke, 1998; Fujiwara et al., 1999a).

In 1993, one of the first reviews discussing the future potential of artificial chromosome transgenesis concluded, with some degree of prudence, that 'bigger is probably better' (Forget, 1993). Indeed, seven years later, after having witnessed the enormous development of these techniques one can undoubtedly state: 'size matters' in animal transgenesis.

Note added in proof

Since submission and acceptance of this review two alternative methods have been described for engineering BACs by homologous recombination [Lalioti and Heath (2001) Nucleic Acids Res. 29: e14; Swaminathan et al. (2001) Genesis 29: 14–21].

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