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Transgenic mice carrying yeast artificial chromosomes

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The generation of transgenic mice with yeast artificial chromosomes (YACs) has proven to be a valuable system to: (1) study gene structure–function relationships; (2) produce mouse models of human disease; (3) complement mouse mutants; (4) generate mice bioreactors; and (5) screen YAC libraries in vivo. Continued refinement of current techniques and development of new protocols should encourage widespread adaptation of this strategy for these and other applications. Use of whole loci as transgenes is an important improvement in murine transgenesis because it results in a more realistic pattern and level of gene expression during ontogeny. Application of this technology to develop human artificial chromosomes (HACs) might provide the next generation of gene therapy vectors that will overcome most of the problems and barriers associated with current vector systems.

Until the advent of transgenic mouse technology, studies of mammalian gene expression and regulation were largely confined to cell lines transfected with constructs containing limited genetic information. Developmental studies were not possible because cell lines are generally locked into one ontogenic stage by virtue of having been immortalised at whatever developmental stage they had reached. By contrast, the establishment of transgenic mouse technology as a routine method in many laboratories facilitated the study of gene function during development and in disease (Refs 1, 2, 3, 4, 5). Transgene expression in mice allowed complementation of existing murine mutants and assessment of other phenotypic effects. In addition, gene expression could be studied throughout development and tissue-specific regulatory elements could be analysed.

Despite the huge importance of transgenic technology, expression of transgenes could be erratic owing to position effects and copy-number-independent expression (Ref. 6). In part, these problems were a consequence of the nature of the transgene constructs: typically, constructs had to be limited in size because of constraints on how large a DNA fragment could be stably cloned into plasmid or cosmid vectors and isolated without degradation before introduction into the mouse. Thus, genes with exons spanning several hundred kilobases, or loci containing multiple genes, could not be used as transgenes, nor could potentially important or unidentified cis-acting sequences be included. cDNAs were often substituted for large genes, or individual genes from a multigene cluster were utilised. However, expression from these constructs was subject to

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the effects of the surrounding chromatin into which they were integrated (Ref. 6). Some improvement was achieved when additional cis elements were included, such as enhancers, introns and polyadenylation signals (Refs 7, 8, 9, 10). Although these extra sequences boosted the level and reproducibility of transgene expression, the truncated constructs lacked their natural regulatory elements, and thus developmental studies did not necessarily indicate how the native gene might be regulated.

Full-size, intact genes or entire loci used as transgenes might improve the utility of transgenic studies, and inclusion of distal regulatory elements as part of the native locus might validate developmental studies and insulate the construct from position effects. By recreating more-native genetic conditions, mouse models of human disease might better recreate the pathogenic phenotype observed in patients. With this in mind, in 1993 several research groups successfully implemented the use of yeast artificial chromosomes (YACs) as transgenes (Refs 11, 12, 13, 14, 15, 16, 17, 18, 19). A number of techniques have been described that are suitable for introducing YACs into transgenic mice (reviewed in Refs 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31). This review discusses the development of YAC-transgenic technology, examples of transgenic mouse models and the types of information that can be obtained from them, and also the development of human artificial chromosomes (HACs) and their potential use in gene therapy.

Cloning and maintenance of large DNA sequences

Although YAC vectors are the primary focus of this review, alternative vector systems are available that can accommodate large DNA fragments. Bacteriophage λ clones can contain up to 22 kb, and cosmid clones up to 40 kb; DNAs of this size are routinely introduced in the production of transgenic animals. Other large-capacity cloning vectors include bacteriophage P1 (Ref. 32), P1 artificial chromosomes (PACs; Ref. 33), fosmids (based on the *Escherichia coli* F factor replicon; Ref. 34) and bacterial artificial chromosomes (BACs; Ref. 35).

YACs have several distinct advantages over other large-capacity cloning vectors. First, the maximum insert size that can be contained in YACs is ~2 Mb (Ref. 36) – regions too large to be cloned intact using traditional bacterial cloning systems. By contrast, P1 can maintain only up to 100 kb, and PACs and BACs have cloning capacities up to 350 kb. Using YACs, intact genes, multigenic loci, distant regulatory sequences and higher-order genomic structure can be studied in the context of native sequences. Because extensive sequences, including coding regions and flanking genomic DNA, can be maintained in an inert state in YAC clones, all sequences having potential regulatory relevance, as well as distances between genes and control elements, are maintained. Second, site-specific mutagenesis can be readily and efficiently performed in vivo using the homologous recombination system of the yeast host instead of recombinant DNA technology (Refs 37, 38, 39, 40). Point mutations, deletions, insertions and replacements can be easily introduced into a YAC without leaving behind foreign DNA, such as selectable marker cassettes, or without producing unwanted alterations. These mutant YACs can then be used to generate transgenic mice in order to study the effect of the mutation on transgene expression.

Although not the focus of this review, mention should also be made of new and diverse methodologies that have been developed to modify BACs accurately in a manner similar to YACs (Refs 41, 42, 43, 44, 45, 46, 47). The most widely used technique is called 'ET-cloning' and several modified versions of this method have appeared (Refs 44, 45, 46, 47).

YAC vectors

Use of YACs for maintenance of exogenous genomic sequences was first described by Burke and colleagues in 1987 (Ref. 36). Since then, a large variety of vector derivatives have been synthesised that are optimised for specific applications, many of which have been catalogued (Refs 21, 31). All YAC clones share several common elements (Fig. 1), consisting of two 'arms' flanking a unique restriction enzyme cloning site for insertion of genomic DNA. The arms contain yeast chromosome sequences essential for vector maintenance such as a centromere (CEN), two telomere (TEL) regions and an autonomous replication sequence (ARS). One yeast gene that allows prototrophic selection for yeast containing the YAC is carried on each vector arm; this is usually *TRP1* (necessary for tryptophan synthesis) and *URA3* (necessary for uracil synthesis).

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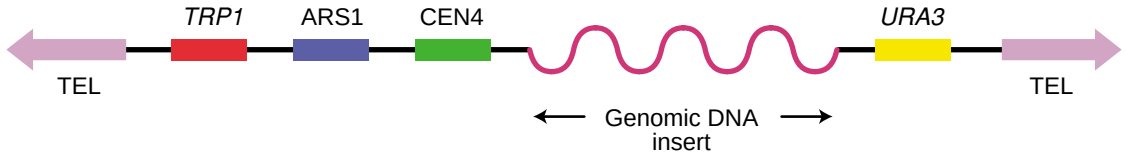
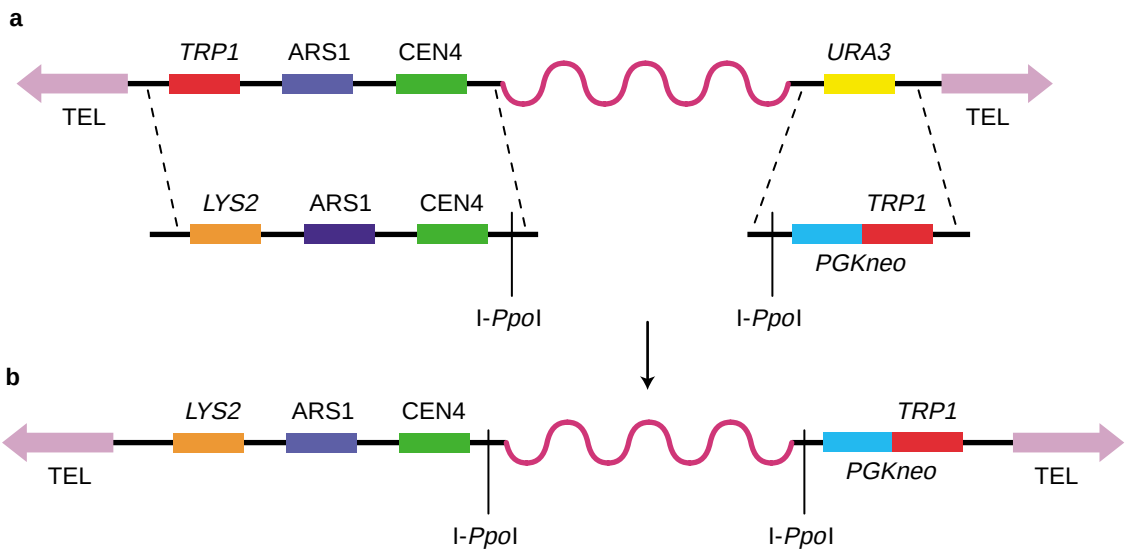


Schematic representation of a YAC clone

Figure 1. **Schematic representation of a YAC clone.** The yeast chromosomal elements on the YAC (yeast artificial chromosome) vector arms are displayed as variously coloured boxes or arrows, based on the pYAC4 vector (11.5 kb). Although there is no minimum fragment size and the maximum size is constrained by limitations of current methodology, reported cloned insert sizes range from 23 kb to over 2 Mb. The centromere, telomere and origin of replication elements are the minimal sequences required to form a chromosome, and are essential for the function of artificial chromosomes in yeast. Abbreviations: ARS1, autonomous replicating sequence; CEN4, centromere; TEL, telomere; *TRP1*, yeast selectable marker for tryptophan prototrophy; *URA3*, yeast selectable marker for uracil prototrophy **(fig001kpk)**.



Retrofitting of the pYAC4 vector

Figure 2. **Retrofitting of the pYAC4 vector.** (a) An unmodified YAC (yeast artificial chromosome) clone can be 'retrofitted' to increase its versatility by homologous recombination with 'retrofitting fragments'. (b) This generates a new vector with improved utility with respect to mutagenesis, structural analysis of inserts and vector selection: it contains the *LYS2* gene instead of *URA3*, rare-cutting I-*Ppo*I restriction enzyme sites near the vector–insert junction, and a *PGKneo* cassette to select transfected embryonic stem (ES) cells (see Ref. 49 for further details). The use of *LYS2* as a selectable marker on the YAC arm instead of *URA3* frees *URA3* for use in yeast-integrating plasmid (YIP)-mediated 'pop-in, pop-out' mutagenesis (Fig. 3) Dashed lines indicate boundaries of homologous recombination. Abbreviations: ARS1, autonomous replicating sequence; CEN4, centromere; I-*Ppo*I, rare-cutting restriction enzyme sites introduced into the YAC vector arms for YAC transgene structural analysis; *LYS2*, yeast selectable marker for lysine prototrophy; *PGKneo*, mammalian selectable marker for resistance to G418; TEL, telomere; *TRP1*, yeast selectable marker for tryptophan prototrophy; *URA3*, yeast selectable marker for uracil prototrophy **(fig002kpk)**.

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Optimisation of YAC vectors for use in generating YAC-transgenic mice

Many YAC clones have been constructed in YAC vectors such as pYAC4, which has the selectable markers *TRP1* and *URA3* (Refs 21, 31, 48). These clones can be used directly to generate transgenic mice, but their versatility is limited for production of transgenics containing mutant YACs. To enhance their utility, three problems must be rectified by modification of the YAC vector arms – a process called 'retrofitting' (Fig. 2). First, although mutagenesis by homologous recombination in yeast has been well documented using the *URA3* marker in a two-step differential selection process (Refs 37, 38, 39, 40), the presence of the *URA3* gene on the arm of the YAC vector precludes its use for this purpose and it must be deleted from the vector. Second, owing to the lack of unique rare-cutting restriction enzyme sites in the YAC vector, structural analysis to determine the integrity of YAC transgenes is difficult without a comprehensive restriction map of the cloned insert. Third, although YACs can also be introduced into embryonic stem (ES) cells for the generation of chimaeric mice, or into other established cell lines, a selectable marker such as the gene encoding neomycin resistance is necessary to select YAC-bearing cell clones.

Retrofitting YAC vectors to incorporate sequence motifs that increase the utility of the vectors is accomplished by homologous recombination. The details of this process have been described elsewhere and many constructs are available for retrofitting YACs (Refs 20, 21, 26, 31, 48, 49). For example, one series of retrofitting constructs alters the YAC vector such that the three problems just described are solved (Fig. 2) (Ref. 49). The *TRP1* gene on the left arm of the YAC is replaced with the yeast *LYS2* gene (necessary for lysine synthesis) and a rare-cutting I-*Ppo*I restriction enzyme site is introduced near the insert–vector junction. The *URA3* gene on the right arm of the yeast vector is replaced with the yeast *TRP1* gene, a *PGKneo* gene and another I-*Ppo*I site near the insert–vector junction. The YAC vector is selected for by growing yeast in the absence of tryptophan and lysine. The *PGKneo* cassette provides a selectable marker for transfected ES cells, and the I-*Ppo*I sites allow structural analysis of intact YAC inserts without the need for a restriction enzyme map of the cloned insert.

Mutagenesis and segregation of YACs

A major advantage to using YACs as transgenes is the ease of site-specific mutagenesis of a target gene or cis-regulatory sequence. Homologous recombination in the yeast host can be used to introduce almost any mutation into the YAC DNA prior to transgenesis. Two methods for this type of manipulation have been described in detail elsewhere (Refs 26, 39, 40). The 'pop-in, pop-out'

Figure 3. Introducing mutations into YAC vectors (*legend; see next page for figure).* (a) The 'pop-in, pop-out' method. In the first step ('pop-in') of this method of homologous recombination in yeast, a yeast-integrating plasmid (YIP) made linear by digestion with a restriction enzyme is transformed into yeast where it recombines with the YAC (yeast artificial chromosome) vector (solid lines indicate crossover regions). The recombinant vector carrying the *URA3* gene is selected using uracil prototrophy. The intermediate construct contains a duplication of the target sequence, one wild-type and one mutant, with the YIP vector sequences, including the *URA3* cassette, in between. In the second step ('pop-out'), uracil is added back to the medium so that the *URA3* gene is no longer required for viability. At a frequency of 10^{-4} to 10^{-5}, spontaneous recombination events occur between the duplicated target sequence resulting in either reversion to wild-type sequence or retention of the mutant sequence. These events can be selected for using 5-fluororotic acid (5-FOA): URA3 metabolises 5-FOA into a toxic compound. Yeast that maintain *URA3*$^+$ YACs are killed, whereas yeast lacking *URA3* are resistant to 5-FOA (5-FOA^R). Thus, YIP integration into the YAC involves only a single crossover event, as does the excision of YIP sequences, and both steps can be selected. For further details of this method, see Refs 39 and 40. (b) The 'sequence-replacement' method. In the first step of this method of homologous recombination in yeast, the targeting fragment contains the desired mutation and is interrupted by the *URA3* cassette. Selection is for uracil prototrophy. In the second step, the targeting fragment also contains the mutation, but lacks the *URA3* cassette. The *URA3* gene is lost following recombination and the mutant target sequence is recreated without interruption. Selection is for 5-FOA resistance. Note that whereas the 'pop-in, pop-out' method uses a single YIP vector that recombines via a single crossover event following only one transformation of YAC-bearing yeast, the 'sequence-replacement' method uses two DNA fragments requiring two yeast transformations, both of which recombine through a double crossover event. For further details of the 'sequence-replacement' method, see Ref. 39 **(fig003kpk)**.

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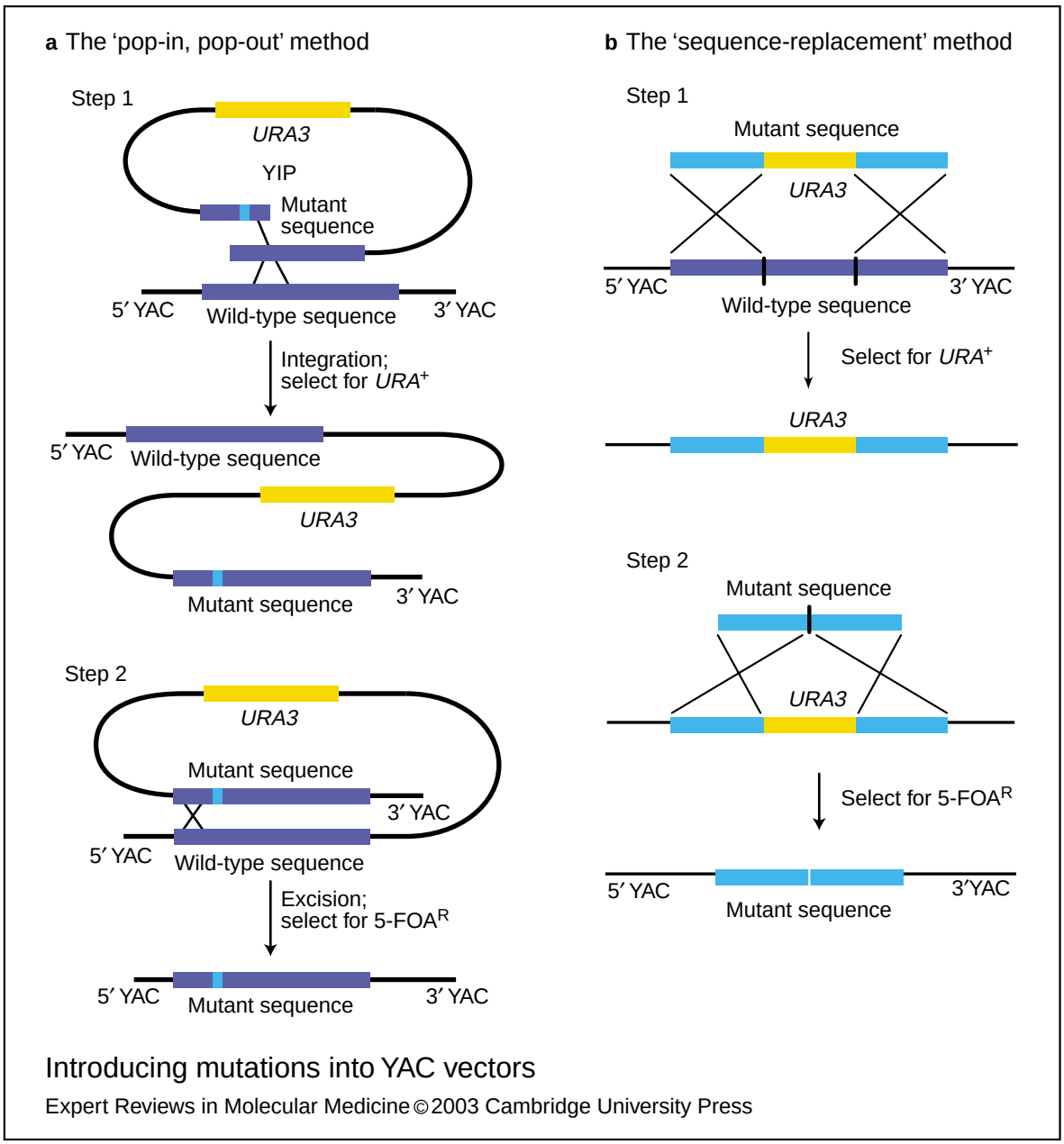

Figure 3. Introducing mutations into YAC vectors (*see previous page for legend*) **(fig003kpk)**.

method has been used most extensively and requires the production of only one construct in a yeast-integrating plasmid (YIP; Fig. 3a) (Refs 39, 40). YIP integration into the YAC involves only a single crossover event, as does the excision of YIP sequences, and both steps can be selected. The 'sequence-replacement' method is advantageous for the introduction of large deletions, but is also applicable for recombining point mutations, small deletions or insertions into the target YAC sequence (Fig. 3b) (Ref. 39). The 'sequence-replacement' method has the disadvantage of needing two constructs. Thus, two transformations of yeast are required to produce a mutant YAC. However, this approach can be used to introduce mutations into the YACs when a convenient scheme cannot be devised using the YIP-mediated 'pop-in, pop-out' method of mutagenesis.

The presence of both mutant and wild-type YACs are frequently observed in the same yeast

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isolate after mutagenesis (Ref. 50). YACs are not replicated and meiotically segregated in strict concordance with yeast chromosomes. Over time, even under selection, daughter cells arise that carry one or more YAC copies. During mutagenesis by recombination, the targeting vector needs to integrate in only one of these YAC copies to establish selection through the marker on the vector. Thus, in a single yeast cell, mutant and normal YACs might co-exist. These YACs should be segregated from one another to avoid co-injection of the normal and modified YACs. One of two schemes is normally used for this: the mutant and wild-type YACs can be meiotically segregated (Ref. 39) or, alternatively, the 'kar-crossing' method can be utilised (Refs 51, 52, 53). In the latter approach, a YAC-containing strain is mated with a *kar1* mutant strain, which is defective in nuclear fusion. Karyogamy does not occur and a heterokaryon is formed with two nuclei. Haploid progeny derived from a heterokaryon share parental cytoplasm, but contain the nucleus from one parent or the other. At very low frequencies, a YAC will be transferred from one nucleus to the other in a heterokaryon. Selection is for the YAC and against the donor nucleus; thus, multiple copies of the YAC can be segregated from one another. A third method is to grow the yeast host in the absence of selection and to allow the YACs to partition asymmetrically into daughter cells during cell divisions [P.A. Navas (Division of Medical Genetics, University of Washington, WA, USA), pers. commun.]. Selection is re-applied and yeast colonies are screened for mutant or wild-type YACs. This approach is less cumbersome and can be performed in parallel to the aforementioned methodologies.

Generation of YAC-transgenic mice

Three basic methods have been described for the production of YAC-transgenic mice (Fig. 4): (1) fusion of yeast spheroplasts with ES-cell protoplasts (Refs 11, 19); (2) lipofection of purified YAC DNA into ES cells (Refs 13, 14, 17, 18); and (3) direct microinjection of purified YAC DNA into mouse oocyte pronuclei (Refs 12, 15, 16). Usually a yeast clone containing the YAC transgene of interest is isolated prior to transgenesis, but some applications described below utilise whole YAC libraries. The first two of these methods introduce YACs into ES cells, which are subsequently used for injection of blastocysts to produce YAC-bearing mice; in the third method, the DNA is instead injected directly into mouse oocyte nuclei to produce YAC-bearing mice. The salient features of each method are outlined below and their advantages and disadvantages are summarised in Table 1. Details of these methods are reviewed elsewhere (Refs 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30). Table 2 provides a comprehensive list of YAC-transgenic mice and the method by which they were produced.

Yeast–ES-cell fusion

YACs are first retrofitted with a *PGKneo* gene cassette by homologous recombination in yeast (see above). Yeast spheroplasts are enzymatically generated and then fused to ES cells using polyethylene glycol (PEG). Selection for G418 resistance (encoded by the *neo* gene) results in up to 40% of the ES cells retaining single or multiple YAC copies that contain most of the YAC, including both vector arms (Ref. 22). Chimaeric mice are generated from the ES cells; these mice are capable of germline transmission of the YAC DNA (Ref. 11). This method does not require purification of the YAC DNA, which could produce some fragmentation of the YAC, especially as the YAC size increases. In fact, yeast genomic DNA is also transferred by this method and has been found in ES cells after fusion; however, yeast DNA does not appear to inhibit ES cells from generating chimaeric mice, nor does it prevent germline transmission (Ref. 11). Because of the size constraints on insert size imparted by the other two methodologies, yeast–ES-cell fusion might be the only method available for transfer of megabase size YAC DNAs such as the 2.3 Mb Duchenne muscular dystrophy gene (Refs 54, 55).

Lipofection of ES cells

Purified YAC DNA is complexed with a lipid reagent and transfected into ES cells. Selection in G418 for retrofitted YAC transductants carrying the *neo* gene is performed as described for spheroplast fusion, or by cotransfection of YAC DNA and *PGKneo*-cassette-containing plasmids. Approximately 10% of G418-resistant ES cells contain an intact copy of the retrofitted YAC DNA (Refs 13, 17); the level drops to 1% for YACs cotransfected with a *PGKneo* plasmid (Refs 14, 18).

Microinjection of murine pronuclei

Production of transgenic mice by pronuclear injection requires highly purified DNA (Refs 12,

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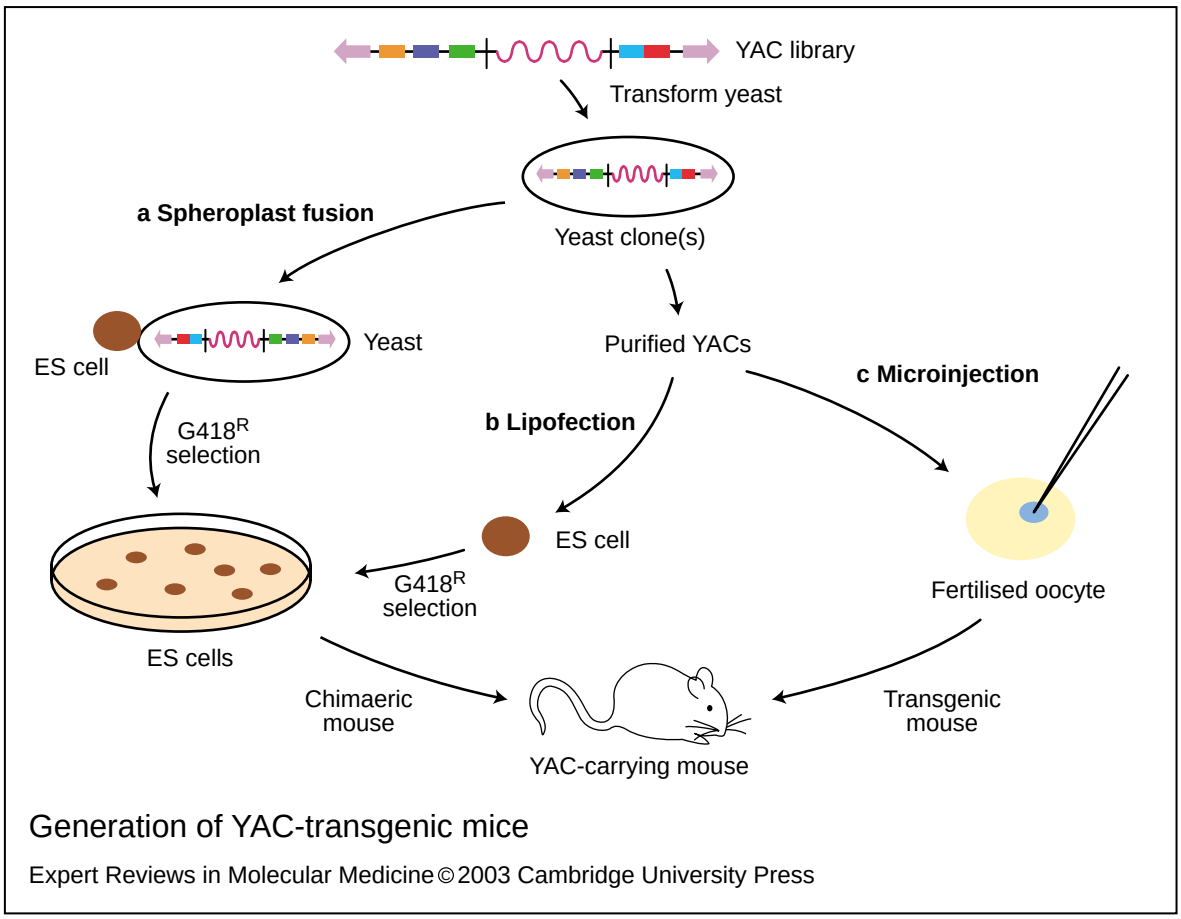

Figure 4. Generation of YAC-transgenic mice. A YAC (yeast artificial chromosome) library is constructed using target genomic DNA, yeast are transformed and desired YAC clones are isolated using standard techniques for library screening. YAC clones can also be identified in existing YAC libraries available commercially or through different laboratories. In some instances, entire libraries are used to produce transgenic mice when the goal is to identify a YAC clone by complementation of a mouse mutation or through a predicated phenotype in mice (see Table 2 for examples). For most applications individual clones are utilised. Three alternative methods can be used for the production of YAC-transgenic mice. (a) YAC-containing yeast spheroplasts can be enzymatically generated and fused to embryonic stem (ES) cells using polyethylene glycol (PEG). Selection for G418 resistance ($G418^R$; encoded by the *neo* gene carried by the YAC vectors) results in up to 40% of the ES cells retaining single or multiple YAC copies that contain most of the YAC, including both vector arms. (b) Purified YAC DNA can be complexed with a lipid reagent and introduced into ES cells by lipofection. Selection in G418 results in approximately 1–10% (depending on the method of selection, see main article) of G418-resistant ES cells containing an intact copy of the retrofitted YAC DNA. (c) Purified YAC DNA can be directly microinjected into fertilised mouse oocyte pronuclei. The efficiency of transgenesis obtained using this protocol has been reported as 10–14%, but only 2–4% contained intact YAC transgenes. In the first two of these methods, ES cells carrying the YAC vector are used for injection of blastocysts to produce YAC-bearing mice (which are chimaeric because they develop from a mixture of recombinant and their own ES cells). In the third method, because the YAC DNA is introduced directly into the oocyte, a transgenic mouse is generated. For further details of these methods, see Refs 11, 12, 13, 14, 15, 16, 17, 18 and 19 **(fig004kpk)**.

15, 16). A minor problem to be overcome during development of this protocol was cleaning of the DNA solution to avoid clogging of the injection needle. Undigested agarose or insoluble particles are removed from the DNA solution by filtration (Refs 15, 56), dialysis (Refs 12, 16) or centrifugation just prior to injection (Refs 15, 57). The efficiency of transgenesis obtained by injecting a 248 kb human β-globin locus YAC using this protocol was 10–14%, a level similar to that achieved with

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Table 1. Comparison of YAC transgenesis methods (tab001kpk)

Method	Advantages	Disadvantages
Yeast–ES-cell fusion	YAC DNA purification not required High percentage of cells harbour intact YAC copies Structural analysis possible in cells before generation of chimaeric mice; functional analysis using ES cells also possible	Effect of yeast genomic DNA on ES cells and transgenic mice is unknown Process to obtain germline-transmitting chimaeric mice is time-consuming
Lipofection of ES cells	Yeast genomic DNA not transferred Transgene structure–function studies possible in cells before generation of mice	Difficult to isolate intact YAC DNA Cells usually contain single or multiple fragmented YAC copies in addition to intact copies Lengthy process to generate chimaeric mice with ES cells
Pronuclear microinjection	Rapid production of transgenic mice relative to ES-cell-generated chimaeric mice Yeast genomic DNA not transferred	Difficult to isolate intact YAC DNA Potential for shear and fragmentation of YAC DNA during microinjection

Abbreviations: ES cell, embryonic stem cell; YAC, yeast artificial chromosome.

injection of smaller plasmid or cosmid constructs (Ref. 15), but only 2–4% contained intact YAC transgenes. Schedl et al. (Ref. 12) reported a 1% yield of transgenic mice containing intact YAC DNA following injection of a 250 kb mouse tyrosinase YAC. Furthermore, for transgenesis by microinjection, the number of mice containing intact YAC copies drops as the YAC vector size exceeds the 500–650 kb range. Larger YAC DNAs will not pass through the bore of the microinjection needle without a high probability of mechanical shearing.

Purification of YAC DNA

Both lipofection of ES cells to produce chimaeric mice and microinjection of murine pronuclei to produce transgenic mice require purified YAC DNA. However, in vitro manipulation of DNA causes physical damage to DNA molecules larger than 40–50 kb, such as deletions of the 5' and 3' ends of YAC clones, and in early studies the major technical difficulty was stabilising the YAC DNA in vitro to minimise shear and denaturation. Gnirke et al. found that solutions of high ionic strength could be used as a protective agent such that intact YAC DNA could be obtained (Ref. 57); other groups found that polyamines or high salt plus polyamines had a similar effect (Refs 12, 58). The second problem that had to be overcome in early studies was the concentration of DNA solutions within a useful range for lipofection or microinjection. Current protocols circumvent this problem by using a final concentration step.

Briefly, the steps used currently in YAC DNA preparation are as follows: (1) preparative pulsed-field gel electrophoresis (PFGE) to fractionate the yeast chromosomes and YAC; (2) enzymatic digestion of the agarose gel slice containing the YAC using agarase or gelase (Ref. 58) in the presence of high salt and/or polyamines to protect against shearing (Ref. 59); and (3) concentration by low-speed ultrafiltration (Refs 15, 17, 57) or dialysis with sucrose (Ref. 12). In some cases, the first step includes a second electrophoresis carried out perpendicular to the first separation, to pre-concentrate the YAC DNA before enzymatic digestion of the gel slice (Refs 12, 60, 61, 62).

Identification and structural analysis of YAC-transgenic mice

Demonstration of YAC integrity in transgenic mice is necessary since fragmentation of YACs can occur during in vitro manipulation and microinjection, as described above. Incomplete YAC copies could affect spatial and temporal patterns of transgene expression as a result of loss of gene structural or regulatory sequences,

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Table 2. Transgenic or chimaeric mice produced with YACs[a] (tab001kpk)

Gene/locus: disease model/goal	Transfer method	Size (kb), structure	Expression	Refs[b]
Human hypoxanthine phosphoribosyltransferase (HPRT) locus: complementation of mutation	Spheroplast–ES-cell fusion	670, probably intact	Tissue-specific, endogenous level	11
Mouse tyrosinase gene: rescue of albino phenotype, structure–function studies	Pronuclear injection	35, 250, intact	Position-independent, endogenous level	12, 92
Mouse α_1(I) collagen (*Col1a1*) locus: complementation of mutation	Lipofection of ES cells	150, probably intact	Endogenous level	13
Human heavy chain Ig gene: human antibody production	Lipofection of ES cells	85	Low level	14, 93
Human β-globin locus: sickle cell disease, β-thalassemias, structure–function studies	Pronuclear injection	248, 150, intact	Tissue-specific, position-independent, endogenous level	15, 16, 56, 66, 72, 73, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112
Human β-amyloid precursor protein (APP) gene: Alzheimer's disease, Down syndrome	Lipofection of ES cells	400, 650, might be intact	Tissue-specific, endogenous level	17, 18, 113, 114, 115
Human Ig light chain: human antibody production	Spheroplast–ES-cell fusion	300, 1300, probably intact	–[c]	19
Human heavy chain Ig gene and light chain κ gene: human antibody production	Spheroplast–ES-cell fusion	220, 170, 800, 1020	High level	74, 75, 116, 117, 118
Human apolipoprotein (a) gene: atherosclerosis, structure–function studies	Pronuclear injection	270, 370, 270, probably intact; 320, not intact	Tissue-specific, probably position-independent, high level	119, 120, 121
Human chromosome 21 region 21q22.2: Down syndrome	Pronuclear injection	430–1100, intact	Position-independent	122, 123
Human apolipoprotein B gene: structure–function studies	Pronuclear injection	108, probably intact	High level	124, 125
Mouse *Xist*/Xic gene: X chromosome inactivation	Pronuclear injection; lipofection of ES cells	350, 450, 460, 480	Not tissue-specific in all animals, position-dependent	126, 127, 128, 129
Human *PMP22* gene: Charcot-Marie-Tooth disease type 1A	Pronuclear injection	560, intact	Tissue-specific, probably position-dependent, high level	76

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Table 2. Transgenic or chimaeric mice produced with YACs (tab001kpk) *(continued)*

Gene/locus: disease model/goal	Transfer method	Size (kb), structure	Expression	Refs
Human membrane cofactor protein (*MCP*), *CD59*, *CD46*: xenotransplantation, measles virus infection, structure–function studies	Pronuclear injection	420	Tissue-specific, position-independent, copy-number-dependent, near endogenous level	130, 131, 132
Human *PAX6* gene: aniridia	Pronuclear injection	420, 310, intact	Tissue-specific, position-independent, endogenous level	68, 133
Human *Huntingtin*: Huntington's disease	Pronuclear injection	350, 600, probably intact	Tissue-specific, position-independent, probably copy-number-dependent, 2–3-times endogenous level	134, 135, 136, 137, 138
Human *IgH*/c-*myc*: Burkitt's lymphoma	Spheroplast–ES-cell fusion	240, probably intact	Tissue-specific	139, 140
Human chromosome 5 5q31 cluster region: gene discovery	Pronuclear injection	350–500	–	141, 142
Human hepatic nuclear factor 3γ (*Hnf3γ*)–*lacZ*: structure–function studies	Pronuclear injection	170, probably intact	Tissue-specific, position-independent, copy-number-dependent	143
Human *CFTR*: cystic fibrosis	Pronuclear injection	310, probably intact	Tissue-specific, position-independent, copy-number-dependent, low level	144, 145, 146, 147
Human Bruton's tyrosine kinase (*Btk*) gene: X-linked agammaglobulinaemia (XLA)	Pronuclear injection	340, probably intact	Tissue-specific, probably position-independent, endogenous level	148
Mouse *H19*, insulin-like growth factor 2 (*Igf2*) region: genomic imprinting	Pronuclear injection	130	–	149, 150, 151
Mouse insulin-like growth factor receptor 2 (*Igf2r*) gene: genomic imprinting	Pronuclear injection	300	Tissue-specific	152
Mouse myogenic factor 5 (*Myf-5*) gene, *Myf-5–lacZ*: structure–function studies	Spheroplast–ES-cell fusion; pronuclear injection	680, fragmentation derivatives of 1000	Tissue-specific	153, 154
Human androgen receptor + CAG repeats: spinal-bulbar muscular dystrophy	Pronuclear injection	450, not intact	No expression	155

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Table 2. Transgenic or chimaeric mice produced with YACs (tab001kpk) *(continued)*

Gene/locus: disease model/goal	Transfer method	Size (kb), structure	Expression	Refs
Mouse *GATA-3, GATA-3–lacZ*: structure–function studies	Pronuclear injection	120, 540, 625, intact	Enhanced tissue specificity as YAC size increases, near endogenous level with 540 and 625	156, 157
Human *SOX9–lacZ*: campomelic dysplasia	Pronuclear injection	350, 600	Partially tissue-specific, enhanced as YAC size increases	158
Mouse *downless* (TNF receptor homologue): autosomal hypohidrotic ectodermal dysplasia (HED)	Pronuclear injection	200	–	159, 160
Mouse *inversin*: inversion of visceral left–right asymmetry, complementation of mutation	Pronuclear injection	450	–	161
Mouse *GATA-2–lacZ*: structure–function studies	Pronuclear injection	120, 200, 250, intact	Tissue specificity enhanced as YAC size increases	162
Human Wilms' tumour 1 (*WT1*)–*lacZ*: nephroblastomas, structure–function studies	Pronuclear injection	280, 470, probably intact	Tissue-specific, probably endogenous level	163, 164
Human *XIST*/XIC: X chromosome inactivation	Lipofection of ES cells	320, 460, 480		165, 166
Human *DAZ*: spermatogenic defects	Pronuclear injection	225, not intact	Tissue-specific, low level	167
Human macrophage scavenger receptor (MSR class A): atherosclerosis	Pronuclear injection	180	Tissue-specific, high level	168
Human presenilin 1 *(PS-1)*: Alzheimer's disease, Down syndrome	Lipofection of ES cells	1000, probably intact	Tissue-specific, probably endogenous level	114
Human asthma *QTL*: asthma	Screening of 5q cluster YAC mice above	400	–	142
Mouse olfactory receptors: allelic inactivation	Lipofection of ES cells	300, probably intact	Tissue-specific, not position-independent	169
Human carnitine transport (*OCTN2*) gene: carnitine deficiency	Deficiency mice bred with 5q cluster YAC mice above	450	Tissue-specific, endogenous level	170

(continued on next page)

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Table 2. Transgenic or chimaeric mice produced with YACs (tab001kpk) *(continued)*

Gene/locus: disease model/goal	Transfer method	Size (kb), structure	Expression	Refs
Human *FMR1*; fragile X syndrome, complementation of mutation	Pronuclear injection	450	Tissue-specific, high level	171
Mouse kainate receptor subunit *KA1-Cre*: structure–function studies	Pronuclear injection	550	Tissue-specific	172
Human *VPAC$_2$R* receptor gene; structure–function studies	Pronuclear injection	117	Tissue-specific, high level	173
Human platelet-derived growth factor receptor α (*PDGFRα*) gene: complementation of mutation	Pronuclear injection	380	Partially tissue-specific, low level	174
Human preprotachykinin-A (*PPTA*) gene: structure–function studies	Pronuclear injection	380	Tissue-specific, high level	175, 176, 177
Mouse glucocorticoid receptor: structure–function studies	Pronuclear injection	290	Tissue-specific, endogenous level	178
Mouse *Smcy* geneL: rescue arrest of spermatogenesis	Pronuclear injection	400	Not tissue-specific	179
Human Friedreich's ataxia (*FRDA*) gene (frataxin): complementation of mutation	Pronuclear injection	370	Tissue-specific, endogenous level	180
Human HLA DR3-DQ2 MHC haplotype region: structure–function studies	Lipofection of ES cells	320	Tissue-specific	181
Human *MJD1* gene +/– CAG repeat (ataxin 3): spinocerebellar ataxia 3 (Machado-Joseph disease)	Pronuclear injection	250	Tissue-specific, position-independent, copy-number-dependent, near endogenous level	182

[a] YAC transgenic mice are listed chronologically, based on their first appearance in the literature.
[b] The first reference for each YAC is the initial report of transgenesis with that construct. After that, references are cited alphabetically by first author, then year.
[c] Dashes indicate no data are available.

Abbreviations: ES cell, embryonic stem cell; YAC, yeast artificial chromosome.

resulting in inaccurate or misleading data. The importance of detailed structural analysis of YAC-transgenic mice cannot be overemphasised, and only transgenic mice with intact copies of the transgene contained within the YAC should be utilised for functional studies to avoid drawing incorrect conclusions about expression from functional data.

Generally, four types of structural analysis should be completed prior to beginning functional

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studies. These analyses include: (1) preliminary identification of transgenic founder animals that contain complete YACs for establishment of transgenic lines; (2) detailed structural analysis of individual YAC copies in each established line; (3) determination of the site of integration in the murine genome; and (4) determination of copy number (see Ref. 26 for a review of these four protocols).

Methods of detailed structural analysis

A number of methods have been applied to determine the continuity of individual YACs within the murine genome. Standard Southern blot analysis of restriction-enzyme-digested transgenic genomic DNA or polymerase chain reaction (PCR) analysis detects the presence of YAC sequences of limited length (~0.1–20 kb). PCR can also be used to demonstrate the presence of the left and right YAC insert–vector junctions (Refs 15, 57). The detection of these two junctions suggests that the entire YAC insert might be integrated into the mouse genome, although YAC vector sequences flanking the insert could still have been lost. However, none of these methods unambiguously demonstrates that YAC clone sequences are contiguous on single molecules. For example, deletions of the 5' and 3' ends of YAC molecules occur as a result of shear. Two YACs, one with a 5' deletion and one with a 3' deletion, could integrate into the murine genome. The aforementioned methods would detect all YAC sequences, implying that an intact YAC copy exists when in fact they are carried on two deleted copies.

Three approaches have been described to determine the continuity of sequence within individual YAC transgene copies: (1) RecA-assisted restriction endonuclease cleavage (RARE) (Refs 63, 64); (2) fibre fluorescent in situ hybridisation (fibre-FISH) (Ref. 65); and (3) long-range restriction enzyme mapping (LRRM) (Refs 26, 50). RARE allows detection of the integrity of the entire insert cloned into the YAC vector, whereas LRRM can be used to analyse the entire insert as a single fragment or internal fragments that encompass all or most of the transgene contained in the YAC insert. RARE and fibre-FISH are technically difficult for structural analysis of many transgenic animals and, because these methods have been described previously (Refs 63, 64, 65), only LRMM is discussed further here.

LRRM is a method for structural analysis of individual YAC copies in transgenics generated using a YAC vector that employs distant, rare-cutting restriction enzymes. This technique is useful when unique restriction enzyme sites flank the transgene within the YAC insert or exist at the YAC insert–vector junctions (see Refs 26, 56, 66 for further details). The approach is particularly applicable if the YAC clone was retrofitted with unique rare-cutting restriction enzyme sites such as that of I-*Ppo*I at the insert–vector junctions prior to transgenesis (Refs 20, 21, 26, 31, 49, 67). I-*Ppo*I is an intron-encoded endonuclease that recognises a 15-nucleotide sequence and is an extremely rare cutter. Loss of one YAC arm as a result of mechanical shear would result in the loss of one I-*Ppo*I site, and the juxtaposition of one nearby in the murine genome would be unlikely. Thus, an intact locus residing on a YAC transgene with a deletion of one of the I-*Ppo*I sites would not be revealed by LRRM unless a murine site was near enough to produce a fragment size that could be resolved by pulsed-field gel electrophoresis. Identification of other rare-cutting restriction enzymes unlikely to digest YAC clones of interest should be a high priority when retrofitting YAC vector arms. Choosing a restriction enzyme whose corresponding restriction sites are more abundant than that of I-*Ppo*I in the mouse genome, yet that still has a low probability of cutting within the YAC, will facilitate structural analysis of YAC-transgenic mice.

Site of integration and copy number

In addition to a structural analysis of individual transgene copies, it is often of interest to determine the site of integration of the transgenes within the genome, as well as the transgene copy number. FISH has been used to examine metaphase chromosome or interphase nuclei spreads to determine the transgene integration site (Refs 50, 68). The presence of tandemly arrayed multiple copies of YACs at a single integration site can also be detected using FISH. Interphase nuclei preparations from transgenic livers are hybridised with a cosmid containing sequence homologous to the centre of the YAC insert. If the cosmid probe is small relative to the YAC and hybridises close to the centre of the YAC, individual copies of the YAC can be discriminated when the chromatin is stretched in interphase nuclei preparations. In addition, by using an ordered array of cosmid probes spanning the YAC from the 5' end to the 3'

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end, the spatial order of each copy with regard to one another (head-to-tail, head-to-head, etc.) and their integrity (presence of all probes within a transgene copy) can be ascertained (Ref. 68).

Determination of transgene copy number is important to assess accurately the per-copy levels of transgene expression. Although FISH analysis of interphase nuclei allows a gross estimate of copy number (see above), the most accurate method is hybridisation intensity. Fragments from the interior of the YAC should be assessed by Southern blot analysis since these will be the same size from all copies of the YAC regardless of the loss of the YAC vector arms in some copies. This information, coupled with LRRM of individual copies and FISH, allows an accurate assessment of numbers of complete and incomplete copies.

Gene expression studies in YAC-transgenic mice

Transgenic mice containing YAC DNA can be used to study intact genes surrounded by large regions of native flanking DNA sequence in all tissues of the mouse at any developmental stage. Several transgenic mice have been produced using both human and murine YACs by the methods described above, and the level and control of expression has been analysed (summarised in Table 2). The remainder of this review highlights the types of studies that can be performed with YAC transgenics. Examples are presented of how YAC-transgenic mice can be used to: (1) study cis regulation of gene expression by YAC mutagenesis; (2) produce mice that function as bioreactors; and (3) generate mouse models of human disease.

Studying cis regulation of gene expression by YAC mutagenesis

A study of transgenic mice bearing the human β-globin locus (β-YAC) was the first report of a multigenic YAC in which gene expression followed the correct developmental progression (Ref. 15). Globin transgene expression was shown to be tissue-specific, position-independent and copy-number-dependent. Expression of the human β-globin genes within these transgenic mice was found to parallel that of the endogenous murine genes yet retain the human expression pattern (Refs 15, 16). Thus, these transgenics represent a good example of the type of analyses that can be carried out using the YAC-transgenic system, and efforts to identify the cis-acting sequences responsible for the developmental regulation of the human multigene locus were undertaken.

The first demonstration that the YAC-transgenic system could be used to study the cis control of developmental expression was carried out by introducing a single base-pair substitution at position –117 relative to the mRNA start site of the $^{A}\gamma$-globin gene of the human β-globin locus (Fig. 5) to mimic a mutation in humans that causes hereditary persistence of foetal haemoglobin (HPFH) in adult blood (Ref. 69). Transgenic lines containing the –117 β-YAC displayed a delayed switch from γ- to β-globin in the foetal liver, as well as detectable expression of γ-globin in adult mice, thus indicating that the –117 mutation prevents γ-globin gene silencing in adult mice, just as in human adults (Ref. 66).

A second series of experiments using β-YACs was undertaken to characterise the function of the DNAse I-hypersensitive sites (HSs) 6 kb upstream of the ε-globin gene called the locus control region (LCR) (Ref. 56). The LCR activates the β-globin locus chromosomal domain, insulates the globin genes from the effects of surrounding chromatin, restricts globin gene expression to cells of the erythroid lineage, and acts as a powerful enhancer directing high levels of globin production in erythroid cells (Refs 69, 70, 71). Studies using β-YAC transgenics with specific deletions of individual HSs have suggested that the individual HSs might mediate interaction between the LCR and specific globin genes during development, but because the effect of these deletions on globin gene expression was minor, there appears to be functional redundancy within the LCR. By contrast, smaller deletions of the core HSs had catastrophic effects on globin gene expression at all stages of development (Refs 72, 73). The reason for these differences is not known, but alteration of LCR sequences might lead to strong position effects and make studies of LCR mutants difficult to interpret. Many other mutant β-YAC-transgenic mice have subsequently been produced (Table 2).

Generating murine bioreactors

YAC-transgenic mice can be used as 'bioreactors' – essentially factories capable of producing high levels of therapeutic proteins (Refs 19, 74, 75). Human monoclonal antibodies to tetanus toxin have been produced in mice deficient in mouse immunoglobulin (Ig) production and containing

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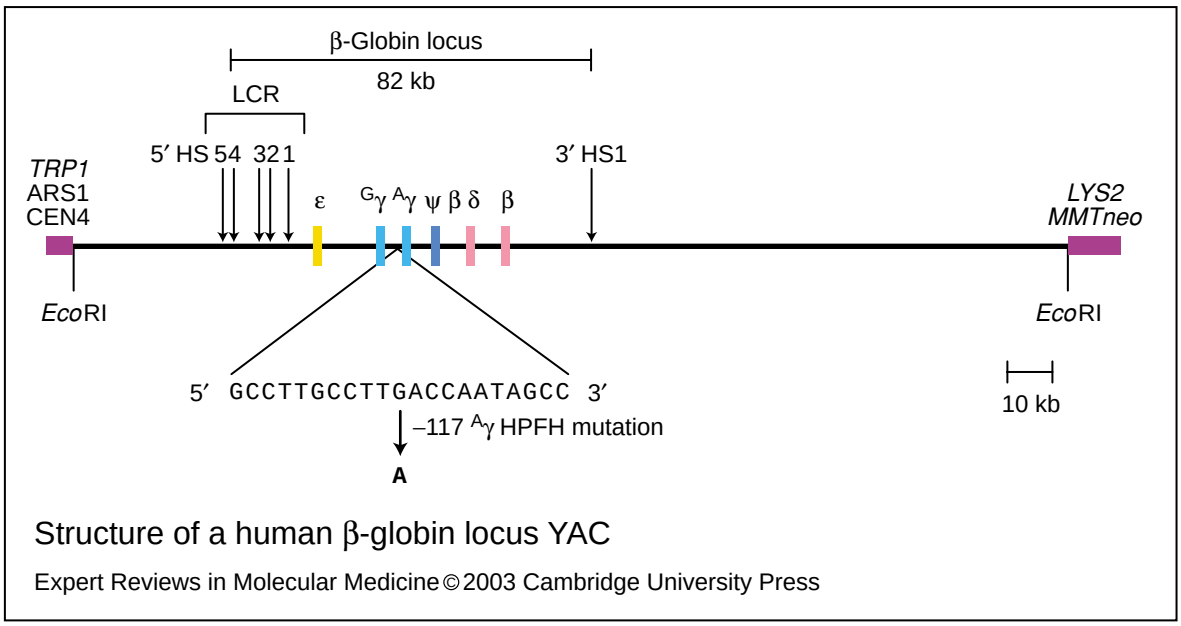

Figure 5. Structure of a human β-globin locus YAC. The 248 kb YAC (yeast artificial chromosome) has a 230 kb insert comprising 82 kb of β-globin locus, 39 kb 5' flanking region and 109 kb 3' flanking region (see Ref. 89 for details of construction). The YAC vector arms are shown as purple blocks with the chromosomal elements or selectable markers (including *MMTneo*, the mouse mammary tumour virus promoter and *neo* gene selectable marker) listed above them (see Fig. 1). Within the locus are five functional β-like globin genes arranged 5' to 3' in the order in which they are expressed during development: ε, $^{G}\gamma$, $^{A}\gamma$, δ and β. The locus control region (LCR) is located 6 kb upstream of the ε-globin gene; it is physically defined by the presence of four erythroid-specific, developmentally stable DNaseI hypersensitive sites (HSs; 5' HS1–4) and one ubiquitous developmentally stable HS (5' HS5) (Refs 90, 91). The LCR activates the β-globin locus chromosomal domain, insulates the globin genes from the effects of surrounding chromatin, restricts globin gene expression to cells of the erythroid lineage and acts as a powerful enhancer directing high levels of globin production in erythroid cells (Refs 69, 70, 71). One other HS, 3' HS1, exists approximately 20 kb downstream from the β-globin gene. Introduction a single base-pair substitution at position –117 relative to the mRNA start site of the $^{A}\gamma$-globin gene of the human β-globin locus mimics a mutation in humans that causes hereditary persistence of foetal haemoglobin (HPFH) in adult blood (Ref. 69). Other mutations have been crossed onto the YAC and transgenic mice produced to study structure–function relationships regarding human β-like globin gene switching (see text and Table 2) **(fig005kpk)**.

human Ig heavy (H)- and light (L)-chain YACs (Ref. 74). The H-chain YAC contained a 220 kb insert comprising the μ and δ constant (C) regions, all six functional joining (J) regions, the major diversity (D) cluster, the intronic enhancer and the five most-proximal variable (V) genes from four V_H families. The L-chain YAC contained a 170 kb insert comprising the κ-deleting element (κde), the intronic and 3' enhancers, the C_κ region, all five functional J regions and the three most-proximal V_κ regions in the B cluster. Both YACs had a hypoxanthine phosphoribosyltransferase (HPRT) selectable marker on the right arm of the YAC vector and were introduced by spheroplast fusion of ES cells. High antibody levels were produced, mostly made up of human H and L chains, and the mice produced a broad repertoire of human Igs. Similar results were obtained by lipofection of ES cells with a 450 kb YAC encompassing V_κ gene segments and two plasmid construct inserts encoding the remaining necessary H- and L-chain Ig sequence elements (Ref. 75).

Generating mouse models of human disease

YAC-transgenic mice can be used as models of human disease (Table 2). Among the first models produced were those for Charcot-Marie-Tooth (CMT) disease type 1A (Ref. 76) and for aniridia (Ref. 68). CMT disease type 1 is characterised by progressive weakness of distal muscles, hand and

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foot deformations, and severe demyelination in the peripheral nervous system including the presence of 'onion bulb' formations. The CMT model was constructed by pronuclear injection of a YAC carrying the 40 kb human *PMP22* gene flanked by approximately 100 kb of upstream sequence and 300 kb of downstream sequence (Ref. 76). One transgenic line containing eight integrated copies strongly expressed the *PMP22* gene in the appropriate tissue-specific manner. Overexpression caused peripheral neuropathy closely resembling the human pathology. This mouse model, and others, can be used to analyse the molecular defect underlying the disease, as well as to test both pharmacological and gene therapy regimens prior to initiation of such trials in humans.

Aniridia in humans is caused by heterozygous mutations of *PAX6*, a gene that encodes a transcriptional regulator with a DNA-binding domain homologous to the *Drosophila* paired gene (Ref. 68). This condition is characterised by a varying degree of iris hypoplasia, corneal opacification, cataracts and glaucoma. Mice with the *Small eye* (*Sey*) phenotype, caused by heterozygous mutations of the *Pax6* gene, have a similar pathology and additionally show a reduction of external eye size. Humans with homozygous *PAX6* mutations and homozygous *Sey* mice lack eyes and nasal cavities, exhibit abnormalities of the brain, and die soon after birth. Thus, the mouse mutant is a good model system for studying the human disorder. Mice were generated with a 420 kb human *PAX6*-YAC and these mice were crossed onto the *Sey* mouse background (Ref. 68). The YAC rescued the mutant phenotype, demonstrating appropriate gene regulation during murine development. In addition, increased *PAX6* gene dosage caused abnormalities of the eye. These data, coupled with those of the *Sey* mutants, suggest alterations of the eye are sensitive to changes in protein level outside a narrow range. Many more models of human genetic maladies have subsequently been generated (Table 2).

Clinical implications/applications: human artificial chromosomes (HACs) and gene therapy

One major block to developing successful gene therapy strategies is the limitations of the current generation of gene-transfer vectors (Ref. 77). Retroviruses can stably integrate into the target cell genome resulting in prolonged transgene expression, but they will infect only proliferating cells, the insert size of the transgene that can be carried by the virus is limited, and integration might result in deleterious mutagenic events (Ref. 78). Adenoviruses will infect nondividing cells, but do not stably integrate. Thus, transgene expression is transient. Repeated administration of a therapeutic adenovirus must be performed, which could result in an inflammatory response in the patient. DNA–liposome complexes are safe, but gene transfer is inefficient and transgene expression is transient when using small constructs (Ref. 79). Therefore, the goal is to design a synthetic, nonimmunogenic gene-transfer vector that would allow long-term, tissue-specific transgene expression. Artificial chromosomes offer one such solution to the expression problem, but more-effective means of delivery are still required (Ref. 80).

The use of artificial chromosomes would resolve many of the aforementioned limitations. An inflammatory response would probably not be mounted against an artificial chromosome since it is naked DNA. In addition, a functional artificial chromosome should exhibit mitotic stability and would not require integration, thereby avoiding insertional mutagenesis. Transient expression would not be a problem because artificial chromosomes carry large inserts that include all of the relevant cis-acting control elements that would insure high-level, tissue-specific gene expression.

Although well-defined, functional, artificial chromosomes have been produced in yeast (Ref. 81), human artificial chromosomes (HACs) have not been generated. Three chromosomal elements are required for artificial chromosomes: an origin of replication, telomeres and a centromere/kinetochore sequence (reviewed in Ref. 82). The origin is required for initiation of DNA synthesis, telomeres are required to stabilise the chromosome ends, and the centromere/kinetochore is required for attachment of the chromosome to the spindle apparatus, which is necessary for mitotic stability. YACs have each of these chromosomal elements. The production of HACs has been hindered by the inability to isolate a complete centromere/kinetochore region, although functional origins and intact telomeres have been identified and characterised (reviewed in Ref. 80). Despite this, a first-generation HAC has been obtained by allowing recombination of

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various human DNA sequences to occur in transfected cells (Ref. 83). Although mitotically stable, the HACs were relatively undefined, limiting their usefulness as a gene-therapy vector. Other recent attempts utilise modification of YACs by substituting putative human chromosome elements for the analogous yeast chromosome elements (Refs 84, 85). These also were mitotically stable, but underwent frequent multimerisation and rearrangement. However, recently, a gene deficiency in human cells was successfully complemented using HAC vectors (Refs 86, 87), demonstrating their potential as therapeutic vectors (Ref. 88).

Research in progress and outstanding research questions

Several YACs containing murine and human genes have been introduced into mice (Table 2), many of which displayed correct stage- and tissue-specific expression. These results have confirmed the expectation that natural levels of gene expression can be achieved with YAC transgenes relative to truncated recombinant vectors. Current techniques, therefore, can be implemented in characterising the regulation of very large genes, possibly even the human dystrophin gene, which spans 2.3 Mb and comprises ~70 exons and five promoters (Refs 54, 55). Mutagenesis of YACs bearing these large genes or multigene loci can be used to identify regulatory sequences, and binary experiments where YAC-transgenic mice are bred with transgenic animals producing specific transcription factors will characterise the role of the trans-acting factors that utilise these sequences. YAC-transgenic mice can be used for the functional analysis of higher-order genomic structures, and specific mutations can be introduced into genes so that phenotypes of human disease can be recreated in mice.

Translation of YAC animal technology into human gene therapy approaches offers a potential alternative to current strategies. Although building HACs is more difficult than anticipated, slow, but systematic, progress has been made and is ongoing. Ultimately, the resultant functional vectors should solve the problems associated with present-day gene-transfer vectors and allow tailored solutions to overcome limitations regarding stable transgene incorporation within the cell, therapeutic levels of gene product expression, and tissue-specific transcription of transgenes.

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Further reading, resources and contacts

The website of Dr Lluís Montoliu at the Centro Nacional de Biotecnología (Madrid, Spain) includes protocols for plasmid and YAC transgenesis, a manual for generation of transgenic mice and a bibliographic survey on YAC/BAC/PAC transgenesis, as well as relevant, useful links to other websites:

http://www.cnb.uam.es/~montoliu/

Dr Gary Silverman (Children's Hospital, Boston, USA) has a protocols web page that includes descriptions of YAC methods:

http://web1.tch.harvard.edu/silverman/protocols/

Features associated with this article

Figures

Figure 1. Schematic representation of a YAC clone (fig001kpk).
Figure 2. Retrofitting of the pYAC4 vector (fig002kpk).
Figure 3. Introducing mutations into YAC vectors (fig003kpk).
Figure 4. Generation of YAC-transgenic mice (fig004kpk).
Figure 5. Structure of a human β-globin locus YAC (fig005kpk).

Tables

Table 1. Comparison of YAC transgenesis methods (tab001kpk).
Table 2. Transgenic or chimaeric mice produced with YACs (tab002kpk).

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