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Transgenic mice generated by pronuclear injection of a yeast artificial chromosome

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ABSTRACT

Transgenic mice have become invaluable for analysing gene function and regulation *in vivo*. However, the size of constructs injected has been limited by the cloning capacity of conventional vectors, a constraint that could be overcome with yeast artificial chromosomes (YACs). We investigated the feasibility of making transgenic mice with YACs by pronuclear injection of a small YAC carrying a gene encoding tyrosinase. Use of a vector with a conditional centromere allowed fifteenfold amplification of the YAC in yeast and its recovery in high yield. The albino phenotype of the recipient mice was rescued demonstrating the correct expression of the tyrosinase gene from the construct. Furthermore, the telomeric sequences added by the yeast integrated into the mouse genome and did not reduce efficiency of integration. Using this technique future experiments with longer YACs will allow the expression of gene complexes such as *Hox* and the globin gene clusters to be analysed in transgenic animals.

INTRODUCTION

Transgenic animals have been produced by pronuclear injection of constructs made in plasmids, cosmids, or λ phages. The cloning capacity of these vectors, however, places a limitation on the size of constructs, which precludes the transfer of genomic fragments greater than 40kb. This constraint is no more acute than in the cases of gene clusters, where regulation might be crucially dependent upon the organization of the genes. This problem could be overcome by YACs (1) in which more than 1000kb of genomic DNA can be cloned (2,3). The development of a technique for the generation of transgenic mice with YACs would therefore be of considerable importance for future research on gene regulation.

Introduction of YAC DNA into fertilized mouse oocytes faces three major problems. 1) The isolation from yeast of sufficiently pure and concentrated material is difficult, since YACs are linear molecules maintained as a single copy per cell and cannot therefore be purified by conventional CsCl gradients. 2) Purification and injection of intact YACs might be compromised by the susceptibility of large molecules to mechanical shearing. 3) The presence of telomeres at either end of the YAC might be detrimental to stable integration. The form although not the precise sequence of telomeric DNA is highly conserved from yeast to mouse (most recently reviewed in (4)). Terminal fragments of mouse chromosomes can provide YAC telomere function in *S.cerevisiae* (5, 6). Furthermore, chromosomes from *S.pombe* can replicate autonomously in mouse fibroblasts indicating that they carry telomeres able to function (7). These observations raise the possibility that YAC telomeres will be recognized in mouse oocytes and hamper the integration process into the mouse genome.

A newly described vector, which allows amplification of YACs in yeast, presents an improvement for isolation of large quantities of YAC-DNA (8). Selective amplification of the YAC vector pCGS966 (ATCC # 68371) depends upon the presence of a conditional centromere. Growth in galactose containing medium results in induction of the *Gal1* promoter and transcription through the YAC centromere, which interferes with centromere function (9,10). As a consequence of incorrect segregation at mitosis specific amplification of the YAC up to twenty-fold is achieved (8). This degree of amplification is maintained to a large extent after reinoculation of these cells into medium containing glucose (8).

To enable us to evaluate purification procedures and assess the influence of YAC telomeres we have designed a 35kb YAC, YtTel25, comprising an amplifiable vector and a mouse tyrosinase minigene (Fig.1). The tyrosinase marker allows us to monitor the frequency of integration and expression of the YAC DNA in albino recipients: Expressing mice are readily identified at birth by eye pigmentation. Here we report the production of germline transgenic mice which carry YAC-DNA and which express the YAC-encoded tyrosinase gene.

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MATERIALS AND METHODS

Strains and Media

E.coli strain HB101 (*supE44 hsdS20*($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) was used for vector construction (Y-RC16). YAC YtTel25 was transformed into *S.cerevisiae* strain CGY2516 (*MATa GAL*$^+$ *ura3-52 trp1-D63 leu2-D1 lys2-D202 his3-D200*; ATCC#74013). Yeast media included YPD (ref. 11), Glc. (0.67% yeast nitrogen base, 1% casamino acids, 3% glucose, with histidine, methionine and adenine each at 50μg/ml), Gal. (Glc. with 3% galactose instead of glucose) and MST (Gal. with thymidine at 0.8mg/ml, methotrexate at 50μg/ml and sulfanilamide at 2mg/ml). For plates the same media were prepared with 1.5% agar. Sphaeroblast transformations were performed essentially as described in (12) and were plated on selective Glc. medium supplemented with 1M sorbitol.

Construction of Y-RC16 and YtTel25

Y-RC16, a yeast artificial chromosome vector, was constructed by cloning the polylinker *Not*I-*Bss*HII-*Eco*RI-*Sal*I-*Not*I-*Bss*HII (oligo A: 5'-AATTGCGGCCGCGCGCGAATTCGTCG-ACGCGGCCGCATGCGCGC; oligo B: 5'-AATTGCGCGCA-TGCGGCCGCGTCGACGAATTCGCGCGCGGCCGC) into the *Eco*RI site of pCGS966 (ref. 8, ATCC#68371). ptrTyr4 (ref. 13) was modified by ligation of *Sal*I linkers to the *Mlu*I linearized plasmid. After recutting with *Sal*I ptrTyr20 was generated by cloning the 18kb tyrosinase fragment into the *Sal*I site of Bluescript (Stratagene). For construction of YtTel25 the 18kb *Sal*I insert of ptrTyr20 was ligated to the *Sal*I/*Bam*HI cut vector Y-RC16. Ligation products were used for transformation of yeast strain CGY2516 with selection on uracil and tryptophan. DNA of yeast clones was prepared according to (14) and the correct structure of the small YAC was confirmed by restriction analysis with *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, and *Xho*I in comparison to digests of ptrTyr20 and Y-RC16. To verify the integrity of the tyrosinase gene the exon containing fragments were subcloned into Bluescript and sequenced using tyrosinase specific primers as described (13).

Amplification, isolation and purification of YtTel25 and generation of transgenic animals

Cultures inoculated with the yeast strain carrying YtTel25 were grown in Glc., Gal. and MST medium to stationary phase. 1:1000 subcultures in Glc. medium were inoculated with MST or Gal. cultures and incubated for an additional 2 days. Yeast DNA was prepared in agarose blocks (80μl/block, 5×10^7 cells/block) essentially as described in (14). Amplification in different media was determined by PFGE analysis on a 1.2% agarose gel carried out in 0.15×TBE at 450V with the following pulses: t_1=0.3s, 1.5h, t_2=0.5s, 1.5h and t_3=0.7s, 2h, 10°C (LKB-Pulsaphor). The corresponding Southern blot was hybridized with a probe synthesized by random priming of ptrTyr20. The density of hybridization signals was measured by a Phosphoimager (Biodynamics).

For preparative isolation of YtTel25 yeast was grown in thymidine kinase (TK) selective medium (MST) for YAC amplification. After 5 days of growth a new culture was inoculated into glucose containing medium (Glc.) and grown for 2 days. DNA containing agarose blocks made from approximately 5×10^8 cells/block (~10μg/block) were loaded onto a 1.2% low melting point agarose (SeaPlaque GTG, FMC) PFGE gel and separation was performed in 0.15×TAE buffer under conditions as described above. Marker lanes on either side of the gel were cut off, stained with ethidium bromide (0.5μg/ml) and a photograph taken. The band containing YtTel25 was excised from the preparative lane and equilibrated in 0.5×TAE, 25mM NaCl. After melting at 65°C the agarose was digested for 2h at 40°C with Gelase (Epicentre) using 0.5U/100mg agarose. DNA was precipitated twice with 0.5vol. 7.5M ammonium acetate and 2.5vol. ethanol and was taken up in 8mM Tris:HCl, pH7.3, 0.1mM EDTA to a final concentration of ~2ng/μl. Microinjection was performed as described (15) using fertilized oocytes from NMRI/Han mice.

Southern blot analysis

DNA was prepared from tails of 3 weeks old mice (15). Approximately 10μg DNA were digested with *Hin*dIII, separated on 1×TAE, 1% agarose gels and transfered to nylon membranes (Biodyne, PALL) as described (13).

A 250bp fragment from exon 5 of the mouse tyrosinase gene was isolated from the cDNA clone pmcTyr63 (16) by *Sau*3A and *Spe*I digestion and cloned into *Bam*HI-*Xba*I digested Bluescript (Stratagene) to generate pTyrE5. An RNA probe was synthesized from pTyrE5 linearized with *Eco*RI by T_7 RNA polymerase. The vector probe comprised two *Hin*dIII fragments (4.5kb and 5.4kb) located next to the telomeres on either arm of Y-RC16. The YAC telomere probe was isolated as a 700bp *Xho*I/*Bam*HI fragment from pYAC-3 (1) and contains the *Tetrahymena* rDNA terminal repeat (identical with Y-RC16 telomeric sequences). Vector and telomere probes were labelled by random priming (17) of gel purified fragments . Hybridization and washing was carried out under standard conditions (18) with a final high stringent washing step at 72°C when hybridizing with the telomere probe.

RESULTS

Construction of an amplifiable tyrosinase-YAC, YtTel25

Isolation of YAC DNA from yeast in concentrations sufficiently high for microinjection into mouse oocytes is a major problem. To increase yield we decided to base a test construct on the amplifiable vector pCGS966 (ref. 8). pCGS966 was modified

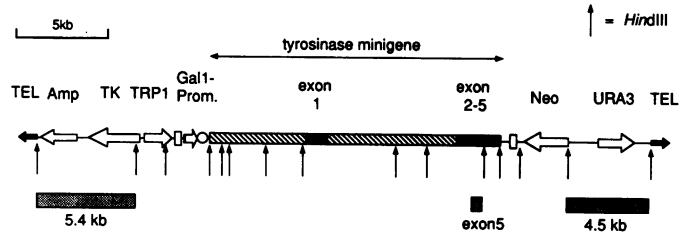

Fig. 1. Structure of the YAC used for microinjection. Telomeres are given as filled arrows labelled with TEL. Open arrows indicate transcription units belonging to the YAC vector and are designated according to (8). Open boxes represent autonomously replicating sequences (ARS1). The open circle indicates the centromere (*CEN4*), which is associated with the *Gal1*-promotor. TK=thymidine kinase gene of herpes simplex virus, Neo=neomycin resistance gene plus the SV40 origin of replication; both arms contain bacterial origins of replication (8). Tyrosinase gene sequences are shown as hatched boxes with filled boxes indicating exon1 (left) and exon 2–5 plus the SV40 polyadenylation signal (right). *Hin*dIII sites are given as vertical arrows. Stippled boxes below the construct represent probes used for hybridization analyses in Fig.3.

by introducing a polylinker containing *Not*I-*Bss*HII-*Eco*RI-*Sal*I-*Not*I-*Bss*HII sites into the *Eco*RI cloning site to generate Y-RC16 (for details see Material and Methods). The presence of flanking *Not*I and *Bss*HII sites on both sides of the *Eco*RI and *Sal*I cloning sites would allow retrieval of the insert, if vector sequences were found to inhibit integration and/or expression of the transgene. As a marker to detect expression in transgenic animals a mouse tyrosinase minigene, adapted from ptrTyr4, was inserted into the *Sal*I site of Y-RC16 (for details see Materials and Methods) to create YtTel25. The minigene comprises genomic sequences from 5kb upstream of the start site to the second exon, with the remainder of the tyrosinase coding region contributed by cDNA sequences and SV40 sequences providing the polyadenylation signal. The insert has been shown to encode functional tyrosinase and to rescue the albino phenotype in transgenic mice (13). YtTel25 transformed yeast clones were analyzed by restriction digestion to confirm the correct structure of the YAC (Fig.1 and data not shown). Linearity of the YAC was demonstrated by *Hin*dIII digests in which the telomeric fragments were released.

Amplification of the construct was tested under various culture conditions (for details see Materials and Methods). DNA prepared from equal numbers of cells imbedded in agarose plugs was separated by PFGE and a filter taken from this gel was hybridized with a tyrosinase probe. The degree of amplification of the YAC was determined by comparing hybridization signals. Taking a culture grown in glucose medium (Glc.) as a standard for a single copy of the YAC per cell (Fig.2) growth in MST medium was estimated to lead to amplification to approximately 11 copies per cell. The copy number of the YAC was approximately fifteenfold after regrowth in glucose medium (Fig.2, MST-Glc.). The greater apparent amplification after regrowth in glucose medium might reflect a lower yield of DNA from MST cultures as a result of increased resistance of the cell wall to lytic enzymes (8). Growth of YtTel25 in Gal. medium led to a lower amplification possibly due to lower selective pressure.

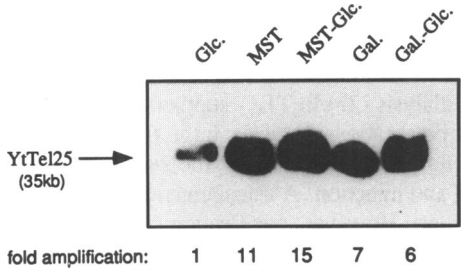

Fig. 2. Amplification of YtTel25. Yeast cells grown in different media were embedded in agarose blocks (5×10^7 cells/block), DNA was separated by PFGE and a Southern blot was hybridized with a tyrosinase probe (ptrTyr4). Lanes are identified: Glc. = glucose as carbon source, Gal. = galactose as carbon source, MST = tk selective medium based on galactose, MST-Glc. = 1:1000 Glc. subculture inoculated with a MST culture, Gal.-Glc. = 1:1000 Glc. subculture inoculated with a Gal. culture. Hybridization signals were quantitated by a Phosphoimager (Molecular Dynamics) and are expressed as fold amplification taking the signal in lane Glc. as 1.

Table 1. Generation of transgenic mice carrying YtTel25. Two independent injection experiments were performed (A, B). Transgenic mice were identified by analysis of tail DNAs with the exon 5 specific probe pTyrE5 (see also Fig.3). Mice expressing tyrosinase are recognized by the presence of pigment in the eye.

experiment	oocytes injected	mice born	transgenic mice	pigmented mice
A	49	10	3	1
B	98	25	7	4

Generation of YAC-transgenic animals

Yeast DNA in agarose blocks was prepared from MST-Glc. cultures and YtTel25 was isolated by preparative PFGE (for details see Materials and Methods). The concentration of YtTel25 within the isolated gel slice was estimated as ~0.3ng/μl by comparison to λ DNA as a standard. Initially, the DNA was concentrated by ethanol precipitation to obtain a concentration normally used for microinjection. Two injection experiments were conducted with a preparation of YtTel25 at ~2ng/μl. Taking the injection volume of 1−2pl and the size of the YAC (35kb) into account approximately 50 copies have been introduced per injection into the oocytes. 35 mice were born from 147 injected oocytes (24%), indicating the absence of harmful substances from the YAC preparation. Five newborn mice (# 1108, # 1279, # 1282, # 1287, # 1293) showed pigmentation in the eye (Table 1).

DNAs were prepared from tails of all 35 mice at 3 weeks and Southern blot analysis was carried out with pTyrE5, a probe recognizing exon 5 of the tyrosinase gene (Fig.1). A 3.4kb *Hin*dIII fragment specific for the transgene was detected in nine of 35 (26%) mice (Table 1; Fig.3A). One additional transgenic mouse did not show the expected fragment (data not shown) and was not analysed further. Comparison of the intensity of the 2.2kb *Hin*dIII fragment arising from the endogenous tyrosinase gene (16) and the transgene-specific 3.4kb fragment implies copy numbers ranging from 1 (# 1284) to ~50 (# 1292). To confirm the integrity of the transgenic construct the same filter was hybridized with the *Hin*dIII fragments from either arm of the

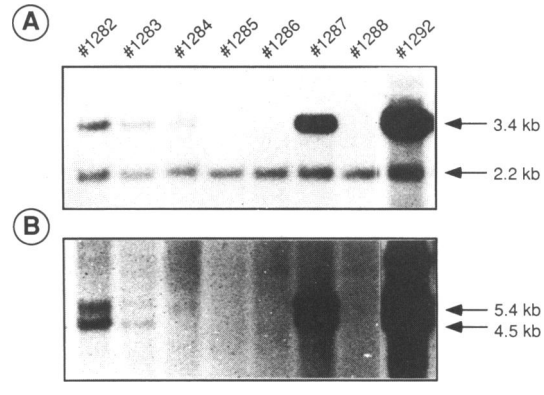

Fig. 3. Southern blot analysis of transgenic animals. Analysis of 5 transgenic founder mice (# 1282, # 1283, # 1284, # 1287, # 1292) and 3 non-transgenic littermates (# 1285, # 1286, # 1288) is shown. *Hin*dIII digests of approximately 10μg tail DNAs were resolved on a 1% agarose gel and blotted onto nylon membrane. **(A)** shows hybridization of the filter to a probe detecting exon 5 of the tyrosinase gene (pTyrE5). The 2.2kb band common to all lanes represents the endogenous gene and the 3.4kb band the transgene. **(B)** The same filter was rehybridized with 4.5kb and 5.4kb *Hin*dIII 'vector' fragments from YtTel25. In DNAs from transgenic mice both *Hin*dIII fragments are detected. Due to the high background within the lanes, hybridization to the vector arms is difficult to see in line # 1284 which contains a single copy of the transgene, but was apparent as faint bands on the original autoradiogram. An additional rearranged band of approximately 10kb can be seen in the multi-copy line # 1292.

YAC vector located next to the telomeres (5.4kb and 4.5kb; Fig.1). 5.4kb and 4.5kb *Hin*dIII bands were detected in all transgenic mice indicating integration of both vector arms without major rearrangements (Fig.3B and data not shown).

The integration of the YAC telomeres was investigated with a telomere probe derived from the YAC vector. The telomere repeat probe also hybridizes to mammalian telomeres (19), but since these are greater than 20kb and do not contain palindromic restriction sites (20, 21), cross-hybridization to mouse DNA in *Hin*dIII digests is confined to the upper part of the gel and a faint band of 4.5kb common to all lanes (Fig.4). Additional, strongly hybridizing fragments were detected in DNAs from mice containing multiple copies of the YAC (# 1287 and # 1292). The length of adjoining telomeres can be investigated in the multiple copy mice assuming that the DNA is integrated in tandem arrays. Each *Hin*dIII endfragment of the YAC vector is 700bp and upon introduction of the YAC into yeast additional 200 to 300bp of telomere sequences are added ((22) and unpublished results). The length of telomeric endfragments is heterogeneous in a given cell population (23, 24). Consequently, a *Hin*dIII fragment spanning the two endfragments might be between 1.8 and 2kb in length. In the multi-copy transgenic mouse # 1292 most of the hybridization detected with the telomere probe was to a band of this size (Fig.4). A similar band was amongst those detected in # 1287 DNA but was absent from non-transgenic DNAs (# 1286 and # 1288). The diffuse nature of the band in # 1292 DNA is consistent with length variation of the telomere containing fragments.

DISCUSSION

Testing the feasibility of generating transgenic animals by pronuclear injection of YACs we have shown that a 35kb YAC is integrated into the mouse genome without obvious rearrangement. The founders so far examined (# 1279 and # 1282) transmitted the transgene to their offspring (7 of 21 newborns had pigmented eyes) indicating stable integration of the YAC DNA. One goal of this paper was to investigate the influence of telomeric sequences on the efficiency of integration of YACs into the mouse genome. The high frequency of transgenic animals (26%) obtained with YtTel25 is comparable to the isolated insert of ptrTyr4 (13) and other injected constructs (25,26), which clearly demonstrates that telomeres do not inhibit integration. The high proportion of transgenes that retained both vector arms of the YAC implies that the presence of telomeres does not promote rearrangements or deletions at or subsequent to integration. Furthermore, the telomeric structures do not prevent the transgene from integrating as multimers. In offspring of the multi-copy line # 1292 the construct is inherited in apparently unrearranged form (data not shown), implying that the inverted telomere repeats are stable through meiosis.

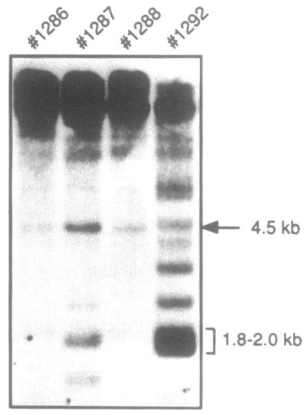

Fig. 4. Telomeric sequences of the YAC are retained in the genomes of transgenic mice. The same Southern blot of *Hin*dIII digested DNAs as in Fig.3 was hybridized with a YAC telomere probe. Lanes containing DNA from two non-transgenic (# 1286, # 1288) and two transgenic (# 1287, # 1292) animals are shown. 1.8 to 2.0kb *Hin*dIII fragments are expected for multi-copy lines with constructs integrated as multimers (see text). The broad appearance of the 1.8–2.0 kb band in # 1292 DNA is compatible with the varying length of the telomeres added by the yeast cell. The arrow indicates a 4.5kb band common to all lanes. The extra intensity of this band in lane # 1287 probably represents a comigrating fragment containing YAC DNA.

Not all transgenic founder mice showed pigmentation and coat colour was weak in all expressing mice. The relatively low degree of pigmentation in comparison to transgenic lines generated with the identical mouse sequence free of vector sequences (13), might be explained by the presence of plasmid and other vector DNA in the YtTel25 construct, sequences known to inhibit expression of associated transcription units in transgenic mice (27). We are presently modifying the vector Y-RC16 by insertion of the 18bp long meganuclease I-*Sce*I recognition sequence (Boehringer Mannheim), which will allow retrieval of almost any insert without internal cleavage and without contamination by digestion products of yeast chromosomes, since the yeast genome does not contain *Sce*I sites (28). Alternatively, the recently published RecA-assisted-restriction-endonuclease (RARE) system (29) could be used to excise DNA fragments cloned into YACs.

The results obtained are encouraging for future experiments with longer YACs which, however, might require more gentle handling. In a preliminary experiment we prepared YtTel25 DNA without precipitation simply by agarase digestion of isolated gel slices equilibrated in a buffer containing polyamines (1×TAE, 25mM NaCl, 0.3mM spermine, 0.7mM spermidine) and subsequent dialysis (2h) in $TE_{0.1}$ supplemented with polyamines. Presence of poly-cations stabilizes large fragments by compaction (30–32) and should minimize mechanical shearing during purification and injection. A transgenic mouse was obtained with this preparation. However, purification procedures might require some improvement since survival of injected oocytes was reduced in comparison to normal injections. Presently we are applying a similar purification strategy to a 250kb YAC covering the genomic tyrosinase locus.

Amplification of YACs seems to be essential to obtain sufficiently high concentrations of DNA in preparative gels to avoid the need to concentrate the isolated construct by precipitation. As most available YACs are not constructed in an amplifiable vector an exchange of the centromere containing vector arm is necessitated. This is feasible by homologous recombination in yeast.

The recent literature presents several examples, in which YACs have been introduced into somatic cells by methods like sphaeroblast fusion (33–35), lipofection (36), calcium phosphate coprecipitation (37) or electroporation (38). In all these cases, however, the entire yeast genome was cotransferred with the YAC, which led to the integration of substantial amounts of yeast sequences into the mammalian genome (33, 34, 36). Although this might be of little consequence for experiments in somatic cells, it is clearly undesirable when seeking to introduce YACs into the mammalian germ line.

Lipofection has been used to introduce a gel-purified 150kb YAC into mouse fibroblast cells (39). This avoided the integration of contaminating yeast chromosomes, but the yield of clones was low and few carried the complete YAC. Although the procedure could be applied to embryonic stem (ES) cells, the generation of transgenic lines via ES cells harbouring YACs is time consuming and subsequent germ line transmission is not guaranteed. Thus, direct injection of isolated YAC DNA into oocytes should be the method of choice to generate YAC transgenic animals. Recent results by Gnirke and Huxley (40) and ourselves (unpublished) showed that even very large fragments (>100kb) can be passed through a microinjection needle without mechanical shearing and can integrate into the mammalian genome.

Introduction of large segments of DNA cloned as YACs into mice has great potential for identification of genes by complementation of mutations and will open the way for experiments on the regulation of gene complexes organized in expression domains like the globin genes and, possibly, the *Hox* gene clusters. The efficiency of homologous recombination in yeast should allow facile manipulation of YACs prior to introduction into mice.

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