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Yeast artificial chromosomes: an alternative approach to the molecular analysis of mouse developmental mutations.

ZOIA LARIN AND HANS LEHRACH*
Imperial Cancer Research Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX

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Summary

Mammalian genetics now allows a molecular study of genomic regions previously analysed by genetic and embryological techniques. To simplify such an analysis, we have established a number of libraries of mouse DNA in Yeast Artificial Chromosome (YAC) vectors, constructed either by partial digestion with *Eco*RI, or by complete digestion with enzymes which cut rarely in the mammalian genome. In this paper we report the construction of complete digest libraries prepared from mouse genomic DNA using the rare cutter enzymes *Not*I and *Bss*HII, and the detection of gene loci from the H-2 complex, the *t*-complex, and other loci from the mouse genome. Due to their large insert size, YAC clones simplify the cloning of extended regions of the mouse genome surrounding known developmental mutations and should, after introduction into the germ line, offer a high probability of correct expression of the genes contained within the cloned region. We hope that this will allow the use of YAC clones to scan regions of interest such as the *t*-complex for specific genes by testing DNA introduced into transgenic mice for the ability to complement mutations localised to this region.

1. Introduction

YAC libraries have been constructed from genomes of organisms such as *Caenorhabditis elegans* (Coulson *et al.* 1988) and *Drosophila* (Garza *et al.* 1989), and have assisted in developing a contiguous map of their genomes. Correspondingly, YACs should contribute significantly to studies of the mammalian genome including the analysis of murine developmental mutations. For example, the *t*-complex region on chromosome 17 contains many interesting embryonal genes which have largely been identified from the phenotype of their mutant forms by classical breeding studies, but molecular studies have been hampered by the size of the region and the extent of its inversions occupying approximately 15 centiMorgans (cM; 30000 kilobase (kb) pairs; Lyon *et al.* 1988). Similarly, the H-2 complex spans 1·5 cM or 3000 kb and is known to contain genes involved in the immune response as well as in embryogenesis (Abe *et al.* 1988). To allow a molecular analysis of such large regions, clones have been generated by chromosome walking (Bender *et al.* 1983), and chromosome jumping (Collins & Weissman, 1984; Poustka & Lehrach, 1986), in conjunction with pulse-field gel electrophoresis (PFGE; Schwartz & Cantor, 1984; Barlow & Lehrach, 1987). In addition, within the *t*-complex, molecular clones have been derived by microdissection and microcloning techniques (Röhme *et al.* 1984) which have provided a significant number of molecular probes. YACs now offer a new way to isolate larger regions in one contiguous piece, and will complement these other techniques in the molecular analysis of specific regions. However, another strategy employing these vectors for localizing murine developmental gene loci that have largely been mapped by traditional genetic methods, is to re-introduce a large cloned portion of the region into the germ line and assay for complementation of the mutant phenotype. This could be done either directly into fertilized embryos or by homologous recombination via embryonal stem cells. In particular this approach will be especially viable where there is an easily visible phenotype such as the Brachyury mutation (Herrmann *et al.* 1990). The position of the gene locus could then be more precisely defined by YAC deletion studies to determine which fragment can then be screened for coding sequences. Since many mammalian genes such as Duchenne muscular dystrophy (Monaco & Kunkel, 1987) are known to be large, the use of large insert clones will increase the probability of appropriate expression of unknown genes; the presence of a larger cloned region

* Corresponding author.

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is also likely to be essential for detection of regulatory elements a large distance from the gene loci (Grosveld *et al.* 1987).

In this paper we discuss the preparation of complete digest libraries where mouse genomic DNA has been digested with the rare cutting enzymes *Not*I or *Bss*HII, and the isolation of sequences from the *t*-complex, H-2 complex, homeo-box domains, and other regions known to contain or be enriched in *Bss*HII or *Not*I sites. Some of these clones are in the process of being prepared for introduction into mouse embryos to complement the mutant phenotype in transgenic mice.

2. Materials and Methods

(i) *Mice*

Spleen tissue of C3H young adult males was used as source material. These mice were inbred from wild mice, and maintained under standard laboratory conditions.

(ii) *Molecular analysis*

Preparation of Genomic DNA

High molecular weight DNA was isolated from fresh mouse spleen cells which were embedded in agarose blocks at a density of $1–2 \times 10^6$ cells per block equivalent to 10–20 μg of total genomic DNA (Herrmann *et al.* 1987). Blocks were incubated at 50 °C in 0·4 M-EDTA, 1% *N*-lauroyl sarcosine, and 2 mg/ml proteinase K for 24–48 h. After treating with phenylmethyl sulfonyl fluoride (PMSF) to inactivate the proteinase K and washing several times in 10 mMTris-HCl (pH 7·5) and 1 mM-EDTA to remove the PMSF, blocks were stored in 50 mM-EDTA and 10 mM-Tris-HCl. For preparative digestion of genomic DNA, blocks were washed twice in 10 mM-Tris-HCl and 1 mM-EDTA, and placed in a restriction digest mix to a final volume of 200 μl. Restriction enzyme was added to a 10-fold unit excess and digests incubated at the appropriate temperature for 4 h, with an additional two-fold unit excess of enzyme after 2 h. The reaction was terminated with the addition of 20 mM-EDTA and 0·5 mg/ml proteinase K at 37 °C for 1 h. Again, inactivation of proteinase K by PMSF treatment is essential prior to ligation and was carried out as described above. After the final wash, blocks were equilibrated in 10 mM-Tris-HCl, 1 mM-EDTA, and 25 mM-NaCl before melting blocks at 68 °C for 5–10 min. The melted agarose was transferred to 37 °C and after a few minutes 100 units of agarase (Calbiochem) was added to each melted block, and the mixture incubated at 37 °C for several hours.

Vector DNA and ligation reaction

Vector DNA was prepared by digesting pYAC RC DNA (Marchuk & Collins, 1988) to completion with the appropriate restriction enzyme in the polylinker sequence, and *Bam*HI. The DNA was then dephosphorylated with an excess of calf intestinal alkaline phosphatase, phenol extracted two times, and concentrated by ethanol precipitation. The efficiency of dephosphorylation of vector ends was assayed by incubating an aliquot of vector DNA in 50 mM-Tris-HCl, 10 mM-$MgCl_2$, 1 mM-Adenosine triphosphate pH 7·5 and T4 DNA ligase (5 U/μl) at 16 °C for 16 h. As an additional control T4 polynucleotide kinase (2 U/μl) in combination with T4 DNA ligase was added to the ligation mixture, to check that vector ends retain the ability to self-ligate.

DNA at a concentration of 0·05 μg/μl was mixed with the linear phosphatased vector in a 1:1 ratio by weight in 50 mMTris-HCl, 10 mM-$MgCl_2$, 1 mM-Adenosine triphosphate pH 7·5 and T4 DNA ligase (5 U/μl) in a volume not greater than 1 ml. The ligation mixture was incubated for 16 h at 16 °C.

Transformation of yeast host and colony hybridization

The yeast strain routinely used is AB1380 (*MATa, ade2-1, can1-100, lys2-1, ura3, trp1, his5,* [*psi*+]; Burke *et al.* 1987), and these cells were spheroplasted according to Burgers & Percival (1987), except that Lyticase was from Sigma. Transformed cells were plated in top agar with sorbitol on 22 × 22 cm plates minus uracil (ura; Rothstein, 1985), and incubated at 30 °C for 4 days. Individual colonies were picked and spotted onto minus ura plates lacking sorbitol. After 48 h at 30 °C filter lifts were taken directly from the plate using Hybond N nylon membrane, placed colony side up on Whatman 3 MM paper soaked in 1 M-Sorbitol, 0·1 M-Sodium Citrate pH 5·8, 10 mM-EDTA, 10 mM-DTT, Novozyme 4 mg/ml (Novo BioLabs), and incubated at 37 °C for 16 h. Filters were then placed on Whatman 3 MM soaked in denaturant (0·5 M-NaOH, 1·5 M-NaCl) for 15–20 min, and allowed to dry on fresh Whatman 3 MM for 5–10 min. Filters were floated colony side up on 1·0 M-Tris-HCl pH 7·6 and 1·5 M-NaCl for 5 min, and then transferred to 0·1 M-Tris-HCl pH 7·6, 0·15 M-NaCl for 5 min. Filters were finally submerged in 0·1 M-Tris-HCl pH 7·6, 0·15 M-NaCl with 0·25 mg/ml Proteinase K and incubated at 37 °C for 30–60 min. Any remaining debris left on the filters was wiped off with soft tissue soaked in 0·1 M-Tris-HCl pH 7·6, and 0·15 M-NaCl. The filters were then placed in 50 mM-Na phosphate pH 7·2, dried on Whatman 3 MM prior to vacuum baking at 80 °C for 10 min, and then UV crosslinked for 2 min. Filters were prehybridized in formamide buffer (50% formamide, 4 × SSC, 50 mM-Na phosphate pH 7·2, 1 mM-EDTA, 10% dextran sulphate, 1% SDS, 0·05 mg/ml salmon sperm DNA, and 10 × Denhardt's solution) for 16 h at 42 °C. Inserts for DNA probes were isolated away from vector sequences on low melting point (1%) agarose gels,

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diluted in 0·5 volumes of 10 mM-Tris-HCl, 1 mM-EDTA, and melted at 68 °C before adding 0·5 U/μl agarase (Calbiochem) and incubating at 37 °C for 2 h. Inserts were then purified by ethanol precipitation. Approximately 30 ng of isolated inserts were radiolabelled by random priming, and hybridized to the filters for 16 h at 42 °C at a concentration of 1×10^6 c.p.m./ml (Feinberg & Vogelstein, 1984) under the same buffer conditions. Filters were then washed in 0·1 × SSC and 1 % SDS before exposure to Kodak X-AR film. Positively hybridizing yeast colonies were directly used to inoculate 10–20 ml minus ura medium cultures for chromosomal DNA minipreps. Yeast cultures were grown to late log phase ($\sim 10^8$ cells/ml) and pelleted at 2000 r.p.m. for 10 min. The cell pellet was resuspended in a volume of 40 μl per ml of starting culture in SCE (1 M-sorbitol, 0·1 M-sodium citrate pH 5·8, 10 mM-EDTA), and mixed with 4 mg/ml final Novozyme, 0·75 % final low melting point agarose to form an 80 μl plug containing approximately 10^8 cells. After 20 min at 4 °C, plugs were incubated at 37 °C for 1 h in SCE (1 ml per plug) and 10 mM-DTT to digest the cell wall. Cells in agarose plugs were lysed in 1 % *N*-lauroyl sarcosine, 0·4 M-EDTA, 2 mg/ml proteinase K, and incubated at 50 °C for 24–48 h. Agarose plugs were washed in 10 mM-Tris-HCl, 1 mM-EDTA, and stored in 10 mM-Tris-HCl and 50 mM-EDTA. DNA in agarose plugs was analysed by CHEF PFGE to detect the presence of recombinant YACs and their mean size, transferred to nylon membranes (Hybond N, Amersham), and hybridized under conditions described above.

3. Results

YAC libraries have been constructed from total mouse genomic DNA digested with the rare cutter enzymes *Not*I, and *Bss*HII. The *Not*I library contains approximately 9500 clones which were individually picked into minus ura medium and 30 % glycerol lacking sorbitol and stored frozen in 96 microtitre well dishes (Table 1). Initially 4000 clones were manually spotted

Table 1. *Rare-cutting enzyme YAC libraries*

Enzyme	Transformation efficiency	Library size (No. clones)	No. clones screened	No. loci isolated
*Not*I	2000/μg	9500	4000	8
*Bss*HII	700/μg	4500	4500	2

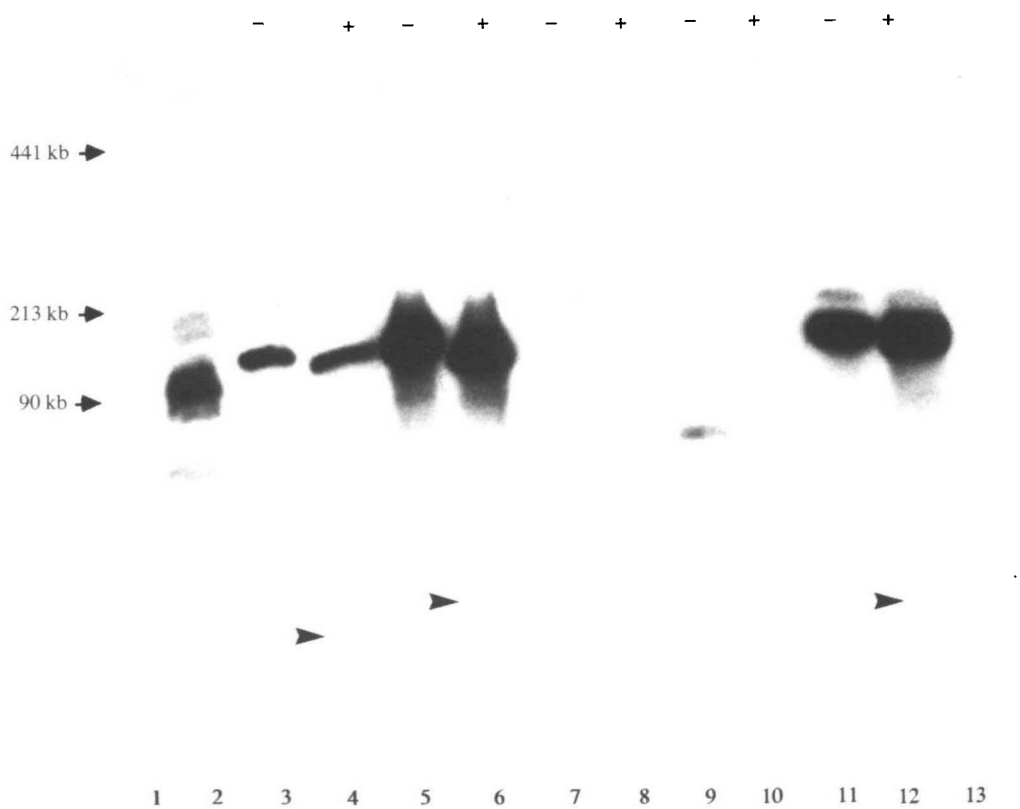

Fig. 1. Pulsed field gel analysis of five YAC clones isolated from the *Not*I library which hybridized to an H-2 class I probe. The pulsed field get was run on a CHEF apparatus (EMBL, Heidelberg), at 170 V and 40 s for 24 h. Lanes 3–12 show each YAC clone before (–) and after cleavage (+) with *Not*I; YAC clone 1 in lanes 3 and 4, YAC clone 4 in lanes 5 and 6, YAC clone 5 in lanes 7 and 8, YAC clone 7 in lanes 9 and 10, and YAC clone 9 in lanes 11 and 12. The hybridization pattern is compared to mouse C3H genomic DNA digested with *Not*I in lane 2. Lanes 1 and 13 contain yeast chromosome markers (prepared as agarose plugs from yeast chromosomal minipreps from the strain YP148). YAC clones 5 and 7 show weak hybridization to the probe on longer exposure. The solid arrows indicate release of vector sequences.

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on two 22 × 22 cm agar plates with a 96 pin capillary device. The mean size of 18 random clones picked was approximately 150 kb. A total of 8 independent loci were isolated by hybridization to only 4000 clones with probes from different regions. Initial characterization of this library was carried out using a cDNA probe encoding an H-2 histocompatibility antigen of the d haplotype (Kvist *et al.* 1981). The probe is a subclone from pH-2d-1 in pBR322 containing a 0·4 kb *Pst*I fragment from the coding region. It recognizes many H-2 class I genes located within the H-2 complex on chromosome 17. Five independent loci have been isolated by hybridization to this probe, thereby exhibiting homology with several H-2 antigens. Fig. 1 shows hybridization of the cDNA probe to a filter transfer of a pulsed field gel of *Not*I-digested mouse genomic DNA against *Not*I-digested YAC clones which were prepared from yeast chromosomal mini-prep cultures. The YAC clones (1, 4, 5, 7 and 9) range from 60–100 kb in size. The hybridization was carried out under conditions of high stringency which may account for the variable intensity depending on the degree of homology between genomic fragment and probe; however, it may also be due to the number of copes of homologous sequences present in each YAC.

Fig. 2 shows *Hin*dIII digested mouse genomic DNA and three YAC clones 1, 4 and 9 hybridized with the same cDNA probe and showing a unique pattern of bands from each clone. The difference in some of the fragment sizes between YAC and genomic DNA is due to loading discrepancies, although adjustments were made to account for the differing complexities between the two genomes. The probe hybridized weakly to YAC clones 5 and 7 in Fig. 1 (data not shown). Two further loci have been isolated which are specific for homeobox domains from two different Hox clusters. One is approximately 120 kb from Hox cluster 2 on chromosome 11 (Rubock *et al.* 1990), and the other is approximately 70 kb from Hox cluster 5 near to the 5·1 gene on chromosome 2 (probe provided by D. Duboule). One other YAC of approximately 90 kb, was isolated from the pink-eyed dilute region on chromosome 7 (probe provided by G. Rinchik).

The *Bss*HII library originally contained 6000 clones which were plated in soft agar onto three 22 × 22 cm plates (Table 1). In this experiment, clones from one plate were then directly transferred or replicated to another supplemented agar plate (lacking sorbitol) via a 22 × 22 cm metal grid containing 40000 closely spaced machined pins (EMBL, Heidelberg), which were pressed onto the surface. Approximately 75% of clones (4500) were transferred in this manner. After 48 h, colony filter lifts using Hybond N membrane were then taken directly from each replicated plate. Transfer of clones using the metal grid device is quicker than individually picking and hand spotting clones; however, not all clones are transferred from within the top agar layer, since yeast colony size is heterogeneous. Retrieval of positive clones which

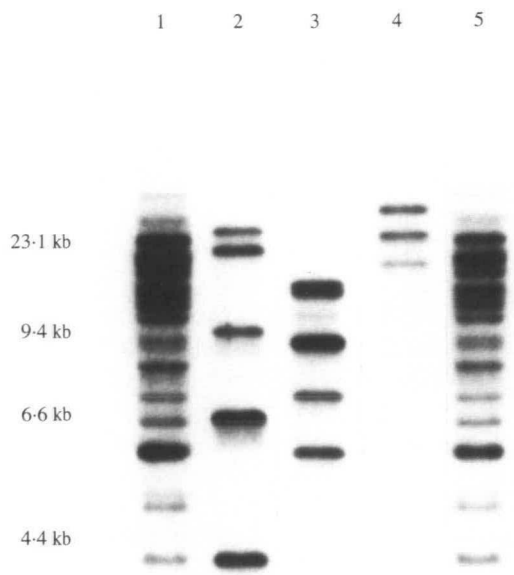

Fig. 2. Hybridization pattern of three *Hin*dIII digested *Not*I YAC clones compared to mouse genomic DNA digested with *Hin*dIII using the same H-2 class I cDNA as probe. In this figure YAC clones 1, 4 and 9 (see Fig. 1) correspond to lanes 4, 3 and 2 respectively. Lanes 1 and 5, contain *Hin*dIII digested mouse genomic C3H DNA. Samples were loaded on a 1% agarose gel in 1 × TAE and run at 2·5 V/cm (in circulating 1 × TAE buffer) for 16 h. Although DNA concentrations of both YAC and genomic DNA were adjusted when loading because of the differing complexity values of their genomes (approximately 1/200), slight differences in fragment migration still occurred in the gel.

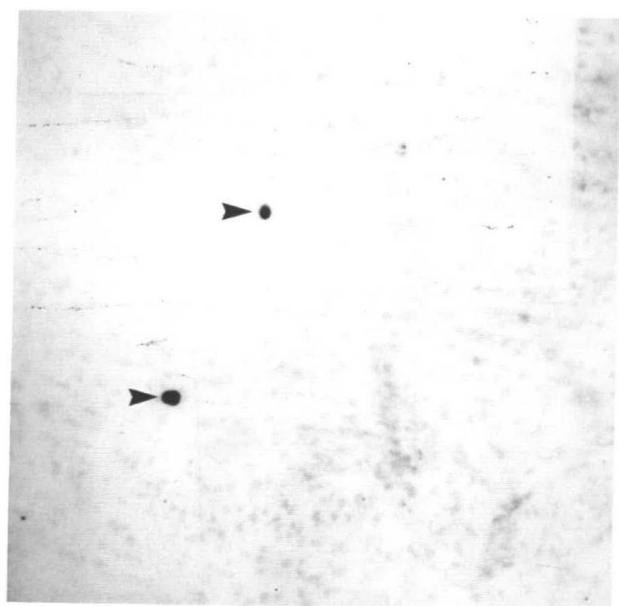

Fig. 3. Colony filter hybridization of *Bss*HII YAC clones using cDNA pme 681 as probe. These filters were lifted from plates containing YAC clones replicated from the original top agar by a 22 × 22 cm metal grid device. Two positive clones were isolated which are indicated by solid arrows. There is some faint background hybridization showing the grid pattern.

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match the grid pattern can be done either from the original replicated plate stored at 4 °C which has a short life span, or from duplicate filters which are stored frozen in minus ura medium and 30% glycerol between thin perspex plates. Ideally, clones should also be picked into 96 well microtitre dishes for long term storage.

Specific YAC clones were isolated by hybridization to these 4500 *Bss*HII clones with probes from the proximal portion of the *t*-complex region on chromosome 17. Fig. 3 shows hybridization to the replicated filters with a cDNA probe (pme 681; provided by B. Herrmann) specific for the Brachury locus. Two YAC clones (of approximately 90 kb) were isolated. One other YAC clone was also isolated with a cDNA probe specific for *tcp-I* (provided by K. Willison) and is approximately 80 kb in size (data not shown).

4. Discussion

We have constructed YAC libraries with the rare cutter enzymes *Not*I, and *Bss*HII, without size selection. Generally, the average insert size of non-size selected DNA using this type of enzyme is about 100 kb, thus isolation of extremely large sequences would not be possible unless a large number of colonies were screened. Libraries generated with rare cutting enzymes are not representative of the entire genome as in a partial digest library, as these sites tend to be clustered; thus some regions will be under-represented and others over-represented. However, we have shown that specific fragments of a particular size range can be isolated from these libraries if the pulsed field restriction map of the region is known, so fewer clones need to be generated and screened than in a partial digest library.

These libraries would be particularly useful in cloning sequences between HTF islands where these sites predominate (Brown & Bird, 1986) and, in conjunction with PFGE, may be important in locating genes or markers known to lie on a particular fragment or be associated with CpG islands. Abe *et al.* (1988) have isolated seven genes within 170 kb of cloned DNA from the K region of the H-2 complex, which were associated with CpG islands and four of these genes are thought to be involved in embryogenesis. This lends support to the observation that CpG islands, known to be associated with 'housekeeping' genes, are also found in the 5′ end of tissue specific genes (Rappold *et al.* 1987). Additionally, sequences tens to hundreds of kilobases away from genes, and which are involved in their regulation, may be isolated by screening for such fragments. In parallel with this, YAC libraries may also prove invaluable in chromosome walks especially where sequences have previously be unclonable by cosmid vectors, and may complement jumping libraries which are also generated with the same rare cutting enzymes.

YAC clones will also be important in assisting the search for novel developmental genes by complementation of the mutant phenotype in mice. Studies are currently in progress to isolate YACs in an intact form containing a large portion of the region of interest. YAC DNA may then be introduced into the mouse genome either by targeted correction via homologous recombination into embryonal stem cells, or by direct integration via microinjection into mouse embryos. By introducing a series of overlapping fragments further sub-localization of the mutation and extent of the gene locus should be possible. Using this approach we aim to analyse regions of the *t*-complex in which many developmental mutations have been located. Isolation and study of the corresponding genes could give an important insight into the development of the mouse. These methods may also assist human research studies in more precisely localizing developmental or disease genes which span large distances, and where conventional cloning approaches have been laborious or proved unsuccessful.

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