Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4123–4127, May 1991
Genetics

Yeast artificial chromosome libraries containing large inserts from mouse and human DNA

(cloning/polyamines/colony hybridization)

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Communicated by Louis M. Kunkel, January 28, 1991

ABSTRACT **Yeast artificial chromosome (YAC) libraries have been difficult to construct with average insert sizes >400 kilobase pairs when DNA is size-fractionated in low-melting-point agarose. By using yeast chromosomes in mock cloning experiments, we found that polyamines should be present whenever agarose containing high molecular weight DNA is melted to protect DNA from degradation. By incorporating polyamines during the cloning procedure, we constructed YAC libraries from mouse and human DNA with average insert sizes of 700 and 620 kilobase pairs, respectively. Several genome equivalents of these YAC libraries were replicated onto the surface of many duplicate agar plates using a 40,000 multipin transfer device. High-density filter replicas were screened by hybridization, and 70 mouse YAC clones from 31 loci and 132 human YAC clones from 49 loci were isolated.**

Yeast artificial chromosome (YAC) libraries have been constructed from several eukaryotic genomes by preparing and size-fractionating high molecular weight DNA in solution by sucrose gradient density separation (1–3) or in agarose by pulsed-field gel electrophoresis (PFGE; refs. 4–6). Insert sizes of YAC clones are larger on average when DNA is prepared in agarose [400 kilobase pairs (kb)], perhaps due to minimizing shear forces. However, we found that the average size of YAC clones generated when preparing DNA in agarose was considerably smaller than the size of DNA fragments selected by PFGE.

This size discrepancy was investigated by conducting a series of mock cloning experiments with yeast chromosomes. Partial degradation of high molecular weight DNA was observed whenever agarose containing DNA was melted during the cloning procedure. The presence of polyamines (5) prior to a melting step prevented the degradation of large DNA fragments. By including polyamines when agarose containing DNA was melted after the first and second size fractionations by PFGE, we constructed mouse and human libraries with average insert sizes of 700 and 620 kb, respectively.

Filter replicas containing YAC clones have been difficult to generate directly from the surface of transformation plates, since regenerating yeast cells must grow within a supportive agar matrix. Most YAC libraries have been assembled by individually picking clones into microtiter dishes and transferring them manually to filters for screening by hybridization. As an alternative approach, we have used a 40,000 multipin transfer device to replicate YAC clones directly from transformation plates onto the surface of many agar plates for filter replicas. By colony hybridization we screened two genome equivalents (one genome equivalent is 5000 clones at 600 kb) of the human library on three 22 × 22 cm filters and three genome equivalents of the mouse library on four filters. The filters were generated in multiple copies for repeated screenings by many probes and one filter was stored frozen at −70°C for retrieval of clones once the library plates were no longer viable at 4°C.

MATERIALS AND METHODS

Preparation of Genomic DNA. High molecular weight DNA from fresh mouse spleen tissue was prepared in agarose blocks, as in Herrmann *et al.* (7), with 2×10^6 cells per block (≈15 μg of DNA). Human genomic DNA was prepared from a lymphoblastoid cell line, GM1416B (National Institute of General Medical Sciences, Human Genetic Cell Repository), containing four X chromosomes per cell. Cells were harvested and embedded in agarose as described above.

Partial Digestion of DNA. Prior to enzyme digestion, blocks were washed in TE (10 mM Tris·HCl, pH 7.5/1 mM EDTA) containing phenylmethylsulfonyl fluoride (40 μg/ml) at 50°C to inactivate the proteinase K and then further washed twice in TE to remove the phenylmethylsulfonyl fluoride. Individual blocks were placed on ice in a restriction digest mixture containing 100 mM NaCl, 100 mM Tris·HCl (pH 7.5), 2 mM $MgCl_2$, 80 μM *S*-adenosylmethionine, bovine serum albumin (0.5 mg/ml), 2.6 mM spermidine trihydrochloride, and 1 mM dithiothreitol in a final volume of 500 μl. A combination of 200 units of *Eco*RI methylase with 1 unit of *Eco*RI (see Fig. 1) was added to the mixture. Blocks were equilibrated in the enzyme buffer for 1 hr before transferring to 37°C for 4 hr. The reaction was terminated with the addition of EDTA and proteinase K to 20 mM and 0.5 mg/ml, respectively, and incubated at 37°C for 30 min.

First Size Fractionation by PFGE. Blocks were pooled, washed in 10 mM Tris·HCl, pH 7.5/50 mM EDTA, and then placed in a trough prepared in a 1% low-melting-point agarose gel (SeaPlaque GTG; FMC) in 0.25× TBE (1× TBE = 0.089 M Tris borate/0.089 M boric acid/0.002 M EDTA, pH 8.0). The gel was subject to electrophoresis at 160 V, using a 30- or 50-sec switch time (for selection of fragments 400 kb or 600 kb and above, respectively) for 18 hr at 15°C in a contour-clamped homogeneous electric field (CHEF) apparatus [European Molecular Biology Laboratory (EMBL) workshop, Heidelberg]. The unresolved portion of DNA, which was focused into a thin band, was excised from the gel by aligning marker lanes (a similar genomic digest and yeast chromosomes stained with ethidium bromide) adjacent to the gel. The slice (1–2 ml) was equilibrated in four 30-min changes of ligation buffer containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 30 mM NaCl, and polyamines (0.75 mM spermidine trihydrochloride/0.3 mM spermine tetrahydrochloride; ref. 5) prior to ligation.

Vector DNA Preparation and Ligation Reaction. The vector pYAC4 was digested with *Eco*RI and *Bam*HI to completion and assayed by gel electrophoresis. Vector ends were de-

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Abbreviations: YAC, yeast artificial chromosome; PFGE, pulsed-field gel electrophoresis; CHEF, contour-clamped homogeneous electric field.

phosphorylated in the presence of calf intestinal phosphatase (0.03–0.06 unit/μg of DNA) and assayed in a ligation reaction at 25°C with and without the addition of T4 polynucleotide kinase (2 units/μl).

The agarose slice containing size-selected DNA was placed in an Eppendorf tube, melted at 68°C for 10 min with vector DNA in a ratio of 1:1 by weight, and cooled to 37°C. Ligase (400 units/μl) was added in ligase buffer with final concentrations of 1 mM ATP (pH 7.5) and 1 mM dithiothreitol and mixed by slow stirring with an additional two or three inversions of the tube. The reaction mixture was kept at 37°C for 0.5–1 hr prior to leaving at room temperature overnight. The reaction was terminated by adding EDTA (pH 8.0) to 20 mM.

Second Size Fractionation by PFGE. The ligation reaction mixture was melted at 68°C and cooled to 37°C. The molten agarose was carefully pipetted (tip bore diameter not less than 4 mm) into a gel trough as described above. The gel was subject to electrophoresis in a CHEF apparatus at 160 V with a switch time of 30 or 50 sec for 18 hr. The unresolved DNA, which was focused into a thin band, was cut from the gel using marker lanes as described above. The slice (in a volume of 2–3 ml) was washed for four 30-min periods in 10 mM Tris·HCl, pH 7.5/1 mM EDTA/30 mM NaCl/polyamines. The agarose slice was melted at 68°C, cooled to 37°C, and 150–200 units of agarase (Calbiochem or Sigma) was added. The molten mixture was incubated for 2–3 hr at 37°C prior to transformation.

Transformation of Yeast Host. Transformation was carried out according to Burgers and Percival (8) by using lyticase (Sigma) to make spheroplasts of *Saccharomyces cerevisae* AB1380 cells (1). Approximately 0.5–1 μg of DNA in digested agarose solution was added to 150 μl of cells in 15-ml tubes at a final concentration of 4.5×10^6 cells per ml. Cells were pooled at the final stage such that 1.5 ml of cells was plated in 50 ml of top agar with sorbitol but lacking uracil (9), onto a 22 × 22 cm plate, and incubated at 30°C for 4 days.

Colony Replication and Preparation of Filters. YAC colonies from up to six regeneration plates were transferred sequentially to the surface of the multipin transfer device containing 40,000 pins (1.2 mm high and 1.2 mm apart; EMBL workshop). Eight copies were made by pressing the device lightly onto the surface of selective agar plates (lacking sorbitol, uracil, and tryptophan) and these copy plates were incubated at 30°C for 2 days. Colony lifts were taken directly from the surface of these plates using Hybond-N+ filters (Amersham) and processed for hybridization. Filters were placed colony side up onto Whatman 3MM paper saturated with 1 M sorbitol/0.1 M sodium citrate, pH 5.8/10 mM EDTA/10 mM dithiothreitol/Novozyme (NovoBiolabs, Copenhagen; 4 mg/ml) and incubated at 37°C for 16 hr. Filters were transferred onto fresh Whatman 3MM paper saturated in 0.5 M NaOH/1.5 M NaCl for 20 min and dried briefly on fresh Whatman 3MM paper for 5 min. Filters were neutralized by floating on 1 M Tris·HCl, pH 7.6/1.5 M NaCl for 5 min and on 0.1 M Tris·HCl, pH 7.6/0.15 M NaCl for a further 5 min. Filters were submerged in fresh 0.1 M Tris·HCl, pH 7.6/0.15 M NaCl/proteinase K (0.25 mg/ml) and incubated while rocking at 42°C for 60 min. The filters were gently wiped with soft tissue soaked in 0.1 M Tris·HCl/0.15 M NaCl to remove any remaining colony debris. Filters were transferred to 50 mM sodium phosphate (pH 7.2) for 5 min, dried on fresh Whatman 3MM paper, vacuum baked at 80°C for 10 min, and UV-crosslinked for 2 min.

One filter containing YAC clones was prepared for storage at −70°C; the filter was placed onto Whatman 3MM paper soaked in YPD medium/30% (vol/vol) glycerol and frozen between two pieces of polycarbonate material. In addition, colonies from one plate were also picked into 96-well microtiter dishes containing YPD/30% glycerol for permanent

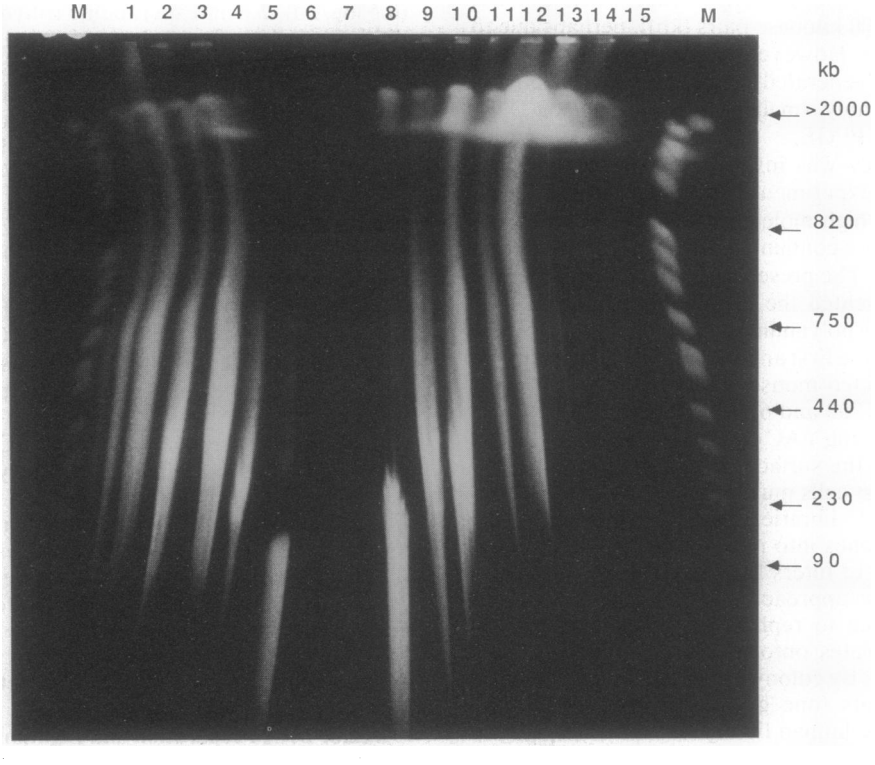

FIG. 1. *Eco*RI partial digests of mouse genomic DNA by using mixtures of *Eco*RI with *Eco*RI methylase. Lanes 1–5 contain genomic DNA (10 μg per block per lane) that was incubated with 1 unit of *Eco*RI and *Eco*RI methylase as follows. Lanes: 1, 2000 units; 2, 1000 units; 3, 500 units; 4, 200 units; 5, 100 units. Lanes 8–15 contain mouse genomic DNA incubated with 200 units of *Eco*RI methylase and *Eco*RI as follows. Lanes: 8, 10 units; 9, 5 units; 10, 1 unit; 11, 0.5 unit; 12, 0.1 unit; 13, 0.05 unit; 14, 0.01 unit; 15, 0.001 unit. Lane 6 contains genomic DNA after incubation in methylase buffer only and lane 7 contains undigested DNA with no apparent ethidium staining in the gel. Lanes M contain yeast chromosomes from *S. cerevisae* YP148 as size markers.

storage at −70°C for future DNA preparations to be spotted in an ordered array by a robot device (Dean Nizetic, Gunther Zehetner, A.P.M., and H.L., unpublished procedure).

Filter Hybridization and Yeast DNA Preparation. Nylon filters were prehybridized and hybridized at 42°C in 50% (vol/vol) formamide/4× standard saline/citrate (SSC)/50 mM sodium phosphate, pH 7.2/1 mM EDTA/8% (wt/vol) dextran sulfate/1% SDS/salmon sperm DNA (0.05 mg/ml)/ yeast tRNA (0.025 mg/ml)/10× Denhardt's solution. Radioactively labeled probes were prepared as described by Larin and Lehrach (10). Filters were washed for two 20-min periods in 0.1× SSC/1% SDS at 65°C and exposed to Kodak XAR film at −70°C for 2–3 days with an intensifying screen. Yeast colonies corresponding to colonies positive by hybridization were picked from the primary plates onto YPD plates, and DNA was prepared in agarose blocks from yeast cultures grown in double selection medium (minus uracil and tryptophan) as in Larin and Lehrach (10). Yeast chromosomes containing YACs were subject to electrophoresis on a CHEF apparatus at 160 V at a 100-sec switch time for 36 hr. The pulsed-field gel was alkali blotted to Hybond N+ and hybridized to the specific probe to verify the size of the positive YAC.

RESULTS

Preparation and Size Fractionation of DNA in Agarose. We have generated mouse and human YAC libraries using high molecular weight genomic DNA prepared in agarose blocks as source material. DNA was partially digested using a combination of *Eco*RI and *Eco*RI methylase, which gave most DNA in the range of 200–2000 kb. Fig. 1 shows mouse DNA in agarose incubated with various ratios of *Eco*RI and *Eco*RI methylase for a 4-hr digestion and assayed by PFGE. We found this procedure to be consistent between DNA sources and concentrations. The partially digested DNA was size-selected on a CHEF apparatus prior to ligation to the vector arms and the ligated material was size-selected a second time prior to transformation, similar to protocols described by Albertsen *et al.* (6). However, we observed by PFGE analysis that partial degradation of DNA had occurred after the initial size fractionation but before the second size-fractionation step.

Control Experiments to Prevent Degradation of DNA in Agarose. To determine the cause of degradation, all buffers used during the cloning procedure were checked to eliminate any possible nuclease effect (data not shown). A series of mock cloning experiments were then conducted using commercial yeast chromosomes (Bio-Rad), which were size-fractionated by PFGE. The unresolved portion of yeast chromosomes >400 kb was excised from low-melting-point agarose, incubated with proteinase K, phenylmethylsulfonyl fluoride-inactivated, washed in ligation buffer (50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/30 mM NaCl) four times, and assayed by PFGE (Fig. 2*A*). The agarose containing DNA was melted at 68°C in the presence of pYAC4 vector, and after cooling to 37°C for 5 min, ATP and dithiothreitol were added to 1 mM and 10 mM, respectively. When the various steps were analyzed by PFGE, it was observed that on melting, yeast chromosomes had degraded to a smear between 50 and 250 kb and there was increased ethidium bromide staining in the gel slots. The degradation was enhanced after prolonged incubation at 37°C. Agarose slices containing size-selected yeast chromosomes were also incubated in buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA/30 mM NaCl) used routinely after the second size fractionation of DNA prior to transformation and assayed by PFGE (data not shown). After heating to 68°C, yeast chromosomes had partially degraded (50–250 kb) and this was enhanced after 16

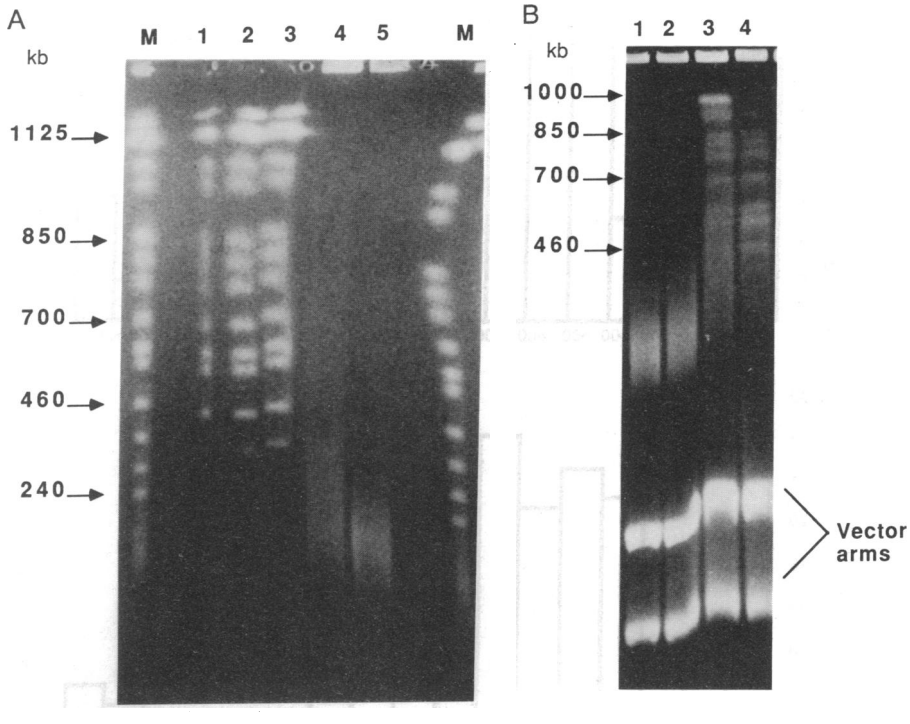

FIG. 2. (*A*) Ethidium bromide-stained 1% agarose CHEF gel of yeast chromosomes after size selection in low-melting-point agarose before and after ligation to pYAC4 vector. Lanes 1–3 show chromosomes in the following preparative stages prior to ligation: proteinase K treatment, phenylmethylsulfonyl fluoride inactivation, and washing in ligation buffer, respectively. Lanes 4 and 5 indicate the effect of yeast chromosomes in ligation buffer after melting at 68°C for 5 min and cooling to 37°C and on prolonged incubation at 37°C overnight with the addition of ATP and pYAC4 vector, respectively. (*B*) Yeast chromosomes were size-selected in low-melting-point agarose to >400 kb and then ligated to pYAC4 in the presence or absence of polyamines. Lanes 1 and 2 in absence of polyamines with and without dithiothreitol (10 mM), respectively. Lanes 3 and 4 in presence of polyamines with and without dithiothreitol, respectively.

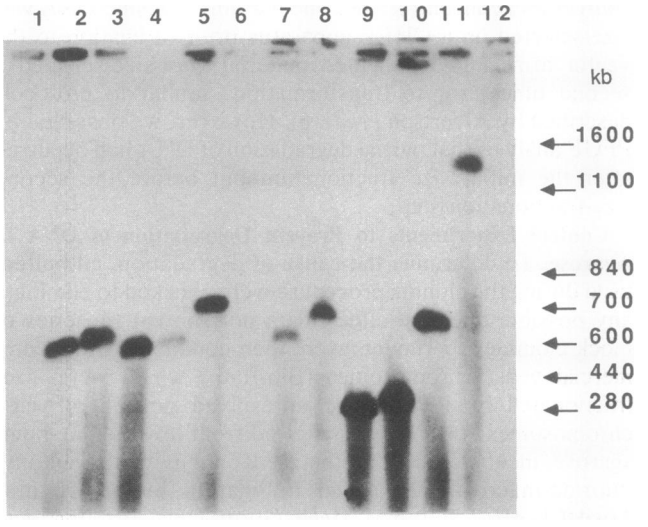

FIG. 3. After PFGE, random YAC clones (lanes 1–12) from the mouse library were hybridized with a B1/B2 repeat sequence probe.

hr at 37°C. Any further increase in NaCl concentration would reduce the transformation efficiency (4).

In separate experiments, protection of yeast chromosomes occurred when $MgCl_2$ at >15 mM was added prior to melting. Therefore, the effect of adding polyamines to protect chromosomes from degradation was tested. Yeast chromosomes remained intact after heating at 68°C when spermidine at 10 mM was added alone (but not at 1 mM) and in a separate reaction when spermine at 1 mM was present alone (but not at 0.1 mM, data not shown). A combination of spermidine and spermine in concentrations reported by McCormick *et al.* (5) (0.75 mM and 0.3 mM, respectively) protected chromosomes from degradation after melting the agarose at 68°C (Fig. 2*B*).

Construction of YAC Libraries. We incorporated polyamines during the YAC cloning procedure in the washing steps after the first size selection prior to melting and ligation and after the second size selection when melting prior to transformation. The presence of polyamines during the transformation procedure (5) was found to be unnecessary to produce large insert YAC clones and reduced the transformation efficiency ≈5-fold. Fig. 3 shows a filter hybridization of random YACs size-fractionated by PFGE from the mouse library with sizes ranging from 250 to 1300 kb. We also analyzed YACs in which the genomic DNA was size-selected using different switch times. In the mouse YAC library construction, both a 30-sec switch time that removed fragments smaller than 400 kb and a 50-sec switch time that removed fragments smaller than 600 kb produced YACs with an average size of 700 kb. Therefore, no appreciable difference was detected between these sizing protocols. The libraries consist of 75% clones generated at a switch time of 30 sec and 25% clones generated at a switch time of 50 sec.

Characterization of YAC Libraries. The mouse library contains 15,000 clones with an average insert size of 700 kb, approximately a 3-fold coverage of the mouse genome. The human library contains 10,000 clones with an average insert size of 620 kb, equivalent to a 2-fold genome coverage, but a 4-fold coverage of the X chromosome. The average size and distribution of 108 mouse and 145 human YACs both randomly selected and specific clones are shown in Fig. 4.

The libraries were replicated at high density by the multipin transfer device and screened by colony hybridization with single-copy probes. Individual positive clones were picked

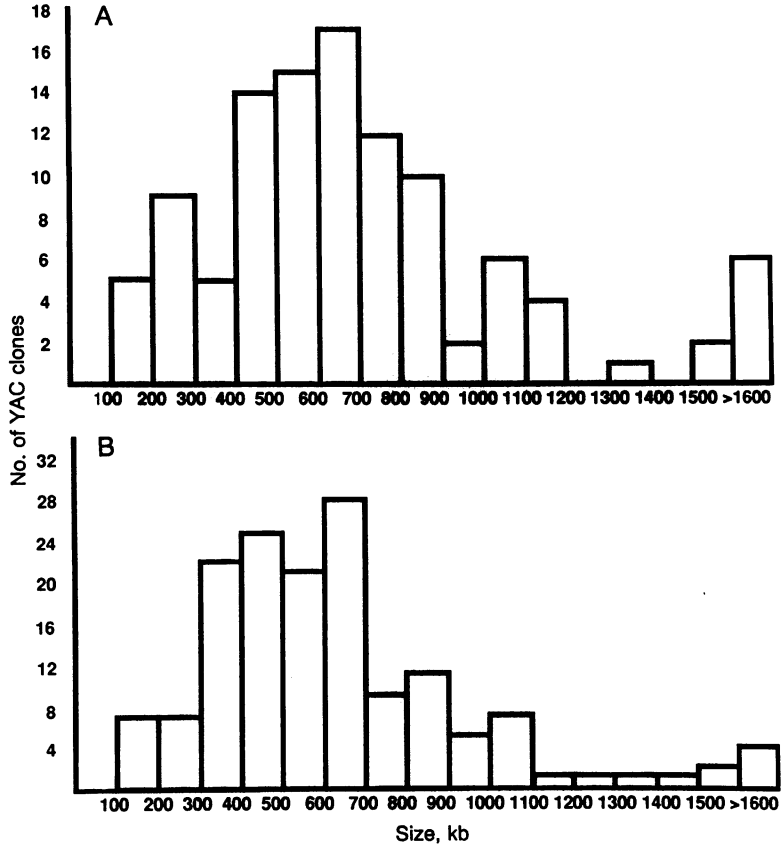

FIG. 4. Histogram showing the size distribution of YACs from the mouse (*A*) and human (*B*) YAC libraries. For the mouse library, 52 randomly selected clones and 56 specific clones (for a total of 108 clones) are represented, and for the human library, 28 randomly selected clones and 117 specific clones (for a total of 145 clones) are shown. The average size of the mouse clones was 700 kb; the average size of the human clones was 620 kb.

from the primary library plates for further analysis by PFGE. Screening the YAC libraries by colony hybridization yielded statistics in relative agreement with the number of genome equivalents represented. From three genome equivalents of the mouse library, 89% of probes were positive (31 out of 35) yielding 70 YAC clones; from two genome equivalents of the human library, 76% of probes located on autosomes were positive (23 out of 30) yielding 40 YAC clones. For the human X chromosome (about four chromosome equivalents), 90% of probes were positive (26 out of 29) yielding 92 YAC clones.

DISCUSSION

In our initial YAC library constructions, in which DNA was size-selected in agarose to >400 kb, we found that our average insert sizes were <200 kb. Many of the resulting clones contained only one arm of the vector indicating that there was degradation of the DNA fragments occurring at some stage during the cloning procedure (unpublished data). By using yeast chromosomes in control experiments, we found that high molecular weight DNA prepared in agarose was partially degraded when heated to 68°C, even with the appropriate amount of NaCl. To eliminate this effect it was necessary to include polyamines in all steps during the cloning procedure prior to transformation, particularly where a melting step at 68°C was involved. In contrast to McCormick *et al.* (5) but in agreement with Albertsen *et al.* (6), we found that there was no appreciable difference in YAC insert size if polyamines were present during the transformation of spheroplasted yeast cells.

Our results indicated that partial degradation of DNA in agarose was not due to nuclease activity since it was proteinase K insensitive and occurred in the presence of EDTA. One possible cause of the partial degradation may be attributed to metal ion cofactors in commercial agarose. Schultz and Dervan (11) showed that Fe^{2+} complexed with EDTA bound strongly to DNA especially in A+T-rich regions and caused cleavage of single- and double-stranded DNA when heated to 68°C in the presence of reducing agents. In this study, we observed that high levels of Mg^{2+} protected DNA in agarose from degradation when heated to 68°C. This may be due to the high ionic strength of Mg^{2+} preventing DNA from denaturing or because Mg^{2+} forms a complex with EDTA and competes with any metal ions for the chelation site. When polyamines were added, DNA was also protected from degradation, allowing us to construct YAC libraries with average insert sizes consistent with the size of DNA fragments selected by PFGE.

The advantage of producing YAC libraries with large insert sizes is that fewer clones are needed for contig building in physical mapping projects and for chromosome walking to genes of interest. Filters containing severalfold genome coverage with YAC clones generated by the multipin transfer device have enabled rapid screening of primary libraries by hybridization. This is comparable to PCR amplification of pools of YAC clones that still must utilize a DNA hybridization step at the last stage to identify the microtiter dish well containing the positive YAC clone (12). Colony hybridization also has the advantage that complex probes such as the Duchenne muscular dystrophy cDNA can isolate large contigs of clones encompassing the gene (2.3 megabase pairs) with two hybridizations to three filters, rather than multiple PCR screenings of the library (A.P.M., unpublished data).

We thank Snezana Drmanac, Iona Millwood, and Tim Evans for excellent technical assistance; Mac Ho for 4× DNA blocks; Lisa Stubbs, Jiannis Ragoussis, Gill Bates, Roger Cox, Mac Ho, Sau-Ping Kwan, and Melissa Rubock for library screenings; and Stephen Goodbourn, Viesturs Simanis, Lisa Stubbs, Gill Bates, Anna-Maria Frischauf, and Peter Goodfellow for many helpful discussions and comments to this manuscript. A.P.M. was supported in part by a research fellowship from the Muscular Dystrophy Association of America.

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