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Streamlined approach to creating yeast artificial chromosome libraries from specialized cell sources

JAY M. FEINGOLD, SHEILA D. OGDEN, AND CHRISTOPHER T. DENNY

Gwynne Hazen Memorial Laboratories, Division of Hematology-Oncology, Department of Pediatrics, and the Jonsson Comprehensive Cancer Center, University of California–Los Angeles School of Medicine, University of California–Los Angeles Medical Center A2-310 MDCC, Los Angeles, CA 90024

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ABSTRACT **The study of tumor-specific chromosomal abnormalities has been severely impeded by an inability to link cytogenetic to molecular data. Restriction fragment length polymorphism mapping of any particular chromosomal rearrangement to the resolution limit of genetic methodology generates sets of probes that frequently are still too widely spaced to render the rearrangement breakpoints accessible to molecular isolation. The stable propagation of genomic fragments of up to one million base pairs in size as yeast artificial chromosomes (YACs) represents an important development in this regard. However, existing YAC libraries have been made from karyotypically normal sources making the localization and cloning of specific rearrangement breakpoints much more difficult. As a solution to this problem, we present an improved method for creating YAC libraries that can utilize specialized tumor-derived materials and that can be executed effectively in a small laboratory setting. Procedures that enabled more consistent DNA insert size selection and enhanced yeast transformation frequency were employed to generate a human YAC library from a neuroepithelioma cell line containing a characteristic t(11;22) chromosomal translocation. Approximately 40,000 colonies with an average insert size of 330 kilobase pairs were created. This library was screened with two single-copy probes that bracket the translocation breakpoint. YAC clones ranging from 370 to 550 kilobase pairs that were specific for each single-copy probe were identified. Specialized YAC libraries will make many more tumor-specific chromosomal abnormalities accessible to molecular isolation.**

Definitive analysis of tumor-specific chromosomal abnormalities has resulted in the discovery of important genes and events involved in malignant transformation (for reviews, see refs. 1 and 2). The molecular isolation of the genetic loci involved in such rearrangements has been hampered by the size and complexity of the human genome. Conventional cloning techniques using phage or cosmid vectors have been limited to fragments less than 50 kilobases (kb). On the other hand chromosome mapping by either karyotypic banding or restriction fragment length polymorphism recombination analysis has a resolution limit on the order of millions of base pairs. This physical disparity poses an obstacle for linking mammalian genetic and molecular data. As a result the complete characterization of tumor-specific chromosomal abnormalities has been limited to those rearrangements that involve a genomic locus that has fortuitously been previously isolated.

The strategy of cloning very large genomic fragments in yeast holds considerable promise of closing the gap between genetic and molecular analyses (3). A few laboratories have reported the construction of recombinant human genomic libraries as yeast artificial chromosomes (YACs) in *Saccharomyces cerevisiae* (4–6). In this process partially digested human genomic fragments are ligated to yeast centromeres and telomeres creating structures that can be stably replicated in yeast as linear DNA molecules. Most YACs range from 100 kb to 500 kb though clones as large as 800 kb have been reported (7). The localization of genomic sequences on such large contiguous fragments of the human genome has made it possible to construct long-range physical maps over large chromosomal domains (8, 9).

Despite the broad utility of such a reagent, the construction of only three complete human genomic YAC libraries has been described (4–6). All these libraries used karyotypically normal human cell lines making them unsuitable for the direct isolation of specific chromosomal rearrangements. We present a feasible streamlined approach for generating individual YAC libraries that contain tumor-specific chromosomal abnormalities. Recent advances in YAC vector construction as well as genomic insert DNA preparation have been applied to create a three-haploid-genomic-equivalent human YAC library from a neuroepithelioma cell line containing a characteristic t(11;22) chromosomal translocation. In contrast to previous YAC library constructions all manipulations of very high molecular weight DNAs were performed in agarose. Modification of the conditions for yeast spheroplast preparation has enhanced transformation efficiency. Clones were grown in 96-well microtiter dishes and screened with two single-copy genomic probes that bracket the t(11;22) translocation breakpoint. Each genomic probe yielded at least one positive YAC clone ranging in size from 370 kb to 550 kb.

METHODS

Preparation of Human DNA. Human DNA was derived from TC-32, a characterized neuroepithelioma cell line (10) containing the t(11;22) translocation: +5,+10, i(1q), t(11;22)(q24;q12). Approximately 4×10^8 washed cells in 5 ml of isotonic phosphate-buffered saline were mixed with 7 ml of 1% low melting temperature agarose (InCert; FMC). The hardened suspension was digested with 0.5% sarcosine/5 mM Tris, pH 9/0.25 M EDTA, pH 8.0/proteinase K (Boehringer Mannheim) (1 mg/ml) at 42°C for 2 days. Agarose plugs were extensively dialyzed and then stored in 100 mM EDTA.

Partial Endonuclease Digestion of Human DNA. For each set of reaction conditions, five 1-mm-thick DNA plugs (total volume, 200 μl) were dialyzed against 5 ml of 1× *Eco*RI–Mg buffer [100 mM NaCl/50 mM Tris/bovine serum albumin (100 μg/ml)] twice for 20 min at room temperature and once at 10°C. To each tube 250 units of *Eco*RI in 1750 μl of 1× *Eco*RI–Mg buffer was added and incubated on ice for 20 min. Various amounts of $MgCl_2$ were added in 50-μl volumes to achieve final concentrations from 0.2 mM to 0.9 mM. After a 10-min preincubation at 4°C, digestion proceeded at 37°C for 1 hr. Tubes were then plunged into ice and immediately dialyzed against 2 ml of ice-cold 100 mM EDTA for two

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Abbreviations: YAC, yeast artificial chromosome; PFGE, pulsed-field gel electrophoresis; PCR, polymerase chain reaction.

15-min periods. Sample plugs from each reaction were analyzed by pulsed-field gel electrophoresis (PFGE).

Ligation and Size-Fractionation of YAC Constructs. The YAC vector pYACneo (5) was prepared by double digestion with *Eco*RI and *Bam*HI followed by treatment with calf intestine alkaline phosphatase (Boehringer Mannheim). Agarose plugs containing DNA partially digested with *Eco*RI were dialyzed against 5 ml of 10 mM Tris, pH 7.5/25 mM NaCl for two 20-min periods at room temperature. Three plugs were combined with 30 μg of prepared vector and heated to 68°C for 5 min then cooled to 37°C. Then 25 μl of 10× ligation buffer [0.5 M Tris·HCl, pH 7.5/0.1 M $MgCl_2$/0.2 M dithiothreitol/10 mM ATP/bovine serum albumin (500 μg/ml)] and 5 μl of T4 DNA ligase (New England Biolabs) were added and carefully mixed with a pipette tip. The mixture was incubated at 15°C overnight. Ligated DNA was melted at 68°C for 5 min and loaded onto a 1% low melting temperature agarose gel (SeaPlaque GTG; FMC) in 0.5× TBE (1× TBE = 89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.3). Standard electrophoresis was performed at 1.5 V/cm for 8 hr and was followed by PFGE on a contour-clamped homogeneous electric field (CHEF) apparatus at 2.8 V/cm, 20-sec pulse time for 40 hr at 14–15°C. DNA species >200 kb migrated as a single band, which was excised from the gel.

Yeast Spheroplast Preparation. A 100-ml culture of AB 1380 was grown in YPD [1% Bacto yeast extract/2% (wt/vol) Bacto Peptone/2% (wt/vol) glucose] to an OD_{600} of 9–10 (2×10^8 cells per ml). Cells were pelleted and washed first with 20 ml of sterile water and then 20 ml of 1 M sorbitol. Cells were resuspended in 20 ml of SPEM (1 M sorbitol/0.01 M sodium phosphate, pH 7.5/10 mM EDTA, pH 8.0/30 mM 2-mercaptoethanol) and divided into two 10-ml portions. Between 20 μl and 30 μl of 20T Zymolyase (100 mg/ml) was added to each and incubated at 30°C for 20 min. Spheroplasts were gently pelleted, sequentially washed with 10 ml of 1 M sorbitol and 10 ml of STC (1 M sorbitol/10 mM Tris·HCl, pH 7.5/10 mM $CaCl_2$), and resuspended in 1 ml of STC.

YAC Transformation. Ligated DNA that had been dialyzed against 25 mM NaCl was melted at 68°C for 5 min and 30 μl was digested with 3 μl of agarase (5.9 units/μl) for 2 hr at 37°C. After heating the agarase to 68°C for 5 min, 10–20 μl of the DNA was transformed into 100 μl of spheroplasts containing 1× polyamines (0.75 mM spermidine/0.30 mM spermine) and 10 μg of herring sperm DNA, as described (11). Spheroplasts were then plated in double drop agar containing 1 M sorbitol, deficient in adenine, and lacking tryptophan and uracil (12). After 3–5 days of incubation, apparent red colonies were picked and placed on double-drop grid plates. Colonies that grew were then picked and placed in 96-well microtiter dishes containing 200 μl of YPD per well. The microtiter dishes were then incubated at 30°C for 2–3 days and a copy of each dish was made using a 96-well replicating device. All microtiter dishes were frozen at −70°C in 15% (vol/vol) glycerol. Nylon membranes (Micron Separations, Westboro, MA) were then inoculated at four microtiter dishes per filter, cells were grown on restrictive medium for 2–3 days and used to make polymerase chain reaction (PCR) DNA pools or for hybridization filters.

Creation of PCR DNA Pools. Yeast colonies grown on filters were scraped into 10 ml of TE (50 mM Tris·HCl, pH 7.5/50 mM EDTA) and the filters were washed twice with 5 ml of TE. The cells were pelleted, resuspended in 10 ml of SPEM, and incubated at 30°C for 50 min with 50 μl of 20T Zymolyase (100 mg/ml). The spheroplasts were pelleted at 800 rpm in a Beckman JS-5.2 rotor for 5 min and lysed in 8 ml of 10 mM Tris·HCl, pH 7.5/50 mM NaCl/100 mM EDTA/proteinase K (1 mg/ml)/1% SDS at 37°C overnight. The suspension was heated to 70°C for 15 min and cooled, 0.8 ml of 7.5 M ammonium acetate was added, and the suspension was left on ice for 30 min. The floculent debris was removed by centrifugation at 8000 rpm in a Sorvall SS-34 rotor for 10 min. The supernatant was extracted first with 8 ml of PCI [phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol)] and then 8 ml of chloroform. DNA was ethanol-precipitated, resuspended in 500 μl of water, and digested with 5 μl of RNase (10 mg/ml) at 37°C for 30 min. After extraction with PCI and chloroform, 150 μl of 7.5 M ammonium acetate was added, cells were left on ice for 20 min, and the debris was pelleted. Addition of 800 μl of isopropyl alcohol yielded a red-tinged DNA pellet. This color could be removed by reprecipitating the DNA with ethanol and resuspending the pellet in 200 μl of water.

Screening of YAC DNA Pools by PCR. Each YAC DNA pool (1 μl) was screened by the PCR using oligonucleotide primers developed from known sequence within two chromosome 22-specific probes D22S1 and D22S15 as described (13). Oligonucleotide sequences were as follows. D22S1, 5′-ATGTGGAATGGTCGACCTGATGCT-3′ and 5′-ATCTGTTGACCTGGTCCACGCATA-3′; D22S15, 5′-CAACAGCTTCATGGCCACCATAATG-3′ and 5′-CGACCACTAATCTCCTTTTCTGGTT-3′. The PCR conditions were as follows: denaturation for 30 sec at 94°C, annealing for 1 min at 65°C, and extension for 90 sec at 72°C for 35 cycles.

RESULTS

YAC Ligation Products Are Created Using Methods Minimizing Degradation. To maximize the number of clonable genomic DNA fragments in the 200- to 1000-kb size range to be inserted into YACs, steps were taken to ensure that these fragments would not suffer degradation secondary to random shear. All biochemical manipulations were performed on very high molecular weight genomic DNAs created by lysis of intact cells suspended in low melting temperature agarose plugs. The agarose matrix acted as a scaffold preventing shear of the DNA but also acted as a diffusion barrier against the influx of restriction endonuclease. Such a barrier could complicate the creation of large partial *Eco*RI restriction fragments to be used as YAC inserts.

To circumvent this impediment in generating reproducible partial *Eco*RI digestion patterns, the reaction was rate-limited by Mg^{2+}, a more diffusible particle. This method differed from others (14) in that enzyme and Mg^{2+} concentrations and preincubation times were adjusted to allow for better equilibration of the restriction endonuclease mixture. Several reactions each using multiple DNA plugs were done in a bulk mixture. Size-fractionation by PFGE of selected plugs reproducibly yielded the typical genomic partial *Eco*RI titration depicted in Fig. 1*A*. The remaining DNA plugs from the digestion producing the most fragments in the 200- to 600-kb size range were melted with an estimated 20- to 40-fold molar excess of the vector pYACneo and ligated overnight.

To select for YACs containing human sequences >200 kb, ligation products were size-fractionated on low melting temperature agarose gels by sequential standard electrophoresis and PFGE. Under these conditions linear molecules >200 kb migrated as a single band that was clearly resolved from the vector arms alone and the lower molecular weight YAC species (Fig. 1*B*). We found that reducing the voltage and increasing the running time resulted in less contamination with YACs lacking human inserts than had been seen (15). To document that the harvested gel slice contained the desired ligation products, a portion of it was analyzed by PFGE. Few species <200 kb were found. The genomic DNA smear >200 kb closely resembled the original partial *Eco*RI digestion pattern, indicating minimal degradation had resulted.

Yeast Spheroplast Transformation Is Optimized Using Agarase. Before attempting to transform yeast with YAC ligation mixtures, the spheroplast transformation procedure was optimized using undigested circular YAC vector as a test frag-

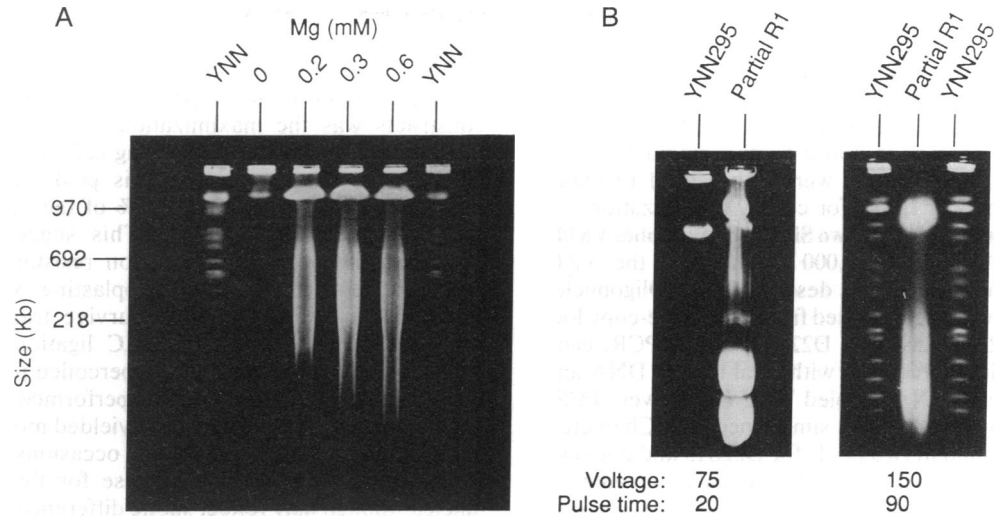

FIG. 1. (*A*) *Eco*RI partial digestion of human DNA by magnesium ion titration. DNA in agarose plugs was dialyzed with restriction enzyme and various concentrations of magnesium prior to incubation at 37°C. The DNA was then subjected to PFGE and the digestion patterns were visualized by ethidium bromide. Increasing magnesium concentration enhanced endonuclease activity resulting in a greater number of lower molecular weight fragments. (*B*) Size-fractionation of ligation products. Size-fractionation of the ligated vector-insert DNA was accomplished by electrophoresis through agarose in a standard horizontal apparatus followed by PFGE. Species >200 kb migrated as a single band under these conditions. The integrity of these products was confirmed when this band was excised from the gel and analyzed by PFGE using parameters that fractionate fragments in the range of 200 kb to 1000 kb.

ment. Zymolyase concentration, cell number, and incubation time were all systematically optimized. Increasing the yeast cell number 5-fold and the Zymolyase concentration 10-fold from published procedures resulted in transformation frequencies of $0.5–2.0 \times 10^7$ colonies per μg, which represented a 5- to 10-fold increase in efficiency (11, 16). Harvesting cells in early or late logarithmic phase for spheroplasting had little influence on subsequent transformation frequencies.

A portion of the YAC ligation gel slice was melted and digested with agarase prior to transformation into yeast spheroplasts. There was considerable variability in the number of YAC colonies from any single transformation by using different portions of the same ligation, indicating that ligated species are not uniformly dispersed throughout the low melting temperature agarose gel slice. Nevertheless, an estimated 10- to 50-fold increase in the number of resultant colonies was obtained by digesting with agarase prior to transformation. Net transformation efficiencies for YAC ligations were 400–2000 colonies per μg.

A Three-Genomic-Equivalent Human Total Genomic YAC Library Was Created. Initial spheroplast transformants were plated in agar on uracil-, tryptophan-, and adenine-deficient plates. Greater than 98% of colonies were red, indicating that these colonies contained the auxotrophic markers from each YAC vector arm and that the *SUP4* gene at the cloning site had been interrupted by a human insert. This was confirmed by PFGE analysis of 80 random colonies. Ethidium bromide staining detected at least one extra chromosome in the majority of putative YAC clones (Fig. 2*A*).

Hybridization of Southern blot transfers to ^{32}P-labeled total human DNA confirmed the presence of human DNA in all but 2 of the 73 colonies analyzed (Fig. 2*B*). Insert sizes ranged from 150 kb to >1000 kb, with an average size of 330 kb (Fig. 2*C*).

In ≈10% of samples more than one YAC was detected. A selection of these transformants were streaked on selective medium. Several single colonies were picked and their chromosomes were analyzed by PFGE. In the majority of cases, the resultant colonies showed only a single YAC, indicating that the original multiple banding pattern was caused by the admixture of different YAC clones. In a minor proportion of clones, the additional bands did not segregate with colony purification. This is consistent with the presence of multiple YACs in the same yeast host.

A total of 38,000 YAC colonies were individually picked and placed in restrictive medium to confirm their phenotype. Viable colonies were then transferred into 96-well microtiter

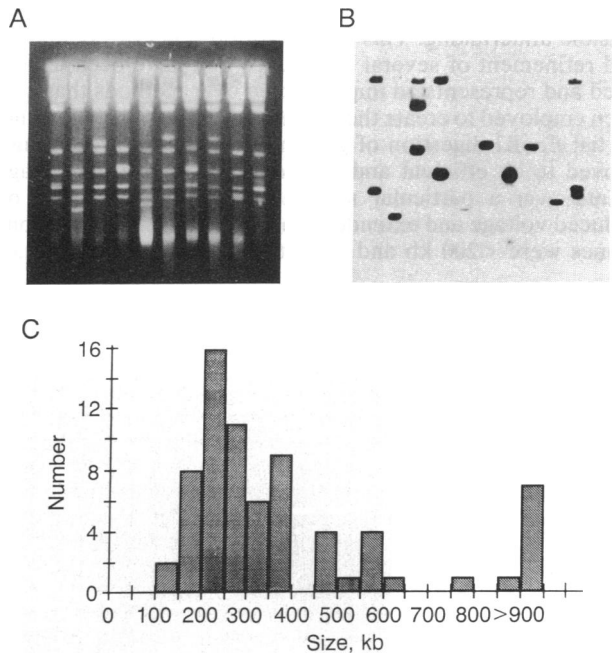

FIG. 2. Analysis of random YAC clones by PFGE. (*A*) Ethidium bromide-stained pulsed-field gel of 10 random YAC clones. At least one extra chromosome is clearly visible in each lane. Size range: 200–650 kb. (*B*) Southern blot of gel in *A* hybridized with a ^{32}P-labeled total human DNA. Each YAC clone specifically hybridized to the extra bands seen by ethidium bromide, indicating the presence of human repetitive sequence. (*C*) Histogram analysis of YAC size. Seventy-three random colonies were analyzed by PFGE, Southern blot transfer, and hybridization to a total human DNA probe. Seven YACs had inserts >900 kb and 2 clones contained no detectable human sequence. Sixty-four YACs were able to be measured accurately and resulted in an average insert size of 330 kb.

dishes containing enriched nonselective medium, grown to saturation, and stored in 15% glycerol. Colonies from each microtiter dish were stamped onto nylon membranes using a 96-prong inoculation device and grown on selective medium. Virtually all colonies grew, indicating that the YAC had not been lost by short passage in nonrestrictive medium. Membranes containing YAC clones were either used to make DNAs for PCR pools or filters for colony hybridization.

YAC Library Screening with Two Single-Copy Probes Yields Positive Clones. A portion (31,000 colonies) of the YAC library was screened by PCR as described (13). Oligonucleotide primer pairs were constructed from two single-copy loci on chromosome 22, D22S1 and D22S15. In the PCR, each pair amplified a single fragment with total human DNA and none with total yeast DNA. Pooled YAC DNAs were PCR-amplified with both primer pairs simultaneously. Characteristic bands were found in two pools for D22S15 and one pool for D22S1 (Fig. 3*A*). Each of these PCR signals segregated to one of five smaller subpools on further PCR screening. Colony hybridization of each subpool localized two of the three clones that were detected by PCR. After colony purification, chromosome DNAs were harvested from these two clones and analyzed by PFGE. Each contained a single additional chromosome that hybridized to total human DNA and to its corresponding single-copy probe (Fig. 3*B*).

DISCUSSION

Specialized YAC libraries have the potential for being the link between genetic and molecular methodologies that is needed if the majority of tumor-specific chromosomal abnormalities are to become accessible to definitive characterization. For this potential to be realized, we have developed a protocol that renders the construction of such libraries a feasible undertaking. This protocol represents the synthesis and refinement of several techniques that have been developed and represents an improvement over methods that had been employed to create the first YAC libraries. Isolation and partial *Eco*RI digestion of genomic DNAs in agarose plugs proved to be efficient and reproducible in generating fragments over a particular size range. Under conditions of reduced voltage and extended running time <15% of random clones were <200 kb and almost no clones lacking human inserts were evident. As a result our library of 38,000 YACs had an average genomic insert size of 330 kb. This compares well with the other total human YAC libraries (4–6).

Equally important to the preparation of size-selected YAC constructs was the maximization of yeast transformation efficiencies obtained by increasing cell number and lyticant (Zymolyase) concentrations. This peak in transformation efficiency occurred when 75–85% of the cells had become spheroplasts (spheroplasting). This suggests that under-spheroplasting yields a population resistant to exogenous DNA uptake whereas over-spheroplasting exerts such a toxic effect that many cells do not survive transformation and reconstitution. Results with YAC ligation mixtures were more variable than those with supercoiled plasmid and were very ligation specific. Ligations performed under the same conditions using similar reagents yielded mixtures that either transformed faithfully on multiple occasions or did not transform at all. The underlying cause for this observation is unclear though may reflect subtle differences in vector-insert mixing prior to ligation. Digestion of ligation mixtures with agarase prior to transformation did reliably increase efficiencies yet transformation rates of supercoiled plasmid were not affected by the presence of agarose. This suggests that transformation inhibition by agarose may be dependent on molecular weight of DNA constructs.

Judging by the size and complexity of our library, the probability that it would contain at least one clone to any single-copy probe was >95% (17). This estimate was confirmed by screening the library with two separate single-copy probes, D22S1 and D22S15. Preliminary mapping of these clones demonstrated the expected linear DNA structures capped by yeast centromeres and telomeres (data not shown). The sizes of these clones varied from 370 to 550 kb, which was well within the expected range based on our analysis of random YAC clones from our library.

Screening with these two probes demonstrates our library's integrity and its utility in approaching the t(11;22) translocation. Genetic restriction fragment length polymorphism analysis had demonstrated that the rearrangement breakpoint fell between two chromosome 22 probes, D22S1 and D22S15, and that the recombination distance between these two loci was zero (18). Though these probes were unresolvable genetically, mapping by PFGE demonstrated

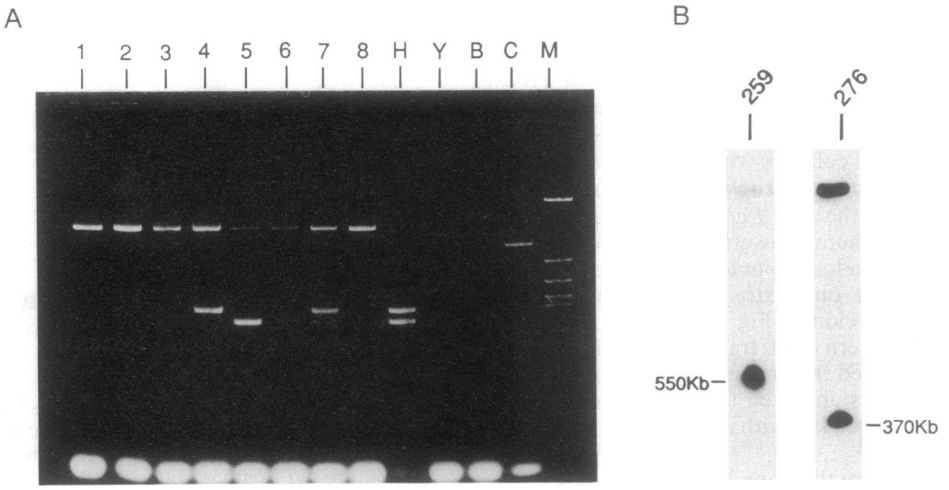

FIG. 3. (*A*) Primary screening of YAC DNA pools containing 1920 colonies. Oligonucleotide pairs derived from D22S1 and D22S15 sequences were simultaneously used as primers in PCRs. A fragment characteristic for D22S15 was present in YAC pools 4 and 7 and a fragment for D22S1 was in pool 5. Lanes: 1–8, eight multifilter YAC pools; H, human total genomic DNA; Y, yeast strain AB 1380; B, buffer blank; C, internal positive control; M, *Hin*fI digest of pBR322. (*B*) Southern blot of PFGE-fractionated YAC clones isolated from D22S1 and D22S15 screens. Chromosome DNAs from clones identified from PCR and colony hybridization screening were harvested and fractionated by PFGE. Gels were blotted onto nitrocellulose and each was hybridized to one single-copy probe. A single 550-kb band is seen in clone 259 when hybridized to D22S15 and a 370-kb fragment is identified in clone 276 when probed with D22S1.

that these two loci were physically distinct and that neither was able to detect the t(11;22) rearrangement (data not shown). A single screening of our YAC library has yielded almost one million base pairs of genomic DNA surrounding these two probes. Employing a chromosomal walking strategy with YAC clones that are hundreds of kb long will enable physical linking of D22S1 and D22S15 and isolation of the t(11;22) translocation breakpoint.

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