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Alu-primed polymerase chain reaction for regional assignment of 110 yeast artificial chromosome clones from the human X chromosome: Identification of clones associated with a disease locus

(DNA fingerprinting/DNA sequencing/Lowe syndrome/somatic cell hybrids)

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ABSTRACT **Over 400 yeast artificial chromosome (YAC) clones were isolated from the human X chromosome, and 110 of these were assigned to regions defined by chromosome translocation and deletion breakpoints. Polymerase chain reaction using *Alu* primers was applied to YAC clones in order to generate probes, to identify overlapping clones, and to derive "fingerprints" and sequence data directly from total yeast DNA. Several clones were identified in regions of medical interest. One set of three overlapping clones was found to cross a chromosomal translocation implicated in Lowe syndrome. The regional assignment of groups of YAC clones provides initiation points for further attempts to develop large cloned contiguous sequences, as well as material for investigation of regions involved in genetic diseases.**

Efforts to characterize the human genome have been greatly assisted by the introduction of DNA cloning vectors capable of retaining large inserts. The development of the yeast artificial chromosome (YAC) (1) has provided a mechanism for the isolation and propagation of human DNA fragments ranging in length from 100 to 1000 kilobases (kb). This methodology now makes feasible the isolation of entire human chromosomal regions in overlapping sets of cloned DNAs. This "genome" approach to chromosome analysis will accelerate the identification of genes important in developmental and disease processes, since a major impediment to such efforts is the difficulty of obtaining regionally localized genomic DNAs.

YAC libraries have been constructed from human cells (2, 3), sorted human chromosomes (4), and somatic cell hybrids (5, 6), providing starting material for large-scale isolation of overlapping cloned regions of the human genome. A drawback of the YAC system is the difficulty of isolating insert sequences from host yeast DNA. The development of a polymerase chain reaction (PCR; ref. 7)-based method for the isolation of human DNA from complex mixtures was reported previously (8) that alleviates this problem for human YACs. This method takes advantage of the presence of the ubiquitous *Alu* repetitive element to prime DNA amplification. In this study, we have employed probes derived from YAC clones prepared from the human X chromosome to sublocalize 110 clones, using somatic cell hybrid mapping panels composed of chromosome breakpoints associated with translocations and deletions. Regionally assigned clones provide starting material for the development of large cloned "contigs," and we describe the use of *Alu* PCR products from YAC clones to identify and characterize overlapping clones and to generate sequence data to define sequence-tagged sites (9) directly from YAC inserts.

MATERIALS AND METHODS

Library Construction and Clone Isolation. Two YAC libraries were constructed in the pYAC4 vector by the method of Burke *et al.* (1). The source of DNA was the hybrid cell line X3000-11.1 (10), which retains the human X chromosome region from Xq24 to Xqter as its only human DNA in a hamster cell, or 4.12 (11), which retains an intact X chromosome as its only human material in the same background. The X chromosome in each hybrid cell line was derived from a patient (GM4025) with fragile X syndrome and demonstrates the fragile site at Xq27.3 under appropriate culture conditions (11). DNA was prepared by partial *Eco*RI digestion, and the vector was digested with *Eco*RI and *Bam*HI and dephosphorylated. YACs were introduced into spheroplasts prepared from the strain YPH274 (4) by the method of Burgers and Percival (12) and selected in medium lacking uracil under agar. Transformation efficiencies ranged from 2000 to 10,000 colonies per microgram of insert DNA. Colonies were picked from the top agar reconstitution medium in duplicate onto rich medium plates, and nylon filters were lifted from one of the two plates. Colonies were further processed by extraction with zymolyase and denaturation with NaOH (13). Clones retaining human inserts were identified by hybridization to radiolabeled total human DNA (14). Agarose plugs (15) were prepared from each clone, and each was sized by field-inversion gel electrophoresis (16) and Southern analysis using total human DNA as probe (14). Total yeast DNA for PCR and hybridization analyses was extracted from a 50-ml culture of YAC-containing cells by standard methods.

***Alu* PCR and *Alu*–Vector PCR.** Amplification of fragments from the YAC inserts was carried out using *Alu* primers (559 and 278) and conditions described previously (primer 559 is identical to TC-65; ref. 8). For general amplification of insert, both primers were used together. For fingerprinting, each was used separately as well. Between 100 and 500 ng of total DNA from the YAC-containing yeast was used. Primers directed to the pYAC4 sequences adjacent to the insert were designed for *Alu*–vector amplification: YAC4L, 5′-CGGAATTCGCCAAGTTGGTTTAAGGCGCAAGAC-3′; YAC4R, 5′-GGAAGCTTGGCGAGTCGAACGCCCGATCTCAAG-3′. These were used separately from one another but in conjunction with either 559 or 278 or both. The single-copy PCR of right end sequences from RS46 used primers 1625 and

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Abbreviation: YAC, yeast artificial chromosome.

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1626 (5′-CTTGCCAACCGTTCAGCCAC-3′ and 5′-ATTTCCTGGAGCACAGACTG-3′, respectively) with PCR conditions as described (8) except for the annealing temperature (65°C) and the extension time (2 min).

Somatic Cell Hybrid Mapping by Southern Hybridization. The somatic cell hybrid lines used for assignment of clones to Xq24–qter have been described (8). In brief, these were X3000-11.1, Xq24–qter; 94-3, Xq25–qter; Lowe-3 (mouse) or F649-5 (hamster), Xq25–qter; RJK 734, Xq26–qter; 8121, Xpter–q27.1; 2384, Xpter–27.2; and Micro 28g, Xpter–q27.3. Additional cell lines used for Xpter–q24 assignments were HY94BT1, Xp22.3–qter (17); AAH, Xp21.2–qter (18); 88H5, p11.21–qter (18); and Ben3B, q21.3–qter (18). Gel electrophoresis and Southern transfer and hybridization were carried out as described (8). PCR products were excised from low-melt agarose gels and labeled by the random priming method (19). Suppression of hybridization by *Alu* and other repetitive elements present in the probes was achieved by prehybridization of Southern filters with sheared, denatured human placental DNA (200–500 μg/ml) for 4–24 hr. In addition, after denaturation by boiling, most probes were pre-annealed to human placental DNA (200 μg/ml) for 20 min at 65°C in hybridization solution [1 M NaCl/1% SDS/10% dextran sulfate (Oncor, Gaithersburg, MD)].

Direct Sequence of *Alu*–Vector PCR Products. Amplified *Alu*–vector fragments were isolated by gel electrophoresis and then subjected to 28 rounds of single-sided PCR utilizing the *Alu* primer (278 for both ends of RS46). Sequence was determined using the ^{32}P-labeled YAC4L or YAC4R vector primer in dideoxy DNA sequencing reactions (20, 21). In general, the sequence analyses were straightforward, provided that the initial PCR product was homogeneous when analyzed by agarose gel electrophoresis. Additional details regarding direct sequence analysis of PCR products can be found in ref. 21.

FIG. 1. Cartoon of *Alu*–vector PCR applied to clones in the YAC vector pYAC4. *Alu–Alu* and *Alu*–vector PCR are shown schematically on a cartoon of a YAC clone containing a human insert.

RESULTS

YAC Library Construction and Clone Characterization. Over 400 human X chromosome YAC clones were isolated, 200 from the intact X (4.12 library) and 220 from the Xq24–qter region (X3000 library). Human positive clones were identified at 0.4% in the X3000 library and ≈2% in the 4.12 library, as expected. Clones were characterized by several methods. Initial analysis involved the preparation of yeast chromosomes embedded in agarose plugs to define the size of the artificial chromosomes. The average size of YAC inserts in the two libraries is 130 kb (data not shown). The clones range in size from 80 to 400 kb; however, the majority of the clones lie in the 120- to 150-kb range. Hybridization of a Southern transfer (22) of the field-inversion gel to a total human DNA probe provided a second check of the validity of the original colony hybridization and was carried out for all clones. Hybridization of Southern filters with total hamster DNA provided a check for clones with hamster sequences either as additional artificial chromosomes or coligated inserts. This check was performed for 25% of the clones. Clones with two artificial chromosomes were found rarely (≈2%), and no clones with concatemeric human–hamster inserts have been identified. Total DNA was prepared from all clones and used for PCR.

***Alu* and *Alu*–Vector PCR.** The ability to isolate the YAC insert (or some portion of it) is key to analysis of the clones. By using two *Alu* primers (559 and 278) allowing amplification to proceed in both the 5′ and the 3′ direction from *Alu* repeats, inter-*Alu* fragments were generated from about 70% of YAC

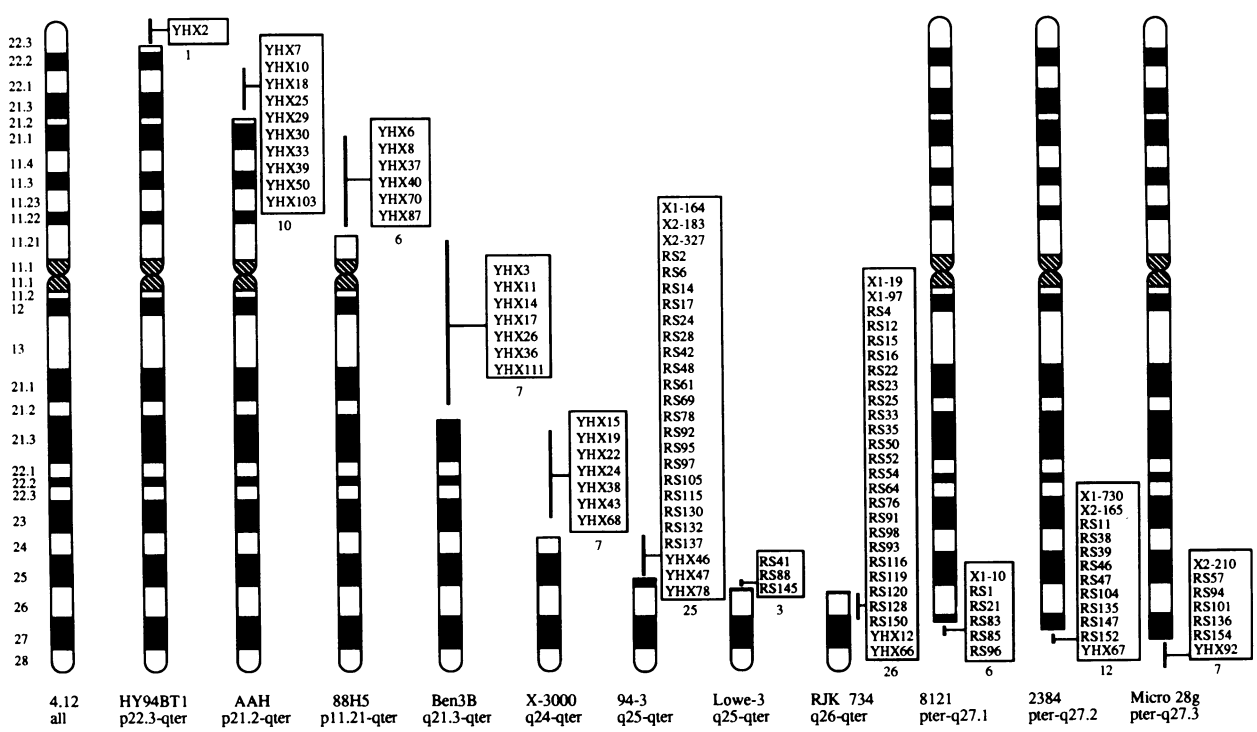

FIG. 2. Summary of mapping information from YAC clones. All YAC clones assigned to date are indicated in the regions between breakpoints in the mapping panels utilized for the two libraries. Clones from the 4.12 library that were found to be located in the Xq24–qter region were further localized using the X3000 library mapping panel. Seventy-one clones from the X3000 library and 29 clones from the 4.12 library have been localized. The names of the hybrid cell lines used for each breakpoint and the regions retained are given below each idiogram. Lowe-3 contains the same translocation chromosome as in F649-5, but in a mouse cell background.

clones. With the addition of primers directed to the pYAC4 sequences adjacent to the insert, products were observed from >90% of clones (Fig. 1). Overall, 60% of clones showed both end fragments amplified.

Regional Assignment of Anonymous YAC Clones. We utilized somatic cell hybrid mapping panels for X chromosome regions to assign clones, using *Alu* PCR products as probes. The mapping panel used and the positions of the 110 clones assigned are shown in Fig. 2. The PCR-product probes contain repetitive DNA due to the *Alu* sequences at their ends; however, with sufficient quenching with human placental DNA (23), these probes can be used readily. With this protocol, only 5–10% of products were refractory to use as probes. These were generally small fragments, and there was most likely little or no single copy sequence between the *Alu* primers. Cross-hybridization of *Alu* PCR probes with rodent sequences present in the hybrid cell lines was observed with <5% of probes under the stringent hybridization and washing conditions used.

One hundred ten clones were assigned, 38 from the 4.12 library, and 72 from the X3000-11 library. Seven of the 4.12 clones fell into the X3000 region and were localized further, giving 79 anonymous clones assigned to intervals in this 40- to 50-megabase (Mb) region of the X chromosome. As the average insert size for YACs in each library is 130 kb, assigned YACs in Xq24–qter represent ≈10 Mb, or 20–25% of the region. Assigned YACs represent ≈10% of the chromosome, although there is considerable bias for Xq24–qter.

Identification of Disease-Associated Clones: Oculocerebrorenal Syndrome of Lowe. One of the somatic cell hybrids in the Xq24–qter mapping panel (F649-5 in a hamster background, Lowe-3 in mouse) contains a translocation chromosome isolated from a female patient with Lowe syndrome (24). Lowe syndrome has been mapped by linkage to markers

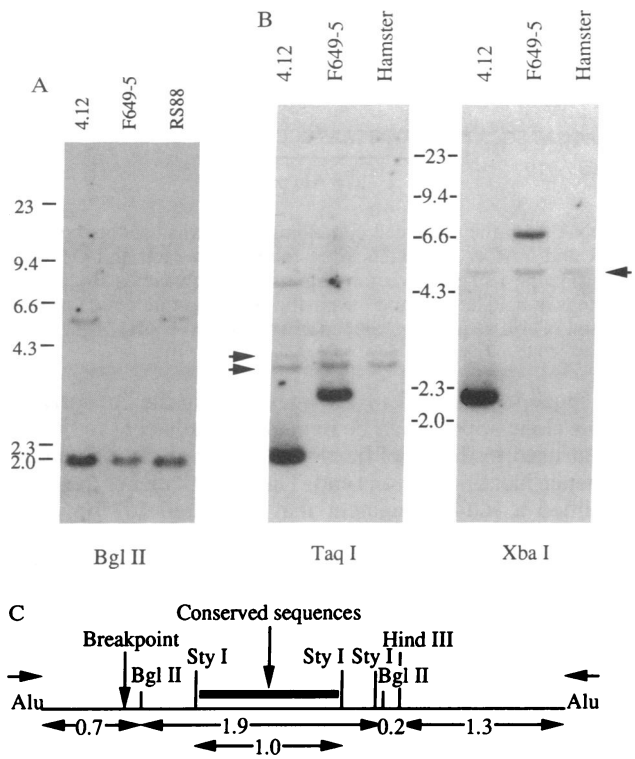

FIG. 3. (*A*) Southern hybridization of an *Alu* PCR-product probe from YAC RS88 detecting the Lowe translocation breakpoint. Ten micrograms of *Bgl* II-digested DNA from 4.12 (X-only hybrid) and F649-5 (Xq25–qter) was electrophoresed with 100 ng of Bgl II-digested DNA from the yeast cell containing RS88, transferred, and hybridized with a probe composed of 2800 base pairs (bp) of the 94-1.4000 *Alu* PCR product from YAC RS88 (between one *Alu* and an internal *Hin*dIII site, see *C*). The 5-kb *Bgl* II band found in the normal X chromosome and the YAC DNAs was absent from the translocation chromosome, while the 1.9-kb band was retained. Final wash was in 0.1 × standard saline citrate at 65°C. Exposure was overnight. (*B*) Southern hybridization of an *Alu* PCR-product probe from YAC RS88 demonstrating altered-size restriction fragments and cross-species homology. Ten micrograms of *Taq* I- or *Xba* I-digested DNA from 4.12, F649-5, and hamster was electrophoresed, transferred, and hybridized with a 1-kb *Sty* I fragment from the 94-1.4000 *Alu* PCR product from YAC RS88. Altered mobilities for the hybridizing fragments in the F649-5 hybrid containing the translocation X;3 chromosome were observed for these and several other enzymes tested. Cross-reacting fragments in hamster DNA are indicated with arrows. Cross-reactivity with this probe was also observed in mouse DNA at these reduced stringencies of washing (0.5 × standard saline citrate, 65°C). Exposure was 4 days. (*C*) Map of *Alu* PCR product 94-1.4000 derived from RS88. A 4-kb *Alu* PCR product derived from 559-primed amplification of the RS88 YAC is shown along with *Bgl* II and *Hin*dIII sites. From hybridization analyses as shown in *A* and *B*, the position of the translocation breakpoint on the X chromosome retained in the F649-5 hybrid was determined. A region defined by a 1-kb *Sty* I fragment exhibited cross-species hybridization to hamster and mouse DNAs and is indicated. Additional *Sty* I sites present outside of the 1.9-kb *Bgl* II fragment are not shown. The orientations of the two *Alu* repeats allowing amplification are shown. Sizes are indicated in kilobases.

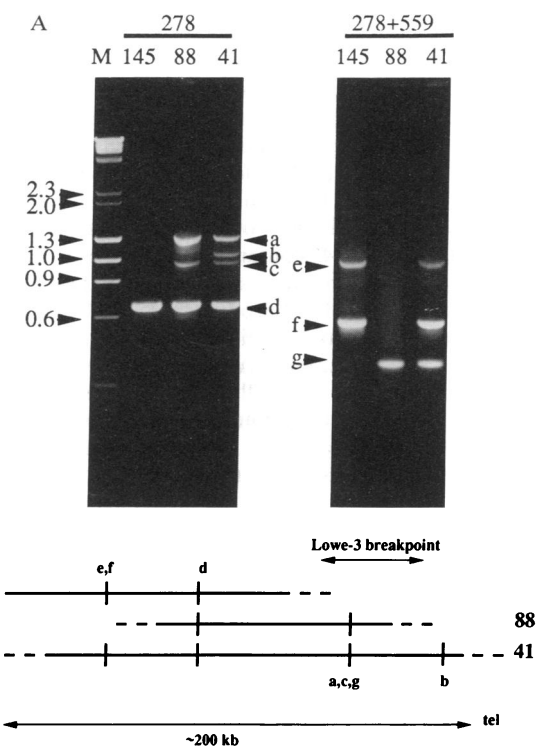

FIG. 4. (*A*) *Alu* PCR fingerprint analysis of Lowe syndrome YACs. The three YAC clones in the region defined by the translocation breakpoint found in F649-5 were amplified with *Alu* primers 278 and 278 plus 559, and one-sixth of the reaction product was electrophoresed through a 1.1% agarose gel. Prominent bands are labeled a–g and were used to construct a map of the clones as shown in *B*. Size markers (lane M) were *Hin*dIII-digested λ phage DNA mixed with *Hae* III-digested φX174 phage DNA, and selected band sizes are shown in kilobases. (*B*) Map of Lowe syndrome YACs and *Alu* PCR fragments. YAC clones RS145, RS88, and RS41 are oriented relative to one another by using the information from the *Alu* PCR fingerprinting shown in *A*. Four regions are defined by the coamplification of templates present in overlapping clones. Distance information is not implied. The region containing the breakpoint in the F649-5 hybrid is defined by products a, c, and g. The position of fragment b is determined by knowledge that the proximal end of RS145 is not contained within RS41 (data not shown). cen, Centromere; tel, telomere.

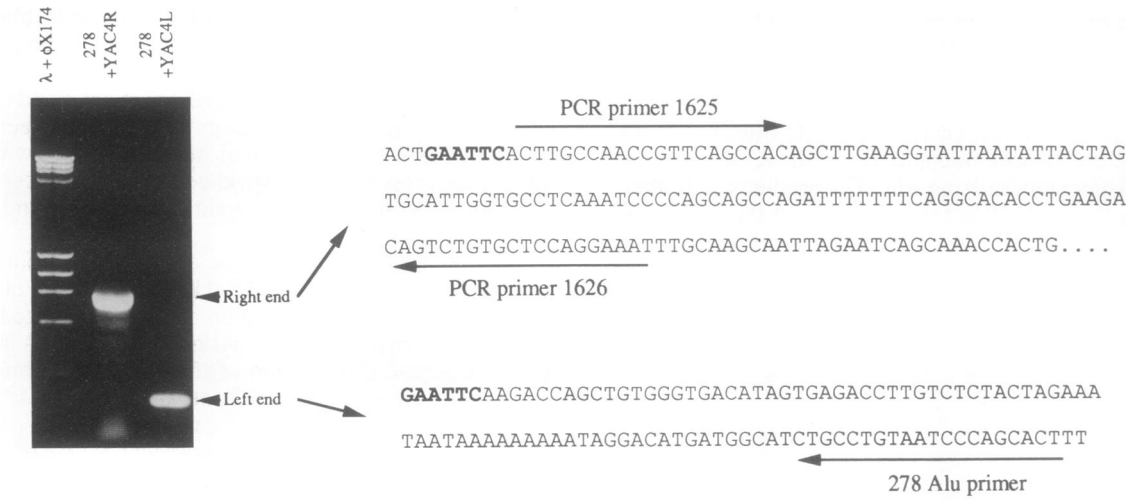

FIG. 5. Direct sequence analysis of *Alu*–vector PCR product from RS46. DNA from the yeast cell containing RS46 was used as template for amplification with the *Alu* primer 278 and pYAC4 vector primers YAC4R and YAC4L. Products were found from each end and their sequences were determined by direct DNA sequencing of asymmetric PCR products, using the vector primers in each case as the labeled sequencing primer. The sequences determined are shown. The *Eco*RI cloning site is in bold letters, and the positions of oligonucleotide primers 1625, 1626, and 278 are indicated. The left end sequence is completely composed of *Alu* sequences. Size markers (lane λ + φX174) are as in Fig. 4*A*.

in Xq25 (24) and is characterized by cataracts, mental retardation, and renal tubule dysfunction. This X;3 translocation (q25;q27) chromosome shows a breakpoint in Xq25 that presumably interrupts (or alters the expression of) the gene defective in Lowe syndrome. YAC RS88 exhibited an unusual pattern of hybridization in F649-5 DNA. The *Alu* PCR-product probe from this clone identified two *Bgl* II fragments in all cell lines of the mapping panel with the exception of RJK 734 (containing Xq26–qter), and only one of the two *Bgl* II fragments was observed in the F649-5 hybrid (Fig. 3*A*). A 4-kb *Alu* PCR product from RS88 (94-1.4000) that contains the breakpoint region was identified. This probe recognized altered-size restriction fragments in the translocation chromosome with all seven enzyme digests tested (Fig. 3*B*), and the breakpoint was localized to sequences immediately proximal to a *Bgl* II restriction site. Cross-hybridization to hamster and mouse sequences was observed with the 94-1.4000 probe, and the sequences responsible were narrowed to a 1-kb *Sty* I fragment (Fig. 3*C*). This cross-species homology may represent the presence of coding sequences for the gene involved in Lowe syndrome.

Fingerprinting by *Alu* PCR. Three overlapping YAC clones were obtained from the X3000 library in the Lowe region by using *Alu* PCR probes derived from the original RS88 clone to hybridize DNA from all the clones in the library. By comparison of *Alu* PCR products derived from the three overlapping clones (Fig. 4*A*), it was possible to construct a map of the clones, allowing the determination of how the clones are oriented relative to one another, and which clones extend furthest in each direction (Fig. 4*B*). Faint bands were also observed; while these were frequently produced, they were not used for fingerprint analyses because they were too variable. Crossing the Lowe syndrome breakpoint also allowed the determination of the proximal/distal orientation of the contig.

Direct Sequence Analysis of YAC Inserts. Sequence derived from the ends of YAC clones is valuable, as it defines a sequence-tagged site (9) for the locus and allows PCR strategies for detecting overlapping clones or mapping to be employed (25). We used the PCR products from *Alu*–vector amplification for direct sequence analysis of YAC inserts. Fig. 5 shows the results of such an analysis on both ends of RS46, a clone from an interval near the fragile X locus. The "left" end of this clone amplified a fragment of 120 bp, which was sequenced. The *Eco*RI site involved in the construction of this clone appeared to be immediately adjacent to the *Alu* repeat used in the amplification. There was no single-copy sequence identified on this end. The "right" end of this clone amplified a 700-bp fragment that generated 157 bp of sequence. Oligonucleotides directed to the sequence were synthesized and used in PCRs (data not shown). The reactions indicate that the sequence is single-copy, show that a product of appropriate length is amplified from the X chromosome, and confirm the map position found by hybridization of PCR products to Southern filters of the panel.

DISCUSSION

Physical characterization of the DNA sequences present in individual human chromosomes will require the isolation of overlapping cloned fragments representing the chromosome. This "genome"-oriented approach provides the materials for study of genes and regions of interest, especially those involved in genetic disorders of unknown etiology. The YAC system will allow this to be accomplished more rapidly due to the large insert sizes capable of being isolated. Several methods have been described for the isolation of insert sequences from artificial chromosomes. These include end rescue (1), subcloning and screening (26), *in vivo* circularization and plasmid preparation (27), the "vectorette" method of Riley *et al.* (28), and inverse PCR (29). We developed a simple method using oligonucleotide primers directed to the ubiquitous *Alu* repeat element (8) that provides insert sequences for use as probes for clone characterization and identification of overlapping clones using crude DNA preparations. The method is rapid and simple and uses small quantities (<1 μg) of total DNA from the yeast cell and can be applied to crude cell lysates as well. This technique is limited to regions surrounded by *Alu* repeats or between *Alu* sequence and the vector sequences, yet only a small percentage of YAC clones in this study were incapable of generating a product.

The fingerprint analysis shown here, while useful for clones known to overlap, is unlikely to be of general use for pairwise comparison of YAC clones originating from complex sources (e.g., total human libraries), since the number of fragments generated is insufficient to generate the data required for confidence in the overlaps predicted. With the

generation of additional fragments via PCR, however, this may represent a feasible approach. Isolation of each end of a YAC insert by *Alu*–vector PCR provides the most sensitive means of determining the presence of coligated molecules, as well as a rapid means of probe preparation for "chromosome walking."

Sequence analysis by direct methods on YAC inserts allows the rapid definition of sequence-tagged sites and provides the opportunity to utilize PCR-based methods for refining map positions and isolating additional clones. Sequence from the ends of YACs is most desirable for the potential identification of overlapping clones; however, sequence can also be determined in this manner from *Alu* PCR products derived from internal sequences. All that is required is that the product to be directly sequenced be generated with different primers at each end, allowing the use of single-sided PCR (20) to generate single-stranded DNA.

The human X chromosome has to date been implicated in 200–300 single-gene disorders (30), which is a larger number than associated with autosomes due to the haploid nature of its inheritance in males. Through the assignment of 110 randomly isolated YAC clones by *Alu* PCR and Southern hybridization, we have identified YACs in close proximity to several disease loci. In the example of the Lowe syndrome region, three YACs defining a region of ≈200 kb span the X chromosome breakpoint in a female with the disease associated with a balanced X;3 translocation, and it is likely that at least part of the defective gene is contained in these clones. Additionally, 12 clones in the region close to the fragile X mutation in Xq27.3 (31) have been identified (Fig. 2), and one of these, RS46 has been further localized to a region ≈150 kb proximal to the fragile site (32).

The use of patient materials for the assignment of anonymous clones in the manner described here allows clones to be assigned to intervals and tested for location at or near disease loci simultaneously. Other methods of regional localization do not provide this potential. The YAC clones presented will likely contribute to the isolation and characterization of several disease loci. There is little doubt that the technology of YAC cloning and PCR characterization has accelerated gene mapping efforts 20- to 50-fold. Simple, direct methods to identify genes in the cloned regions will provide a similar increase in the rate of disease-gene analysis.

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