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Use of yeast artificial chromosome clones for mapping and walking within human chromosome segment 18q21.3

(plasminogen activator inhibitor type 2/polymerase chain reaction/DNA cloning/human genome)

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ABSTRACT **Well-characterized large genomic clones obtained from yeast artificial chromosome (YAC) libraries provide the framework to localize genes and approach genetic disease. We developed universally applicable approaches to establish authenticity, localize and orient internal genes, map restriction sites, and rescue the distal ends of large human genomic DNA inserts. We selected human chromosome segment 18q21.3 as a model system. Molecular cloning of this segment was initiated by characterizing three plasminogen activator inhibitor type 2 (PAI-2) clones [290, 180, and 60 kilobases (kb)] isolated from a YAC library. Comparison of YAC and bacteriophage λ genomic DNA clones confirmed the fidelity of the PAI-2 locus. Detailed rare cutting restriction maps were generated by ramped contour-clamped homogeneous electric field electrophoresis. The PAI-2 locus was located and oriented within the YACs, which span a distance 70 kb 5′ to 220 kb 3′ of PAI-2. Moreover, both left and right ends of the YAC genomic DNA inserts were rescued by amplifying circularized cloning sites with an inverted form of the polymerase chain reaction. These unique terminal genomic DNA fragments were used to rescreen the YAC library and isolate overlapping clones that extend the map. These approaches will enable neighboring loci to be definitively linked and establish the feasibility of using YAC technology to clone and map chromosomal segments.**

Genes located on human chromosome segment 18q21.3 have been implicated in the pathogenesis of several diseases. The most common form of human lymphoma, follicular B-cell lymphoma, commonly possesses a specific interchromosomal translocation, the t(14;18)(q32;q21) (1). This translocation juxtaposes the *Bcl-2* protooncogene from 18q21.3 next to the immunoglobulin locus from 14q32 resulting in a deregulated *Bcl-2*–immunoglobulin fusion gene (2–4). The 18q$^-$ syndrome, a congenital abnormality characterized by craniofacial anomalies, mental retardation, and humoral immunodeficiency is the most common nonlethal chromosomal deletion syndrome (5). The deleted region common to these patients is 18q21.3 (6, 7). In addition, allelic deletions of 18q within colorectal carcinoma cells suggest that a tumor suppressor gene maps within or telomeric to 18q21.3 (8). A complete physical map and overlapping clones of this estimated ≥4-megabase region would provide the framework for better understanding of the molecular basis of these disorders.

We initiated the molecular dissection of this segment by using pulsed-field gel electrophoresis (PFGE) to construct a rare cutting restriction endonuclease (RE) map of five loci that map to 18q21.3 [*Bcl-2* (2, 4), plasminogen activator inhibitor type 2 (PAI-2) (9), OLVIIE10 (10), c-*yes-1* (11), and gastrin-releasing peptide (12)]. This map placed *Bcl-2* exon III centromeric to *Bcl-2* exon II and identified a 350-kilobase (kb) intron separating these exons (4). However, no certain linkage of the other loci could be established confidently by this approach. Long-range restriction maps constructed from digests of high molecular weight DNA in genomic cell plugs are frequently incomplete and potentially in error because of the technical problems of rare cutting REs (13) and the limited number of available probes.

These difficulties could be overcome by the isolation of large overlapping molecular clones of this segment. Routine technologies of bacteriophage λ or cosmid-based DNA cloning show marked limitations when applied to large expanses of the human genome (14). Yeast artificial chromosome (YAC) libraries of human and *Caenorhabditis elegans* genomes have permitted the isolation of large regions of DNA (15, 16). However, the usefulness of YAC isolates in the analysis of complex genomes depended on the development of routine approaches to assess their fidelity in representing genomic structure, locate and orient internal genes, map restriction sites, and rescue their distal ends. We developed methodologies that accomplished these goals and enabled the extensive characterization of three YAC isolates possessing the PAI-2 locus.

MATERIALS AND METHODS

DNA Analysis by PFGE. High molecular weight yeast DNA from YAC clones or human genomic DNA from the lymphoblastoid cell line CGM-1 (established from the same source used to construct the library) was prepared in low-melting point agarose plugs as described (17, 18). After digestion with 100 units (≈10 units per μg of DNA) of the appropriate RE and buffer, restriction fragments were separated in a 1% agarose gel using a contour-clamped homogeneous electric field (CHEF) apparatus (17). Electrophoresis conditions included a 6 V/cm (160 V) field strength, 0.5× TBE buffer (19) at 12°C, and a 24- to 30-hr running time. An 80-sec switch interval was used to separate fragments ranging from 50 to 1500 kb. Resolution in the region between 20 and 300 kb was enhanced by using a ramped switch interval that started at 25 sec and ended at 50 sec. Restriction fragments were transferred to reinforced nitrocellulose (Nitroplus 2000, 0.45 μm; Micron Separations, Westboro, MA) by routine Southern blotting techniques (19, 20). Filters were baked (80°C for 2 hr) and then hybridized to a random-primed ^{32}P-labeled (21) probe in a hybridization mixture containing formamide and

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Abbreviations: CHEF, contour-clamped homogeneous electric field; PAI-2, plasminogen activator inhibitor type 2; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RE, restriction endonuclease; YAC, yeast artificial chromosome.

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dextran sulfate at 42°C. Blots were washed in 0.2× SSC/0.1% SDS at 50°C (19). Autoradiograms were developed between 1 and 7 days. Filters were stripped of a probe by incubation in 0.1% SDS at 80°C. Fragment sizes were determined by comparison to concatemers of λ phage DNA and the electrophoretic karyotype of *Saccharomyces cerevisiae* yeast strains AB972 and AB1380 (22).

DNA Analysis by Conventional Electrophoresis. Either 0.1 μg of total yeast DNA or 15 μg of total human genomic DNA was digested with either *Eco*RI or *Bam*HI. The difference in amount of yeast to human DNA reflects the lower (1/200) complexity (C value) of the yeast genome. Fragments were separated at 20 V on a 0.9% agarose gel, in 2× TAE (19) at 12°C for 48 hr.

Genomic End Rescue Polymerase Chain Reaction (PCR). YACs were separated from the natural yeast chromosomes of *S. cerevisiae* strain AB1380 by CHEF electrophoresis in a 1% low-melting point agarose gel (BRL). The desired band was excised and dialyzed overnight in 5.0 ml of TE (pH 7.4) (19). The agarose plug was placed in a microcentrifuge tube and melted by incubation at 65°C for 5 min. A 40-μl aliquot was digested with either *Hae* III, *Eco*RV, or *Taq* I for left (L) arm rescue or *Acc* I or *Hin*cII for right (R) arm rescue. These enzymes cut at sites several hundred base pairs (bp) away from the *Eco*RI cloning site within the vector arm and at unknown locations within the insert. After a 2-hr incubation, the enzymes were heat inactivated at 65°C for 30 min. Monomer circles were formed by an overnight incubation at 14°C under dilute ligation conditions [10 μl of restriction digest, ligation buffer, and 400 units of T4 ligase (New England Biolabs) in a total vol of 50 μl]. A set of sense and antisense universal primers in opposite orientation were synthesized for both the L and the R arms of the vector. After circularization, the reoriented primers permitted amplification of the intervening genomic insert. PCR was performed in a DNA thermal cycler (Perkin–Elmer/Cetus) with 1 set of primers, 3 units of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Cetus), 10 μl of ligation mixture, buffer, and nucleotides (as recommended by the supplier) in a total vol of 100 μl. Thirty to 40 cycles of denaturation (94°C for 2 min), annealing (61°C for 3 min), and extension (72°C for 3–5 min) were performed.

RESULTS

PAI-2 Genomic Map and Fidelity of YAC Isolates. The characterization of large genomic YAC clones would be facilitated by any prior data concerning the organization of internal loci. To assess the validity and extent of the YAC isolates, the restriction map surrounding the PAI-2 locus (9) was expanded. Routine 6-bp recognition RE sites and rare cutting RE sites were placed within ≈20 kb of bacteriophage λ genomic DNA clones carrying the PAI-2 locus (Fig. 1*a*). *Eco*RI, *Bam*HI, *Nae* I, and *Sma* I sites proved particularly useful. In addition, the sizes of rare cutting RE fragments containing PAI-2 were determined within genomic cell plugs by CHEF analysis. This indicated that the *Nae* I and *Sma* I rare cutting sites located within the phage clones were not detectable within digests of genomic cell plugs (data not shown). Mammalian cells frequently methylate such CpG-containing restriction sites, thereby inhibiting their digestion (13). This is representative of the limitations of mapping rare cutting RE sites within genomic cell plugs.

Three clones were isolated by colony hybridization to a PAI-2 cDNA probe from 13,000 clones of a YAC library prepared from a size-selected *Eco*RI partial digest of human genomic DNA (24). Total yeast genomic DNA from A27D8 (290 kb), A24E4 (180 kb), and C3E8 (60 kb) was digested with *Eco*RI and *Bam*HI and compared to CGM-1, a human cell line established from the same source that was used to construct the library (Fig. 1*b*). The two larger YAC clones contained the four *Eco*RI and the three *Bam*HI fragments that span the PAI-2 locus (Fig. 1*a*). In contrast, clone C3E8 possessed only the 5′ 9.1- and 5.1-kb *Eco*RI and the 5′ 11.4-kb *Bam*HI fragments. Furthermore, only the 5′ but not the 3′ PAI-2 probe hybridized with C3E8 (data not shown). These data indicated that C3E8 contained only the 5′ region of PAI-2 and suggested that the insert originates at the *Eco*RI site 5′ to exon IV (Fig. 1*a*).

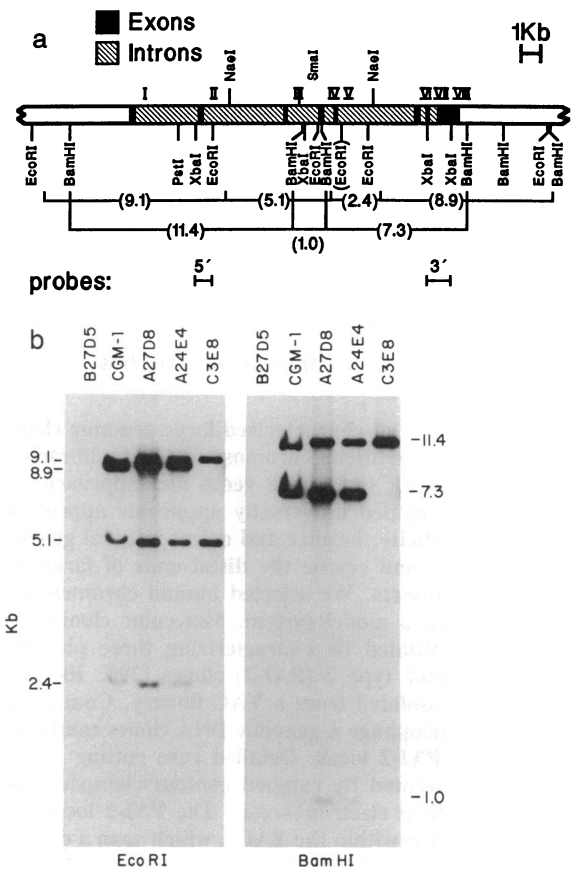

FIG. 1. Genomic organization and restriction map of the PAI-2 locus and fidelity of YAC clones. (*a*) Location of eight exons and restriction sites identified by analysis of overlapping genomic phage clones (9). The (*Eco*RI) site is polymorphic. Unique 5′ and 3′ probes were obtained by gel purifying a 0.8-kb *Xba* I/*Eco*RI and a 0.9-kb *Xba* I fragment (9), respectively. Sizes (kb) are shown beneath restriction sites. (*b*) Autoradiogram of the *Eco*RI (*Left*) and *Bam*HI (*Right*) restriction digests of yeast genomic DNA from three PAI-2-containing YAC clones (A27D8, A24E4, and C3E8), one anonymous human DNA-containing YAC clone (B27D5), and human genomic DNA from a lymphoblastoid line (CGM-1). The blot was hybridized with the ^{32}P-labeled, 1.9-kb, full-length PAI-2 cDNA (23).

Mapping and Alignment of PAI-2 YAC Clones. A variety of approaches could be used to map large genomic YAC clones. The indirect end-labeling of DNA partially digested by routine cutting 6-bp REs with probes specific for the R or L vector arm (15) effectively placed *Eco*RI and *Bam*HI sites within the 60 kb of C3E8 (data not shown). However, the accuracy of this approach was markedly diminished when attempting to place such sites in inserts >100 kb. This prompted an alternative approach in which complete digestion of YAC DNA by rare cutting REs and size fractionation on ramped CHEF gels was used. Transferred blots from these gels were hybridized to probes derived from the 5′ and 3′ portion of PAI-2 (Fig. 1*a*) and the R and L arm of the YAC vector (Fig. 2). The known *Nae* I and *Sma* I sites between the 5′ and 3′ PAI-2 probes were exploited to locate and orient that

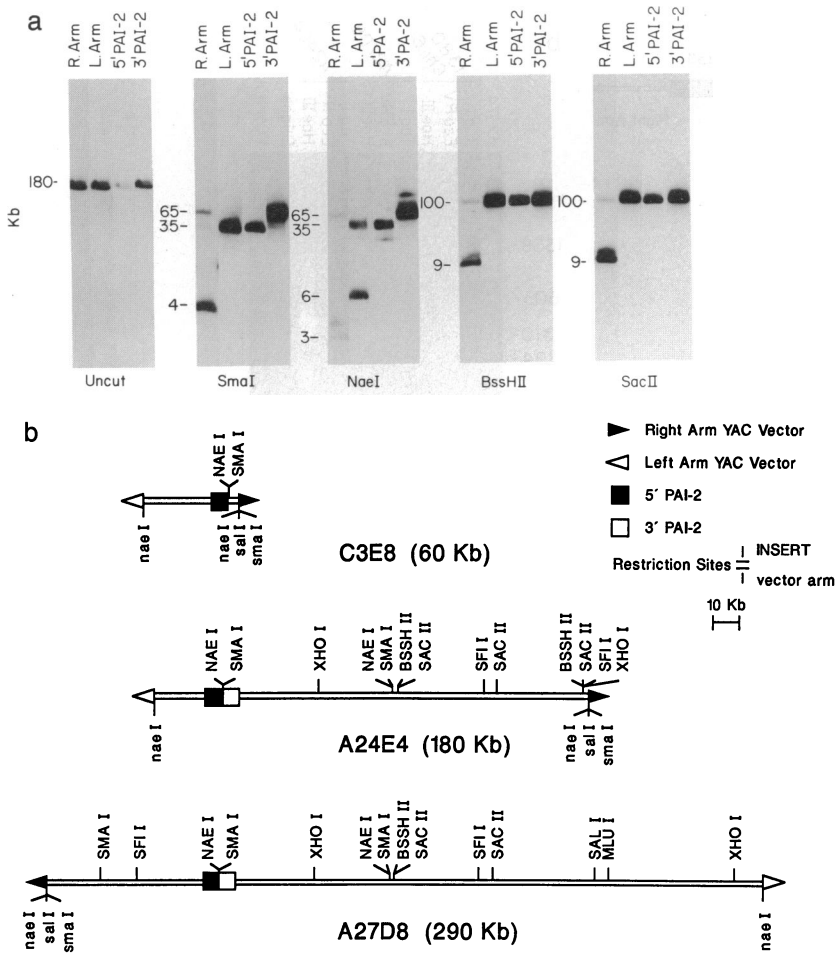

FIG. 2. Mapping of rare cutting RE sites within PAI-2 YAC clones by ramped CHEF gels. (*a*) Autoradiograms of a single blot containing lanes of uncut and *Sma* I, *Nae* I, *Bss*HII, and *Sac* II digested DNA from clone A24E4. The blot was sequentially hybridized to ^{32}P-labeled probes (top of panels) specific for the R (1.7-kb *Pvu* II/*Bam*HI pBR322 fragment) and L (2.7-kb *Pvu* II/*Bam*HI pBR322 fragment) arms of the YAC vector (15) and the 5′ and 3′ region of PAI-2. The minor bands present in the R arm probe lanes represent the incomplete removal of the 3′ PAI-2 probe. (*b*) Position and alignment of restriction sites and the PAI-2 gene within YAC clones. Clones were aligned relative to the 5′ to 3′ orientation of the PAI-2 locus. Note that the genomic insert of A27D8 was cloned into the YAC vector in an orientation opposite that of clones A24E4 and C3E8.

gene within the YAC clones (Fig. 2). Analysis of A24E4 (Fig. 2*a*) revealed that both the 5′ PAI-2 and the L arm probe hybridized to a 35-kb *Sma* I fragment, whereas the 3′ PAI-2 and the R arm probe recognized 65- and 4-kb fragments, respectively. A probe of total human DNA hybridized to 35-, 65-, and 70-kb *Sma* I fragments (data not shown). As expected, only the 5′ PAI-2 probe hybridized to a 35-kb *Nae* I fragment (*Nae* I sites exist in both vector arms). Thus, the 5′ portion of PAI-2 was 35 kb from the L arm, while the 3′ portion was 135 kb from the R arm. Similarly, sites for *Xho* I, *Sac* II, *Bss*HII, *Mlu* I, and *Sal* I were mapped within the YAC clones. This revealed the presence of clustered CpG-containing restriction sites (*Nae* I, *Sma* I, *Bss*HII, *Sac* II) 60 kb 3′ to PAI-2, which was not appreciated from routine PFGE analysis of genomic cell plugs (Fig 2). Overall, the YAC isolates span a distance 70 kb 5′ to 220 kb 3′ of PAI-2. The locations of most of the rare cutting RE sites were in agreement in A24E4 and A27D8 (Fig. 2*b*). However, A24E4 did demonstrate an additional CpG island (clustered *Bss*HII, *Sac* II, *Sfi* I, *Xho* I sites) adjacent to the R arm of the YAC vector that was not found in A27D8. A human genomic DNA probe neighboring the L vector arm was prepared from A27D8 (as described below). This A27 L end probe mapped to chromosome 18 when analyzed on a somatic cell hybrid panel. This probe also recognized the same sized 1250-kb *Mlu* I and 1400-kb *Not* I fragments as PAI-2 (data not shown). Thus, the rescued L end of A27D8 appears to be appropriately located 3′ to the PAI-2 locus. The rescued R genomic end of A24E4 is not present within A27D8, but reiterated human sequences within this probe compromise its further localization. Thus, the additional CpG island near the R end of A24E4 may represent a cloning artifact such as an unrelated insert or an interstitial deletion.

Genomic End Rescue PCR. To extend the physical map and link overlapping YAC clones, a universal method was needed to isolate both ends of YAC genomic DNA inserts. The pYAC4 vector was designed to facilitate plasmid end rescue of the L arm only (15). This procedure was dependent also on generating a compatibly sized *Xho* I or *Nde* I fragment for plasmid propagation. We developed a form of PCR that has consistently permitted the rescue of genomic DNA sequences contiguous to the *Eco*RI cloning site (Fig. 3*a*). Purified YACs are digested with a series of frequent cutting REs, which cut at known locations on the vector side of the *Eco*RI cloning site and at unknown sites on the side of the genomic DNA insert. Fragments containing the cloning site are circularized by ligating the digested YACs in dilute conditions. The vector–insert junctions are subsequently amplified by an inverted form (28) of PCR by using common primers of vector sequences that now flank the rescued end. As an example, the L arm rescue of purified A27D8 and A24E4 YACs yielded amplified products in one of three RE choices for A27D8 and in two of three choices for A24E4 (Fig. 3*b*). In contrast, isolated chromosome 6 DNA from the parental yeast strain AB1380 revealed no such products. Correct PCR products were identified by hybridization to an oligonucleotide probe specific for amplified YAC vector sequences adjacent to the *Eco*RI cloning site (Fig. 3 *a* and *c*). The human origin and single copy nature of the rescued ends were assessed by radiolabeling the PCR products and hybridizing them to conventional human genomic DNA blots (Fig. 3*d*). Finally, successfully rescued R and L arm fragments were subcloned into the Bluescript plasmid vector and the DNA sequence was determined by using T3 and T7 primers and standard dideoxynucleotide chain-termination methods (26) (Fig. 3*e*). Known YAC sequences were present

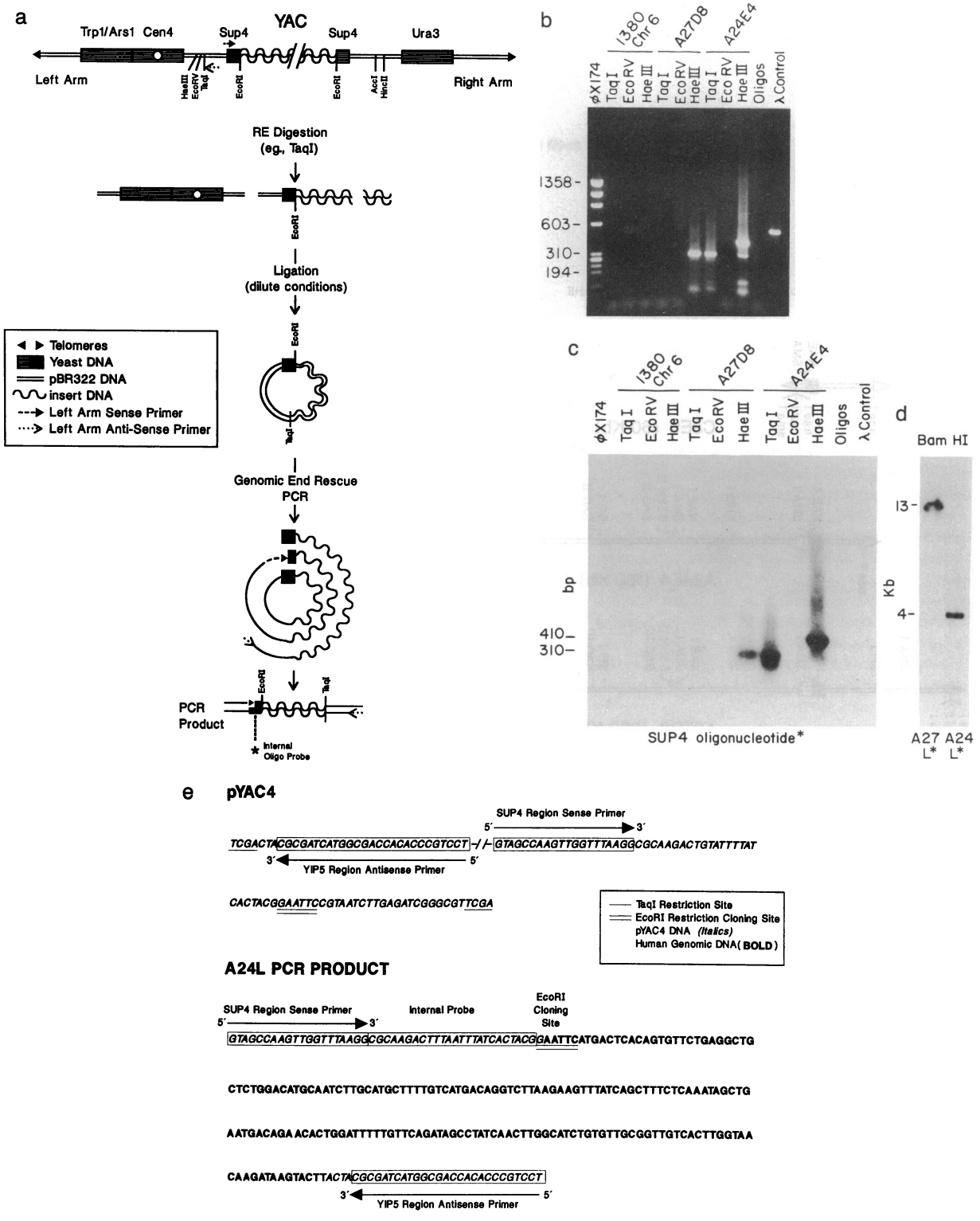

FIG. 3. End rescue of genomic inserts by an inverted PCR. (*a*) Schematic representation of an L arm end rescue PCR. (*b*) L arm rescue products of YAC clones A27D8 and A24E4. Ethidium bromide-stained 1% agarose gel of PCR products (10 μl) generated after digestion with either *Taq* I, *Eco*RV, or *Hae* III and circularization. PCR controls included AB1380 chromosome 6 (chromosome 6 is ≈290 kb and was copurified in the initial preparation of A27D8), oligonucleotide primers alone, and a 500-bp product derived from a control template and primers (λ DNA). (*c*) Specificity of PCR products. The blot was hybridized to a ^{32}P-end-labeled oligonucleotide probe specific for the amplified portion of the *SUP4* gene within L vector arm (shown in *e*). (*d*) Examination of PCR products for human origin. Southern blot of *Bam*HI-digested human DNA hybridized with ^{32}P-labeled PCR product from the L arm of A27D8 (A27L*, *Hae* III digest) or A24E4 (A24L*, *Taq* I digest). (*e*) DNA sequence of the PCR product. pYAC4: sequence of the L arm of the vector including the *Taq* I restriction sites 5′ and 3′ of the *Eco*RI cloning site (15, 25–27). The sense primer was specific for a region of the *SUP4* locus and oriented such that DNA synthesis would proceed through the *Eco*RI cloning site. The antisense primer was specific for the pBR322-related sequences of the YIP5 region of the vector and oriented such that synthesis could proceed to the sense primer only if the restriction fragment was circularized. A24L PCR product: Sequence of the product obtained after *Taq* I digestion, circularization, and L arm amplification of A24E4.

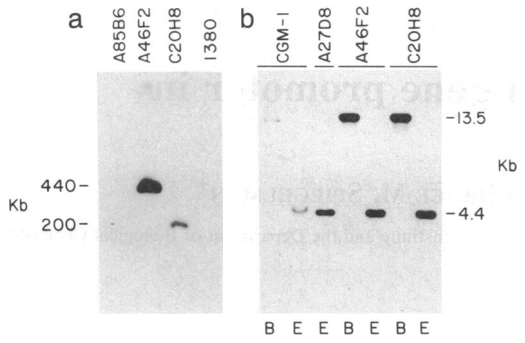

FIG. 4. Identification of overlapping YAC clones by a genomic end rescue probe. (*a*) Autoradiogram of size-fractionated chromosomes from host strain AB1380 and clones A46F2 and C20H8. A85B6 is a *Bcl-2*-containing YAC clone. The probe was a ^{32}P-labeled A27 L arm probe. (*b*) Autoradiogram of the *Eco*RI (E) and *Bam*HI (B) digests of total yeast DNA from YAC clones (A27D8, A46F2, and C20H8) and human genomic DNA (CGM-1) hybridized with the ^{32}P-labeled A27 L arm probe.

flanking the *Eco*RI cloning site and the secondary ligation site (e.g., *Taq* I, Fig. 3*e*), while the internal sequence was not of vector origin. This confirmed that the rescued products were the distal ends of the YAC inserts.

Rescued Genomic Ends Identify Overlapping YAC Clones. The DNA sequence from the rescued genomic ends of the PAI-2-containing inserts made it possible to rescreen the YAC library and isolate neighboring clones. Oligonucleotide primers synthesized from the 5′ and 3′ portions of the rescued L end of A27D8 were used to rescreen the library by a conventional PCR approach (E. D. Green and M. V. Olson, personal communication). Aliquots of DNA extracted from a total of 23,000 YAC clones were screened. DNA from clones A46F2 and C20H8 generated correctly sized amplified fragments. The identity of the clones was confirmed by colony hybridization with a radiolabeled A27L probe.

Electrophoretic karyotypes of A46F2 and C20H8 revealed the presence of 440-kb and 200-kb YACs, respectively, which hybridized to the radiolabeled A27L probe (Fig. 4*a*), but not to the A24L or PAI-2 probes (data not shown). Total yeast genomic DNA from A46F2 and C20H8 was digested with *Bam*HI and *Eco*RI and compared to human genomic DNA (CGM-1). Both YAC clones possessed the correctly sized *Bam*HI and *Eco*RI fragments, thereby confirming their true identity (Fig. 4*b*). Thus, A46F2 and C20H8 constitute overlapping isolates that extend the molecular clones of 18q21.3 further 3′ of the PAI-2 gene.

DISCUSSION

Attempts to accurately map genes and to identify disease loci within human chromosome segments such as 18q21.3 emphasize the necessity to bridge the gap in resolution between cytogenetics and conventional molecular cloning techniques (14). Significant advances have been made in the electrophoretic separation of large DNA fragments (17, 18, 29). However, such PFGE analysis relies extensively on rare cutting REs that often recognize CpG motifs. Unfortunately, these sites are unpredictably methylated in higher eukaryotes (13, 30). They also tend to cluster and therefore are not randomly distributed throughout the genome (30). These limitations coupled with a sparse number of available probes frequently make it impossible to construct complete long-range maps of chromosomal segments.

Characterization of large overlapping genomic regions cloned into YAC vectors could overcome these limitations. While preliminary studies indicated that YAC libraries can yield large genomic fragments containing single-copy genes (24), the application of YAC technology to genomic mapping was untested. The approaches developed here represent uniformly applicable methods to locate and orient genes and map RE sites within YAC isolates. Moreover, the genomic end rescue PCR provides an efficient method to routinely obtain the R and L distal ends of the inserts. These rescued ends are often unique, but even those with reiterated sequences may possess identifiable single-copy regions. Rescued ends are a crucial reagent needed to rescreen the library and isolate overlapping clones. This provides a practical approach to march to neighboring loci.

Accurate physical maps and large contiguous clones are required to order the known genes placed within a chromosome segment by classical approaches. YAC isolates provide a substrate to identify new genes and explore the relationship of topography to gene regulation and function. Correctly ordered genes, restriction fragments, and clones are also a necessary prerequisite to sequencing large expanses of the human genome. The results of this study establish the feasibility of using YAC technology to clone and map 18q21.3 and to search for loci associated with human disease.

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