Jpn. J. Human Genet. **36**, 229–243, 1991

SINGLE DNA MARKER GENERATED BY "YAC-*Alu* PCR" THAT IS END-SPECIFIC

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Summary A simple strategy for the rapid preparation of an end-specific linking-DNA probe from the YAC-human chromosome 21 DNA recombinant clone and the characterization of this single DNA probe are described. Synthetic oligodeoxynucleotide primers, based on the consensus *Alu* sequence, and the Sup4 DNA fragment in the YAC arms were used to amplify end-specific DNA sequences by the polymerase chain reaction (PCR) for screening of the linking YAC recombinant clones ("YAC-*Alu* PCR"). Nucleotide sequencing of the product of PCR from human genomic DNA in a YAC insert confirmed the boundary between the vector and the insert and the presence of the 3'-end *Alu*-like structure. The probe R1, prepared by "YAC-*Alu* PCR" amplification, was assigned to chromosome 21 by Southern hybridization of somatic cell hybrid DNAs. *In situ* hybridization allowed localization of the R1 DNA probe to the human chromosome 21q21-q22.1 region. Thus, this approach has significant advantages not only for isolation of a single DNA probe specific for human chromosome 21 but also for the screening of YAC linking recombinant clones for mapping of the human genome.

Key Words DNA marker, YAC contigs, YAC-*Alu* PCR

INTRODUCTION

The recent development of yeast artificial chromosome (YAC) cloning systems allows the cloning of large fragments of human DNA that are several hundred kilo-

Received, May 28, 1991; Accepted June 27, 1991.
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bases in length (Burke *et al.*, 1987; Brownstein *et al.*, 1989; Little *et al.*, 1989; Traver *et al.*, 1989; McCormick *et al.*, 1989; Anand *et al.*, 1989; Imai and Olson, 1990). We have constructed a human chromosome 21 equivalent library from a line of human-rodent somatic hybrid cells (Kai *et al.*, 1990). The average size of inserts in the library is between 155 and 165 kb and individual YAC clones can be isolated by conventional hy bridization techniques (Brownstein *et al.*, 1989) with human repetitive sequence *Alu* and total human DNA as probes (Kai *et al.*, 1990). Such a YAC-cloned library should be assist in efforts directed towards the mapping of the human genome. The cloning of the targetgenetic loci produces a series of "contigs" —groups of overlapping clones that collectively span a particular part of the region. Thust he development of "YAC contig" methodology is a prerequisite for attempts to link individual YAC recombinant clones to complete the map of the genome.

The construction of "YAC contigs" would be straightforward if DNA probes derived from the termini of the inserts could be isolated. The *Alu*-mediated polymerase chain reaction (PCR) provides a method for generating specific fragments of human DNA from the DNA of somatic cell hybrids. Genomic DNA from a line of human/rodent hybrid cells can be used as the template for a polymerase chain reaction (Saiki *et al.*, 1988) in which a human *Alu*-based oligodeoxynucleotide is used as the primer. *Alu*-mediated PCR provides a vast improvement over present standard recombinant-DNA methods. It eliminates the need for screening human clones by hybridization with total human DNAs; it reduces the need for fractionating the human clones into repetitive and single-copy sequences; and it sllows faster chromosomal localization of the new markers has previously been possible.

Alu-mediated PCR has already been demonstrated to be an effective method for producing human-specific DNA fragments from the DNA of human/rodent somatic cell hybrids (Nelson *et al.*, 1989). Recently this method was used for the rapid amplification and cloning of human DNA (Cotter *et al.*, 1989, 1990; Baldini and Ward, 1991; Bernard *et al.*, 1991) and human chromosome-specific DNA (Brooks-Wilson *et al.*, 1990; Cotter *et al.*, 1991; Guzzetta *et al.*, 1991). We reported previously that PCR technology with a combination of primers from a YAC-arm vector and an *Alu* repeated sequence can facilitate the isolation of terminal-specific probes from YAC recombinant clones for "YAC contigs" (referred to as "YAC-*Alu* PCR"; Yokoyama and Soeda, 1989).

Now we have expanded our approach to the direct selection and cloning of a terminal-DNA single probe from YAC recombinant clones. To verify the utility of this method we performed a series of pilot experiments in which we prepared the end-specific single DNA-linking probe from YAC-human chromosome 21 DNA recombinant clones. *In situ* hybridization and nucleotide sequence analysis of such linking single clones demonstrated that this probe is of human origin and can be localized to chromosome 21q21-q22.1. Thus, we conclude that this technique is useful for the mapping and linking of YAC-recombinant clones.

MATERIALS AND METHODS

Yeast strains, plasmids, and chemicals. *Saccharomyces cerevisiae* strain AB-1380 (Mat-α, ade2-1, can 1-100, lys2-1, trp1, ura3, his5[psi$^+$]), pYAC55, and the HY-1 YAC clone [125-kb DNA fragment from human peripheral lymphocytes (CGM1)] transformed with EB virus were kindly provided by Dr. M.V. Olson (Burke *et al.*, 1987). Restriction enzymes, bacterial alkaline phosphatase (BAP), and DNA ligase were purchased from Toyobo, Inc., Tokyo, Japan. All other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Cells and tissue culture. The Chinese hamster ovary cell-human chromosome 21 hybrid cell line, 153E9a3 (Patterson and Schandle, 1983) was kindly given by Dr. D. Patterson and cultured in Ham's F12 Medium (Nissui, Tokyo) supplemented with 7% dialyzed fetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.). A total of 1×10^7 153E9a3 cells were radiolabeled with 1 mCi of [^{3}H]thymidine (20 Ci/mmol; Amersham, Buckinghamshire, England).

Plasmids and DNAs. Cloned probes used for hybridizations included a pBLUR-8 probe (*Alu* probe; Deininger *et al.*, 1981) and a pBR322 probe. All probes were purified as restriction fragments: the *Bam*HI fragment of pBLUR-8 and the *Pvu*II/*Bam*HI fragment of pBR322. These probes were radiolabeled with [^{32}P]-dCTP by the random-primer method of Feinberg and Vogelstein (1983). DNA from human placental tissue was used as the human genomic probe. DNA from Chinese hamster ovary (CHO) cells was lysed as the hamster genomic probe. Both genomic DNAs were nick-translated (Maniatis *et al.*, 1982) for use as hybridization probes.

Contour-clamped homogenous electric field (CHEF) gel electrophoresis. CHEF gel electrophoresis was performed on 1% agarose gels as described elsewhere (Chu *et al.*, 1986), using a Pulsarphor™ Electrophoresis Unit (Pharmacia LKB, Uppsala, Sweden). The 1% agarose gels in 0.5 × TBE [45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA (pH 8.0)] were prepared by pouring 130 ml of agarose (Agarose NA, Pharmacia AB, Uppsala, Sweden) into a 15×15 cm^2 frame. Electrophoresis was carried out in 0.5 × TBE as running buffer at a constant temperature of 12°C, which was maintained by recirculation of the buffer through a heat exchanger, at a constant voltage of 170 v for 20 hr. The stepped switching interval was 60 sec for the first 12 hr and 90 sec for the next 8 hr.

Southern blot analysis. Genomic DNA was isolated from the nuclei of somatic cell hybrids and CHO cells by standard treatment with sodium dodecyl sulfate (SDS) and proteinase K and subsequent phenol-chloroform extraction. Yeast cells were embedded and lysed in agarose (agarose plugs) by the method of Carle and Olson (1984). High-molecular-weight DNA was extracted from yeast colonies and YAC recombinant clones as described elsewhere (Carle *et al.*, 1984; Yokoyama *et al.*, 1990; Kai *et al.*, 1990; Imai and Olson, 1990). After digestion with

*Eag*I or *Eco*RI, 10 μg of DNA were subjected to electrophoresis on a 1% agarose/Tris-acetate gel and transferred to a nylon membrane. Gels were treated with 0.25 N HCl for 15 min, then with a solution of 0.4 N NaOH and 0.6 M NaCl for 15 min, and finally with a solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5), before transfer to nylon membranes, as described elsewhere (Carle *et al.*, 1986), and subsequent crosslinking (Church and Gilbert, 1984). Hybridization was carried out in a solution of 1 M NaCl, 10% dextran sulfate and 1.0% SDS with 100 μg/ml of yeast tRNA as carrier and approximately 10^6 cpm/ml of ^{32}P-labeled *Eag*I-digested product of "YAC-*Alu* PCR" or other probes at 65°C for 20 hr. Membranes were washed twice with 2×SSC at room temperature for 10 min, then once with 2×SSC supplemented with 1.0% SDS at 65°C for 30 min, and finally once with 0.1×SSC at room temperature for 30 min. Autoradiography was performed at −80°C for one day using Kodak XAR-5 film.

Construction of the YAC library. The YAC library specific for human chromosome 21 DNA had been prepared previously, as described in detail elsewhere (Kai *et al.*, 1990). In brief, the DNA from 153E9a3 cells were entrapped in agarose beads or agarose films as described (Yokoyama *et al.*, 1990; Kai *et al.*, 1990). We estimated that there were approximately 50 μg of genomic DNA per ml of agarose beads (or films) that contained approximately 8×10^6 cells. High-molecular-weight DNA was digested with *Eag*I or *Not*I and ligated to the arms of pYAC55 to yield products with an average size of 150 to 160 kb. Screening of yeast colonies with a probe for human *Alu* DNA was performed as described previously (Yokoyama *et al.*, 1990; Kai *et al.*, 1990).

Amplification by "YAC-Alu PCR." Enzymatic amplification was carried out by the polymerase chain reaction (PCR) using the DNA polymerase from *Thermus aquaticus* (Taq; Saiki *et al.*, 1988) and an automated DNA thermal cycler (Perkin-Elmer Cetus Co., Emeryville, CA., U.S.A.). Prior to addition of the DNA for amplification, the reaction mixture was prepared, for a final volume of 100 μl, with oligodeoxynucleotide primers (2 mM total), 200 mM each of the deoxyribonucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), 1.0 unit of Taq DNA polymerase (Amplitaq; Cetus, Norwalk, CT, U.S.A.), gelatin (0.01%, w/v), and Taq buffer [50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$]. One hundred nanograms of DNA from a human DNA YAC recombinant clone were used for reaction. Initial denaturation was allowed to proceed for 4 min at 94°C. 1.0 unit of Taq DNA polymerase was added and then 35 cycles were conducted for 2 min each at various temperatures (37°C to 62°C; annealing, Fig. 1A), with 2 min at 72°C (extension), and 1 min at 94°C (denaturing). The final extension period at 72°C was lengthened to 10 min. Aliquots (5 μl) from the reaction mixture were analyzed by electrophoresis on 2% agarose gels (in TBE buffer) which were stained with ethidium bromide. Primers for the PCR were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA, U.S.A.). Primer sequences for PCR were as follows: *Alu* primer #1, 5′-CCA AAG TGC TGG GAT

TAC AGG-3′; and *Alu* primer #2, 5′-GGG TTC AAG CGA TTC TCC TGC-3′, anv *Alu* primer #3, 5′-ATC TCG GCT CAC TGC AA-3′. Primers #1, #2, and #3 were also synthesized in the opposite orientation. The YAC-left arm (Sup4) primer had the sequence 5′-AAC CGA TCT TGG AAG GAC CGG-3′. The total number of combinations of both YAC-arm primers and *Alu* primers for generating the products of PCR was 12.

Cloning of DNA fragments from 14C1 by "YAC-Alu PCR." The products of "YAC-*Alu* PCR" were gel-purified, precipitated in ethanol and resuspended in 15 μl TE. Aliquots (100 ng) were "blunt-ended" by treatment with the Klenow fragment of DNA polymerase I from *Escherichia coli*. After digestion with *Sma*I, the DNA was inserted into the *Sma*I site of pBluescript SKII⁻ (Stratagene, La Jolla, CA., U.S.A.). Products of ligation were used to transform *E. coli* strain XL-1 Blue (Stratagene) and transformants were selected on LB agar plates that contained ampicillin and X-Gal/IPTG.

In situ hybridization to chromosomes. The product of "YAC-*Alu* PCR" designated R1 was radiolabeled to a specific activity of approximately 5×10^8 cpm/μg by random oligonucleotide priming (Feinberg and Vogelstein, 1983) with ^{3}H-labeled deoxynucleotides (dCTP, dATP, and TTP). Metaphase chromosomes from normal human lymphocytes were hybridized with 90 ng of probe per milliliter of hybridization mixture by the method of Harper and Saunders (1981), and Zabel *et al.* (1983) with modifications (Nakai *et al.*, 1986). Conditions of relatively low stringency were used with a temperature of 37°C for the hybridization and 35°C for the washes. Slides were exposed for 5 to 10 days and all silver grains on or touching chromosomes were scored. G-banding was performed as described by Zabel *et al.* (1983).

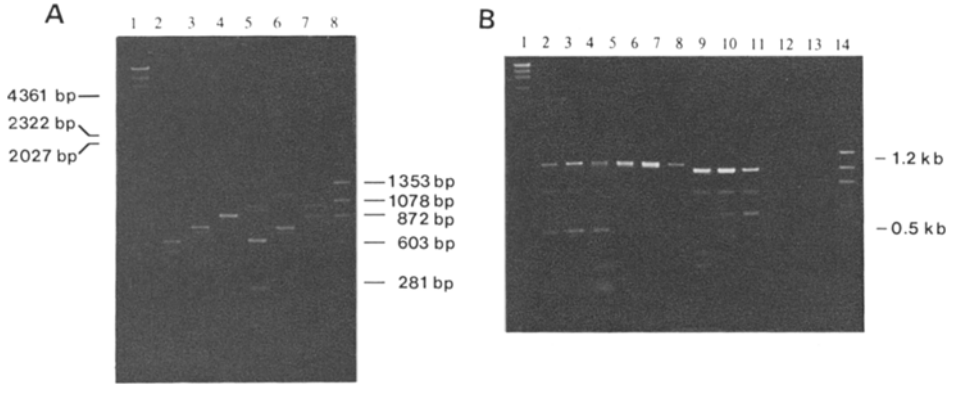
Nucleotide sequence analysis. The nucleotide sequence of R1 was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977). The reaction mixture was fractionated on a 6% polyacrylamide gel.

RESULTS

Amplification of an end-specific DNA probe from YACs

Construction of a yeast artificial chromosome (YAC) library specific for human chromosome 21 was carried out by a new method that involves entrapping cells in agarose beads (or agarose film) and digesting the DNA with the restriction enzymes *Not1* or *Eag1* (Yokoyama *et al.*, 1990; Kai *et al.*, 1990). Approximately 1.0 μg of DNA from the somatic hybrid cell line 153E9a3 generated $2–4 \times 10^6$ transformants. More than 90% of the transformants were recombinants, as determined by Southern blot hybridization of pulsed-field gels, which also revealed the average size of the YACs to be 155–165 kb (Kai *et al.*, 1990). About 1–2% of the recombinants were expected to contain DNA from human chromosome 21 and recombinants were screened by colony hybridization with human repetitive *Alu* sequences and total human DNA as probes. The total length of the human DNA-YACs was

equal to more than 15 megabases and represented approximately 31% of chromosome 21. We next tried to construct the "YAC contigs." Previously, we developed a new method for preparing end-specific single-DNA probes using PCR amplification with *Alu* consensus sequences and vector sequences as primers (Yokoyama and Soeda, 1989). The oligodeoxynucleotide 20-mer, corresponding to the 3′-end of the Sup4 region DNA of the YAC-left arm was synthesized and used as the "YAC-arm primer" for amplification by PCR. We selected the 5′-end sequence of Sup4 DNA to determine whether, as anticipated, the amplified products of PCR extended from the 5′-end to the 3′-end *Alu* primer. In an attempt to confirm the correct extension we performed two experiments: hybridization of the products of



3' Primer	Position (nt)	Nucleotide Sequences
#1	22 - 43	5'-CCAAAGTGCTGGGATTACAGG-3'
#2	187 - 207	5'-GGGTTCAAGCGATTCTCCTGC-3'
#3	220 - 240	5'-ATCTCGGCTCACTGCAA-3'
5' Primer	YAC-sup4	5'-AACCGATCTTGGAAGGACCGG-3'

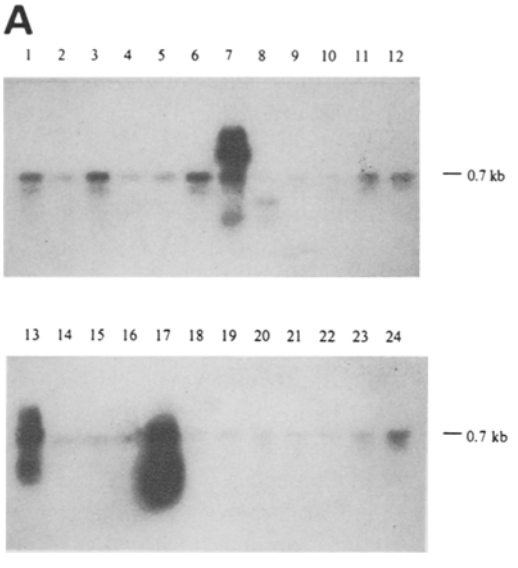
Fig. 1. Amplification by PCR of end-DNAs from YAC clones by use of YAC-arm Sup4 and *Alu* primers. Products of "YAC-*Alu* PCR" (see text) were separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. (A) Comparative fingerprinting study of products of "YAC-*Alu* PCR" in which three different *Alu* primers were used (#1, #2, #3; see text). Lane 1, *Hin*dIII-digested λ DNA size markers; lanes 2–4, template DNA from 6H1; lanes 5–7, template DNA from 3G6; lanes 2 and 5, *Alu* primer #1; lanes 3 and 6, *Alu* primer #2; lanes 4 and 7, *Alu* primer #3; lane 8, *Hae*III-digested ø×174 DNA size markers. (B) Comparative study of products of "YAC-*Alu* PCR" (YAC-Sup4 primer and *Alu* primer #1 were used) generated at various annealing temperatures. Lane 1, *Hin*dIII-digested λ DNA; lanes 2–7, 14C1 as template DNA; lanes 8–13, 15D1 as template DNA, lane 14, *Hae*III-digested ø×174 DNA. The following annealing temperatures were used: lanes 2 and 8, 37°C; lanes 3 and 9, 42°C; lanes 4 and 10, 47°C; lanes 5 and 11, 52°C; lanes 6 and 12, 57°C; lanes 7 and 13, 62°C.

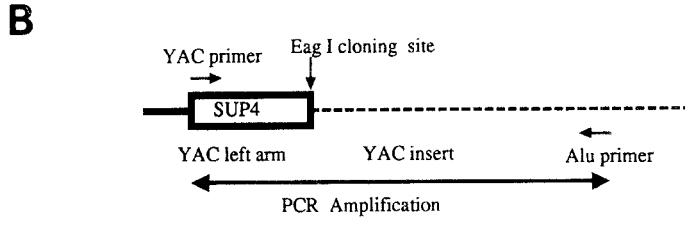
PCR with the Sup4 DNA probe; and digestion with *Eag*I to decrease the expected size of the DNA products of the PCR.

Results of one example of such experiments are shown in Fig. 1. Three oligodeoxynucleotide pairs, #1, #2, and #3, with sequences based on the recently revised consensus *Alu* sequence (Bains, 1986; Kariya *et al.*, 1987), were surveyed in an initial experiment designed to amplify the end-specific DNAs from two YAC recombinant clones, 6H1 (insert size: 250 kb) and 3G6 (insert size: 260 kb). The range of sizes of YAC-derived DNA fragments amplified from 100 ng of DNA is shown in Fig. 1A. It can be seen that the *Alu* primer #3 yielded a major single band in the case of the 6H1 clone (lane 4) and several bands in the case of the 3G6 clone (lane 7). *Alu* primer #3 is identical in sequence to primer #517, but in the opposite orientation to primer TC-65, as described by Nelson *et al.* (1989). This sequence is located within a 31-bp sequence that is unique to the second monomer of primate *Alu* repeats (Jelinek and Schmid, 1982). Nelson *et al.* (1989) reported that this primer served to amplify human DNA specifically. We confirmed their conclusion that primer #3 is suitable for the specific amplification of human DNA in "YAC-*Alu* PCR," as shown in Fig. 1A (lanes 4 and 7). By contrast, the DNA products obtained with *Alu* primer #2 migrated so close together on a gel that we were unable to distinguish and isolate the expected bands for further procedures, such as subcloning (lanes 3 and 6). The DNA products with *Alu* primer #2 or #3 contained the 5′-ends of the *Alu*-derived repeated sequences, which caused the non-specific hybridization of human DNA to prevent isolation of the end specific DNA fragment from YAC clones. The DNA products obtained with *Alu* primer #1 yielded major distinct bands of the expected size (lanes 2 and 5) and were free of *Alu*-derived repeated sequences. Primer #1 is identical in sequence to primer #278 described by Nelson *et al.* (1989). Comparison of the *Alu* consensus sequences from human and hamster revealed that primer #1 was directed to a region of the repeat that is highly conserved between rodents and primates. However, we always detected the human-specific product of PCR using this primer (data not shown). From an analysis of more than 100 YAC clones, we concluded that primers #1 and #3 (in the opposite orientation) were the best primers for the amplification of DNA. No products were detected after control reactions in which no primers were included.

We next examined the appropriate temperatures for the annealing of primers to substrate DNA during "YAC-*Alu* PCR." We show here one set of results using two YAC clones, 14C1 and 15D1 with 145 kbp and 136 kbp of DNA, respectively (Fig. 1B). The optimal annealing temperature of primers in the case of 14C1 was 57°C and that in the case of 15D1 was 42°C. After analyzing other YACs, we concluded that a temperature of 42°C is optimal for annealing of the *Alu* primer #1, the YAC-Sup4 primer and template DNA. To demonstrate that the amplified DNA was correctly elongated to the *Alu* primer from the YAC-left arm primer in the Sup4 region, we used two approaches to examine the product of the PCR; Southern hybridization of PCR-generated DNAs with a Sup4 DNA probe and

direct nucleotide sequencing of the PCR product. Of 24 YAC clones examined, only four were positive for hybridization with Sup4 DNA (Fig. 2A, lanes 7, 8, 13, and 17). The common bands of 0.7 kb were derived from yeast DNA. These four clones were apparently extended correctly from the 5′-end Sup4 region of the 3′-end *Alu* primers. Approximately 10 to 20% of YAC clones examined were



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5'    YAC Primer     3'
AACCGATCTT GGAAGGACCG GATAATTATT TGGAATCTCT TTTTCAATTG TATATGTGTT ACGTAGTATA CTTTGTCTTC
                                                                  ↓ Eag I  site
AACAATTAAG TACTCTCGGT AGCCAAGTTG GTTTAAGGCG CAAGACTGTA TTTTATCACT ACGCGGCCGC TGATTCTCCG

GCGAGCTCTT GTCCTCAGAG GACCAGCCGC CTGGGTGCGT GGAGACGGAG CTGGCTGGGT AGAGGTGGCA GCTCGCCCCG
GCACAGCTTT TGCCGACCGT CTGCGGTAGG TTCTCACGGG ACCTACGCCA AGATGGTTAG TTGCTCACAA TATTAAGGTG
GGCTGTTGTC TTCGAATAGG TCTCCCCCGG TCGTAGAGCT CTCACCACAG TGTTGCATGG AATCTCAGAG TGTGTGCAGA
GGGCGATCCT TTATTCCTCC AGGGTTTACG GGGGACAGCG AGGGATGCAA AGAAAAAAAA GCCTGCTGCT CTCCTCCCTC
CCGTTCTCCA TTTCCGACAC AGATCTGCTT GTGGACTGGA AGAGAGCAGG AGAGCGAGAT GGGAAGCTGC GTGAATTGTT
CTTT•••
                                        •••C TGAAACTCTG AGATCATGCA AACGTGGTGC GAGTTACTCG
GACCTATTCG AAGCCACTGC CCCTTAATAT TGTGAGCCAC TAACCATCTT GGCGTCGGTC CCGTGAGCCC CTACCGCATG
ACGGTCGGTA AAAGCTGTGC CGGGCGAGCT GCCACCTCTA CCCAGCCAGC TCGTCTCCAC GCACCAGCGG CTGTCCTCTA
AAGAAGGTTG GCTTTGAAGT CTGGTGAATG TCGGGATGAA TGCTGGTTAC TGTGCTATTG GAATAGGTGG GTACACTCTG
GGGAATCCAT GCAAGTATAG CCAGACACAT ATTCCGGATA GCAAGTTAAC TATAAACCTG ATAAAATATA AAAAAATGTT
GGACTGGAGA GATGGATGGC TCCACAGTTA AGAGCACTGG CTGGGCGCGG CCGCTCACGC CTGTAATCCC AGCACTTTGG
                                                  3'    Alu Primer    5'
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positive for hybridization with Sup4 DNA. Final confirmation by nucleotide sequencing was carried out to confirm the correct extension of the PCR product designated R1 (Fig. 2B). The nucleotide sequence of R1 derived from 14C1 confirmed that both sequences of the 5′-end YAC-Sup4 primer and the 3′-end *Alu* primer were present intact and the *Eag*I cloning site was also maintained in the product of PCR (Fig. 2B). No repetitive *Alu*-like sequence was detected within the nucleotide sequence of R1. Thus, specific products of amplification can be synthesized from the termini of the YAC insert, and it appears that both primers were very suitable for obtaining the end-specific probe by PCR amplification.

Hybridization study with the product, R1, of the "YAC-Alu PCR"

In an attempt to confirm the specificity of the product of "YAC-*Alu* PCR," designated R1, Southern hybridization of DNAs from various YAC clones and

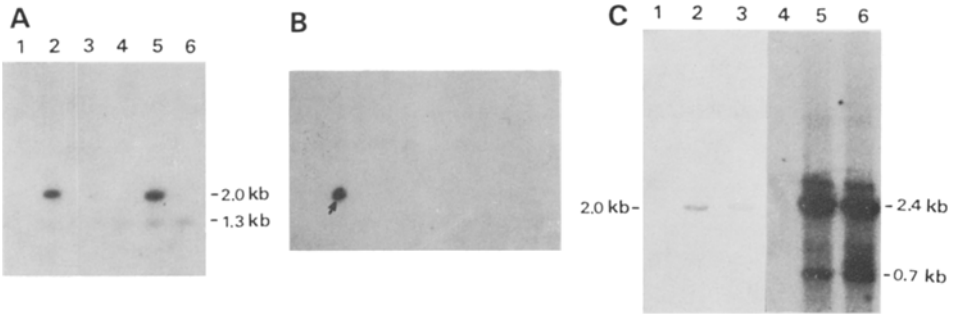

Fig. 3. Characterization of R1, a product of "YAC-*Alu* PCR." (A) Southern hybridization analysis using the *Eag*I-digested R1 probe and *Eco*RI-digested DNAs (10 μg each) from CHO cells (lane 1), 153E9a hybrid cells (lane 2), HY-1 YAC (lane 3), 4E11 YAV (lane 4), 14C1 YAC (lane 5) and AB1380 (lane 6). (B) Dot hybridization of 96 YAC clones with ^{32}P-labeled R1 DNA as probe. Hybridization was carried out as described in MATERIALS AND METHODS. The linking YAC clone that overlapped with 14C1 was 31F9 (indicated by an arrow). (C) Southern hybridization of DNAs from 14C1 and 31F9 using *Eag*I-digested R1 as probe. *Eco*RI-digested DNAs (10 μg each) from AB1380 (lane 1), 14C1 (lane 2), and 31F9 (lane 3). *Dde*I-digested DNAs from AB1380 (lane 4), 14C1 (lane 5), and 31F9 (lane 6). The sizes of hybridized fragments are indicated.

←Fig. 2. Hybridization and nucleotide sequence analysis of the products of "YAC-*Alu* PCR." (A) Southern hybridization of PCR products from YAC clones with the primer specific for the 5′ end of the Sup4 region. Lane 1, AB1380; lanes 2–24, YAC clones, as follows: lane 2, 1B3 (210 kb); lane 3, 3F2 (200 kb); lane 4 4E11 (180 kb); lane 5, 5E7 (250 kb); lane 6, 12E4 (200 kb); lane 7, 14C1 (145 kb); lane 8, 17A4 (125 kb); lane 9, 18B12 (180 kb); lane 10, 19D3 (120 kb); lane 11, 20E1 (120 kb); lane 12, 23F3 (200 kb); lane 13, 31E8 (200 kb); lane 14, 32C4 (220 kb); lane 15, 36H3 (230 kb); lane 16, 40A1 (190 kb); lane 17, 41H8 (125 kb); lane 18, 42A1 (140 kb); lane 19, 42D4 (155 kb); lane 20, 43B3 (180 kb); lane 21, 50C1 (115 kb); lane 22, 52C8 (155 kb); lane 23, 56D1 (140 kb); lane 24, 57E10 (155 kb). (B) Nucleotide sequence of R1, a product of "YAC-*Alu* PCR." The positions of the YAC left-arm Sup4 primer, the *Alu* primer and the *Eag*I sire are indicated. (EMBL accession number, X56557).

from rodent and hybrid cells was carried out using a product of PCR after digestion with *Eag*I to remove the YAC-arm portion. As shown in Fig. 3A, only one *Eco*RI fragment (2.0 kb) was detected in DNAs from 153E9a3 (lane 2) and none was detected in DNA from CHO cells (lane 1). Only the 14C1 YAC clone generated the samesized band (lane 5) and DNAs from other YAC clones did not hybridize to this product of PCR (lanes 3 and 4). AB1380 was negative for hybridization with this probe (lane 6). These data indicate that the product of "YAC-*Alu* PCR," R1, was human in origin and was a human-specific single-DNA probe. We used the PCR screening method of Green and Olson (1990a) to localize the positive clone and then by colony hybridization to find the "contig YAC clones" as described elsewhere (Kai *et al.*, 1990). A new YAC clone, 31F9, was identified by this approach (Fig. 3B). By Southern hybridization analysis of both YAC clones 14C1 and 31F9, we found that the *Eco*RI fragment (2 kb; lanes 2 and 3) and the *Dde*I

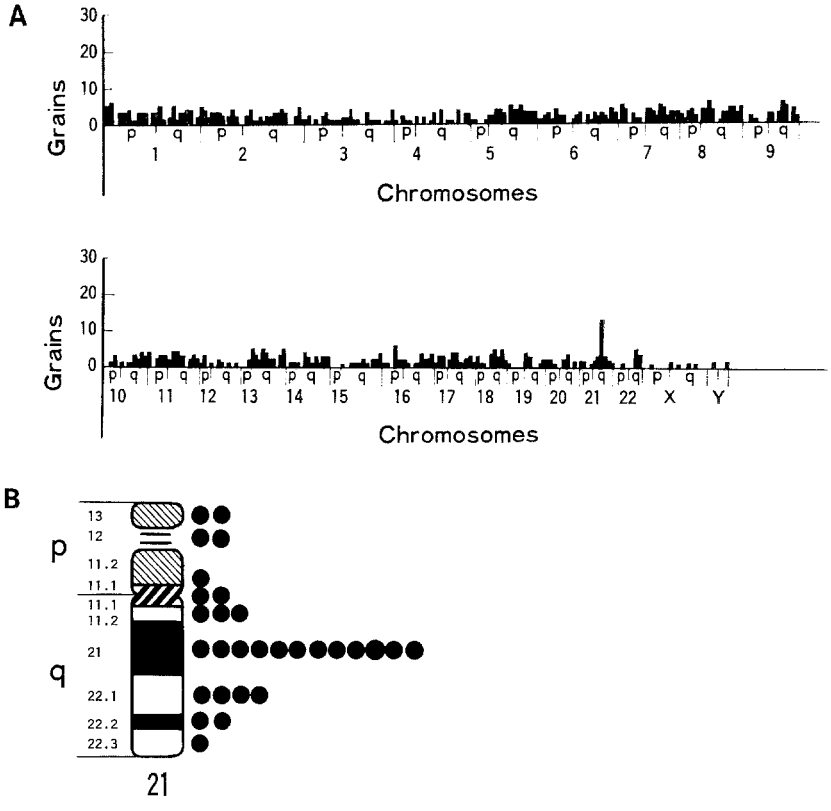

Fig. 4. *In situ* hybridization of the R1 DNA marker. (A) Histogram showing the distribution of silver grains over all chromosomes when they were allowed to hybridize with the radiolabeled R1 DNA probe. Results were compiled from an examination of 350 cells. (B) Schematic diagram of G-banded chromosome 21 showing the maximum accumulation of grains over the region 21q21-q22.1.

fragments [3.1 kb (this band was the result of incomplete digestion), 2.4 kb, 0.7 kb; lanes 5 and 6] were shared by both clones (Fig. 3C). These results suggest that the YAC clone 31F9 is linked to YAC 14C1.

Chromosomal mapping by in situ hybridization

The regional localization of the product, R1, of PCR was confirmed and further clarified by *in situ* hybridization to human metaphase chromosomes. Figure 4A shows a histogram of the distribution of silver grains over a full set of chromosomes. Results were compiled from an analysis of 350 cells. The specific hybridization was strong in the 21q21-q22.1 region and the maximum density of grains was seen at q21 (29 grains: 4.0% of the total 717 grains), as shown in the schematic representation of chromosome 21 in Fig. 4B. The combination of results from Southern blot hybridization and *in situ* hybridization using the R1 probe allows assignment of the locus to be 21q21-q22.1 region of human chromosome 21.

DISCUSSION

A number of recent studies using *Alu*-mediated PCR have demonstrated the selective amplification of human specific DNA clones or human-chromosome specific DNA clones from hybrid cells (Nelson *et al.*, 1989; Benham *et al.*, 1989; Cotter *et al.*, 1989, 1990, 1991; Brooks-Wilson *et al.*, 1990; Baldnini and Ward, 1991; Bernard *et al.*, 1991; Guzzetta *et al.*, 1991). The general concept of the "YAC-*Alu* PCR" method for ordering of "YAC contigs" has been reported previously (Yokoyama and Soeda, 1989; Breukel *et al.*, 1990). We have developed this protocol and determined the precise conditions for amplification by PCR for preparation of an end-specific DNA marker from YAC clones. Probes derived from "YAC-*Alu* PCR" should be valuable in studies designed to "walk" along the chromosome. We have examined various 3′-end *Alu* primers for the appropriate synthesis of DNA molecules with a 5′-primer specific for the YAC-left arm Sup4 region. The use of a primer from the extreme 5′-end of the *Alu* repeat (#1 in Fig. 1A) allowed us to amplify DNA molecules that are largely free of *Alu*-derived repeat sequences, and the frequency of synthesis of the desired product of PCR was higher than was obtained with any other *Alu* primer (Fig. 1A). This primer sequence lies within the region (bases 23–47) previously identified by Kariya *et al.* (1987) as the most highly conserved among *Alu* families. The temperature for annealing of primers and template DNAs is also important. An annealing temperature of 42°C was optimal for preparation of larger amounts of R1, the product of PCR (Fig. 1B). Other parameters, such as the number of cycles, and the conditions for denaturation and extension, were optimized by reference to the standard conditions of the basic protocol (manuals from the manufacturer, Perkin-Elmer Cetus Co.).

It has been estimated that there are about 5×10^5 copies of *Alu*, each approximately 300 bases long, which represent about 3–6% of the genome. The ampli-

fication relies on the occurrence of adjacent *Alu* repeats that are sufficiently close to the cloning site of YACs, with sufficient homology to the primer, and with an appropriate orientation that permits successful amplification under the elongation conditions. The efficiency of amplification of different *Alu* sequences may vary and such variation would be reflected in the products of PCR. The frequency with which any given product of PCR is produced will depend ultimately on the interplay between factors such as sequence homology, placement and orientation of *Alu* families. It has been estimated that individual *Alu* families are 86% homologous to one another and to the consensus sequence (Bains, 1986). We have, therefore, used primers derived from the consensus sequence to increase the proportion of *Alu* sequences that are ampified.

We observed that the frequency of with which the end-specific DNA probe is generated from a YAC recombinant clone by "YAC-*Alu* PCR" is ten-fold higher than that when the "*Alu*-mediated PCR" method is employed (data not shown). Thus, we can conclude that the method of "YAC-*Alu* PCR" is more advantageous than the "*Alu*-PCR" method.

Moyzis *et al.* (1989) reported that the *Alu* family of sequences could be distributed into two classes of genomic DNA: "*Alu*-rich" domains and "*Alu*-poor" domains. Within each domain the placement of *Alu* sequences was considered to be random. This model predicted that, within an *Alu*-rich domain, the average spacing would be approximately 1 kb, while within an *Alu*-poor domain it would be approximately 10 kb, with an overall average of 4 kb. Thus, the frequency of the generation of the products of PCR from YACs can be expected to be lower in the case of YACs that contain *Alu*-poor domains. To circumvent these difficulties, we recommend the use of primers that correspond to other repeated sequences, such as the L1 repeated sequences and GT: AC families (Hwu *et al.*, 1986; Rinehart *et al.*, 1981). The inverse PCR technology (Ochman *et al.*, 1988; Triglia *et al.*, 1988; Silverman *et al.*, 1989) and the linker-specific amplification technique (Riley *et al.*, 1990) are other useful tools for preparation of linking probes.

Southern hybridization with the Sup4 DNA probe showed that the frequency of generation of positive YACs was approximately 5 to 10% (Fig. 2A). The nucleotide sequence analysis of R1, the product of "YAC-*Alu* PCR," provided a novel method for experimentally examining the 5′-end of the junction sequence of the cloning site and the 3′-end of the *Alu* sequence (Fig. 2B). It was apparent that the products of PCR hybridized to the 2-kb restriction fragment generated by *Eco*RI from DNAs of 153E9a3 and the YAC 14C1 clone (Fig. 3A). R1 was found to be particularly useful as a single DNA probe for genome-mapping studies. The dot hybridization of YACs with this product demonstrated the possibility of obtaining the "contig YAC" clone (Fig. 3B). Southern hybridization of 14C1 and 31F9 YACs with the R1 DNA probe gave identical bands of restriction fragments (Fig. 3C). Thus, we conclude that this approach is extremely efficient for the preparation of end-specific DNA probes for linking.

In situ hybridization allowed assignment of R1 to chromosome 21q21-22.1. A series of experiments confirmed the significant advantages of the "YAC-*Alu* PCR" method for preparing an end-specific single DNA probe for screening experiments designed to isolate overlapping YAC clones. Recently, Olson *et al.* (1990) proposed the use of "sequence tagged sites" (STSs) to generate landmarks for mapping. The method described here provides a convenient technique for generating such markers.

Acknowledgments We thank Dr. M.V. Olson for helpful discussions and advice. We also thank Dr. R. Di Nicolantonio and Dr. L. Siqin for their critical reading of the manuscript and Ms. T. Yamauchi and Ms. K. Oka for editing the manuscript.

This work was supported by the Life Science Research Project of RIKEN and The Science and Technology Agency of Japan.

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