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ESTABLISHMENT OF SEQUENCE-TAGGED SITES ON 15q11-q13 BY *ALU*-VECTOR PCR CLONING OF YAC-GENERATED FRAGMENTS

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SUMMARY

Angelman syndrome (AS) is caused by the loss of function of undefined gene(s) on human chromosome 15. The majority of subjects have deletions involving maternally-derived chromosome 15q11-q13, and the shortest region of deletion overlap (SRO) has been localized to the region between D15S10 and D15S113. In this study, yeast artificial chromosomes (YACs), 6G-D4, 9H-D2 and 37D-F9, mapping within the AS SRO, were isolated from the ICI YAC library. *Alu*-vector PCR products were amplified from the YACs and from YACs A229A2 and A33F10 which had been obtained from the St. Louis YAC library. The PCR products were cloned and sequenced, and three new sequence-tagged sites were generated within the AS SRO, facilitating the characterization of gene(s) involved in the Angelman syndrome.

KEY WORDS *Alu*-Vector PCR YAC STS

INTRODUCTION

Two unrelated disorders, Angelman (AS) and Prader-Willi syndrome (PWS) have been shown to map to chromosome region 15q11-q13. The AS is a complex disorder characterized by severe mental retardation, absence of speech, epilepsy, ataxia and hypopigmentation. An interstitial deletion of maternal origin, involving 15q11-q13, is present in 75% of subjects (Knoll *et al.*, 1989). PWS is phenotypically distinct from AS and is characterized by neonatal hypotonia, developmental delay with mild mental retardation, short stature, dysmorphic features and obesity associated with an insatiable appetite. It is also associated with an interstitial deletion of 15q11-q13, but in this case, it is paternal in origin (Nicholls *et al.*, 1989). Subjects with variable deletions involving different subsets of loci in 15q11-q13 have allowed the establishment of a linear order of probes mapping to loci present in this region and the identification of minimal deletion intervals in the two syndromes. The linear order of loci (probes) is CEN-D15S9(ML34)-D15S11(IR4-3R)-D15S13(189-1)-D15S63(PW71)-SNRPN-D15S10(3-21)-D15S113(LS6-1)-GABRB3-GABRA5-D15S78(MN47)-D15S12(IR10)-TEL. The shortest regions of deletion overlap (SRO) in PWS and AS map to D15S63-SNRPN (Kuwano *et al.*, 1992) and D15S10-D15S113 (Reis *et al.*, 1993) respectively.

Mammalian genomes contain many short interspersed repetitive DNA sequences. In man, and other primates, the major family is denoted *Alu* repeat element (Jelinek and

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Schmid, 1982). These repeats are ubiquitous with over 900,000 copies being present in the haploid human genome with an estimated average distance of 4 kb between repeat elements (Nelson *et al.*, 1989). Repetitive sequences homologous to human *Alu* repeats are not found in the yeast genome and this difference can be exploited to isolate yeast artificial chromosome (YAC) sequences from yeast DNA sequences. Primers complementary to both *Alu* repeats and YAC telomere sequences can be used to specifically amplify YAC ends by PCR elements (Nelson *et al.*, 1989).

The PWS SRO is well characterized and a substantial amount of data is now available, however, only limited data on the characterization of the long range physical structure of the AS SRO are available. To facilitate the detection of small deletions and the location and characterization of candidate gene(s) involved in AS, a large number of markers are needed in the AS SRO. In this study three new sequence-tagged sites (STSs) were established by *Alu*-vector PCR cloning of YAC fragments mapping within the AS SRO. These STSs can also serve as anchor points to facilitate the ordering of large genomic clones and as initiation points for continuous sequencing of large genomic fragments.

MATERIALS AND METHODS

YACs

Forty primary pools of the ICI YAC library were received from the Human Genome Mapping Project Resource Centre (Harrow, U.K.), as agarose blocks containing DNA. The blocks were melted at 65°C for 20 minutes and diluted 1:10 with water. Forty PCR were carried out in 25 µl reactions containing 5.0 µl of the diluted DNA. A pair of primers (Nicholls *et al.*, 1989) mapping to D15S10 were used. An appropriately sized 180 bp product was amplified from three of these pools. The information obtained from these pools was sent to the HGMP Resource Centre where the second and third round of screenings of the library were performed. Three more YACs, A229A2 and A33F10, and 307A12, used in this study, were obtained from the St. Louis YAC library (St. Louis, MO, USA) and the CEPH YAC library (Paris, France) respectively, in the form of single yeast colonies on agar slopes.

All of the YACs used in this study have been shown to map exclusively to 15q11-13 by hybridisation to a panel of mouse-human cell lines and by fluorescence *in situ* hybridisation to human metaphase spreads. A number of other YACs isolated for the same region were excluded from further study because they were chimeric.

All of the YACs have been constructed with the pYAC4 (Burke *et al.*, 1987) cloning vector and were maintained as a single copy chromosome in *Saccharomyces cerevisiae* strain AB1380. The presence of the *Trp* and *Ura3* genes allows selection for yeast strains containing YACs by growth in a medium deficient in tryptophan and uracil (double drop-out medium) (Brownstein *et al.*, 1989). *S. cerevisiae* grows optimally at 30°C. The host strain was grown on YPD (yeast extract 10 g/l, tryptone 20 g/l and dextrose 20 g/l) agar plates and strains containing YACs on double drop-out medium agar plates. Double drop-out medium was made with drop-out base medium (BIO 101), 27 g/l, supplemented with complete supplement mixture lacking in tryptophan and uracil (BIO 101), 0.7 g/l. Total yeast DNA was prepared from the YAC clones as previously described (Anand *et al.*, 1990).

Alu-Vector PCR

PCR was carried out in 50 µl reactions containing 100 ng of total yeast DNA, 20 pmol of each primer, 50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin, 100µM dNTPs and 0.75 units of *Taq* polymerase (AmpliTaq, Perkin-Elmer Cetus). After an initial denaturation at 94°C for 3 min, amplification was carried out with 35 cycles of denaturation (92°C, 45 s), annealing (55°C, 60 s) and extension (72°C, initially 60 s with an additional 6 s for each subsequent cycle). The *Alu* primers (Lengauer *et al.*, 1992) and YAC telomere primers pYAC4 (Burke *et al.*, 1987) were designed to contain *Bam*HI and *Hind*III sites respectively to facilitate subsequent cloning steps. The sequences of the *Alu* primers are, CL1: 5'TAGTAGGATCCCAAAGTGCTGGGATTACAG3' and CL2: 5'TAGTAGGATCCTGCACTGCAGCCTGGG3', and the YAC telomere primers are, pYAC4-R: 5'TAGTAGAAGCTTCAACTTGCAAGTCTGG3' and pYAC4-L: 5'TAGTAGAAGCTTCTCGGTAGCCAAGTTGG3'. Twenty µl samples were electrophoresed in 1% agarose gels in 0.5xTBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0) for 4 h at 80 volts. The gels were stained with ethidium bromide and the DNAs were visualized by illumination with short-wave UV. The desired *Alu*-vector PCR products were excised out of the gel, and the DNAs were purified using silica matrix (Geneclean, BIO101). The cloning and sequencing of the *Alu*-vector PCR products were done following the standard methods (Sambrook *et al.*, 1989).

STS PCR

STS PCR was carried out in 50 µl reactions containing 100 ng of human genomic DNA, 20 pmol of each primer, 50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin, 100µM dNTPs and 0.75 units of *Taq* polymerase. The PCR conditions for the STS primers were: 25 cycles of denaturation (94°C, 1 min), annealing (55°C, 2 min) and extension (72°C, 2 min).

RESULTS

Three YAC clones 6G-D4, 9H-D2 and 37D-F9, mapping to D15S10, were isolated from the ICI YAC library. Three more YACs, A229A2 and A33F10, and 307A12, were obtained from the St. Louis YAC library and the CEPH YAC library respectively. YACs A229A2 and A33F10 mapped to D15S113, and 307A12 to D15S13 (Kuwano *et al.*, 1992). A simple and rapid method based on PCR amplification of YAC ends was applied to all the YAC clones. Oligonucleotides corresponding to the 5' and 3' ends of the *Alu* consensus sequence were synthesized and used in conjunction with primers based on YAC-telomere sequences to amplify DNA from total yeast DNA containing YACs. The *Alu* primers, which amplify only human DNA, together with the YAC vector primers (one for each telomere) specifically amplified DNA sequences at the ends of each YAC. Amplification of YAC A229A2 DNA with the CL1 primer alone generated several discrete bands (Figure 1). However, when the CL1 primer was used in conjunction with the pYAC4-R primer in a separate PCR reaction an extra band of 700 bp was also produced (Figure 1). The generation of the 700 bp *Alu*-vector PCR product was verified by repeating the PCR reaction using the isolated fragment itself as the template for amplification. Only the combination of the two primers produced an identically-sized PCR product. The 700 bp fragment was digested with *Bam*HI and *Hind*III and subcloned into pBluescript (Stratagene). Sequencing was performed on the recombinant DNA

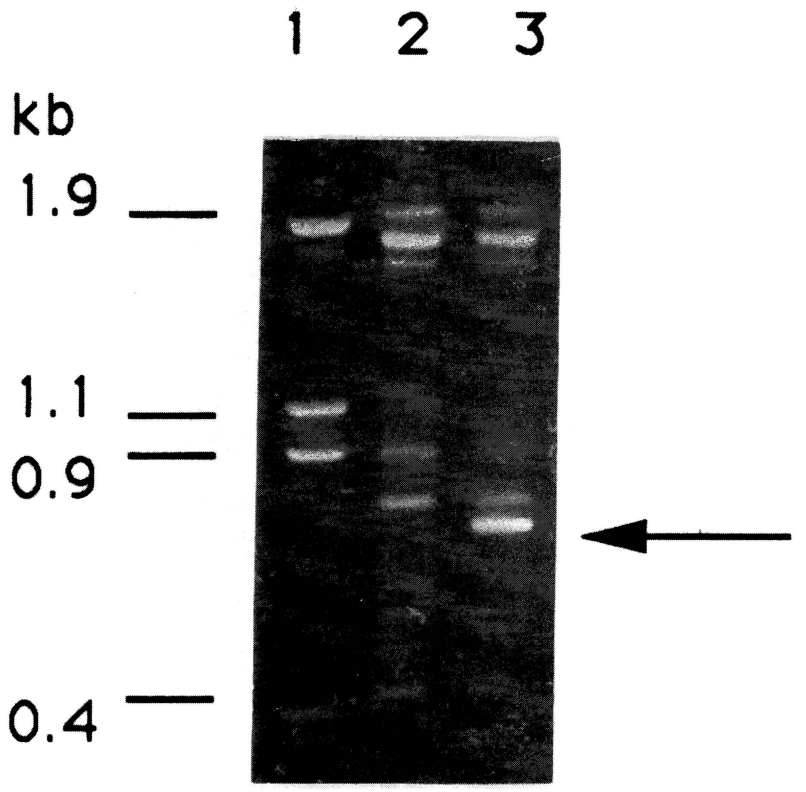

Figure 1. The *Alu*-vector PCR amplification of YAC A229A2. Lane (1) pBR322-*Bst*N1 marker (bands at 1.9, 1.1, 0.9 and 0.4 kb); (2) amplification with the CL1 primer alone; and (3) amplification with the CL1 primer in conjunction with the pYAC4-R primer. The 700-bp *Alu*-vector PCR product is arrowed.

clone using the M13 forward (-20) and reverse primers. Comparison of the derived sequence with the CL1 and pYAC4-R primers, the *Alu* consensus sequence and the YAC right-arm telomere sequence allowed the identification of novel sequences that corresponded to human DNA derived from the end of the cloned YAC insert. A new pair of primers was then synthesized from this sequence, A229R1: 5'TCTTTAATTCCAGTTTGGCC3' and A229R2: 5'TGCTGAGATTGAACTTCAGG3', and were used to amplify a predicted 507 bp product from YAC A229A2 DNA. The primers were also used to demonstrate amplification of a single band of size 507 bp from human genomic DNA.

Alu-vector PCR products were also generated from YAC A33F10 (510 bp) using CL1 and pYAC4-L, and YAC 6G-D4 (550 bp) using CL2 and pYAC4-L. The *Alu*-vector PCR products were all subcloned and sequenced. Pairs of primers were synthesized from the

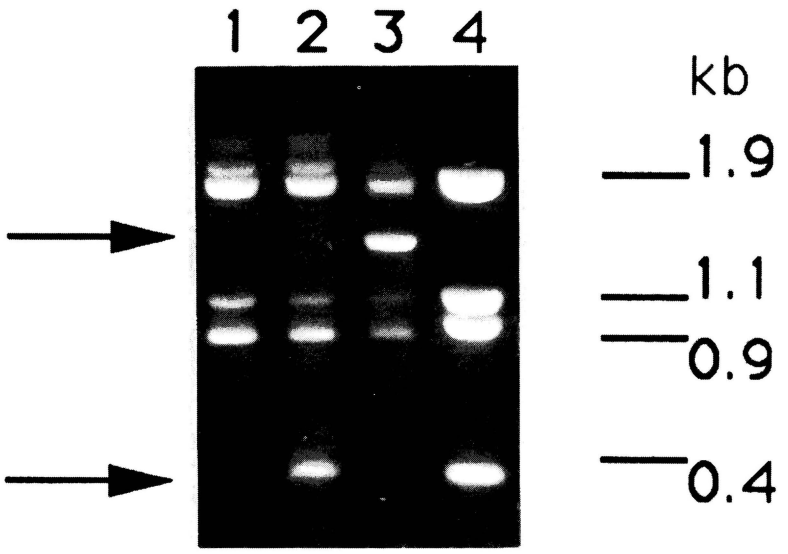

Figure 2. The *Alu*-vector PCR amplification of YAC 307A12. Lane (1) amplification with the CL2 primer alone; (2) amplification with the CL2 primer in conjunction with the pYAC4-L primer; (3) amplification with the CL2 primer in conjunction with the pYAC4-R primer; and (4) pBR322-*Bst*N1 marker (bands at 1.9, 1.1, 0.9 and 0.4 kb). The *Alu*-vector PCR products are arrowed.

each sequence and were used to verify the amplification product in each case. The primers and their product sizes are, A33L1: 5'GCACTTTGGGAGGCTCAGGT3' and A33L2: 5'CCACCTGAGCCTCCCAAAGT3' (331 bp); and 6GL1: 5'GTCCAAGAATGAGAGAAACTGAG3' and 6GL2: 5'CTGCAGGAATTCGATATCAAGCTT3' (344 bp) respectively. These primers were also used to demonstrate amplification of a single band of exact size from human genomic DNA. Figure 2 shows amplification of YAC 307A12 with primers CL2 and pYAC4-L, and also with primers CL2 and pYAC4-R.

DISCUSSION

PCR primers were designed to utilise regions of the *Alu* repeat that were both highly conserved and located close to the end of the consensus sequence, minimising the amount of repetitive DNA in the amplified product. They also contained a restriction endonuclease site at their 5' ends to allow convenient subcloning of the *Alu*-vector PCR product. A number of different techniques have been described for the isolation of terminal sequences from YAC DNA inserts. While the method described here is constrained by the need for an *Alu* element in the proximity of the insert ends, this condition was met in these cases, and they demonstrated the ease with which *Alu*-vector PCR cloning can be used to isolate such sequences.

The selection of PCR primers capable of amplifying the ends of the YAC insert allows the generation of sequence-tagged sites that are of use in genome mapping and analysis,

and in this study, facilitating the characterization of the AS SRO, which contains the gene(s) responsible for the Angelman syndrome. The STSs can serve as anchor points for constructing contigs of genomic clones and also as initiation points for continuous sequencing of large genomic fragments.

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