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
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).
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**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 MgCl₂, 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 BamHI and HindIII sites respectively to facilitate subsequent cloning steps. The sequences of the Alu primers are, CL1: 5’TAGTAGGATCCCAAAGTGCTGGATTACAG3’ and CL2: 5’TAGTAGGATCCCTGCAGCCTGGG3’, 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 MgCl₂, 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 BamHI and HindIII 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-BstNI 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’TGCTGAGATTGAAGTTCAGG3’, 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-BstNI marker (bands at 1.9, 1.1, 0.9 and 0.4 kb). The Alu-vector PCR products are arrowed.

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.

REFERENCES


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