Am. J. Hum. Genet. 55:372–378, 1994

A YAC Contig Encompassing the Treacher Collins Syndrome Critical Region at 5q31.3-32

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Summary

Treacher Collins syndrome (TCOF1) is an autosomal dominant disorder of craniofacial development the features of which include conductive hearing loss and cleft palate. Previous studies have localized the TCOF1 locus between D5S519 (proximal) and SPARC (distal), a region of 22 centirays as estimated by radiation hybrid mapping. In the current investigation we have created a contig across the TCOF1 critical region, using YAC clones. Isolation of a novel short tandem repeat polymorphism corresponding to the end of one of the YACs has allowed us to reduce the size of the critical region to ~840 kb, which has been covered with three nonchimeric YACs. Restriction mapping has revealed that the region contains a high density of clustered rare-cutter restriction sites, suggesting that it may contain a number of different genes. The results of the present investigation have further allowed us to confirm that the RPS14 locus lies proximal to the critical region and can thereby be excluded from a role in the pathogenesis of TCOF1, while ANX6 lies within the TCOF1 critical region and remains a potential candidate for the mutated gene.

Introduction

Treacher Collins syndrome (TCOF1) is an autosomal dominant disorder of craniofacial development that has an incidence of ~1/50,000 live births (Rovin et al. 1964; Frazen et al. 1967). The clinical features are usually bilaterally symmetrical and include (i) abnormalities of the external ears, frequently with atresia of the external auditory canals and anomalies of the middle ear ossicles, resulting in bilateral conductive hearing loss, (ii) hypoplasia of the facial bones, particularly the mandible and zygomatic complex, (iii) downward slanting of palpebral fissures with colobomata of the lower eyelids and a paucity of lid lashes medial to the defect, and (iv) cleft palate (Rovin et al. 1964; Frazen et al. 1967). While nonpenetrance is rare (Dixon et al. 1994), diagnosis and subsequent genetic counseling can be extremely difficult, as expression of the gene is extremely variable.

On the basis that the tissues affected in TCOF1 arise from the first and second branchial arches during early embryonic development, it has been proposed that the condition results from an abnormality of neural crest cell migration (Poswillo 1975). The genetic defect, while as yet unknown, has been mapped to human chromosome 5q32-33.1 (Dixon et al. 1991; Jabs et al. 1991), and flanking short tandem repeat polymorphisms (STRPs) defining the TCOF1 critical region have been identified (Dixon et al. 1993). Multipoint linkage analysis has placed the TCOF1 locus in a 2.1-cM interval between D5S519 and SPARC, a region of 22 centirays as estimated by radiation hybrid (RH) mapping (Loftus et al. 1993). As these markers are formatted for use with PCR, they have provided the ideal starting points for the creation of long-range continuity across the TCOF1 critical region, via the use of YAC clones. Moreover, RH mapping has placed a sequence-tagged site (STS) for the ANX6 locus in the middle of this region (Loftus et al. 1993), thereby providing an additional starting point for bidirectional walking toward the flanking markers.

In the current study we have used the STRP/STS markers at these loci to isolate YAC clones. While the density of the markers was not high enough to permit map closure by using this approach alone, the creation of additional STSs, representing the ends of the YACs, has allowed us to assemble a contig encompassing the entire TCOF1 critical region. The creation of an additional STRP, corresponding to the end of one of these YACs, has allowed us to estimate the size of this region as ~840 kb, which is in close agreement with data derived from RH mapping (Loftus et al. 1993). In addition, the results of the present investigation have allowed us to confirm that the RPS14 locus lies proximal to the critical region and can thereby be excluded from a role in the pathogenesis of TCOF1, while ANX6

Received February 7, 1994; accepted for publication April 15, 1994.

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0002-9297/94/5502-0020$02.00

lies within the TCOF1 critical region and remains a potential candidate for the mutated gene.

Material and Methods

Isolation and Initial Characterization of YAC Clones

YAC clones were isolated from the ICI (Anand et al. 1990) and CEPH (Albertsen et al. 1990) libraries by using a PCR-based approach. The primers used in the initial screening of the libraries have been detailed elsewhere (Dixon et al. 1993; Loftus et al. 1993). In order to achieve gap closure, the libraries were also screened with primers constructed from sequence data generated from the YAC ends. Positive clones isolated via these methods were streak purified to obtain a single positive clone, and YAC plugs were prepared according to the method of Anand et al. (1990). YAC clones were sized on a 1% agarose gel in 0.5 × Tris-borate EDTA buffer at 200 V and 14°C by using a contour-clamped homogeneous electrophoresis field (CHEF) (DRII; Bio-Rad). DNAs were fractionated using either a ramp from 6.7 to 8.5 s over 18 h (to resolve from 0 to 100 kb) or a ramp from 6.7 to 26.3 s over 30 h (to resolve from 50 to 400 kb).

Isolation of YAC Ends

YAC insert termini were isolated using a modification of the Vectorette system, which exploits the asymmetry of the pYAC4 vector (Riley et al. 1990). The YACs were digested with a panel of restriction enzymes (*Alu*I, *Eco*RV, *Pvu*II, and *Rsa*I) to produce blunt-ended fragments, which were then ligated to the Vectorette oligonucleotide cassette (Cambridge Research Biochemicals) to produce Vectorette libraries. The terminal sequences were PCR amplified using a linker-specific (5′ TCT CCC TTC TCG AAT CGT AAC CGT TCG TAC 3′) and a pYAC4 vector-arm-specific primer (left arm, 5′ GTG TTA TGT AGT ATA CTC TTT CTT CAA C 3′; and right arm, 5′ ATA TAG GCG CCA GCA ACC GCA CCT GTG GC 3′). To increase the specificity of the reaction, a second round of PCR was performed using nested primers (linker-specific primer, 5′ CGA ATC GTA ACC GTT CGT ACG AGA ATC GCT 3′; left arm primer, 5′ CTT CAA CAA TTA AAT ACT CTC GGT AGC C 3′; and right arm primer, 5′ CTT GCA AGT CTG GGA AGT GAA TGG AGA C 3′). The left (L) and right (R) arms of pYAC4 were defined as those containing the trp and ura genes, respectively. Insert termini were therefore designated "L" or "R" by their proximity to one or the other of the YAC arms. To confirm that amplification had occurred across the cloning site in pYAC4, the PCR products from the secondary reactions were digested with *Eco*RI and were resolved against the corresponding uncut secondary PCR product. True Vectorette products are those which have been digested by *Eco*RI because of removal of the amplified vector sequence segment.

Vectorette products were cloned into T-tailed M13mp18 and were sequenced. The sequence data so derived were used to design oligonucleotide primers that were used (i) to screen a somatic cell hybrid panel (Bios Corporation) to confirm their chromosomal location and (ii) for further screening of the YAC libraries.

Restriction Mapping of YACs

Each YAC was restriction mapped by partial/complete digestion with a panel of rare-cutting restriction enzymes (*Bss*HII, *Eag*I, *Nae*I, *Not*I, *Sac*II, and *Sfi*I) as detailed by Butler et al. (1992). The resulting restriction fragments were fractionated by CHEF as above. The gels were stained, photographed, and blotted onto Biodyne A nylon membrane (Pall) according to the manufacturer's instructions. The filters were hybridized with 2.7-kb and 1.7-kb *Pvu*II/*Bam*HI pBR322 fragments corresponding to the left and right arms of the YAC vector respectively (Silverman et al. 1989) and then with any available internal probes. The membranes were washed in 0.5 × SSC, 0.1% SDS at 65°C. Autoradiography was performed at −70°C with double intensifying screens for 1–4 d with Fuji RX film. All filters were stripped of radiolabeled probe and were reexposed to X-ray film prior to hybridization with subsequent probes. The data were used to construct consensus rare-cutter restriction maps of each YAC.

Isolation of STRPs

*Eco*RI-restricted Vectorette products (i.e., those free of pYAC4 vector sequences) were radiolabeled by random priming (Feinberg and Vogelstein 1983) and were used to screen a pooled chromosome 5 cosmid library (Dr. L. Deaven, Los Alamos National Laboratory) by using standard methods (Sambrook et al. 1989). Positive colonies were cloned and screened with a (dC-dA)(dG-dT)$_n$ probe (Pharmacia). (CA)$_n$ repeat–containing clones were digested with *Sau*3AI and cloned into the *Bam*HI site of M13mp18. Recombinant plaques were rescreened with (dC-dA)(dG-dT)$_n$ and were sequenced. The optimization and utilization of polymorphic dinucleotide repeats for genotyping have been described elsewhere (Dixon et al. 1992). Negative controls were established for all reactions.

Results

The YAC libraries were initially screened with primer sets defining (*a*) the polymorphic loci IG90 (D5S519) and SPARC, which previously have been shown to flank the TCOF1 locus (Dixon et al. 1993), and (*b*) the locus RPS14, which has been mapped proximal to D5S519 (Loftus et al. 1993). The libraries were also screened with an STS defining the ANX6 locus, which has been shown, by RH analy-

Table 1

STS Primer Sequences Corresponding to the YAC Insert Termini

STS	Forward Primer	Reverse Primer	Size (bp)
RPS14	CTC ACT GGG ATA TTC TCA TTC T	ACG TAG GTC AGT GAC TGA GG	160
D5S519	TAC AGA GTG GAA AGC CCA GT	CTT CCC ACA TAG CAC TCA CA	100
ANX6	GCA CTT CTG CCA AGA AAT GG	ACA GAC AGA GGT TCA GGA TG	350
SPARC	TAT GTT CAC AAG AGG GTG TC	ATC TCG CCA CTG TAC TCT AC	160
699C4R	TTA CGG GTA TCT TTT CAG TGC	TCA TTA TTT CAT GAG GCG CAC	124
699C4L	AAT AAT GTG GTC TTT GGT GCC	CTA TCT CAT AAA CTG GCC TGA	86
29CC10R	AAA CCC TAA TCA GTG TCG GT	CTT CAA CAA CAG GTA TTG GC	150
29CC10L	GGA GAC AGT GGC TAT AAA GG	CTT GCT GCT TTT GCA GTC TG	120
3G3R	TTT CTC AGA AGT CAG ACC TTG	TGT GAC TTC TGG ATG AAG GC	100
3G3L	ACT GAT CTG GGC TTC TCT GA	TGA ACA CAA ATT GCC TGT CAG	145
1ED1R	TCT ACA CTG TGC TAT GGT GC	GAT TCT GTT AGG TCC AGA CC	212
1ED1L	AGC AGA CAT GCC AAT GCG AT	ATC TGA ACA GGT TCT GTG CC	114
21FC7R	TTG CGT TGA TTA CGT CCC TG	ATT CTC CGC TCT GAG ATG GA	120
21FC7L	GCC TTT ATC CTA GAT CCC TG	ATA GGC TGT GAA CCA ACC TG	160
37FF12R	TAG TAA CCC CTG CCT TCT GT	ACC ACG TCC AGC CTA AAC AT	92
37FF12L	CAG ATA AAG GAC TCC CTT CC	TTC TCC AGC AAC ATG AGG AG	185
8FH10R	CAT GTG GCT CTA TTT CTG GG	AAT AGA GCT GAA GGC ACC AC	138
8FH10L	CAG GGT CTT CCT GGT AAG TT	AAG CCT CCA TCC AGT GTA AG	226
23FD6R	TAC TTT GGT TGA GTT TAC ACA G	CTT GAT AGC CCC TCT ACT TG	133
23FD6L	CTG ATA GCT GCT AAA CCA CC	ATT TGG AGA CGG TAA ACA TCC	188
45D8R	ATC CTG CTT GTG GGC ATT TG	TGG AAG AGG TGG TGT ATG GA	96
45D8L	AAA TTA CGG GTC CAG AGA GG	GAT GGG CTG TAC ATT GTT GG	187

sis, to map between these markers (Loftus et al. 1993). This initial strategy identified one YAC (699C4) for RPS14, two YACs (29CC10 and 3G3) for IG90 (D5S519), one YAC (45D8) for SPARC, and three YACs (21FC7, 37FF12, and 8FH10) for ANX6; however, none of these clones contained any of the other loci. As the density of the markers was not high enough to permit map closure by using this approach alone, the insert termini of the YACs were isolated to provide additional STS sites, which could be used to rescreen the YAC libraries and to achieve gap closure (table 1). With the exception of 699C4 and 29CC10, all of the YAC ends were found to reside on chromosome 5 when used to screen a somatic cell hybrid panel, indicating that they are unlikely to be chimeric. The left end of 699C4 (699C4L) was found to be from chromosome 6, while the right end of 29CC10 (29CC10R) was from chromosome 2. Both 29CC10L and 3G3L were found to amplify 699C4; however, 29CC10L must lie closer to RPS14 than does 3G3L, as the latter amplifies the former, but the converse situation is not true (fig. 1). These YAC ends also lie within 40 kb of one another, as they recognize the same cosmids when used to screen a pooled chromosome 5–specific library (data not shown). 3G3R in turn recognizes 21FC7 and 37FF12, both of which contain the ANX6 locus, but not 8FH10, which also contains ANX6. Screening of the ICI YAC library with 3G3R also detected one additional clone, 1ED1, which is ANX6 negative (fig. 1).

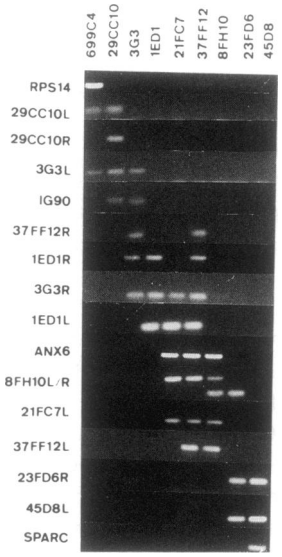

Figure 1 STS content analysis of the nine YACs documented in the present study. The YACs are listed across the top of the figure. Each row represents the result of colony PCR using primers for the STS indicated. In the case of 8FH10, primers recognizing both ends of the YAC were used in a duplex reaction. In all cases, total yeast DNA containing the YAC of interest was used as a substrate in the PCR. The sizes of the PCR products are given in table 1.

The primers designed from the right and left ends of 37FF12 amplified 3G3 and 8FH10, respectively, suggesting that this clone entirely encompasses 21FC7 and 1ED1. This was confirmed by the observations that 1ED1R detects 3G3 and 37FF12; 1ED1L detects 21FC7 and 37FF12; and 21FC7L detects 37FF12 and 8FH10. Interestingly, primers designed from the sequence of 21FC7R amplified total yeast DNA in the PCR. Hybridization probes produced from 21FC7R were further found to hybridize to 3G3, 1ED1, and 37FF12, as well to yeast chromosome 12. Sequence derived from 21FC7R proved to be 100% identical to yeast 18S rRNA, suggesting that this YAC has a small piece of yeast DNA at its right end.

As 8FH10 appeared to extend the contig further distally, 8FH10L and R were used to screen the clones isolated from the region in the initial screening strategy. 8FH10L recognized 21FC7 and 37FF12, but 8FH10R was negative in this assay. This primer set was therefore used to screen the YAC libraries, and 23FD6 was isolated, the right end of which detected the SPARC-containing YAC 45D8 (fig. 1). Completion of the YAC contig allowed us to confirm that the order of loci is cen–RPS14–D5S519–ANX6–SPARC–tel.

In an attempt to further reduce the size of the critical region, *Eco*RI-restricted (vector-free) 8FH10R was used to screen a pooled chromosome 5 cosmid library. The resulting positive clones were screened for the presence of STRPs. A $(CA)_n$ repeat was identified and was shotgun cloned into M13mp18. Sequence analysis identified a highly imperfect repeat $(GT)_3(GA)_8(GT)_{14}(GA)_9$ (Weber 1990). Primers flanking the repeat sequence were designed: 5′-AAA AGA TGC AGA AAG AAA CTT GTT-3′ and 5′-TCC CAA GGT ATT TAT GAA GGC-3′. Analysis of 50 CEPH parents revealed a total of nine alleles, with frequencies as follows: 219 bp, .12; 217 bp, .06; 215 bp, .03; 213 bp, .02; 205 bp, .03; 199 bp, .02; 197 bp, .15; and 195 bp, .57. The heterozygosity value of this locus was found to be .62. This STRP was used to genotype those TCOF1 families which define the recombination boundaries of the TCOF1 critical region; a single recombination event was identified in an affected individual (fig. 2) who exhibited downward-slanting palpebral fissures plus hypoplasia of the mandible and zygomatic complex. An identical result was obtained from two independent blood samples taken from this patient on different occasions.

A rare-cutter restriction map was constructed of those YACs covering the TCOF1 critical region (figs. 3 and 4); YACs 23FD6 and 45D8 are not included in this map, as they lie outside the critical region. The map presented is a consensus map derived from data generated, for the most part, from a number of overlapping clones. This has proved to be important, as some sites produced only weakly hybridizing bands in some clones. In such cases, data from additional clones permitted confirmation of the

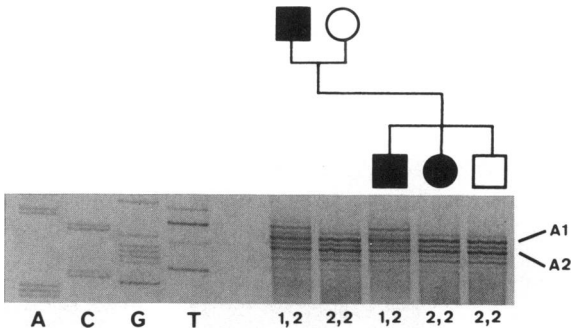

Figure 2 Partial pedigree of a TCOF1 family, showing a recombination event between 8FH10R and TCOF1 in an affected individual. Genotypings are indicated under each family member. Allele sizes were obtained by comparison with an M13mp18 sequencing ladder.

map and simultaneously allowed us to check for gross internal deletions/rearrangements within the YACs. In order to increase the accuracy of the map, we have found it advantageous to perform two separate CHEF runs, the first in the 0–100-kb range and the second in the 50–400-kb range (see Material and Methods). This has aided the identification of restriction sites, particularly those close to the ends of the YACs. This proved to be important in the identification of the HTF island associated with ANX6, which is situated 40 kb from the left end of 8FH10 (fig. 3*A* and *B*).

This map confirms the overlaps predicted from the STS content of the various clones and indicates that the contig contains minimal deletions or rearrangements. The map has further permitted us to assess the amount of overlap between adjacent YACs and to estimate the size of the TCOF1 critical region as being ~840 kb. This analysis simultaneously allowed us to identify any potential HTF islands located within the critical region. In total, 17 clusters of two or more rare-cutter sites were detected, 7 islands of which were found to contain three or more sites. The highest densities of rare-cutter restriction sites were found to be in the 150-kb interval immediately adjacent to the left ends of 3G3/29CC10, close to the D5S519 locus, and in a region of 150 kb between ANX6 and 8FH10R.

Discussion

In the present study we have used the relatively high density of markers around the TCOF1 locus to screen YAC libraries, using PCR. While the density of the markers was not high enough to permit map closure by using this approach alone, the creation of additional STSs representing the ends of the YACs has allowed us to assemble a contig encompassing the entire TCOF1 critical region. The STS sites corresponding to the YAC insert termini were also used to screen a somatic cell hybrid panel to check the

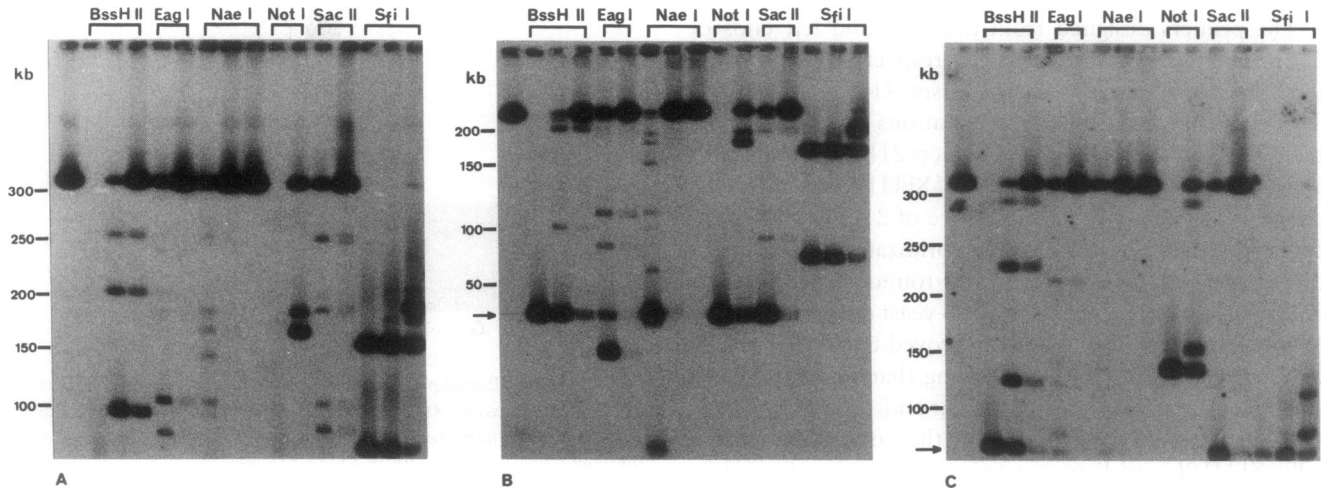

Figure 3 Restriction-mapping autoradiographs of YAC 8FH10. Yeast blocks were digested with decreasing amounts (left to right) of the enzymes indicated. The filters were probed with (*A* and *B*) a 2.7-kb *Pvu*II/*Bam*HI pBR322 fragment corresponding to the L arm of PYAC4 and (*C*) a 1.7-kb *Pvu*II/*Bam*HI PBR322 fragment recognizing the R arm of PYAC4; in panels *A* and *C* they were resolved from 50 to 400 kb, whereas in panel *B* they were was resolved from 0 to 150 kb. Clusters of rare-cutter restriction sites (potential CpG islands) are indicated by arrows.

clones for chimerism. Of the nine YACs tested, two were found to be chimeric—one each from the CEPH and ICI libraries. In addition, one YAC (21FC7) was found to have a small piece of yeast DNA at its right end.

In an attempt to further reduce the size of the TCOF1 critical region, the right end of YAC 8FH10 was used to screen a chromosome 5–specific cosmid library. The isolated clones were found to contain a highly informative STRP that shows recombination with TCOF1 in an affected individual. This marker defines the distal recombination boundary of the TCOF1 critical region, which is estimated to be ~840 kb. This estimate is in close agreement with the data generated by RH mapping and genetic linkage analysis (Loftus et al. 1993), confirming previous findings for this set of RHs in the region of the SMA locus (Francis et al. 1993; Thompson et al. 1993). In this region of the genome, however, 1 centiray is equivalent to ~46 kb, as opposed to both the 17 kb found in the SMA region (Thompson et al. 1993) and the 32 kb found in the Huntington disease region (Altherr et al. 1992) when the same RH panel is used. Previous estimates of the physical distance/centiray conversion for 5q31.2-qter have suggested that 1 centiray is equivalent to 51 kb (Warrington et al. 1992), which is very close to the estimate in the present study.

The combined results of genetic linkage analysis, RH mapping, FISH, and YAC contig assembly have allowed us to establish that the order of loci relative to TCOF1 is cen–RPS14–D5S519–ANX6/TCOF1–SPARC, confirming that RPS14 can be excluded from a causative role in

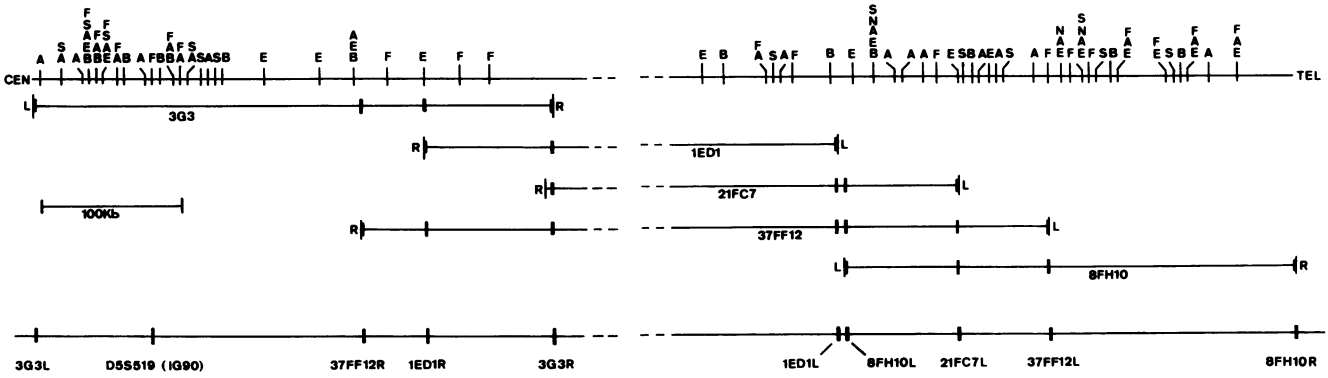

Figure 4 Rare-cutter restriction map of those YACs encompassing the TCOF1 critical region, showing the position and orientation of each clone. The STS content of each YAC is shown by black boxes at the bottom of the figure. The ANX6 STS lies in the interval between 8FH10L and 21FC7L. Restriction sites are as follows: A = *Nae*I; B = *Bss*HII; E = *Eag*I; F = *Sfi*I; N = *Not*I; and S = *Sac*II.

the pathogenesis of TCOF1 (Loftus et al. 1993; present study). Furthermore, we have demonstrated that ANX6 lies in the middle of the TCOF1 critical region, confirming previous results from RH mapping and linkage analysis (Loftus et al. 1993). The ANX6 locus is now being assessed as a potential candidate for being the TCOF1 gene. In addition, cosmids surrounding ANX6 are being screened for STRPs, which will be used to type recombinants in TCOF1 affected families, which could reduce the size of the candidate region significantly.

The distal region of chromosome 5 contains a high density of genes encoding growth factors, growth-factor receptors, hormone receptors, and neurotransmitter-receptor genes (Warrington et al. 1991, 1992). It is interesting that we have observed a high density of clustered rare-cutter restriction sites within our contig, as this suggests that a number of as-yet-unidentified genes may lie in the TCOF1 critical region. Now that the TCOF1 critical region has been cloned in YACs and a detailed physical map of the region has been created, recently described methods for the isolation of coding sequences can commence (Elvin et al. 1990; Buckler et al. 1991; Lovett et al. 1991; Parimoo et al. 1991), with a view to identifying further candidate genes that can be tested for disease-specific mutations.

Acknowledgments

The technical support of Miss Sara Edwards is acknowledged. We thank the Treacher Collins families for their interest and cooperation, without which the present study would not have been possible. We should also like to thank those clinicians who collected samples on our behalf. The financial support of Wellcome Trust grant 036797/Z/92/Z, Medical Research Council grants G9010336CB and G9204430, the Hearing Research Trust, the Independent Order of Odd Fellows, and the Oral and Dental Research Trust is gratefully acknowledged.

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