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Narrowing the Position of the Treacher Collins Syndrome Locus to a Small Interval between Three New Microsatellite Markers at 5q32-33.1

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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. The TCOF1 locus has been localized to chromosome 5q32-33.2. In the present study we have used the combined techniques of genetic linkage analysis and fluorescence in situ hybridization (FISH) to more accurately define the TCOF1 critical region. Cosmids IG90 and SPARC, which map to distal 5q, encompass two and one hypervariable microsatellite markers, respectively. The heterozygosity values of these three markers range from .72 to .81. Twenty-two unrelated TCOF1 families have been analyzed for linkage to these markers. There is strong evidence demonstrating linkage to all three markers, the strongest support for positive linkage being provided by haplotyping those markers at the locus encompassed by the cosmid IG90 (Z_{max} = 19.65; θ = .010). FISH to metaphase chromosomes and interphase nuclei established that IG90 lies centromeric to SPARC. This information combined with the data generated by genetic linkage analysis demonstrated that the TCOF1 locus is closely flanked proximally by IG90 and distally by SPARC.

Introduction

Treacher Collins syndrome (TCOF1) is an autosomal dominant disorder of craniofacial development, which has an incidence of 1/50,000 live births. The clinical features include (1) abnormalities of the pinnae (external ears), which are frequently associated with atresia of the external auditory canals and anomalies of the middle-ear ossicles (bilateral conductive hearing loss is therefore common) (Phelps et al. 1981); (2) hypoplasia of the facial bones, particularly the mandible and the zygomatic complex; (3) downward slanting of the palpebral fissures, with colobomata (notching) of the lower eyelids and a paucity of lid lashes medial to the defect; and (4) cleft palate. Expression of the gene is variable, but there is usually a reasonable degree of bilateral symmetry in the clinical features (Kay and Kay 1989). Some individuals are so mildly affected that it is difficult to reach a diagnosis and provide accurate genetic counseling. The mutated gene occasionally appears nonpenetrant, although, in most cases where this is suspected, careful examination of the obligate carrier frequently reveals minor stigmata of TCOF1 (Dixon et al. 1991*a*). In addition, only 40% of cases have a previous family history; the remaining 60% appear to arise as the result of de novo mutations (Connor and Ferguson-Smith 1988).

The gene mutated so as to cause TCOF1 has been mapped to the long arm of chromosome 5 at 5q31-34 (Dixon et al. 1991*b*). This localization was subsequently confirmed by Jabs et al. (1991). It is, however, likely to prove difficult both to refine this linkage by using "classical" markers and to detect genetic heterogeneity, because of a relative shortage of suitable pedigrees, the low informativity of the majority of RFLPs, and the nonrandom distribution of certain classes of highly informative markers (Willard et al. 1986; Royle et al.

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1988). To date, recombination between polymorphisms in a number of candidate genes and the TCOF1 phenotype in affected individuals would appear to have excluded these genes from a causal role in the etiology of TCOF1 (Dixon et al. 1991*b*; Jabs et al. 1991). In the absence of further candidate genes or of focusing cytogenetic abnormalities, the establishment of markers that closely flank the TCOF1 locus will be integral to the isolation of the mutated gene itself.

In this paper we report the identification of highly informative microsatellite markers (Weber and May 1989) that closely flank the TCOF1 locus. These markers have allowed us to make our families more informative for linkage and thereby refine the genetic position of the TCOF1 locus.

Subjects and Methods

Pedigrees

The pedigrees of 12 of the 22 families used in the current study have been presented elsewhere (Dixon et al. 1991*b*). The pedigrees of the remaining 10 families are presented in figure 1. All patients were examined by experienced clinicians and were scored as affected if they presented with the clinical signs noted above. Venous blood samples were taken with informed consent from 164 individuals, of whom 95 were affected. In all cases paternity was confirmed genetically.

Isolation of Cosmids

Three different cosmids were utilized in the experiments described in the present study. The first cosmid, SPARC, was obtained by screening a total human genomic cosmid library in pWE15 (Stratagene Cloning Systems) by using standard procedures (Sambrook et al. 1989) with a 0.6-kb *Eco*RI fragment of an osteonectin cDNA clone. The *Eco*RI fragment was derived from the 5′ end of the gene (Young et al. 1990). The gene designation for osteonectin is "SPARC." Positive primary clones were purified by two additional rounds of screening. Validity of the positive SPARC clones was confirmed by sequencing. The two remaining cosmids, IG90 and IG52, were derived from a cosmid library constructed using DNA from the somatic hybrid cell line HHW983, which contains chromosomes 5 and 10 as the sole human components. The isolation and physical mapping of clones from this library have been described elsewhere (Klinger et al. 1991).

DNA Analysis

The identification and optimization of polymorphic dinucleotide repeats encoded within the SPARC cosmids and IG90, as well as the utilization of these simple repeat polymorphisms for genotyping, were as described elsewhere for IG52 (Dixon et al. 1992). Negative controls were established for all reactions.

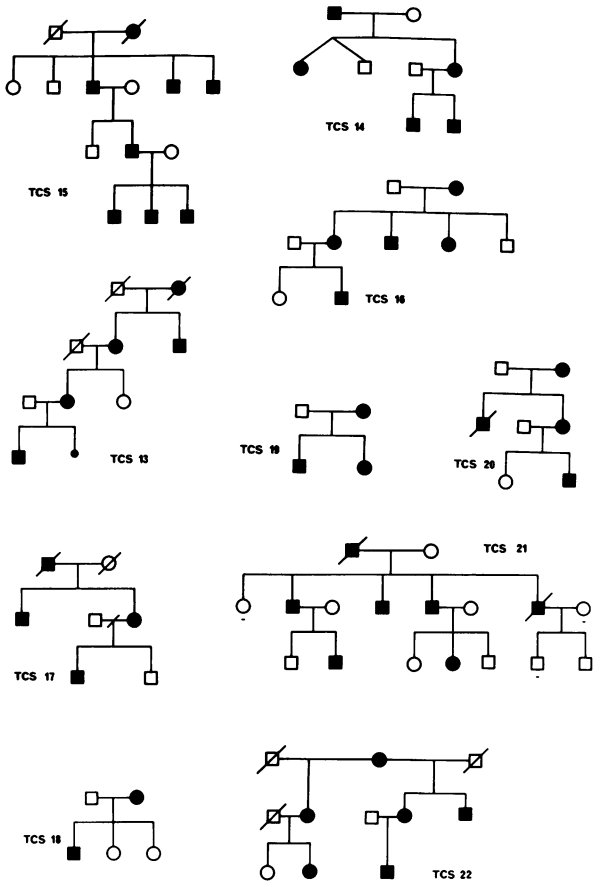

Figure 1 Pedigrees of the additional 10 TCOF1 families used in this study. The original 12 pedigrees have been presented elsewhere (Dixon et al. 1991*b*).

Linkage Analysis

The alleles were scored, and the data were coded in linkage format. To facilitate the linkage analysis, the genotype results were processed to produce a maximum of four alleles at each marker locus while preserving linkage information, as described by Ott (1978). The TCOF1 locus was modeled as an autosomal dominant, two-allele system. The gene frequency and the penetrance in heterozygotes were taken as .00001 and .99, respectively. Simulations have previously shown that altering the penetrance between .90 and .99 makes little difference to the results obtained (Dixon et al. 1991*a*). Pairwise analysis was performed using the

MLINK routine of the LINKAGE package (Lathrop et al. 1984, 1985). Maximum-likelihood estimates of sex-averaged recombination were calculated using ILINK. Significance was evaluated using the standard criterion ($Z > 3.0$).

Fluorescence In Situ Hybridization (FISH)

Probe haptenization and FISH were performed as described elsewhere (Lichter et al. 1990; Klinger et al. 1991; Curran et al. 1992). DNAs were labeled with either biotin-11-dUTP (Sigma, St. Louis) or digoxigenin-11-dUTP (Boehringer-Mannheim, Indianapolis). Biotinylated probes were detected with FITC-avidin (Vector Laboratories, Burlingame, CA) or cy3-streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), while digoxigenylated probes were detected with FITC-labeled anti-digoxigenin Fab (Boehringer-Mannheim). Slides were viewed using a Zeiss epifluorescence microscope equipped with filter sets for the visualization of DAPI alone and FITC plus cy3 simultaneously.

Cosmids IG52, IG90, and SPARC were mapped to metaphase chromosomes by using the method of Lichter et al. (1990). The chromosomal subregional localization for each cosmid was expressed as FLpter% values, which denotes the distance of the hybridization signal from pter relative to the contour length of the hybridized chromosome. Distance measurements were made using custom computer software (Mr. Timothy Rand), from digitized images displayed on a high-resolution color monitor. The FLpter% values were determined from 10 metaphase chromosomes and then were averaged.

Ordering of cosmids IG52, IG90, and SPARC was done by performing multicolor FISH using metaphase and interphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes. Cloned DNAs IG90 and SPARC could not be resolved from each other by using prometaphase chromosomes but could be distinguished from uniquely haptenated cosmid IG52. The order of colocalized IG90/SPARC (biotin) and digoxigenin-labeled IG52 relative to the centromere and telomere was determined by analyzing 50 hybridized metaphase chromosomes. Two parallel experiments, utilizing interphase multicolor FISH, were performed to resolve IG90 and SPARC and then to order this pair of markers relative to IG52. Digoxigenin-labeled IG90 and biotinylated SPARC were used for both experiments. IG52 was haptenated with biotin for the first experiment and with digoxigenin for the second. Two hundred hybridized interphase chromosomes were enumerated for the order of red and green hybridization signals.

Images of hybridized metaphase and interphase chromosomes were taken with a Zeiss epifluorescence microscope equipped with a cooled CCD camera (Photometrics, Tucson). Gray-scale images were captured with specific filter sets (DAPI, fluorescein, and cy3), processed with an automated thresholding routine (Recognition Technology, Holliston, MA), and merged to form a composite image. Photographs were taken from images displayed on a high-resolution color monitor.

Results

We have previously reported genetic linkage of the SPARC locus to TCOF1, with a Z_{max} of 3.75 and a θ of .038, by using an osteonectin cDNA probe to type both *Taq*I and *Msp*I RFLPs (Dixon et al. 1991*b*). To increase the genetic informativity of the SPARC locus, as well as to refine its physical localization via FISH with large cloned inserts, we screened a total human genomic cosmid library with the osteonectin cDNA clone. Three overlapping clones were identified. This small contig encompasses approximately 65 kb of genomic DNA. The validity of the osteonectin cosmid clones was confirmed by sequencing exon 5 of the gene, the nucleotide sequence that was generated being in perfect agreement with that already published (Villarreal et al. 1989).

To refine their localization on chromosome 5, the osteonectin cosmids were used as FISH probes against metaphase chromosomes. The chromosomal location of the hybridized probe, expressed as FLpter% (see Subjects and Methods), ranged from 82.2% to 89.9%, with an average of 85.0% This interval is within a chromosomal region to which we have previously mapped 13 other cosmids (Klinger et al. 1991), including the cosmids IG90 and IG52.

Screening of the osteonectin clones and the anonymous cosmid IG90 by hybridization with the poly(dC.dA)(dG.dT) probe revealed the presence of one (CA)n microsatellite in the former and two in the latter. Sequencing of these microsatellites revealed three perfect repeats; the SPARC cosmid contained a (CA)17, while both repeats present in the IG90 cosmid were (AC)24. The length and structure of the repeats suggested that they would be highly polymorphic (Weber 1990). Oligonucleotide primers were therefore constructed to the unique sequence flanking the repeat for use in the PCR (table 1). The sequences of the remaining markers have been presented elsewhere (Dixon et al. 1992).

Table 1

New Chromosome 5 Microsatellites

Clone Name	Locus	Primer Sequences	Primer Annealing Temperature	No. of Alleles	Allele Frequencies	Heterozygosity
IG90-1	D5S519	TACAGAGTGGAAAGCCCAGT CTTCCCACATAGCACTCACA	56°C	7	113 = 2.3, 109 = 5.3, 107 = 25.8, 105 = 41.7, 103 = 20.5, 101 = 3.7, 99 = .7	.72
IG90-3	D5S519	CTACTACCAGCAGCATTCTC ATCTGCAGTGTGAGGCAATG	57°C	8	128 = 6.7, 126 = 12.6, 124 = 24.2, 122 = 5.9, 120 = 16.3, 118 = 20.8, 116 = 12.6, 114 = .7	.82
SPARC	SPARC	TATGTTCACAAGAGGGTGTC ATCTCGCCACTGTACTCTAC	54°C	10	179 = 1.5, 177 = 17.8, 175 = 18.5, 173 = 33.3, 171 = 19.3, 169 = 5.3, 167 = 2.2, 165 = .7, 163 = .7, 157 = .7	.80

All three microsatellites were shown to be inherited in a codominant Mendelian fashion and are highly polymorphic, with heterozygosity values ranging from .72 to .81, as assessed from 60 unrelated individuals (table 1). The most informative locus is that recognized by the probes IG90-1 and IG90-3, as the combined use of these microsatellites can generate 56 possible haplotypes. The heterozygosity of this locus is .92. Segregation analysis in the 22 unrelated TCOF1 pedigrees revealed that all three markers showed highly significant evidence of linkage to the TCOF1 locus (table 2). Haplotyping the two microsatellites in the cosmid IG90 gave a Z_{max} of 19.65 at $\theta = .010$, providing the strongest support for positive linkage. The microsatellite at the SPARC locus was also very tightly linked to TCOF1 ($Z_{max} = 19.52$ at $\theta = .010$). All the families that have been studied in the present investigation suggest linkage of the TCOF1 locus to the markers IG90 and SPARC, with no families showing unequivocal evidence of nonlinkage. Inspection of those families that were informative for these markers revealed a single recombination event for each locus, both of which involved an affected individual (table 3). Linkage data between the markers 2D10 and IG52 and 15 TCOF1 families have been presented elsewhere (Dixon et al. 1992); the combined data from the analysis of these families together with the seven additional kindreds are presented in table 2.

Examination of the recombinant individuals revealed by pairwise linkage analysis (table 3) indicates that the TCOF1 locus is flanked proximally by the probes 2D10 and IG90 and distally by SPARC and IG52, all informative meiotic events supporting this order. These data are in agreement with those presented by Dixon et al.

Table 2

Pairwise Z Values for TCOF1

	Z AT θ =								
PROBE	0	.05	.10	.15	.20	.30	.40	Z_{max}	$\hat{\theta}$
IG90-1	6.53	12.28	10.92	9.41	7.86	4.76	2.00	12.91	.014
IG90-3	8.00	13.08	11.63	10.01	8.33	4.96	1.98	13.77	.013
IG90-H[a]	13.10	18.57	16.60	14.43	12.15	7.49	3.15	19.65	.010
SPARC	14.95	18.48	16.56	14.42	12.20	7.62	3.31	19.52	.010
2D10	6.81	10.12	9.19	8.01	6.71	4.03	1.63	10.32	.026
IG52	−9.35	11.44	11.19	10.19	8.85	5.73	2.52	11.50	.063

[a] Represents the data derived from haplotyping the results obtained with probes IG90-1 and IG90-3.

Table 3

Analaysis of Individuals Showing Recombination between Chromosome 5q Markers and the TCOF1 Locus, Indicating That the Likely Position for TCOF1 Is in the Interval between IG90 and SPARC

Recombinant, Clinical Description	cen-2D10-IG90-SPARC-IG52-tel			
1, Clinically normal	R	NR	NR	NR
2, Clinically normal	NR	NR	NR	R
3, Severely affected	NR	NR	NR	R
4, Moderately affected	R	NR	NR	NR
5, Moderately affected	...	NR	NR	R
6, Moderately affected	NR	NR	NR	R
7, Severely affected	...	NR	R	R
8, Mildly affected	...	R	NR	NR

(1992). The markers that most closely flank TCOF1 are IG90 and SPARC, with two meiotic events supporting this order and, again, none supporting other orders. Both recombinant individuals were clinically affected; recombinant 7 (table 3) was severely affected with down-slanting palpebral fissures, severe zygomatic and mandibular hypoplasia, and anomalies of the pinnae. This individual has also had an affected pregnancy terminated (Nicolaides et al. 1984). Recombinant 8 (who was not included in the recombinants listed in Dixon et al. 1992) appears to be only minimally affected with down-slanting palpebral fissures and mandibular hypoplasia. The results of the marker-marker pairwise linkage analysis derived from the TCOF1 pedigrees are presented in table 4. They showed that the two flanking markers IG90 and SPARC are tightly linked (Z_{max} = 23.62 at θ = .026). The confidence limit (1-lod support interval) for the estimate of recombination between IG90 and SPARC is θ = .007–.065 (Conneally et al. 1985).

Physical Mapping and Ordering by FISH

Attempts at confirming the genetic order of SPARC and IG90 by using multicolor FISH on metaphase chromosomes were unsuccessful (data not shown). These results suggest that these loci may be within 1 Mb of each other, the resolution limit of FISH on metaphase chromosomes. To circumvent this limit in resolution, we chose to order SPARC and IG90, genetically and physically, relative to a third polymorphic cosmid from this region, IG52 (Dixon et al. 1992). Genetic linkage analysis in the 22 TCOF1 pedigrees favored the order cen-IG90-SPARC-IG52-tel.

This proposed order was tested by cohybridizing digoxigenin-labeled IG52, biotinylated SPARC, and biotinylated IG90 to metaphase chromosomes (fig. 2, *top left panel*). Biotinylated probes were detected with cy3-streptavidin and emitted a red fluorescence, while digoxigenin-labeled probes were visualized with FITC-conjugated antidigoxigenin Fab fragments and exhibited a green fluorescence. In 42 of 50 metaphase chromosomes, IG52 mapped distal to colocalized SPARC and IG90; red and green hybridization signals were colocalized in the remaining 8 metaphase chromosomes. These results are consistent with those obtained from the coarse metaphase map, which showed IG52 to be distal to IG90 and SPARC (Klinger et al. 1991).

To enhance the resolution, we performed two parallel multicolor hybridization experiments and analyzed hybridized interphase nuclei. Biotinylated IG52 (red) was used in the first experiment, and digoxigenin-labeled IG52 (green) was used in the second. Biotinylated SPARC cosmid (red) and digoxigenylated IG90 (green) were used in both hybridizations. Examples of hybridized nuclei are presented in the bottom left (experiment 1) and top right (experiment 2) panels of figure 2. Two hundred hybridized interphase chromosomes were analyzed for the order of red and green hybridization signals. In experiment 1, 148 (74%) of the chromosomes showed the order red-red-green, while only 52 were seen with the order red-green-red. When IG52 was labeled with digoxigenin rather than with biotin (experiment 2), 150 (75%) of the chromosomes showed the order green-red-green. Collectively, the interphase results support the order IG90-SPARC-IG52. When these results are combined with the metaphase hybridization results and the genetic linkage data, the chromosomal order of these markers relative to TCOF1 is cen-IG90-TCOF1-SPARC-IG52-qter.

Discussion

In the present investigation we report the isolation of two cosmids that contain highly informative microsatel-

Table 4

Marker-Marker Pairwise *Z* Values (below Diagonal) and Corresponding θ Estimates (above Diagonal)

	2D10	IG90	SPARC	IG52
2D10	—	.016	.026	.076
IG90	18.14	—	.026	.089
SPARC	16.62	23.62	—	.070
IG52	7.31	11.78	14.26	—

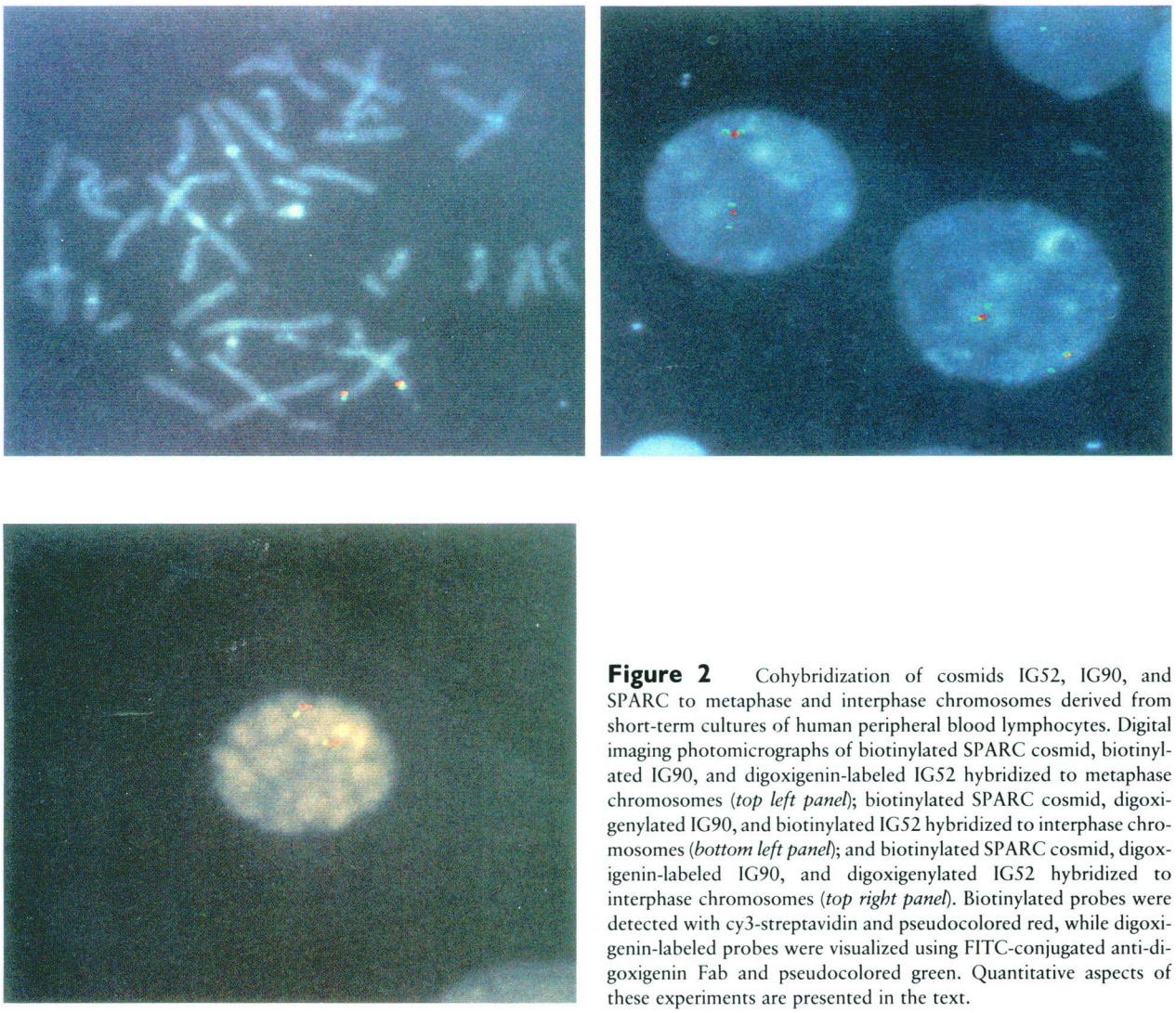

Figure 2 Cohybridization of cosmids IG52, IG90, and SPARC to metaphase and interphase chromosomes derived from short-term cultures of human peripheral blood lymphocytes. Digital imaging photomicrographs of biotinylated SPARC cosmid, biotinylated IG90, and digoxigenin-labeled IG52 hybridized to metaphase chromosomes (*top left panel*); biotinylated SPARC cosmid, digoxigenylated IG90, and biotinylated IG52 hybridized to interphase chromosomes (*bottom left panel*); and biotinylated SPARC cosmid, digoxigenin-labeled IG90, and digoxigenylated IG52 hybridized to interphase chromosomes (*top right panel*). Biotinylated probes were detected with cy3-streptavidin and pseudocolored red, while digoxigenin-labeled probes were visualized using FITC-conjugated anti-digoxigenin Fab and pseudocolored green. Quantitative aspects of these experiments are presented in the text.

lite markers, both of which show highly significant evidence of linkage to the TCOF1 locus. The evaluation of key recombination events is of critical importance in the fine-mapping stage of linkage analysis. The identification of close flanking markers is virtually a prerequisite for the isolation and characterization of mutated genes, particularly in the absence of candidate genes or focusing cytogenetic anomalies. Indeed, the lack of flanking markers has hampered the search for a number of disease genes. Analysis of recombinant individuals in our TCOF1 families suggests that the TCOF1 locus is closely flanked by IG90 and SPARC.

The markers presented in this paper have been physically mapped to the long arm of chromosome 5, at 5q32-33.2, by FISH (Klinger et al. 1991; Le Beau et al., in press; present investigation). The construction of coarse metaphase maps followed by multicolor FISH to both metaphase and interphase chromosomes has provided a means of ordering two cloned DNAs near the TCOF1 locus. The results established that cosmid IG90 lies centromeric to SPARC. The combined genetic and physical mapping data have therefore allowed us to demonstrate that the TCOF1 locus is flanked proximally by IG90 and distally by SPARC. Genetically, IG90 appears to map within 2.6 map units of SPARC; however, it was not possible to resolve these loci by FISH using metaphase chromosomes. This observation suggests that these loci may lie within 1 Mb of one

another (the limit of resolution of FISH on metaphase chromosomes).

To date, only a limited number of TCOF1 families have been studied (Dixon et al. 1991*b*, 1992; Jabs et al. 1991). All the families that have been analyzed (approximately 23 families) suggest linkage of the disease locus to markers in the region 5q31-34, with none showing unequivocal evidence of nonlinkage. The presentation of the additional seven families in the present investigation brings the total studied to date to 30, and the data generated support genetic homogeneity. Dixon et al. (1992) have previously reported microsatellites that flank the TCOF1 locus; however, these are of limited use for diagnostic predictions. Currently, prenatal diagnosis is only possible using either ultrasound or fetoscopy in midtrimester (Nicolaides et al. 1984). Moreover, while the gene mutated so as to cause TCOF1 occasionally appears nonpenetrant, in most cases where this is suspected, careful examination of the obligate carrier usually reveals minor stigmata of the syndrome (Dixon et al. 1991*a*). Nevertheless, some individuals are so mildly affected that it is difficult to reach a diagnosis and thereby provide accurate genetic counseling. The linkage data reported in the current investigation with close flanking markers permits first-trimester prenatal DNA diagnosis and also enables diagnostic predictions in certain mildly affected and apparently unaffected individuals.

To date, a number of candidate genes would appear to have been excluded from a causal role in the etiology of TCOF1 (Dixon et al. 1991*b*; Jabs et al. 1991). Nonetheless, the large number of growth factors and receptor genes in the region 5q21-35 makes it a possible source of other, as yet unidentified genes encoding growth factors or receptors that have arisen by duplication and divergence. This is a particularly interesting possibility in that it has been proposed that the development of the face involves a complex interaction of growth factors and extracellular matrix molecules (Ferguson 1988). Now that the TCOF1 locus has been assigned to a small genetic interval, the construction of a detailed physical map of the critical region can commence. In this regard, the fact that the markers documented in the present study are formatted for the PCR means that they can be used as sequenced tagged sites, thereby permitting the combination of the genetic and physical maps of the region (Olson et al. 1989). For instance, these markers can rapidly be placed on the existing radiation-reduced hybrid maps of the region (Warrington et al. 1991, 1992), thereby permitting the identification of candidate loci that lie within the TCOF1 critical region. Furthermore, they can be used to screen yeast artificial chromosome libraries (Anand et al. 1990) as a prelude to the creation of long-range continuity between the flanking markers. These markers will also prove useful in the refinement of the localization of other disease loci that have been mapped close to TCOF1, including diastrophic dysplasia and hyperekplexia (Hastabacka et al. 1991; Ryan et al. 1992).

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