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Molecular Characterization of the Marker Chromosome Associated with Cat Eye Syndrome

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Summary

Cat eye syndrome (CES) is associated with a supernumerary bisatellited marker chromosome which is derived from duplicated regions of 22pter-22q11.2. In this study we have used dosage and RFLP analyses on 10 CES patients with marker chromosomes, by using probes to five loci mapped to 22q11.2. The sequences recognized by the probes D22S9, D22S43, and D22S57 are in four copies in all patients, but the sequences at the more distal loci, D22S36 and D22S75, are duplicated only in some individuals. D22S36 is present in three copies in some individuals, and D22S75 is present in two copies in the majority of cases. Only three individuals have a duplication of the most distal locus examined (D22S75), and these individuals have the largest marker chromosomes identified in this study. From the dosage analysis it was found that the marker chromosomes are variable in size and can be asymmetric in nature. There is no obvious correlation between the severity of the phenotype and the size of the duplication. The distal boundary of the CES critical region (D22S36) is proximal to that of DiGeorge syndrome, a contiguous-gene-deletion syndrome of 22q11.2.

Introduction

Cat eye syndrome (CES) is highly variable in phenotype, but criteria for diagnosis include ocular coloboma (of the iris and/or retina); anal atresia (with or without fistula); preauricular skin tags and pits; heart defects (especially total anomalous pulmonary venous return); dysmorphic features, such as hypertelorism and down-slanting palpebral fissures; urogenital defects; and mental retardation (mild to moderate) (Schinzel et al. 1981). Cytogenetic analysis of CES patients is usually characterized by the presence of a supernumerary bisatellited marker chromosome derived from an inverted duplication of the short arm and proximal long arm of chromosome 22 (inv dup 22pter-22q11.2) (McDermid et al. 1986). This marker chromosome results in partial tetrasomy for this region. Previous dosage analysis of CES marker chromosomes revealed that all six CES individuals studied had four copies of the D22S9 locus, which maps to 22q11.2 (McDermid et al. 1986).

The 22q11.2 region is also associated with a deletion that results in DiGeorge syndrome (DGS), a developmental defect of the third and fourth pharyngeal pouches. It is typically characterized by absent or hypoplastic thymus and parathyroids, as well as by conotruncal cardiac malformations (DiGeorge 1965; Robinson 1975; Conley et al. 1979; Greenberg et al. 1988). Microdeletions of 22q11.2 have been detected, and a critical region for DGS has been delimited (Carey et al. 1992; Driscoll et al. 1992*a*). Velocardiofacial syndrome is also associated with similar deletions of this region and shares some phenotypic features with DGS (Driscoll et al. 1992*b;* Kelly et al. 1993).

Since DGS and CES map to the same cytogenetic region, there has been speculation that gene(s) deleted in DGS may be overexpressed in CES. It has been assumed that these gene(s) are responsible for at least some of the phenotypic features of CES (Sharkey et al. 1992). In order to determine the extent of 22q11 duplication associated with CES and the relationship of the duplicated region to the region commonly deleted in DGS, we have molecularly characterized the marker chromosomes in 10 CES cases. Dosage analysis was carried out with five previously described DNA probes assigned to the proximal region of 22q11.2 (see Material and Methods). We show that the marker chromosomes vary in size, that at least some are asymmetrically duplicated, and that the CES critical region is distinct from that of DGS.

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Material and Methods

Clinical and Cytogenetic Evaluation

Clinical information was obtained either from the referring physicians or from the literature in cases CM05, CM06 (cases 2 and 5, respectively, in Schinzel et al. 1981), CM10 (Buckton et al. 1985), and CM01 (Rosenfeld et al. 1984). Cytogenetic analysis was performed using standard techniques at the respective clinical cytogenetics laboratories.

Cell Lines/DNA

A DNA sample from CM02 was provided by Dr. Cheryl R. Greenberg. A previously established lymphoblastoid cell line for CM10 was obtained from Dr. Veronica Van Heyningen. The other CES lymphoblastoid lines were established from blood samples obtained from Dr. Albert Schinzel (CM05 and CM06), Dr. Michael Baraitser (CM09), Dr. Ram S. Verma (CM01), Dr. Richard Stallard (CM04), Dr. William J. Rhead (CM03), and Dr. Jacqueline Siegel-Bartelt (CM07 and CM08). Three control cell lines were obtained from the Coriell Institute for Medical Research (NIGMS). GM03657 is a normal lymphoblastoid line, GM07106 is a trisomy 22 CVS line, and GM02325 is a fibroblast line with partial trisomy 22 [47,XX, +der(22)t(16,22)(p13.3;q12.2)] (Sutherland et al. 1989).

Probes

The 22q11.2 probes used in this study have been described elsewhere. D22S9 (P22/34) was isolated from a chromosome 22–enriched library and localized by in situ hybridization (McDermid et al. 1986). D22S43 (H32), D22S57 (H98), and D22S36 (H11) were isolated from flow-sorted chromosome 22 library LL22NS01 from American Type Cell Culture/NIH (Budarf et al. 1991). D22S75 (N25) was isolated from a *Not*I end-clone library (McDermid et al. 1989). D21S110 (P21-4U), a chromosome 21–specific probe (Spinner et al. 1989) was used as the reference to normalize ratios for dosage analysis.

The order of the loci, proximal to distal, on chromosome 22q11.2 is as follows: D22S9–D22S43–D22S57–D22S36–D22S75. This was determined by genetic mapping in CEPH pedigrees (Fibison et al. 1990; Driscoll et al. 1992*a*). Deletion mapping in DGS confirmed the order of D22S36 relative to D22S75 (Driscoll et al. 1992*a*). These five loci span a genetic distance of 14.6 cM (sex-averaged) (Fibison et al. 1990).

D22S43 detects a *Taq*I polymorphism with alleles of 5.0, 4.0, or 3.5 kb. D22S57 detects a *Bst*XI polymorphism with alleles of 2.6 or 2.0 kb. D22S75 detects a *Taq*I polymorphism with alleles of either 3.3 or 1.0 kb, in addition to a 1.6-kb constant band.

For FISH analysis, cosmids specific to D22S36 (cosS36), D22S181 (cosS181), and D22S39 (cosS39) were used. All three cosmids were isolated from the LL22NC03 library (Human Genome Center, Lawrence Livermore National Laboratory). D22S181, D22S36, and D22S39 have been described elsewhere (Budarf et al. 1991; Lekanne Deprez et al. 1991). A cosmid specific to D22S57 was unavailable, so cosS181 was used, because of its close proximity (<600 kb) to D22S57 (authors' unpublished observations). D22S39 maps to the distal tip of the long arm of chromosome 22 and was used as a control locus, to identify the normal chromosomes 22.

DNA Studies

DNA was extracted for all cell lines by routine methods. The DNA was digested with the appropriate restriction enzymes as recommended by the manufacturer (New England Biolabs or GIBCO/BRL). DNA was separated by 0.8% agarose gels and transferred to GeneScreen Plus (DuPont) by the method of Southern (1975).

DNA probes were isolated via digestion with appropriate enzymes and purified in 0.8% low-melt agarose (Sea Plaque, FMC) by gel electrophoresis. These probes were labeled with (α-^{32}P)dCTP by the random-primer method (Feinberg and Vogelstein 1984). D21S110 and D22S75 were preannealed with sonicated placental DNA (Litt and White 1985).

Hybridizations were performed at 65°C for 18–24 h in a roller-bottle hybridization oven (Tyler Research) with 6.6% SDS, 1 mM EDTA, 0.25 M sodium phosphate pH 6.5, 4.7 × Denhardt's solution, and herring-sperm DNA (100 μg/ml) (slight modification of Church and Gilbert 1984). Blots were washed with 2 × SSC/0.2% SDS at room temperature for 10 min, 0.2 × SSC/0.2% SDS at 65°C for 15 min, followed by a final wash with 0.1 × SSC/0.2% SDS at 65°C for 15 min. Blots were then exposed to Kodak XAR-5 film at −70°C for an appropriate length of time (typically 24–48 h). Prior to rehybridization, bound probe was removed by boiling the blots for 20–25 min in a large volume of 0.1 × SSC/1% SDS (as recommended by DuPont).

Dosage Analysis

Two methods were utilized to determine copy number of the 22q11 test probes—quantitative dosage analysis and RFLP analysis.

Quantitative dosage analysis.—Quantitative hybridization is associated with several variables, which makes copy-number determination problematic. Prior studies in the lab of H. E. McDermid indicated that analysis using small sample sizes, mean ratios, and arbitrary cutoff points to determine copy number was a relatively unreliable method of analysis. The approach taken to reduce the potential errors was to increase the number of replicates (sample size) and to analyze the resulting data with a nonparametric statistical method. Seven replicates of DNAs

from a normal disomic control, a control trisomic for chromosome 22, and three patients with CES were loaded onto a single gel. Concentrations of DNA samples were previously determined such that approximately equal amounts were loaded in each lane. The resulting filters were hybridized to the test probes and the nonsyntenic reference probe. Hybridization signals were quantified from the autoradiograms by the Biophotonics Gel Print 2000i system with the Gel Print Toolbox software (version 2.0). To avoid the variability that may occur across a single band, signals were calculated as the average intensity across the whole band. Standardized signal ratios for all 35 gel lanes were calculated as the ratio of the test probe to the reference probe, thereby correcting for variable DNA concentration across the lanes.

For each test probe, data sets comprising a maximum of seven standardized ratios were established for each of the disomic and trisomic control DNAs and for the respective CES patients. If nonspecific background interfered with the analysis of bands on an autoradiogram, those ratio values were removed from the data set. For each test probe, the two control data sets (disomic and trisomic) were compared statistically via the Wilcoxon rank sum test (Wilcoxon 1945; Wilcoxon and Wilcox 1964). Occasionally, when the *s*um of *r*anks of the *t*risomy control data set (SR_T) was not significantly greater than that of the disomic control data set (SR_D), then all associated data were eliminated. If the SR_T was significantly greater than the SR_D, then the CES patients' data sets (SR_P) were compared with those of the controls. It was assumed that, for each of the five test loci investigated, patients possessed two, three, or four copies of the sequence. Copy number was then determined from the statistical results by using the following criteria: (i) *two copies,* if the SR_P is not significantly greater than the SR_D and is significantly less than the SR_T, (ii) *three copies,* if the SR_P is significantly greater than the SR_D but is not significantly greater than the SR_T, and (iii) *four copies,* if the SR_P is significantly greater than both the SR_D and SR_T. In all cases a probability of .05 was used as the level of significance.

RFLP analysis.—In RFLP analysis, three or four copies are more readily discernible, because in heterozygotes they are distributed between two or more alleles. This distribution results in a higher relative signal ratio than is obtained by other more conventional means. For example, three copies may be represented by two alleles in a heterozygote, with a 2:1 signal ratio or a 100% signal difference. If the same duplication is quantified using test and reference probes, a 3:2 signal ratio is observed, with only a 50% signal difference. Typically there is no need for a reference locus to standardize for DNA concentration with RFLP analysis, because the alleles of a given polymorphic locus act as internal controls. However, in the present study, a heterozygote that shows two alleles of equal intensity indicates only an even copy number for this locus (either two or four copies). These cases were easily resolved by the results obtained from the test/reference probe dosage analysis. The major drawback of RFLP analysis is the requirement for heterozygosity. RFLP analysis was performed with the polymorphic probes for the loci D22S43, D22S57, and D22S75. The intensities of the polymorphic bands were quantified from the autoradiograms, as described above. In each case, three or four replicates were used, and the ratios calculated for the band intensities in heterozygous CES individuals were compared with those of disomic and trisomic heterozygous controls in order to determine copy number.

FISH.—Metaphase spreads were prepared from the lymphoblastoid cell lines CM01, CM03, CM04, CM07, CM09, and CM10. They were then cohybridized with biotinylated-11-dUTP–labeled test probes (cosS36 or cosS181) and the control probe (cosS39) and were visualized with fluorescein-labeled avidin by methods described elsewhere (Lichter et al. 1990; Driscoll et al. 1993).

Results

Clinical and Cytogenetic Analysis

All the patients were referred with a diagnosis of presumed CES because of the presence of some or all of the cardinal phenotypic features of CES (coloboma, skin tags/pits, and heart and anal defects). Routine cytogenetic analysis for all patients in this study revealed a supernumerary marker chromosome (karyotype 47,+mar). CM07 and CM08 are the only related cases in this study (CM07 is the mother of CM08). In the 10 cases there was a typical wide spectrum of features, with the major clinical findings summarized in table 1.

Dosage Analysis

DNA was analyzed by quantitative dosage to determine copy number for the probes to the five loci D22S9, D22S36, D22S43, D22S57, and D22S75. An example of an autoradiogram used in the dosage analysis is shown in figure 1. In this case the standardized ratios were calculated for the test probes to loci D22S43 and D22S36, relative to the reference probe D21S110. This autoradiogram indicates that all three patients shown demonstrate the presence of four copies for D22S43. CM03 and CM04 are in three copies for D22S36, but CM05 is in two copies for this locus. The data analysis for these three patients for determination of D22S36 copy number is presented in table 2. The results for all patients and loci are summarized in table 3. It was assumed that all individuals have two, three, or four copies of each locus investigated. In all cases in this study, patients demonstrate four copies using the probes to the proximal three loci D22S9, D22S43, and D22S57. The probe to the locus D22S36 is present in at

Table 1

Summary of Major Clinical Findings in 10 CES Patients

	Patients									
	CM01	CM02	CM03	CM04	CM05	CM06	CM07	CM08	CM09	CM10
Cardinal features:										
Coloboma	−	−	+	+	+	+	−	−	+	+
Preauricular tags/pits	+	+	+	+	+	+	+	+	+	?
Anal defects	+	+	−	+	+	+	+	+	?	?
Heart defects[a]	−	−	pda	pda	−	tap	−	m	tof	?
Developmental status[b]	N	N	?	DD	LN	LN	LN	LN	N	MR
Dysmorphic features	+		+		+	+				
Urogenital defects		+		+		+		+		
Intestinal anomalies			+			+				
Skeletal defects			+							

NOTE.—A plus sign (+) indicates presence of the phenotypic characteristic; a minus sign (−) indicates absence of the feature. A question mark (?) indicates that no information was available. Ages at developmental classification were as follows: CM01, 4 mo; CM02, 21 mo; CM04, 4 years; CM05, 15 years; CM06, 30 years; CM07, 31 years; CM08, 4 years; and CM09, 28 years. No information was available for CM03 and CM10. Dysmorphic features included down-slanting palpebral fissures (CM01, CM03, CM05, and CM06), high-bridged nose (CM04 and CM05), epicanthal folds (CM05 and CM06), hypertelorism (CM04, CM05, and CM06), and micrognathia (CM01 and CM04). Urogenital defects noted were undescended testis (CM02), small genitalia (CM04, CM06, and CM08), and an absent kidney (CM06). Intestinal anomalies were Hirschsprung disease (CM03) and malrotated gut (CM06). The only skeletal defect identified was Wormian bones and large fontanels (CM03). Blank space indicates that the presence of the feature was not reported in the clinical information.

[a] Heart defects are classified as follows: pda = patent ductus arteriosus; tap = total anomalous pulmonary venous return; tof = tetrology of fallot; and m = presence of a murmur but no specified defect.

[b] Developmental status is classified as follows: N = normal development; LN = low-normal range in IQ; DD = severe developmental delay; and MR = mental retardation.

least three copies in 4 of 10 cases and in two copies in 6 of 10 cases. The probe to D22S75 is present in two copies in 7 of 10 cases.

RFLP Analysis

DNA samples were analyzed for the polymorphic probes to D22S43, D22S57, and D22S75. Copy number was determined for heterozygous individuals for the respective probes. All 10 individuals were informative for D22S43, and the ratio of allele signal intensities was calculated and compared with those of the disomic and trisomic heterozygous controls, to approximate copy number. Figure 2 shows a composite autoradiogram of Southern blots for seven of the genomic DNAs digested with *Taq*I and probed with D22S43, which shows three alleles. CM01, CM05, and CM09 were heterozygous with two alleles of approximately equal band intensity, suggesting an even copy number (two or four). When this information is used in conjunction with the test/reference-probe method of quantitative dosage analysis, it was apparent that these individuals demonstrated the presence of four copies of this locus. CM03, CM04, CM07, and CM10 were heterozygous, with two alleles of unequal band intensities indicating three or four copies (CM03 and CM10 are not shown). Densitometric analysis, calculation of ratios, and comparison with the controls revealed that these ratios were indicative of four copies for this locus (significantly greater than a 2:1 ratio). These findings confirmed prior dosage analysis. CM02 (not shown), CM06, and CM08 possessed three alleles, and, in all cases, two of the allele bands were of equal intensity, and the third was approximately twofold greater, indicating a total of four copies at this locus.

Only four individuals were heterozygous for the polymorphic alleles produced by *Bst*XI and detected by D22S57 (not shown). CM03 and CM04 showed allele bands of equal intensity (two or four copies), indicating four copies when quantitative dosage analysis results are included. CM06 and CM09 were heterozygous, with two alleles of unequal band intensities demonstrating a 3:1 ratio, confirming that this DNA sequence was present in four copies.

D22S75 was informative for only three individuals. CM06 and CM08 were heterozygous for the *Taq*I polymorphic alleles, with bands of equal intensity indicating two copies of D22S75 when compared with the previous dosage findings. CM02, however, showed bands of unequal intensity, with one allele being approximately twice the intensity of the other, confirming D22S75 to be in three copies in CM02. The results of both the dosage methods are shown in table 2.

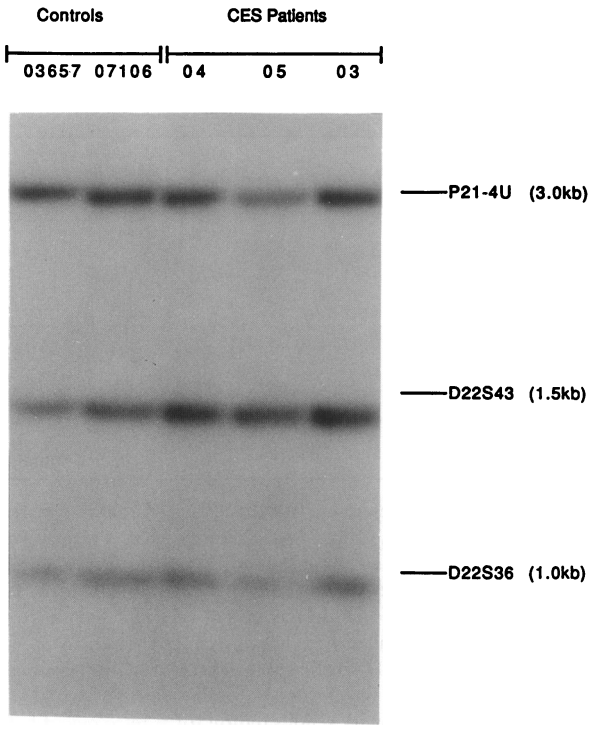

Figure 1 Autoradiogram showing one replicate set from a Southern blot of *Hin*dIII-digested DNA from a normal control (GM03657), trisomy control (GM07106), and three CES patients—CM04, CM05, and CM03. The blot was hybridized with the reference probe D21S110 (P21-4U, 3.0 kb) and the test probes to the loci D22S43 (1.5 kb) and D22S36 (1.0 kb). When standardized signal ratios are calculated (test probe signal/reference probe signal), it can be seen that all three patients show four copies for D22S43, whereas CM03 and CM04 are in three copies for D22S36, and CM05 shows two copies for D22S36 (table 2).

FISH Analysis

In order to confirm the localization of the duplicated sequences on the marker chromosomes, CM01, CM03, and CM04 were cohybridized with the cosmid probes cosS36 and cosS39 (CM02 cells were unavailable). These three patients were previously shown to have duplications of D22S36, by DNA dosage analysis. The cosS36 signals were observed on the marker chromosomes of CM01 and CM03, as expected. However, no cosS36 signal was observed on the marker chromosome of CM04. Instead, it was noted that one of the intact chromosomes 22 consistently had greater signal intensity for cosS36, while the distal reference cosmid, cosS39, appeared to be of similar intensity on both homologues. Furthermore, the two chromosomes 22 of CM04 could be distinguished from one another on the basis of an observed cytogenetic polymorphism of the short arm (fig. 3). In 84% (21/25) of metaphase spreads examined, one chromosome 22 demonstrated greater fluorescent signal for cosS36. The brighter signal was always on the chromosome with the smaller short arm, chromosome "a" in figure 3. These results suggest that, in addition to a marker chromosome, CM04 has an interstitial duplication encompassing D22S36 on one of the chromosomes 22. When CM04 was cohybridized with cosS181 and cosS39, a cosS181 signal was observed on the marker chromosome, as expected. Probe cosS181 also hybridized to the marker chromosomes of the other cell lines tested (CM07, CM09, and CM10).

Discussion

From the two methods of dosage analysis used in this study, the extent of duplication (three or four copies) in 10 CES marker chromosomes was determined. The smallest marker chromosomes span the loci D22S9–D22S57 (in CM05, CM06, CM07, CM08, CM09, and CM10); the largest span at least D22S9–D22S75 (in CM01, CM02, and CM03). If the supernumerary chromosome associated with CES were a true isodicentric chromosome, then one would expect tetrasomy for all loci present on the marker chromosome. Thus the finding of trisomy for some 22q11.2 loci suggests breakpoint heterogeneity and asymmetry. This study has demonstrated breakpoint asymmetry in at least two marker chromosomes of unrelated individuals. This has also been observed with chromosome 15–derived markers (Robinson et al. 1993). The implication from such findings is that these derivative chromosomes may possibly be a product of misalignment and exchange over a considerable distance. Despite the variability of marker chromosome sizes, there appears to be clustering of breakpoints. One such region lies between D22S57 and D22S36, where 75% of the breakpoints occur. The size of this region is not currently known. Isolation and cloning of rearrangement breakpoints will help clarify the mechanism involved in marker formation.

DNA dosage analysis can determine the presence of additional copies of a unique DNA sequence, but it does not indicate the location of the duplicated sequences. We initially hypothesized that duplications of chromosome 22 loci would be associated with the CES marker chromosome. To test this hypothesis, limited FISH studies were performed. By FISH analysis, all samples tested demonstrated hybridization to the marker chromosome with cosS181 (D22S181), as expected. In addition, in two of the three cases tested, the marker chromosome also hybridized to cosS36. For case CM04, a signal for cosS36 was not observed on the CES marker. In this patient, it is assumed, on the basis of the differential hybridization intensity of cosS36 on the chromosomes 22, that two of the three copies of D22S36 were located on one of the cytogenetically normal chromosomes. These results suggest an interstitial duplication in CM04. The presence of an interstitial duplication in combination with a CES marker is an intriguing result, and further studies may elucidate a possible mechanism for marker-chromosome generation.

Table 2

Example of Data Used in Dosage Analysis

	Normalized Ratios for the Test Probe D22S36				
Ranking	Disomic Control	Trisomic Control	CM04	CM05	CM03
1	.58	.78	.74	.53	.81
2	.60	.79	.79	.57	.82
3	.66	.85	.90	.64	.91
4	.68	.89	.96	.66	.91
5	.68	.90	.98	.69	.95
6	.73	.95	1.08	.70	.96
7	.78	.98	1.14	.72	1.00
Average	.67	.88	.94	.64	.91
Copy no.	2	3	3	2	3

Note.—The above sample of normalized ratios for D22S36 was calculated from quantified autoradiographic bands (band intensity for D22S36/band intensity for D21S110) (see fig. 1). The averages are also shown for each of the normalized-ratio data sets. The control data sets show very little overlap. Considerable overlap between the CM03/CM04 data sets with the trisomic control data set indicates three copies in these patients for this locus. The CM05 data set shows overlap with the disomic control data set, which indicates two copies for D22S36. The Wilcoxon rank sum test confirms these conclusions statistically.

Previous studies (McDermid et al. 1986) identified a CES patient who possessed only three copies of the 22q11.2 region because of a visible interstitial duplication (Reiss et al. 1985). Therefore, the critical region of CES can be defined as the region that exists in at least three copies in all CES individuals. We have shown that the CES critical region lies proximal to D22S36, as it is not duplicated in 6 of the 10 cases in this study. There are no physical estimates of the size of the region involved in the CES minimal duplicated region, but from genetic linkage data it is in the order of 14 cM (Fibison et al. 1990). The minimal defined CES critical region is clearly distinct from that of DGS,

Table 3

Summary of Dosage and RFLP Analyses

	Patients									
Locus and Analysis	CM01	CM02	CM03	CM04	CM05	CM06	CM07	CM08	CM09	CM10
D22S9:										
Dosage	4	4	4	4	4	4	4	4	4	4
D22S43:										
Dosage	4	4	4	4	4	4	4	4	4	4
RFLP	2/4	4	4	4	2/4	4	4	4	2/4	4
Combined	4	4	4	4	4	4	4	4	4	4
D22S57:										
Dosage	4	4	4	4	4	4	4	4	4	4
RFLP	NI	NI	2/4	2/4	NI	4	NI	NI	4	NI
Combined	4	4	4	4	4	4	4	4	4	4
D22S36:										
Dosage	4	3	3	3	2	2	2	2	2	2
D22S75:										
Dosage	4	3	3	2	2	2	2	2	2	2
RFLP	NI	3	NI	NI	NI	2/4	NI	2/4	NI	NI
Combined	4	3	3	2	2	2	2	2	2	2

Note.—The Table shows copy number determined for the five loci shown to the left. Loci are shown proximal to distal (top to bottom). NI = not informative for RFLP analysis. In the RFLP analysis, the notation 2/4" refers to heterozygotes revealing two bands of equal intensity, indicating an even copy number. Final conclusions on copy number are shown underlined.

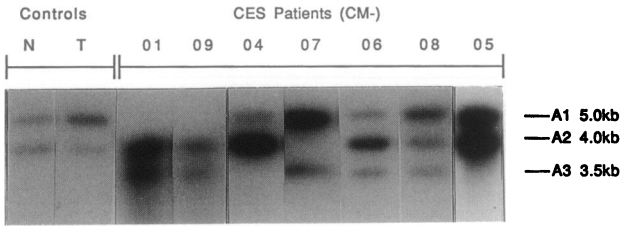

Figure 2 Composite autoradiogram of Southern blots of *Taq*I-digested DNA samples, including normal (GM03657; lane N) and trisomic (GM07106; lane T) controls and seven of the informative CES patients, hybridized to D22S43, a probe that reveals three alleles. Differences in the amount of DNA loaded between lanes precludes direct comparison between lanes. Signal ratios between polymorphic bands in heterozygous individuals were compared with such ratios in heterozygous controls in order to determine copy number. Note that the signal for allele band A2 is typically 20%–30% more intense than the signals for A1 and A3. CM01, CM05, and CM09 show approximate 1:1 signal ratios, indicative of an even copy number (two or four). CM04 and CM07 show signal ratios indicative of a 3:1 distribution (four copies). CM06 and CM08 show an approximate 1:1 signal ratio (four copies).

which is associated with microdeletions spanning the region defined by the proximal locus D22S75 and the distal marker R32 (Driscoll et al. 1992*a*). The CES critical region is therefore proximal to the DGS critical region (DGCR), indicating that these syndromes are not related. The duplications of CM01, CM02, and CM03 do extend at least partially into the DGCR. Although CM03 has a slightly more complex phenotype, including Wormian bones, large anterior/posterior fontanels, and Hirschsprung disease, CM01 and CM02 show only typical CES features. Further analysis of the extent of these marker chromosomes may indicate whether duplications of the loci from within the DGCR have any further effect on the CES phenotype.

Further analysis of marker chromosomes with more probes will enable precise definition of the distal boundary of the critical region. This process will be aided by the somatic cell hybrid X/22-3311TG, derived from the translocation-carrying cell line GM11220A (Guerts van Kessel et al. 1980; Driscoll et al. 1992*a*). The X/22 translocation breakpoint lies between D22S57 and D22S36 (the boundary between CES and DGS) and hence will enable probes to be mapped to the CES critical region. Mapping of the proximal boundary of the critical region requires the identification of CES patients with cytogenetically normal chromosomes, in order to characterize potential interstitial duplications that are smaller than those previously studied.

Parental origin of de novo marker chromosomes could not be determined in the present study. Previous studies reported maternal origin in the three cases investigated (Gabarrón et al. 1985; Magenis et al. 1988). Mother-to-child transmission of the CES marker chromosome has also been reported (Schinzel et al. 1981; Ing et al. 1985). In the present study, CM04 and CM08 each inherited the marker from their mothers, and CM09 transmitted the marker to her affected offspring. However, paternal transmission has also been documented (Noël et al. 1976). Further studies are needed to determine whether parental origin of the CES marker is of any significance.

The phenotypic variability of CES that has been previously reported in the literature (Schachenmann et al. 1965; Schinzel et al. 1981) is also observed in this study. One offered explanation is that the phenotypic variability correlates with corresponding variability in the size of the duplication. This explanation is not supported in our findings. The results indicate that the size of the duplication is not obviously correlated with the severity of the phenotype. CM06, with one of the smallest duplications, has all cardinal features of CES, including a serious heart defect. In contrast, CM01 and CM02, with two of the largest duplications, have no coloboma or heart defect. Our results agree with observed familial cases of CES, where individuals presumably have the same marker chromosome, yet phenotypic variability is often still observed. Noël et al. (1976) described one such familial case where the father displayed facial dysmorphology, coloboma, and renal defects but normal intelligence. His son lacked colobomata but presented with renal, anal, and heart defects and

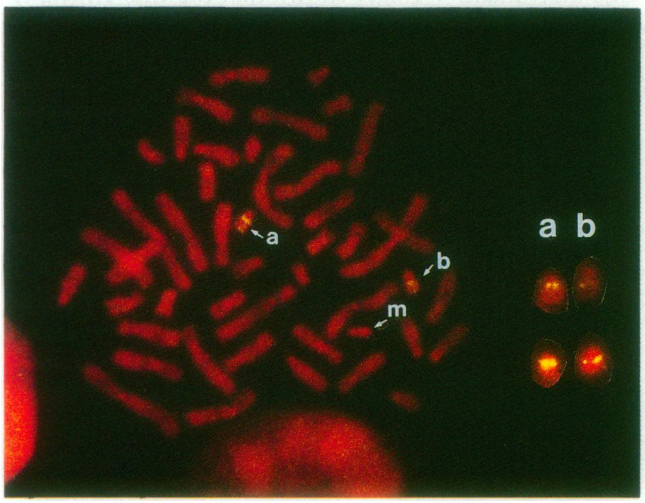

Figure 3 FISH with chromosome 22 cosmids. The test probe is a cosmid for D22S36, cosS36, and the chromosomes 22 are identified with cosS39 marking the distal long arm. The metaphase chromosomes are from patient CM04. Hybridization signal for cosS36 is seen to be greater on the chromosome 22 indicated as homologue "a," compared with the other chromosome 22, indicated as homologue "b." The marker chromosome is indicated by an "m." Two additional partial metaphase chromosome 22 pairs are shown to the right of the metaphase spread. In all three chromosome 22 pairs shown in this figure, the chromosome 22 with the smaller short arm was the one found to have the greatest intensity of signal for cosS36.

showed developmental delay. In our study, patient CM04 has a mild heart defect (patent ductus arteriosus), but a sibling with the same marker died soon after birth as a result of multiple congenital malformations, including total anomalous pulmonary venous return (A. Sommer, personal communication). CM08 inherited the marker chromosome from the parent CM07 but, unlike the parent, has a potential heart problem indicated by the presence of a murmur. It appears therefore that other factors, such as stochastic influences, may cause much of the variability observed with this syndrome. Although prenatal detection of the marker chromosome by using FISH can be readily performed, the correlation to outcome is likely to remain difficult.

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