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Molecular Definition of 22q11 Deletions in 151 Velo-Cardio-Facial Syndrome Patients

C. Carlson,[1] H. Sirotkin,[1] R. Pandita,[1] R. Goldberg,[1,2] J. McKie,[4] R. Wadey,[4] S. R. Patanjali,[5] S. M. Weissman,[5] K. Anyane-Yeboa,[3] D. Warburton,[3] P. Scambler,[4] R. Shprintzen,[6] R. Kucherlapati,[1] and B. E. Morrow[1]

[1]Department of Molecular Genetics, Albert Einstein College of Medicine, [2]Center for Craniofacial Disorders, Montefiore Medical Center, and [3]Division of Clinical Genetics, Department of Pediatrics, Columbia Presbyterian Medical Center, New York; [4]Molecular Medicine Unit, Institute of Child Health, London; [5]Boyer Center for Molecular Medicine, Yale University School of Medicine, New Haven; and [6]Communication Disorder Unit, Center for the Diagnosis, Treatment and Study of Velo-Cardio-Facial Syndrome, State University of New York Health Science Center, Syracuse

Summary

Velo-cardio-facial syndrome (VCFS) is a relatively common developmental disorder characterized by craniofacial anomalies and conotruncal heart defects. Many VCFS patients have hemizygous deletions for a part of 22q11, suggesting that haploinsufficiency in this region is responsible for its etiology. Because most cases of VCFS are sporadic, portions of 22q11 may be prone to rearrangement. To understand the molecular basis for chromosomal deletions, we defined the extent of the deletion, by genotyping 151 VCFS patients and performing haplotype analysis on 105, using 15 consecutive polymorphic markers in 22q11. We found that 83% had a deletion and >90% of these had a similar ~3 Mb deletion, suggesting that sequences flanking the common breakpoints are susceptible to rearrangement. We found no correlation between the presence or size of the deletion and the phenotype. To further define the chromosomal breakpoints among the VCFS patients, we developed somatic hybrid cell lines from a set of VCFS patients. An 11-kb resolution physical map of a 1,080-kb region that includes deletion breakpoints was constructed, incorporating genes and expressed sequence tags (ESTs) isolated by the hybridization selection method. The ordered markers were used to examine the two separated copies of chromosome 22 in the somatic hybrid cell lines. In some cases, we were able to map the chromosome breakpoints within a single cosmid. A 480-kb critical region for VCFS has been delineated, including the genes for *GSCL, CTP, CLTD, HIRA,* and *TMVCF,* as well as a number of novel ordered ESTs.

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Address for correspondence and reprints: Dr. Bernice Morrow, Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461. E-mail: morrow@aecom.yu.edu

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Introduction

Velo-cardio-facial syndrome (VCFS; MIM 19243) is a genetic disorder with an estimated frequency of 1/4,000 live births (Burn and Goodship 1996). Most VCFS cases are sporadic, but, when inherited, it has an autosomal dominant pattern of transmission. This disorder is characterized by conotruncal heart defects, cleft palate, learning disabilities, and mild facial dysmorphology. Since its original description in 1978 (Shprintzen et al. 1978), >40 physical anomalies, including hypocalcemia, blood-vessel anomalies such as tortuous retinal vessels, thrombocytopenia, mental retardation, and hypotonia have been described to occur in association with VCFS (Goldberg et al. 1993). A newly recognized finding associated with VCFS is the development of severe psychiatric illness in patients as they reach adulthood (Goldberg et al. 1993; Chow et al. 1994; Pulver et al. 1994; Karayiorgou et al. 1995; Papolos et al. 1996; Carlson et al. 1997). The clinical features of VCFS overlap significantly with those of DiGeorge syndrome (DGS) (DiGeorge 1965). DGS includes the anomalies of VCFS, and the patients also manifest thymic aplasia or hypoplasia and severe hypocalcemia (Goldberg et al. 1985; Stevens et al. 1990). Both VCFS and DGS are associated with interstitial deletions of 22q11 (de la Chapelle et al. 1981; Greenberg et al. 1988; Driscoll et al. 1992*a*, 199*b*; Scambler et al. 1992).

The relatively high incidence of sporadic cases of VCFS associated with 22q11 deletions suggests that specific regions in 22q11 might be susceptible to rearrangements. To ascertain the molecular basis for the deletions, we used a set of 15 highly polymorphic markers located in 22q11 to genotype 151 VCFS patients and to perform haplotype analysis on 105. Loss-of-heterozygosity analysis with these markers revealed that 83% of the patients had an interstitial deletion. The patients who had deletions could be classified into three classes. More than 90% of them had a large, 3-Mb deletion. Most of the remaining cases had a smaller, 1.5-Mb deletion, whereas the rest contained unique nested deletion breakpoints.

We were not able to make any correlations between the size of the deletion and the severity of the syndromic manifestations.

To more precisely define the chromosome breakpoints, we developed a high-resolution physical and transcript map within 22q11, containing a set of deletion breakpoints. Somatic hybrid cell lines were developed from a subset of the patients. By separating the two copies of chromosome 22 in the somatic hybrid cell lines, we were able to utilize all of the PCR based markers, including simple tandem repeat polymorphic (STRP) markers and sequence-tag site (STS) markers that were developed during construction of the physical map, to define the deletion endpoints. On the basis of the unique nested deletion endpoints in the patients, we were able to identify for VCFS a critical region that is estimated to be 480 kb in size. The critical region for VCFS contains several recently discovered genes and ESTs.

Material and Methods

Preparation of DNA from VCFS Patients for Deletion Analysis

Each of the patients met the diagnostic criteria for VCFS, including velo-pharyngeal insufficiency, cleft of the secondary palate, developmental delay, characteristic facies, learning disabilities, and slender tapered digits, among others (Shprintzen et al. 1978; Goldberg et al. 1993). We found that 61% of the VCFS patients had a heart or major-vessel defect. Genomic DNA was prepared from blood or buccal scrapings from each of the VCFS patients and family members, by use of the Puregene protocol (Gentra). The blood samples from each of the individuals in this study were collected with the assistance of many genetic counselors, physicians, and scientists, through an Internal Review Board–approved program.

Genetic Markers and Genotype and Haplotype Analysis

Fifteen STRP genetic markers were used for genotyping. Eleven of these markers (D22S420, D22S427, D22S941, D22S944, D22S264, D22S311, D22S306, D22S308, D22S425, D22S303, and D22S257) have been described elsewhere (Morrow et al. 1995). A new set of primers for D22S944 was developed because of a sequence polymorphism corresponding to one of the primers (P. Bridge, personal communication). The four new STRP markers—D22S1638, D22S1648, D22S1623, and D22S1709—were developed from fosmid clone A11-22 and cosmid clones c443, 36F3, and 107D7, respectively, as described elsewhere (Morrow et al. 1995). The methodology to genotype the DNA from patients and family members has been described elsewhere (Morrow et al. 1995). The size and number of alleles, as well as the frequency of heterozygosity of each marker, were determined by analysis of DNA from a number of normal relatives of VCFS patients. To define the deletion at the individual patient level, we conducted detailed haplotype analysis on 105 patients and their parents. The genotype of the patient and parents, for each of the markers, was determined. The haplotypes of the parents were deduced from the genotype of the patient. Failure to inherit an allele from one of the parents was reflected by a loss of heterozygosity for that particular locus.

Hybridization Selection

A composite short-fragment cDNA library prepared from RNA from a total fetal abortus (8–10 wk gestation), fetal brain, and four adult tissues (brain, spleen, testes, and thymus) was used (Parimoo et al. 1991; Sirotkin et al. 1996, 1997*a*, and 1997*b*). The YACs Y20E9, Y5All, and R10BE5 (Collins et al. 1995; Morrow et al. 1995) were used separately as substrates to select cDNAs. The DNAs from the six-member fosmid contig consisting of H8-68, A4-73, A11-22, A9-100, C5-94, and E2-21 were pooled and used as a substrate for the selection. Forty independent clones from each selection were sequenced by use of linker primers, by automated sequencing machines (ABI 377), after PCR amplification and cloning into the bacteriophage lambda GT10 (Parimoo et al. 1991). After sequence analysis, clones that contained overlapping sequence were assembled into cDNA contigs. STSs were developed from the sequence of the cDNA contigs and were used for PCR analysis.

End-Specific Sequence-Tagged Sites

The DNA from each cosmid clone was purified on Qiagen T-100 columns (Qiagen) and was used directly for end sequencing with T3 and T7 vector primers, by use of an ABI 377 automated sequencing machine. After sequence analysis, a homology search of the GenBank/EMBL database was performed by use of the computer programs BLASTN and BLASTX (National Center for Biotechnology Information). Primer pairs were developed (by the PRIMER program) from unique genomic sequences. The PCR conditions used for all of the primers were one cycle at 94°C for 2 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and 1 cycle at 72°C for 2 min, with use of standard PCR reaction mixture (Perkin-Elmer).

Construction of a PAC/Cosmid Contig in 22q11

The high-resolution physical map was generated by use of cosmid clones from the LL22NC03 cosmid library and a genomic PAC library provided by Dr. P. DeJong (Roswell Polytechnical College and Institute, Buffalo). The libraries were arrayed on high-density gridded membranes, and the DNAs were screened with pools of 5–

Table 1

Markers in 22q11

Name	Primer Sequence	No. of Alleles	Heterozygosity
D22S1638	GACAACAGCAAATTGCACATT TCACGCCACTACCCTCCAG	9	.78
D22S1648	AGTTGTCAGATGCCTAAGAGA CAGATGCTTCAGGAGAAGTG	5	.33
D22S944N	CGACCATAACTACTGAAAATAAAGG ATCCCATGCTCCTCCCCAT	9	.68
D22S1623	AGGTAAATCTCATACCATGTAAAT CACAACTCCTGGGCTCAAGCT	6	.64
D22S1709	CTCTTCCAAGTTCAGTGCTCT CACTTCAGCAAGAACAGCAGA	10	.73

8 ^{32}P-radiolabeled STSs (Random Primed DNA Labeling Kit; Boehringer Mannheim). The positive clones were isolated from their individual plates, and DNA was prepared (Qiagen). Marker content of individual clones was established by PCR (50-ng template) under standard conditions. The initial assembly yielded 10 sets of overlapping bacterial clones. To connect the individual cosmid/PAC contigs, STSs were developed from the ends of the clones by direct sequence analysis and were used as hybridization probes to rescreen the cosmid and PAC library. This walking procedure was repeated until all clone contigs were connected.

Somatic-Cell Hybrids

Somatic-cell hybrids were developed by fusion of Epstein-Barr virus–transformed lymphoblastoid cell lines from patients BM41, BM308, BM293, and BM8 from the Albert Einstein College of Medicine VCFS Patient Repository and patient G in the study by Levy et al. (1995), with either the hypoxanthine ribosyltransferase (HPRT)–deficient Chinese hamster–ovary fibroblast cell line GM 10658 (National Institute of General Medical Sciences [NIGMS]) (patients BM8 and BM75) or the HPRT-deficient Chinese hamster–ovary cell line CHTG49 (for patients BM41, BM308, and BM293 and patient G), as described elsewhere (Carlson et al. 1997). DNA was prepared and used for PCR analysis as described elsewhere (Carlson et al. 1997).

Results

Genotype Analysis of VCFS Patients by Means of 15 STRP Markers

Four new polymorphic markers were developed in the 22q11 region (table 1). We used the 15 consecutive STRP markers mapped to 22q11 to genotype 151 VCFS patients. For each marker, we compared the level of heterozygosity in the patients with that in their unaffected relatives. Both the order of the markers and the results from this analysis are shown in table 2. A subset of the patients were genotyped earlier with 11 of the markers (Morrow et al. 1995). The level of heterozygosity among the unaffected family members was $>.5$ for each of the 15 markers, except for D22S1648, which was .33. The levels of heterozygosity of the two most proximal markers, D22S420 and D22S427, and of the five most distal markers were indistinguishable between the VCFS patients and their unaffected relatives. For each of the eight interstitial markers (D22S1638–D22S1709), the level of heterozygosity in the VCFS patients was considerably lower than that observed in the unaffected population (table 2). These results suggest that a large proportion of VCFS patients are hemizygous for the region covered by the eight polymorphic markers, and they placed the most commonly proximal breakpoint between D22S427 and D22S1638 and placed the most commonly distal breakpoint between D22S1709 and D22S306/308.

Definition of Deletions in VCFS Patients

Haplotype analysis with the 15 polymorphic markers was performed on 105 cases of VCFS with one or both parents available. Among the 105 cases, five families inherited the deletion; the rest of the cases were sporadic. We found that 90% of the patients had a similar deletion flanked by D22S427 and D22S306/308, which was consistent with the results obtained from genotype analysis (table 2). We estimate the size of this interval to be 3 Mb. We have identified 12 deleted VCFS patients who had nested deletion endpoints. Nine VCFS patients had a proximal breakpoint similar to that of the patients with the 3-Mb deletion and had a nested distal deletion breakpoint between the STRP markers D22S1623 and D22S264. The deletion flanked by D22S427 and D22S264 spans an interval with an estimated size of 1.5

Table 2

Order of Markers and Results of Analysis

	D22S420	D22S427	D22S1638	D22S941	D22S1648	D22S944	D22S1623	D22S264	D22S311	D22S1709	D22S306	D22S308	D22S425	D22S303	D22S257
No. of unaffected relatives	89	164	192	194	137	193	114	191	96	81	92	90	81	70	72
No. of VCFS patients	90	126	151	150	119	150	98	150	93	79	90	90	84	77	81
Heterozygosity in unaffected relatives	.74	.6	.78	.7	.33	.68	.64	.83	.74	.73	.53	.51	.54	.81	.6
Heterozygosity in VCFS patients	.71	.56	.21	.15	.07	.17	.17	.22	.18	.27	.54	.59	.39	.64	.57
χ^2	.1	.49	63.38	64.18	24.73	54.22	33.36	67.59	39.98	23.6	.02	1.07	3.48	2.99	.12
P			>.95	>.95	>.95	>.95	>.95	>.95	>.95	>.95					

Mb (fig. 1; Lindsay et al. 1993; Collins et al. 1995; Morrow et al. 1995).

One VCFS patient, whose cell line is referred to as "GM00980" (NIGMS Cell Repository), carried an unbalanced translocation with a breakpoint at 22q11 (Fu et al. 1976). Elsewhere, this patient had been shown by FISH to have a unique nested distal deletion breakpoint distal to the TUPLE1/HIRA gene and proximal to the D0832 cosmid (fig. 2; Halford et al. 1993*a*). To further define the distal deletion endpoint in GM00980, we performed genotype analysis. One allele was present for markers D22S420–D22S941 and two alleles, alleles 4 and 6 for D22S944. These results were consistent with a chromosomal breakpoint between D22S941 and D22S944.

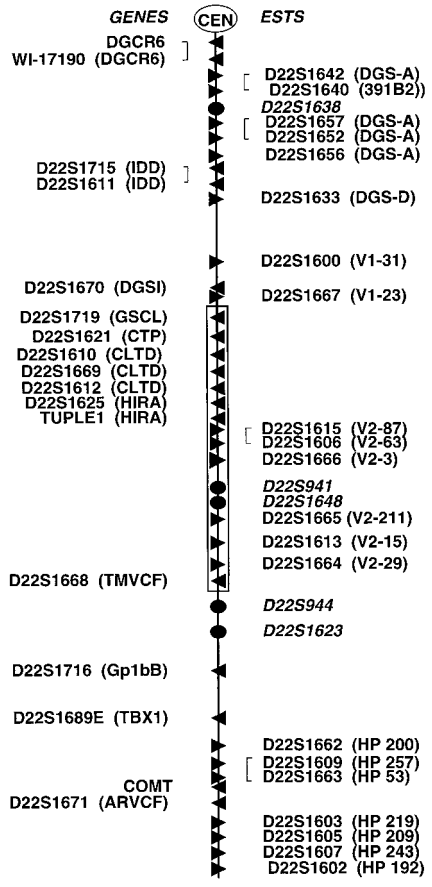

Figure 1 Gene/EST map of the DGCR6–D22S933 interval in 22q11. Genes and ESTs (*triangles*) are indicated to the left and right, respectively, of the line representing the 22q11 region. TBX1 is a member of the T-box family of transcription factors (Chieffo et al. 1996). "COMT" denotes the catechol-O-methyl transferase gene (Grossman et al. 1992). The genetic markers, D22S1638, D22S941, D22S1648, D22S944, and D22S1623 are indicated (*ovals*). The critical region is boxed.

We found that 17% of the patients did not have a detectable deletion at the resolution of the 15 STRP markers. In addition, we did not identify any nondeleted cases VCFS that were inherited, suggesting that the nondeleted sporadic cases of VCFS may be due to new mutations in 22q11, be due to genetic heterogeneity, or have nongenetic causes.

Hybridization Selection of cDNAs from the Region Flanked by D22S427 and D22S264

A hybridization selection methodology (Parimoo et al. 1991) was used to isolate short-fragment cDNAs corresponding to genes within the D22S427–D22S264 interval. The map position of each of the 12 genes and 18 ESTs, with respect to the genetic markers, is shown in figure 1. Comparison of the sequences corresponding to DNA on chromosome 22 with those in public databases revealed that three of them corresponded to known genes, *IDD* (integral membrane protein deleted in DGS; Wadey et al. 1995), *COMT* (catechol-O-methyl transferase; Grossman et al. 1992), *HIRA* (related to yeast repressors of histone gene transcription, HIR1 and HIR2; Lamour et al. 1995), and *DGSI/ES2* (Lindsay et al. 1996; Gong et al. 1997). We isolated three distinct full-length cDNAs corresponding to three short-fragment cDNAs. One of them encodes a novel clathrin heavy-chain gene (*CLTD;* Sirotkin et al. 1996); the second encodes a new member of the catenin gene family, which has armadillo repeats termed "*ARVCF*" (arma-

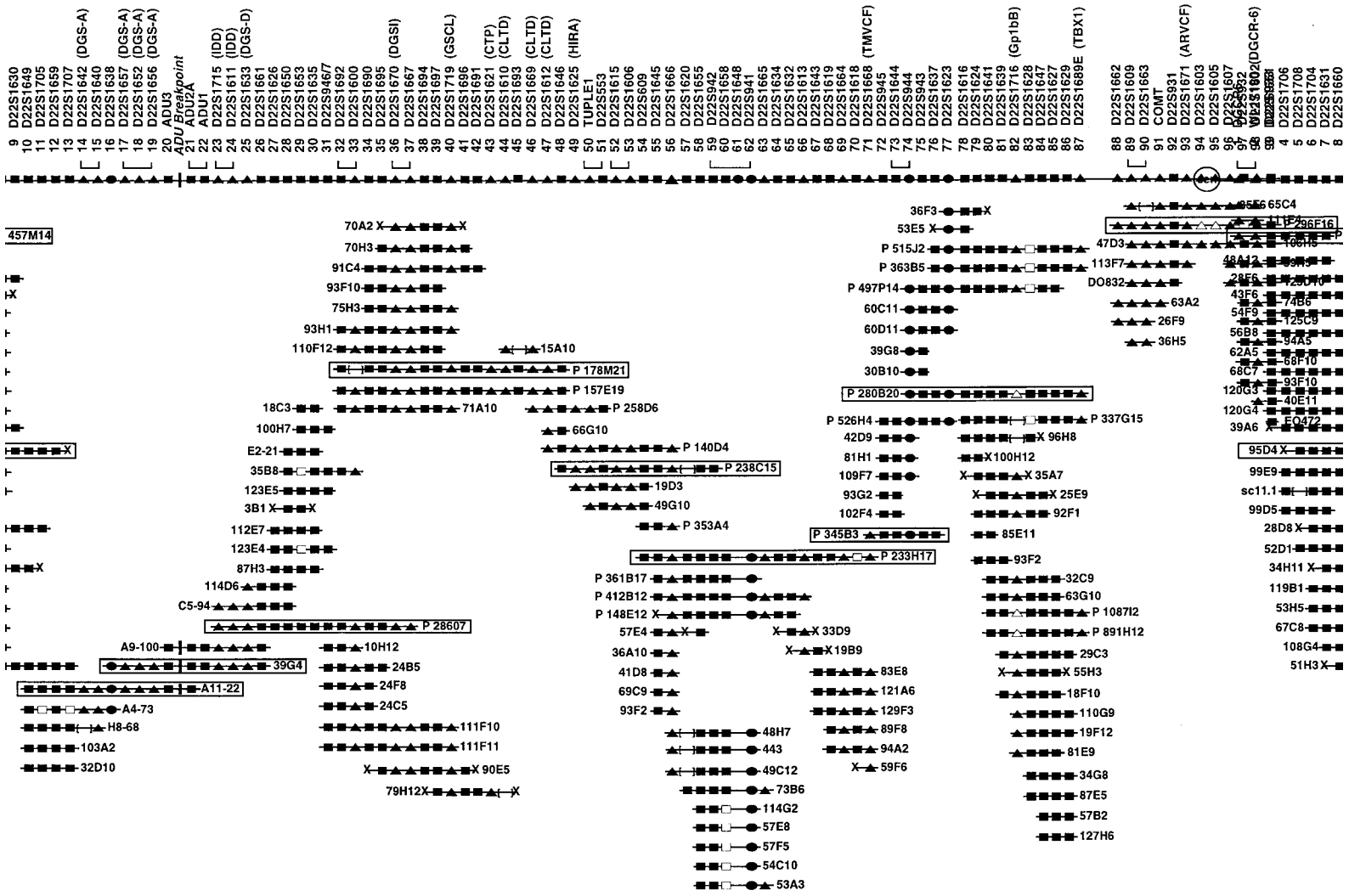

Figure 2 High-resolution physical map of the DGCR6–D22S933 interval. The markers used to construct the physical map are indicated above the line representing chromosome 22q11. The orientation of the map is centromere to telomere, left to right. The polymorphic STSs (*ovals*), monomorphic STSs (*squares*), and gene or EST markers (*triangles*) are shown. All of the STSs are numbered consecutively. The STSs derived from the ends of bacterial clones are denoted by an "X." The blackened symbols denote markers that were tested with the particular bacterial clones and that were present; and the unblackened symbols denote markers was not tested with the particular bacterial clone. The brackets under some of the markers indicate regions where the markers could not be unambiguously ordered with respect to each other. The bacterial clones represented below the line consist of LL22NC03 cosmids and PACs ("P" followed by the address). Brackets within bacterial clones indicate a negative PCR result for that particular marker. The clones that form the minimal tiling path across the region are boxed.

dillo repeat-containing gene deleted in VCFS; Sirotkin et al. 1997*b*); and the third encodes a novel transmembrane protein with homologies to a cloned rat cDNA, termed "*TMVCF*" (transmembrane protein deleted in VCFS; Sirotkin et al., 1997*a*). A few of the other short-fragment cDNAs and cDNA contigs correspond to ESTs that recently have been described elsewhere (Gong et al. 1996); they include DGS-A (D22S1642, D22S1657, D22S1652, and D22S1656) and DGS-D (D22S1633) (Gong et al. 1996). In addition to all of these, we have 18 unique ESTs, each of which might correspond to genes in 22q11. We prepared primer pairs for each of the genes and ESTs and used them for construction of the high-resolution physical map.

Construction of a High-Resolution Physical Map of the D22S427–D22S264 Interval

We constructed a high-resolution cosmid and PAC contig, using a YAC contig (Collins et al. 1995; Morrow et al. 1995) as the framework for the map shown in figure 2. The map contains 125 cosmids, 6 fosmids, and 22 PACs. There are 99 STS markers, numbered consecutively on the map, and they include 5 STRP genetic markers (D22S427 and D22S264 flank the map), 38 gene or EST-based markers, and 56 monomorphic markers. The cosmid sc11.1, used routinely for FISH to detect 22q11 deletions (Lindsay et al. 1993, 1995*a,* 1995*b*), was integrated into the physical map and was found to be located between STRP markers D22S427 and D22S1638. The cosmid 79H12 contains the gene for the mitochondrial citrate-transport protein (*CTP*), as well as the D22S75 locus that comprises the N25 probe commonly used to detect deletions in VCFS/DGS patients (Goldmuntz et al. 1996). The cosmid and PAC clones that constitute the minimal tiling path across the region from *DGCR6* to D22S933 are highlighted in figure 2. The approximate size of the interval can be calculated by adding the average size of each of the clones that constitute the minimal tiling path. There are eight PAC clones (120 kb × 8 = 960 kb) and three cosmid/fosmid clones (40 kb × 3 = 120 kb). We estimate this distance to be ~1,080 kb. The map contains 99 STS markers, and they provide an average resolution of 11 kb (1,080 kb/99 markers).

Somatic-Cell Hybrid Analysis to Define the VCFS Deletion Endpoints

The resolution of the 15 STRP markers than span the 5 Mb of 22q11 region is 330 kb. To more precisely define the breakpoints in VCFS patients, we separated the two copies of chromosome 22 by generating human-hamster somatic-cell hybrids. Somatic hybrid cell lines were obtained by fusion of lymphoblastoid cells from patients BM41, BM308, BM293, BM8, and BM75 from the Albert Einstein College of Medicine VCFS Patient Repository and patient G from the study by Levy et al., with hamster fibroblasts. Clones that retained chromosome 22 were detected by use of D22S1604, a marker that maps between D22S420 and D22S427 (fig. 3) and is not deleted in VCFS patients. To determine which copy of chromosome 22 was present in each cell line, the hybrids were genotyped with all or a subset of the three STRP markers D22S420, D22S1638, and D22S303 (data not shown). Appropriate hybrids were screened with markers that are contained within the D22S420–D22S306/308 region. The results of this analysis are summarized in figure 3.

STRP marker analysis of patients BM41, BM308, and BM293 revealed that they had the commonly occurring 3-Mb deletion flanked by D22S427 and D22S306/308. All three deletions occurred on the maternal chromosome 22. Using STS markers, we determined that the proximal breakpoint occurred between D22S1714 and DGCR6 (Demczuk et al. 1996), in a gap of unknown size in the physical map. The distal chromosomal breakpoint occurred between D22S935/D22S936 and D22S1702. Since these distal markers are present within a single cosmid, we conclude that in these three patients the distal breakpoints lie within the same 40-kb region. Included in this figure are the results of STRP marker analysis of VCFS patient BM14, who represents a subset of VCFS patients who had the same proximal chromosomal breakpoint as the majority of VCFS patients—that is, between D22S427 and D22S1638—but had a nested distal deletion endpoint between D22S1623 and D22S264, creating a deletion of ~1.5 Mb.

A DGS patient termed "ADU" carries a t(2;22) balanced translocation that disrupts the 22q11 region (Augusseau et al. 1986). The breakpoint junction has been cloned (Budarf et al. 1995; Demczuk et al. 1995; Wadey et al. 1995), and we have integrated the breakpoint within the physical map (figs. 2 and 3). Patient BM8 was shown by haplotype analysis to have a unique nested proximal deletion between STRP markers D22S1638 and D22S941, the interval that contains the ADU breakpoint. To precisely define the breakpoint interval of BM8, we developed somatic hybrid cell lines and found that the proximal breakpoint of BM8 lies ~5 kb telomeric to that of ADU, within cosmid 39G4 (fig. 2).

A second DGS patient termed "G," was found to have a unique proximal breakpoint, also telomeric to that of ADU (Levy et al. 1995). Analysis of somatic hybrid cell lines from patient G revealed that the proximal deletion breakpoint occurred between D22S1697 and D22S1621, both present on cosmid 79H12 (fig. 3). The distal breakpoint of patient G was also unique, and it occurred between the markers D22S311 and D22S1709, both of which are present on the CEPH mega-YACs 792f9 and 861d9 (Collins et al. 1995; Morrow et al. 1995). On the basis of the analysis of patient G and

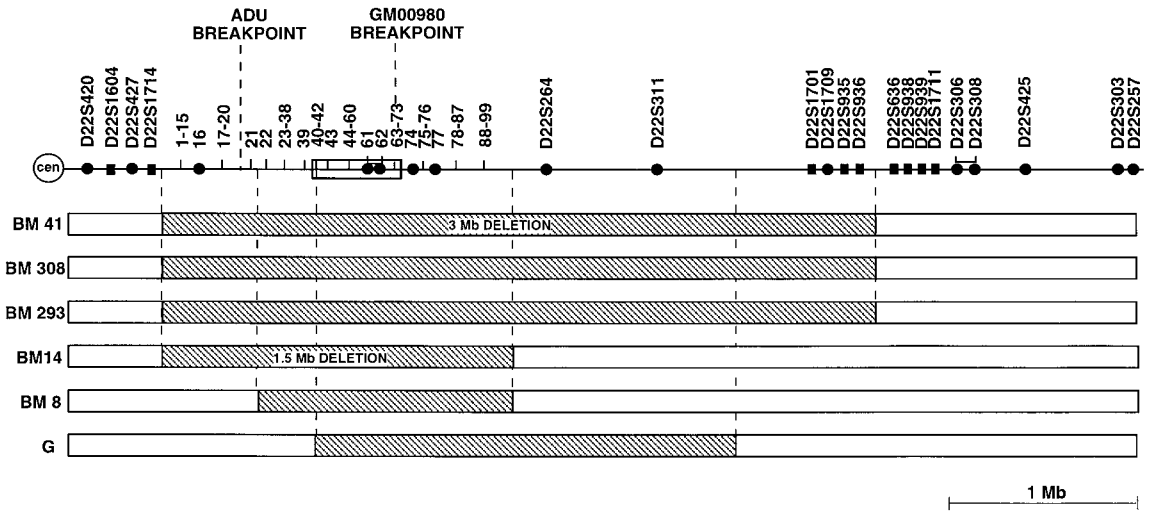

Figure 3 Definition of the deletion endpoints in VCFS patients. All of the markers are shown above the line representing chromosome 22q11. The map is drawn to scale and was based on the YAC-contig physical map (Collins et al. 1995; Morrow et al. 1995). All of the markers in the DGCR6–D22S933 interval are numbered consecutively (fig. 2). Both the genetic markers a(*circles*) and the monomorphic STSs (*squares*) are shown. The 3-Mb and 1.5-Mb deletions are indicated. The broken vertical lines denote various chromosomal breakpoints. The hatched region represents the deleted interval determined by STS analysis for patients BM41, BM308, BM293, and BM8 and patient G (or STRP analysis, for BM14). The critical region is boxed.

FISH, as well as genotype analysis of GM00980 (Halford et al. 1993*a*), the critical region for VCFS must lie within the D22S1694–D22S944 interval. The size of the critical region is estimated to be 480 kb and is based on the minimal tiling path of four PACs—178M21, 238C15, 233H17, and 345B3 (fig. 2). The proximal end of this critical region is 120 kb distal to that of ADU.

VCFS patient BM75 was tested with 71 markers that span the 1,080-kb interval. All of these markers were present on both the paternal and the maternal chromosome 22 (data not shown). These results show that BM75 did not have a deletion that can be detected by these 71 markers, which provide an average resolution of 15 kb (1,080/71). Two other VCFS patients, examined elsewhere (Carlson et al. 1997), also did not have a detectable deletion in 22q11.

Discussion

Many of the tissues and structures affected in VCFS/DGS derive from the pharyngeal arches of the developing embryo. During embryonic development, neural crest cells migrate into the pharyngeal arches and participate in the formation of the craniofacial region, the neck, and the conotruncal region of the heart. It is possible that a defect in either neural crest cells or their migration is responsible for the main clinical findings in VCFS/DGS. To identify candidate genes for VCFS, we have defined the deletions among a large number of VCFS patients, have identified a new critical region for the disorder, and have isolated genes within the interval. However, not all of the >40 clinical findings associated with VCFS (Goldberg et al. 1993) can be attributed to a defect in neural crest cells. It is possible that some of the associated anomalies of VCFS occur as the result of haploinsufficiency or because of uncovering of recessive mutations in additional genes outside the critical region but are deleted in the great majority of patients. For example, the expression of the *GpIb*β gene, a gene that maps within the commonly deleted region but is outside the critical region, is eliminated in one VCFS patient affected with a rare bleeding disorder, termed "Bernard-Soulier syndrome" (Budarf et al. 1995). It is possible that a recessive mutation in the *GpIb*β gene is responsible for the etiology of Bernard-Soulier syndrome in this patient.

We have genotyped 151 well-diagnosed VCFS patients and have performed haplotype analysis on a large number of them, using 15 highly polymorphic markers. Four classes of patients were identified: nondeleted patients, patients with a 3-Mb deletion, patients with a 1.5-Mb deletion, and patients with unique deletions. There was no correlation between the phenotype and the presence or size of the deletion in this patient population.

To detect unique small deletions among the patients who did not have a deletion detectable by haplotype analysis, we analyzed somatic hybrid cell lines that were generated from three patients (BM26 and BM102 from the report by Carlson et al. [1997] and BM75 from the present report), using STS markers across the 1,080-kb interval, with an average between-marker spacing of 15

kb, but we did not find a deletion. It is possible that these patients have a mutation in a critical gene located in 22q11 or that a gene(s) elsewhere in the human genome is involved in the etiology. Genetic heterogeneity for VCFS has been suggested (Greenberg et al. 1988; Daw et al. 1996). Alternatively, the nondeleted patients are phenocopies with a teratogenic (Lammer and Opitz 1986), maternal diabetes (Wilson et al. 1993), or other fetal insult.

The most common deletion that we observed in the VCFS patients maps to a 3-Mb region in 22q11, flanked by the genetic markers D22S427 and D22S306/308. At the resolution of the genetic markers, 90% of the VCFS patients with a detectable deletion had the same proximal and distal chromosomal breakpoints. Analysis of the individual copies of chromosome 22 in somatic hybrid cell lines from three patients, by means of STS markers, confirmed the finding derived by use of genetic markers—that is, that the deletion breakpoints may be within the same interval. These results suggest that sequences at these sites may be susceptible to chromosomal rearrangements. Molecular cloning and sequencing of these regions may reveal the basis for this susceptibility to breakage. A small proportion of the patients have deletions that have the same proximal breakpoint as is seen for the 3-Mb deletion, but they have a nested distal deletion chromosomal breakpoint resulting in a deletion that is estimated to 1.5 Mb. It is possible that the D22S1623–D22S264 interval also contains sequences that confer susceptibility to rearrangement.

Detailed analysis of several unique patients allowed us to define a critical region for VCFS. The critical region is defined by the breakpoints in patients G (Levy et al. 1995) and GM00980 (Fu et al. 1976; Halford et al. 1993*a*). Examination of somatic hybrid cell lines from patient G revealed that the proximal breakpoint in this patient is located between marker D22S1697 and the citrate-transport protein (*CTP*), which is defined by D22S1621. This region is 120 kb distal to the balanced translocation breakpoint in patient ADU (Budarf et al. 1995). These results confirm that the disruption of a gene containing the ADU breakpoint is not directly responsible for VCFS/DGS. The distal boundary of the critical region is defined by the breakpoint in patient GM00980. We estimate the size of the critical region to be 480 kb.

The critical region defined by us contains five genes and six ESTs. The genes are *GSCL* (*goosecoid-like* [*GSCL*]; Gottlieb et al. 1997; B. Funke, B. Saint-Jore, A. Puech, H. Sirotkin, S. Raft, L. Edelmann, C. Carlson, et al., unpublished data), *CTP* (mitochondrial citrate-transport protein; Heisterkamp et al. 1995; Goldmuntz et al. 1996), *CLTD* (clathrin heavy-chain gene D; Lindsay et al. 1996; Kedra et al. 1996; Sirotkin et al. 1996), *HIRA* (related to yeast repressors of histone-transcription genes, HIR1 and HIR2; Halford et al. 1993*a*; Lamour et al. 1995) and *TMVCF* (transmembrane protein deleted in VCF; Sirotkin et al. 1997*a*). Several of these genes have been suggested to have a role in the etiology of VCFS. *GSCL* is the second member of the goosecoid class of homeodomain-containing transcription factors (Blum et al. 1992, 1994; Gottlieb et al. 1997; B. Funke, B. Saint-Jore, A. Puech, H. Sirotkin, S. Raft, L. Edelmann, C. Carlson, et al., unpublished data). The *GSCL* and *goosecoid* genes are members of the larger, *Drosophila bicoid* gene family (Nusslein-Volhard et al. 1987; Blum et al. 1992, 1994). The mouse *goosecoid* homologue, located on human chromosome 14, has been inactivated by gene targeting, and the mice have been shown to develop craniofacial and rib anomalies (Rivera-Perez et al. 1995; Yamada et al. 1995). It is possible that *GSCL* may also be involved in embryonic development. *CLTD* is highly homologous to the clathrin heavy-chain gene located on chromosome 17 (Sirotkin et al. 1996; Kedra et al. 1996). The *CLTD* gene is expressed predominantly in adult skeletal muscle (Sirotkin et al. 1996). A patient with some of the clinical findings of VCFS/DGS was recently described to carry a t(21;22) balanced translocation that disrupts the *CLTD* gene, suggesting that *CLTD* may have a role in at least some of the anomalies of VCFS (Holmes et al. 1997). *HIRA* has significant sequence homology with two repressors of histone gene transcription, HIR1 and HIR2, in the yeast *Saccharomyces cerevisiae* (Lamour et al. 1995). The *HIRA* gene is expressed during mouse embryonic development and, in particular, in the cephalic region of the embryo and the limb buds (Wilming et al. 1997). Expression studies in the chick have revealed expression in the developing neural plate, neural tube, head mesenchyme, and neural crest (Roberts et al. 1997). A newly described gene, *TMVCF,* encodes a putative transmembrane protein of unknown function (Sirotkin et al. 1997*a*). We have identified six ESTs in the critical region, and they may correspond to yet additional genes.

Although the precise role of all of these genes is not yet understood, the fact that they are always hemizygous in VCFS patients carrying deletions suggests that haploinsufficiency of one or more of these genes may play a role in the etiology of the main clinical findings of VCFS. Although the current effort is focused on identification of genes in the 480-kb critical region, it is important to identify all of the genes encoded by the 3-Mb region. Functional analysis of all of these genes would provide valuable clues to the full understanding of all of the anomalies associated with VCFS.

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