Am. J. Hum. Genet. 56:1391–1403, 1995

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

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Summary

Velo-cardio-facial syndrome (VCFS) is a common genetic disorder among individuals with cleft palate and is associated with hemizygous deletions in human chromosome 22q11. Toward the molecular definition of the deletions, we constructed a physical map of 22q11 in the form of overlapping YACs. The physical map covers >9 cM of genetic distance, estimated to span 5 Mb of DNA, and contains a total of 64 markers. Eleven highly polymorphic short tandem-repeat polymorphic (STRP) markers were placed on the physical map, and 10 of these were unambiguously ordered. The 11 polymorphic markers were used to type the DNA from a total of 61 VCFS patients and 49 unaffected relatives. Comparison of levels of heterozygosity of these markers in VCFS patients and their unaffected relatives revealed that four of these markers are commonly hemizygous among VCFS patients. To confirm these results and to define further the breakpoints in VCFS patients, 15 VCFS individuals and their unaffected parents were genotyped for the 11 STRP markers. Haplotypes generated from this study revealed that 82% of the patients have deletions that can be defined by the STRP markers. The results revealed that all patients who have a deletion share a common proximal breakpoint, while there are two distinct distal breakpoints. Markers D22S941 and D22S944 appear to be consistently hemizygous in patients with deletions. Both of these markers are located on a single nonchimeric YAC that is 400 kb long. The results also show that the parental origin of the deleted chromosome does not have any effect on the phenotypic manifestation

Received January 20, 1995; accepted for publication March 9, 1995.

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0002-9297/95/5606-0018$02.00

Introduction

Velo-cardio-facial syndrome (VCFS; MIM 19243), originally described in 1978 (Shprintzen et al. 1978), is the most common syndrome associated with cleft palate and is estimated to have an incidence of 1/5,000 (Scambler 1993). Although a large proportion of VCFS cases are sporadic, some inherited cases have been reported, and they have an autosomal dominant pattern of inheritance (Williams et al. 1985). VCFS patients exhibit a wide spectrum of anomalies; and the most frequent ones include cleft palate, congenital heart defects of the conotruncal type, facial dysmorphology, and learning disabilities (Shprintzen et al. 1978, 1981, 1985; Young et al. 1980; Arvystas and Shprintzen 1984; Lipson et al. 1991; Jedele et al. 1992). Some of the patients manifest more complex phenotypes with varying severity, such as delayed growth, lymphoid hypoplasia, microcephaly, mental retardation, and psychiatric disorders (Golding-Kushner et al. 1985; Williams et al. 1987; Goldberg et al. 1993; Chow et al. 1994; Pulver et al. 1994). VCFS is phenotypically related to the more severe disorder, DiGeorge syndrome (DGS) (DiGeorge 1965; Goldberg et al. 1985; Wilson et al. 1992), which is associated with the symptoms of VCFS and, in addition, with thymic aplasia or hypoplasia and hypocalcemia. Both VCFS and DGS affect multiple organ systems, many of which are derived from the third and fourth pharyngeal arches of the developing embryo (LeDouarin 1980; Kirby et al. 1983; Kirby and Bockman 1984; Bockman and Kirby 1987). Therefore it is possible that VCFS/DGS may arise as a result of a developmental field defect.

Cytogenetic studies revealed that ≤20% of VCFS and DGS patients have cytologically detectable hemizygous deletions of a part of chromosome 22 that includes the 22q11 region (de la Chapelle et al. 1981; Kelley et al. 1982; Augusseau et al. 1986; Rouleau et al. 1989; Scambler et al. 1992). A number of molecular markers from DNA in 22q11 have been identified (Budarf et al. 1991, Carey et al. 1990; Collins et al. 1992; Van Biezen et al. 1993), and studies based on dosage of some of these molecular markers substantiated and extended the observations about deletions and revealed that 76% of

VCFS and 88% of DGS patients are hemizygous for part of 22q11 (Driscoll et al. 1993). It also has been shown that deletions not detectable by cytogenetic methods can be detected by alterations in dosage of specific bands by using Southern blot hybridization. This observation has suggested that the size of the deletions varies between patients (Scambler et al. 1991, 1992; Driscoll et al. 1992*a*, 1992*b*). It is possible that the reduced dosage of one or more genes in the region commonly deleted among the VCFS and DGS patients underlies the etiology of these disorders. Although estimates about the size of the commonly deleted region in the VCFS and DGS patients have been made, the lack of a high-resolution physical map of the region, the low density of markers, and the low reliability of dosage studies make these estimates inaccurate.

In an effort to define the molecular basis of VCFS, we constructed a physical map of 22q11 in the form of a sequence-tagged site (STS) content map of overlapping DNA fragments cloned into YACs covering 5 Mb. The map presented here contains a total of 61 highly polymorphic, monomorphic, and gene-based markers. The markers are present at an average interval of 82 kbp. The map includes 10 ordered highly polymorphic microsatellite markers. To delineate the extent of loss of heterozygosity within the 22q11 region in each patient, we typed the DNA of a total of 61 VCFS patients and 49 relatives, with each of the polymorphic markers. Results from these experiments, documented here, show that two new microsatellite markers, D22S941 and D22S944, are hemizygous in 82% of the VCFS patients. This analysis also permitted us to conclude that the size of the deletions varies in different patients and permitted us to define, with a high degree of definition, the sites at which breaks have occurred to cause deletions.

Material and Methods

YACs

YAC clones containing sequences from 22q11 were identified by examination of databases that store information principally on CEPH YACs that contain markers derived from this region (Quickmap [CEPH, Paris]; Philadelphia Genome Center for Chromosome 22; MIT Genome Center at Whitehead Institute; Hudson et al. 1994). Additional YACs known to contain markers in 22q11 were obtained by PCR screening of the ICI Diagnostics and Imperial Cancer Research Fund YAC libraries (Larin et al. 1991). A total of 384 YACs were used in the analysis. Yeast cultures were grown in AHC medium (Brownstein et al. 1989), and total genomic DNA was prepared by conventional methods. For purposes of screening, DNA from individual YACs was pooled. The first level of pooling involved mixing DNA from 96 unrelated YACs arranged randomly in the format of a 96-well microtiter dish. Each of these pools was mixed to prepare four superpools encompassing the entire region and all 384 YACs. Pools of DNA from each column and row from each plate were also prepared. To identify individual YAC addresses positive for a set of PCR primers, plates that were positive for the marker were identified, and row-and-column pools were screened to obtain unique addresses. The results were confirmed by screening individual YAC DNAs for the presence of the marker.

STSs

The primer sequences for each of the new STSs described in this report, as well as the conditions used for amplification, are shown in table 1. Primers for eight polymorphic markers (D22S420, D22S427, D22S264, D22S311, D22S306, D22S425, D22S303, and D22S257) were purchased from Research Genetics. The primer pairs that amplify STSs publicly available through the Genome Center at the Whitehead Institute (Hudson et al. 1994) either were purchased from Research Genetics or were synthesized ([D22S272 [WI-405], D22S601 [WI-290], D22S543 [WI-403], D22S580 [WI-254], D22S627 [WI-352], D22S730 [WI-307], D22S553 [WI-91], D22S609 [WI-326], D22S663 [WI-328], D22S636 [WI-362], D22S585 [WI-265], D22S647 [WI-385], D22S626 [WI-351], D22S563 [WI-167], D22S556 [WI-445], and D22S654 [WI-402]). New STSs were generated by two methods. In one case, DNA from cosmids known to be derived from 22q11 was digested with *Eco*RI and *Hin*dIII and were fractionated on agarose gels, and specific individual fragments were subcloned and subjected to sequencing. Markers D22S942, D22S943, D22S933, D22S934, D22S939, and D22S940 were derived by this method, from cosmids c443, cN60C11, cE0472, cN122B5, cN77F7, and cN87E9, respectively. A second set of markers, corresponding to ends of YACs, were obtained by a PCR-based rescue of YAC ends (Riley et al. 1990) and sequence analysis. Markers derived from YACs B20E9, R37AF5, R16IH4, R14FC6, 842D2, Y5A11, 849E9, 881H10, and 8H4A10 were developed in this fashion. The PCR reactions for all STSs were conducted by using a standard PCR reaction mixture. The PCR was performed for 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 40 s and 57°C for 30 s, followed by 1 cycle at 72°C for 2 min.

Development of Polymorphic Markers D22S941 and D22S944

DNA from cosmids c443 and cN60C11 was digested with *Sau*3AI and was subcloned into pcDNAII vector. The resulting colonies were hybridized with a nick-translated $(CA)_{18}$ probe (LeBlanc et al. 1994). DNA from hybridizing colonies was sequenced, and primer

Table 1

Markers for Physical Mapping

GDB Locus	Name	Left Primer 5′–3′	Right Primer 5′–3′	Product Size (bp)
D22S930	848D2L	ACACAGTCTACTAGATCTCTG	CAAGCCACTTCTTCATGCCT	115
TUPLE1	TUPLE1	GAGACACTGGCAGGAGATGT	TCTGCTGTAATACCTAACGCT	292
D22S941	CA 443	CAGGTTACAAAGTACATTAACTT	CAAGAAATGGTTGGAGCTGGT	224–260
D22S942	443	AGTTGAGGCTTTTGCCCAGT	CTGGAGTACAATGGTGCGC	285
D22S943	N60C11	TTGCCTGCTGTCGAAAGAG	TTGAGGATTTGGAGCACCTT	249
D22S944	CA N60C11	CATGTGAAAGATGCTACTTCC	ATCCCATGCTCCTCCCCAT	158–178
D22S945	37AF5R	GTAGACCAAGACTCTATCTCTAA	AGGCAGAGCCCCTGTAGTCTG	178
D22S946	R14FC6L	GAGGACAGAGACCACTGTTGATAG	ACATCGTGGCCATCTCTGAGGC	152
D22S947	Y5A11L	CATATTGCTCGGTGACCTTCT	GCCTTCCAGAGCGTGCATC	195
D22S931	HD7K	GTGAGATGGACCGGAACTTTG	CTACCAGGGCAATCCTGAGC	124
D22S932	HP500	CTCCCAACCCTGCCGATCTC	GGTATAGAATCACGGCTGGGTC	460
D22S933	E0472	AATCACTTGGGACTTTGGTGT	CTGGTATTCTTAGAGGACGG	267
D22S934	N122B5	ACAGCTGGTCAGCCTCCTC	TTTGAGTTAAAGTTTCTCCCAAC	138
D22S935	849E9R	CCCACAGCCTTCTGTGTCT	TTCTGCAATGCAGAGACAGG	132
D22S936	881H10R	CAGCATCTTCCTGGTGGCC	CAATCTTGGCAGCCAGTTTAG	179
D22S937	8H4A10R	AATTCCTCATCTCTACACA	GTCATGGCGTGATCATG	178
D22S938	849E9L	CAATACCATGTCTTCTGTCTG	GAGGAGACTGAAGTGTGTTG	132
D22S939	N77F7	ATATATTGCACAAAGAATTGCATA	CCTATTATCACAGACACACAGT	170
D22S940	N87E9	TTTAAGCTGTTAAGGCCCTAC	CCTACTTAATGTTACAGTTCCT	202

pairs corresponding to unique flanking sequences were synthesized (table 1).

Human Tissue Samples, Cell Lines, and DNA Extraction

Blood samples from 110 individuals who are either VCFS patients or relatives of VCFS patients were collected with the assistance of many genetic counselors, physicians, and scientists (see Acknowledgments). The program was approved by the local institutional review board. Genomic DNA was prepared from 3 ml of human peripheral blood by using the Puregene DNA isolation kit (Gentra). The DNA was diluted 1:50, and 1 μl was used for a PCR reaction. Lymphoblastoid cell lines were established by using conventional procedures.

Genotyping

To genotype DNA from individuals, highly polymorphic short tandem-repeat–containing sequences were used. The methodology for this analysis has been described elsewhere (LeBlanc et al. 1994). The cycling conditions for the PCR consisted of 1 cycle at 94°C, followed by 25 cycles for 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by 1 cycle at 72°C for 2 min. The PCR product was denatured by adding 20 μl of stop solution consisting of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol (Sequenase Version 2.0; USB) and then heating for 10 min at 65°C, followed by electrophoresis of 4 μl of each sample, through 6% denaturing polyacrylamide gels. A DNA sequence ladder was used to determine the size of the alleles.

Results

High-Resolution Physical Map of 22q11

To construct a high-resolution physical map of 22q11, we assembled a set of YACs that might contain sequences from this region and a set of markers. Fifty-two of the 84 YACs used in constructing the map were derived from the CEPH II (mega YAC) library (Chumakov et al. 1992). The average insert size of these YACs is >900 kb. The choice of YACs used for constructing the physical map was based on information contained in a public database called "Quickmap." The Quickmap database contains the addresses of YACs that contain the genetically mapped Généthon polymorphic markers (Gyapay et al. 1994). These YACs are referred to as "anchor YACs." Addresses of YACs related to anchor YACs by virtue of sharing either DNA fingerprint profiles (Bellanné-Chantelot et al. 1992) or Alu-PCR products, referred to as "level 2 neighbors," are also found in Quickmap. Adjacent polymorphic markers may be linked by YACs that share the two markers, level 2 neighbors, or higher-level neighbors. Different groups estimated the genetic length of chromosome 22; and the values range from 49 cM (Gyapay et al. 1994) to 93.5 cM (Cooperative Human Linkage Center [CHLC]). In the CEPH/Généthon map, marker D22S420 is the most proximal, at 0 cM, D22S427 is at 3 cM, and D22S425 is at 9 cM, on the genetic map. Using the Quickmap database, we selected YACs that are potentially in the interval defined by the markers D22S420 and D22S425. The order of additional genetic markers (F8VWFP,

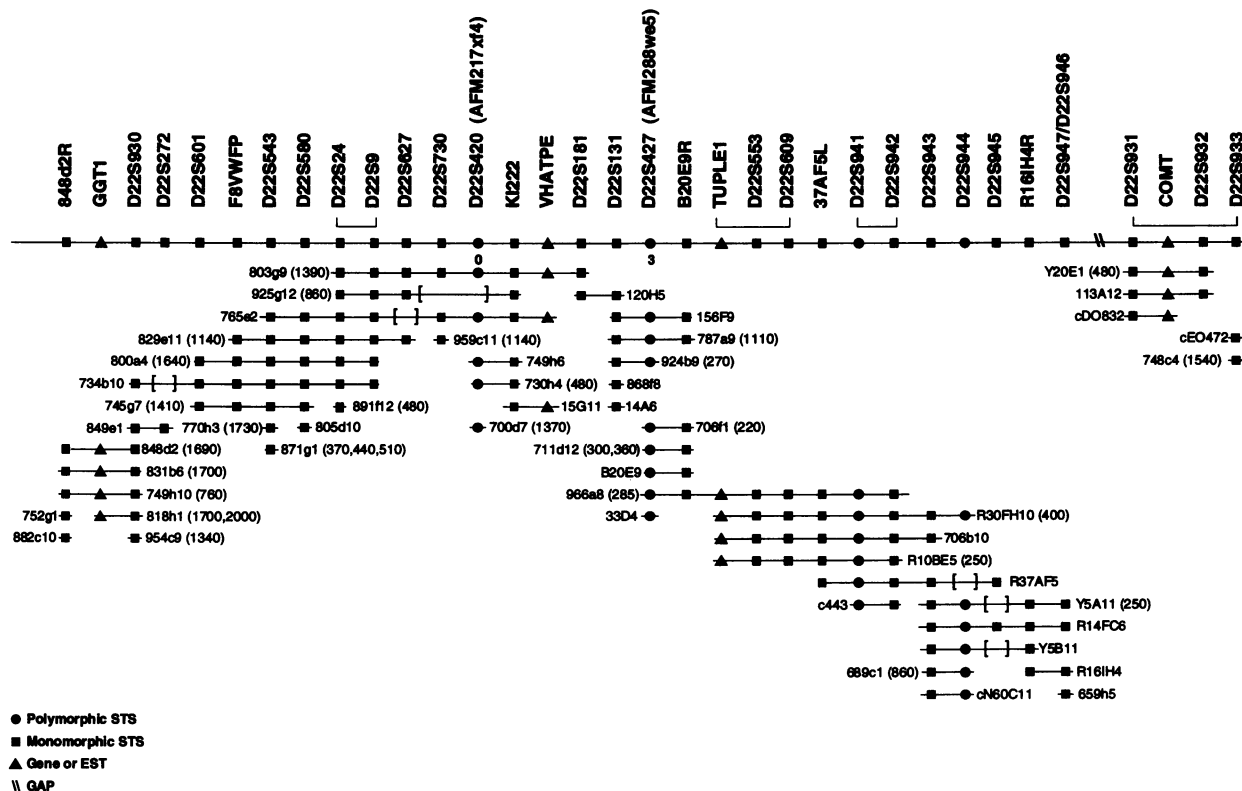


Figure 1 Physical map of 22q11. The markers used to construct the physical map are indicated above the line. The left end is centromere proximal, and the right end of the map is distal. Polymorphic STSs are denoted by the circles; and monomorphic STSs are denoted by the squares; and gene or EST markers are denoted by the triangles. The brackets underneath some of the markers indicate regions where the markers could not be unambiguously ordered with respect to each other. Each of the two gaps present within the contig is indicated by a double diagonal lines (\\). The 84 YACs are represented below the line. The designations of the YACs, as well as their sizes (shown [in kb] in parentheses) are shown. Cosmids are indicated by the prefix "c'" (e.g., c443).

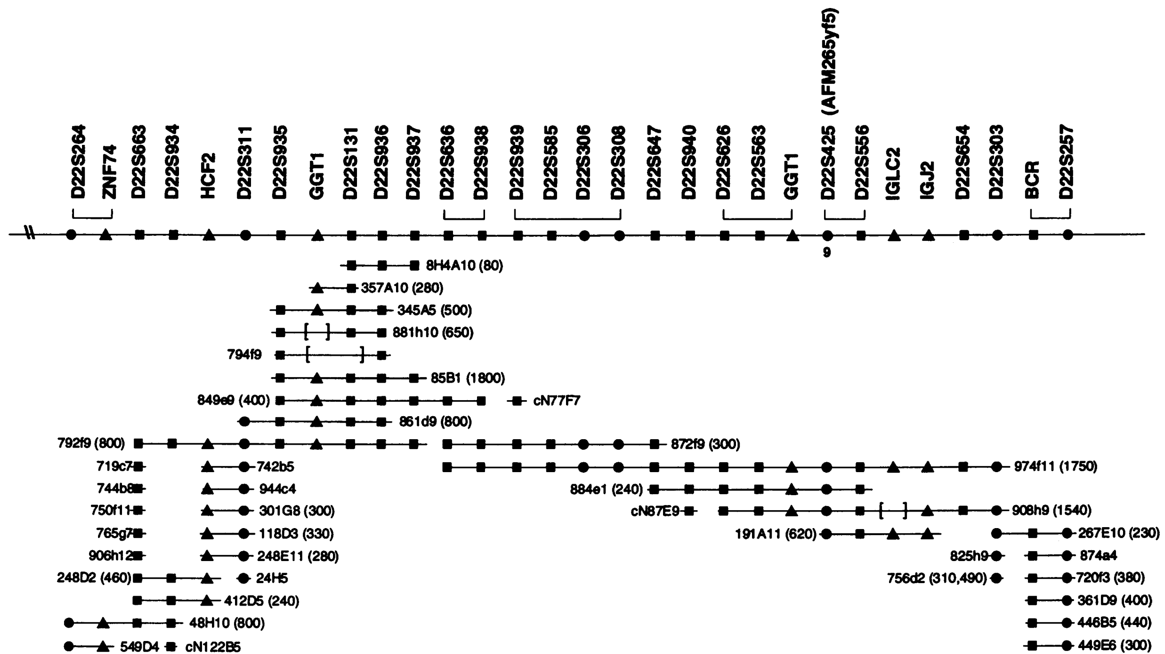
COMT, D22S264, D22S311, D22S306, D22S303, and D22S257), deduced by CHLC, provided the framework for assembling overlaps between groups of YACs. Additional YACs obtained by screening the CEPH, ICI, and ICRF libraries with several PCR-based STS markers were also included in the study (Carey et al. 1990; Budarf et al. 1991; Collins et al. 1992; Buetow et al. 1993; Porter et al. 1993). Each new marker was used to screen the YACs, as described in Material and Methods. The STS content of the YACs was used to establish overlaps between individual YACs.

If two YACs share one or more markers, they are considered to have sequence overlap. A continuum of partially overlapping YACs is referred to as a contig. The YAC contig map deduced by STS-content analysis is shown in figure 1.

The map contains 84 YACs and is composed of 64 markers, of which 11 are short tandem-repeat polymorphism (STRP) markers and 53 are monomorphic and gene-based markers from different sources (Collins et al. 1992; Buetow et al. 1993; Porter et al. 1993; Gyapay et al. 1994; present report). As a result, it was possible to integrate all of the markers into a single map. The average density of YACs that are positive for each marker throughout this map is four. This is significantly less than the 10 genome equivalents that constitute the CEPH mega YAC library, suggesting that this part of the genome may be underrepresented in the library. Because the map is only four YACs deep, it was not possible to order all of the markers unambiguously. Of the 64 markers that constitute the map, we have been able to order 48 markers.

The map contains three overlapping sets of YACs and two gaps. The order of the contigs is based on the established genetic map for chromosome 22. The most centromeric contig extends from the 1,690-kb CEPH mega YAC 848D2 that contains a GGT1 locus to a 250-kb YAC Y5A11 (ICRFy901A115). This region consists of 44 YACs. The gene for TUPLE1 (Halford et al. 1993*b*) is present in a group of markers flanked by D22S427 and D22S941.

The second contig is relatively small, consisting of three YACs—Y20E1 (ICRFy901E0120), 113A12 (ICRFy900A12113), and 748C4—and two cosmids,



cD0832 and cD22S933. This contig contains the gene COMT, TI0 (Halford et al. 1993*c*), and three other markers. The relative position of this contig was established by FISH mapping of cosmid D0832 and EO472, from which the markers HD7K, COMT, and D22S933, respectively, were derived (Lindsay et al. 1993).

The third contig spans the genetic markers D22S264 and D22S257 and contains 37 YACs and 30 markers. One of the anchor points for this contig is D22S131, which is present on YACs 792F9, 849E9, 861D9, and 881H10. This region comprises several genes including the immunoglobulin lambda locus, ZNF74, HCF2, two GGT1 repeats, and BCR. We observed that the YACs within this contig are prone to rearrangements.

Although the precise size of the two gaps between the three contigs could not be determined, it was possible to estimate the size of the region covered by the markers. This length was obtained by adding the sizes of YACs that are least likely to be chimeric (containing the largest number of consecutive markers) and that constitute a minimal tiling path. The size was 5 Mb. Since this size can only be an overestimate (because of chimerism and overlaps), the average marker resolution in this region is ⩾78 kb (5,000/64). The map encompasses genetic markers D22S420 and D22S425, which are 9 cM apart in the sex-averaged CEPH/Généthon genetic map. We estimate the size of the interval between the two markers to be 3.5 Mb. Therefore, in this part of the chromosome, 1 cM of genetic distance is approximately equivalent to 400 kb.

An additional feature of this map is that certain markers are present in two or more sets of unrelated YACs. The marker D22S131 is present within the proximal and distal contig. Similarly, a marker derived from the gamma glutamyl transferase (GGT1) gene recognizes three separate sets of YACs in 22q11. Reports of patterns of repeat sequences of GGT (Heisterkamp and Groffen 1988) and other markers on chromosome 22 have appeared elsewhere (Heisterkamp and Groffen 1988; Halford et al. 1993*a*).

Polymorphic Markers in 22q11

To facilitate genetic analysis of VCFS, we assembled a set of polymorphic markers and placed them on the physical map. We ordered eight of the nine polymorphic markers generated by other investigators. In addition, we generated and mapped two additional markers. These were derived from cosmids c443 and cN60C11 and were designated "D22S941" and "D22S944," respectively. Primers for D22S941 amplify a 224–260-bp product (table 1), which has at least eight alleles (sizes 224, 229, 239, 251, 254, 256, 258, and 260 bp) in the population, as determined by analysis of unaffected family members of VCFS patients, and reveals .66 heterozygosity (table 2). The primer pairs for D22S944 amplify a 158–178-bp product, which has nine alleles (sizes 158, 160, 162, 164, 166, 168, 170, 176, and 178 bp) in the population and has .70 heterozygosity (table 2). These two markers were placed on the map and are contained in the proximal contig (fig. 2). The 11 polymorphic markers were used to define the deletions in VCFS patients.

Analysis of Polymorphic Markers in VCFS/DGS Patients

To provide a molecular definition of deletions in VCFS patients, we isolated DNA from 61 VCFS patients

A

	PATIENT					MARKERS											CLINICAL FEATURES						
		420	427	941	944	264	311	306/308	425	303	257	FACE	VSD	TOF	rt. AA	IAA	ASD	PDA	HEART	SMCP	OSMCP	CP	LD
1	BM 51											X		XX	XX						XX		XX
2	GM07939											X							TA	NT	NT	NT	NT
3	BM 6											XX							BAV	XX			XX
4	AP 1056											XX								XX			XX
5	AP 1079											XX	XX		XX						XX		XX
6	BM 60											XX	XX							XX			NT
7	BM 34											XX							VR				NT
8	BM 72											XX							VH		NT		XX
9	AP 1074											XX								XX			NT
10	BM 11											XX	XX							XX			XX
11	BM 29											XX	XX									XX	
12	VCF3											XX						XX		XX			XX
13	BM 7											XX							BAV/AAA		NT		XX
14	BM 65											XX	XX			XX				XX			NT
15	VCF1											XX				XX				XX			XX
16	VCF2											XX	XX							XX			NT
17	AP 1012											XX								XX			XX
18	AP 1015											X		XX	XX					XX			XX
19	AP 1018											XX	XX							XX			XX
20	AP 1019											XX		XX	XX					XX			XX
21	AP 1141											XX	XX									XX	NT
22	AP 1201											XX			XX							XX	XX
23	BM 23											XX	XX							XX			NT
24	BM 45											XX			XX						NT		XX
25	BM 2											XX									XX		XX
26	BM 3											XX		XX						XX			NT
27	BM 17											XX						XX		XX			XX
28	BM 18											XX	XX								XX		NT
29	BM 37											XX	XX								NT		NT
30	AP 1011											XX			XX					XX			XX
31	AP 1036											XX							SM		XX		XX
32	AP 1136											XX					XX			XX			XX
33	AP 1229											XX									NT		XX
34	BM 1											XX									XX		NT
35	BM 5											XX	XX						MVA, LSV		XX		XX
36	BM 10											XX	XX			XX	XX			XX			NT
37	BM 41											X								XX			NT
38	BM 50											XX	XX								XX		NT
39	AP 1014											XX		XX	XX							XX	XX
40	AP 1017											XX							LSA	XX			
41	AP 1035											XX	XX									XX	XX
42	AP 1088											XX									XX		XX
43	BM 43											X											NT
44	BM 58											XX							MVP	XX			NT
45	BM 54											XX	XX										XX
46	BM 8											XX								XX			NT
47	BM 13											X									NT		NT
48	BM 14											XX	XX			XX	XX	XX		XX			NT
49	BM 15											XX										XX	NT
50	BM 26											X	XX							XX			XX
51	BM 4											XX		XX						XX			NT
52	BM 16											XX										XX	MR
53	BM 22											X	XX					XX		XX			NT
54	BM 31											XX								XX			XX
55	BM 32											X								XX			XX
56	BM 66											XX								XX			XX
57	BM 75											X								XX			NT
58	MK 400390											XX	XX								NT		NT
59	AP 1013											XX										XX	XX
60	AP 1016											XX	XX							XX			XX
61	AP 1139											XX	XX				XX		PS	XX			XX

Figure 2 *A*, Major clinical features and polymorphic marker analysis of 61 VCFS patients. Each patient who participated in the study is listed by a code name. The DNA from each individual was tested for heterozygosity of the 11 STRP markers indicated above each column. Two alleles, denoted by the shaded boxes, indicate heterozygosity at a given locus; an unshaded box denotes that there is a single allele at the locus. The clinical features of each patient are shown. A black bar separates the patients who are heterozygous for at least one of the markers D22S941 and D22S944 (patients 51–61). Typical facial dysmorphology (FACE) is denoted by "XX, and more subtle dysmorphology is denoted by "X." The conotruncal cardiac anomalies, including ventricular septal defect (VSD), tetralogy of Fallot (TOF), right-sided aortic arch (rt. AA), interrupted aortic arch (IAA), atrial septal defect (ASD), patent ductus arteriosus (PDA), along with other heart anomalies, are indicated. The presence of submucous cleft palate (SMCP), occult submucous cleft palate (OSMCP), and cleft palate (CP) are denoted by "XX." Learning disabilities (LD) are also indicated (XX). Other abbreviations are as follows: TA = truncus arteriosis; VR = vascular ring; VH = right ventricular hypertrophy; BAV = bicuspid aortic valve; AAA = probable aortic arch anomalies; SM = systolic murmur; MVA = mitral valve anomaly; LSV = anomalous left superior vena cava; LSA = left subclavian artery; and MVP = mitral valve prolapse. *B,* Polymorphic analysis of 49 unaffected family members of VCFS patients. Their relationship to the patient is indicated.

B

	RELATIVE	MARKERS: 420	427	941	944	264	311	306/308	425	303	257	RELATIONSHIP TO PATIENT
1	BM 9											Father of BM 8
2	BM 12											Mother of BM 14 & 15
3	BM 19											Mother of BM 18
4	BM 20											Twin of BM 4
5	BM 21											Brother of BM 4
6	BM 24											Mother of BM 4, 20 & 21
7	BM 25											Father of BM 4, 20 & 21
8	BM 27											Father of BM 26
9	BM 28											Mother of BM 8
10	BM 30											Mother of BM 29
11	BM 33											Mother of BM 32
12	BM 35											Father of BM 37
13	BM 36											Mother of BM 37
14	BM 38											Sister of AP 1079
15	BM 39											Mother of AP 1079, BM 38 & 40
16	BM 40											Sister of AP 1079
17	BM 42											Mother of BM 41
18	BM 44											Mother of BM 26
19	BM 46											Father of BM 41
20	BM 48											Father of BM 50
21	BM 49											Mother of BM 50
22	BM 52											Mother of BM 51
23	BM 53											Father of BM 51
24	BM 55											Mother of Vcf 1
25	BM 56											Brother of Vcf 1
26	BM 57											Father of Vcf 1
27	BM 59											Father of BM 58
28	BM 61											Mother of AP 1018
29	BM 62											Father of AP 1018
30	BM 63											Father of AP 1079,BM 38 & 40
31	BM 64											Mother of BM 60
32	BM 67											Mother of BM 66
33	BM 68											Father of BM 66
34	BM 74											Father of BM 72
35	BM 76											Mother of BM 75
36	MK 010763											Father of MK 400390
37	MK 041567											Mother of MK 400390
38	MK 062292											Sister of MK 400390
39	AP 1089											Mother of AP1088
40	AP 1090											Mother of AP 1035
41	AP 1091											Uncle of AP 1035
42	AP 1137											Mother of AP 1136
43	AP 1138											Father of AP 1136
44	AP 1140											Father of AP 1139
45	AP 1142											Mother of AP 1141
46	AP 1199											Father of AP 1201
47	AP 1200											Father of Vcf 3
48	AP 1222											Mother of AP 1201
49	AP 1230											Mother of AP 1229

and 49 unaffected relatives and typed their DNA, with each of the 11 polymorphic markers. The results from this analysis are shown in figure 2*A* and are summarized in table 2. These data provide clues to the identity of the region that is commonly deleted among VCFS patients. If a VCFS patient has two distinguishable alleles at a locus, that individual is not hemizygous at that locus. If a single allele is present, the individual is homo- or hemizygous at that locus. Significant deviation from the expected levels of heterozygosity at a locus suggests that the region may be hemizygous among the patients. When all VCFS patients are compared with their unaffected relatives (fig. 2*B*), they show the expected levels of heterozygosity of the proximal markers D22S420 and

Table 2

Determination of Significance of Loss of Heterozygosity of STRP Markers in VCFS Patients

	D22S420	D22S427	D22S941	D22S944	D22S264	D22S311	D22S306	D22S308	D22S425	D22S303	D22S257
Normal heterogeneity[a]	.82	.76	.63	.65	.88	.67	.57	.53	.71	.82	.63
VCFS heterogeneity[b]	.77	.59	.15	.16	.29	.26	.53	.54	.70	.69	.59
χ^{2c}	.18	2.31	22.54	22.17	23.72	15.13	.09	.01	.00	.72	.15
P			>.95	>.95	>.95	>.95					

[a] Frequency of heterozygosity in 49 unaffected parents of VCFS patients.
[b] Observed frequency of heterozygosity in VCFS patients.
[c] Used to determine significance of deviation from expected levels of heterozygosity.

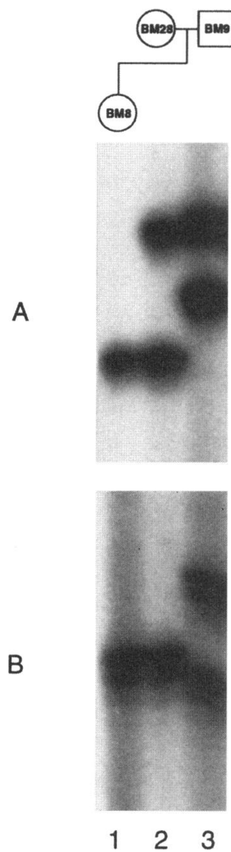


Figure 3 Denaturing gel electrophoresis of STRP markers D22S941 and D22S944: STRP analysis of DNA purified from Epstein-Barr virus–transformed lymphoblastoid cell lines from the proband BM8 and her parents BM28 and BM9. *A,* D22S941. *B,* D22S944.

D22S427 and distal markers D22S306, D22S308, D22S425, D22S303, and D22S257. The levels of heterozygosity observed for the markers D22S941, D22S944, D22S264, and D22S311 are significantly lower than the expected values (table 2), suggesting that regions containing these four markers are deleted among VCFS patients.

The polymorphic marker typing data were used to classify the VCFS patients as those who have a single allele at D22S941 and D22S944 and those who have two alleles at one or both of these loci (fig. 2). Of the 61 patients analyzed, 50 (82%) of them, designated 1–50 in figure 2*A,* had a single allele of these two loci, suggesting that the region encompassed by these two loci might be commonly deleted among VCFS patients. The other 11 patients (designated 51–61; fig. 2*A*), as well as the unaffected family members (fig. 2*B*), did not show any consistent homozygosity. Comparison of the data from the 50 patients who have a single allele at D22S941 and D22S944 with the rest of the VCFS patients and the unaffected parents also revealed patterns of possible hemizygosity at several contiguous loci. In this group of 50 patients, D22S420 and D22S427 have the expected level of heterozygosity, which abruptly changes for the next marker, D22S941. These results suggest that, among the VCFS patients who have deletions, the proximal breakpoint lies between D22S427 and D22S941. Seven of these patients, from five different families, had a single allele at D22S941 and D22S944 and had two alleles at the next distal marker, D22S264.

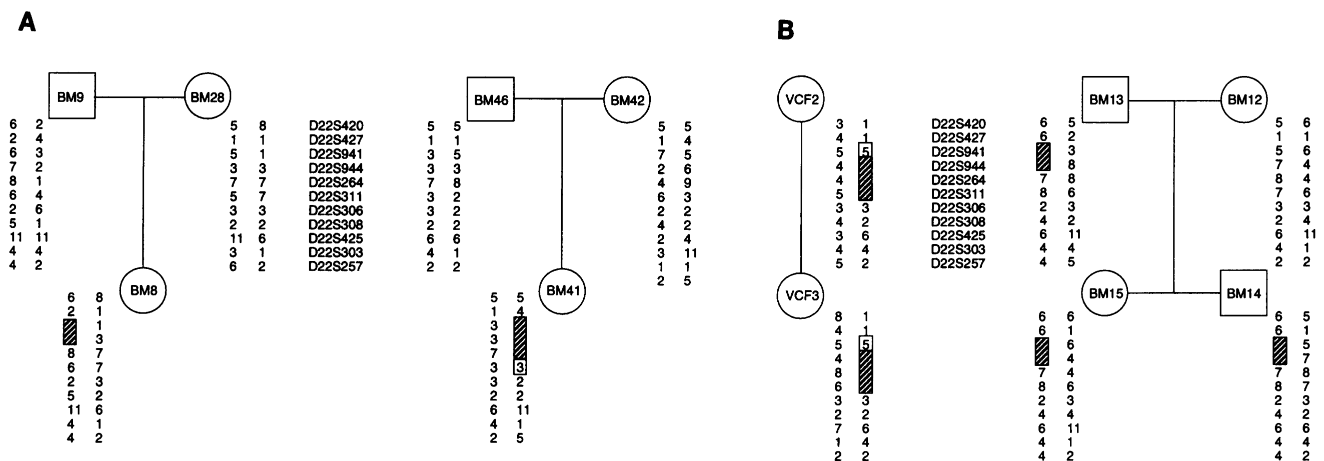


Figure 4 *A,* Genotypic analysis of BM8 and BM41. Haplotypes at the 11 polymorphic loci were deduced for two families with unaffected parents and VCFS children. The hatched box represents loss of heterozygosity of markers derived from the paternal chromosome for BM8 and from the maternal chromosome for BM41; and the unhatched box, for BM41 with marker D22S311, represents an uninformative marker for this family. *B,* Genotype analysis of VCFS3, BM14, and BM15. Haplotypes at each of the 11 polymorphic markers are shown. The hatched box represents the hemizygous region; and the unhatched box denotes that the marker was uninformative in the family.

This result suggests that among these patients the distal breakpoint lies in the region flanked by D22S944 and D22S264. Forty-three of the patients have one allele at the four consecutive loci D22S941, D22S944, D22S264, and D22S311, suggesting that the distal breakpoint in this group of patients lies between markers D22S311 and D22S306/D22S308.

Determination of Deletion Boundaries by Haplotype Analysis

To confirm that VCFS patients indeed have deletions and to define the deletion boundaries, we examined 15 families in which the child is affected while the parents were unaffected. The genotyping information from the two parents and the child was examined to deduce the haplotypes of the pairs of chromosome 22. Representative results are shown in figure 3, and the summary of data from two such families is shown in figure 4*A*. In the first case, BM8 is the patient, and BM9 is the father, and BM28 is the mother. The haplotypes at each of the 10 consecutive polymorphic loci are shown. Typing with D22S427 revealed that the mother had one allele, designated 1, and that the father had two alleles, designated 2 and 4. The patient also had two alleles, 1 and 2, 2 derived from the father and 1 derived from the mother. The typing with the next marker, D22S941 (fig. 3*A*), revealed that the mother had alleles 1 and 5 and that the father had alleles 3 and 6. The patient had only allele 1, showing that the paternal gamete did not contain this locus. At locus D22S944, the next consecutive marker (fig. 3*B*), the mother has allele 3 and the father has alleles 2 and 7. The patient has only allele 3, again showing that the paternal gamete did not contain this locus. At D22S264, the fifth marker on the map, the mother had allele 7 and the father had alleles 1 and 8. The patient had alleles 7 and 8, one allele contributed by each parent. All of the other distal markers are informative, and BM8 showed heterozygosity at all these markers. On the basis of these data, we conclude that BM8 is hemizygous at D22S941 and D22S944. These data also show that the proximal breakpoint leading to the deletion occurred between markers D22S427 and D22S941 and that the distal breakpoint was in the DNA flanked by markers D22S944 and D22S264.

In the second family shown in figure 4*A*, BM41 is the affected child, BM42 is the mother, and BM46 is the father. In this case, D22S420 was uninformative but BM41 was heterozygous for D22S427. At D22S941, the mother was heterozygous, with alleles 5 and 7, and the father was also heterozygous, with alleles 3 and 5. The child had only allele 3, suggesting that the maternal gamete did not contain this locus. At D22S944 and D22S264, BM41 contained the paternal alleles but had no contribution through the maternal gamete. At D22S311, the locus was uninformative, and D22S306 and D22S308 show clear heterozygosity. On the basis of these data, we conclude that BM41 was hemizygous for part of chromosome 22. The proximal breakpoint in this case occurred between markers D22S420 and D22S941, the same location as in BM8, but the distal breakpoint was between D22S264 and D22S306/D22S308. The relative order of D22S306 and D22S308 has not been established.

Parental Origin of the Deleted Chromosome

Comparison of BM8 with BM41 revealed two significant features. In BM8 the paternal gamete carried the deletion, while in BM41 the maternal gamete contained the deletion, indicating that the parental origin of the chromosome containing the deletion had no affect on the manifestation of the syndrome. The second feature is that in these two individuals the extent of the deletion is different. In both cases, the proximal breakpoint lies between D22S427 and D22S941. BM8 has a smaller deletion, with the distal breakpoint between D22S944 and D22S264, while BM41 has a larger deletion, which certainly encompasses D22S264 and possibly encompasses D22S311.

Haplotype analysis of 15 patients with unaffected parents who had deletions revealed that 5 of these patients received the deleted chromosome from their father, while in the rest the deleted chromosome was maternal in origin. Examination of haplotype data together with results presented in figure 2 revealed that the most likely location for the proximal breakpoint, in all patients who had deletions, is between D22S427 and D22S941, whereas the distal breakpoints can be classified into two groups. For example, in BM8 and BM26 the distal breakpoint definitely lies between D22S944 and D22S264. In BM50 and BM37 the deletion breakpoint lies between D22S264 and D22S311.

Since all of the patients were clinically evaluated, it was possible to ascertain if any correlations could be made between the size of the deletion and the degree of severity of the phenotypes. The major clinical features of each of the patients are summarized in figure 2. For example, AP1056, who has the larger deletion (fig. 2, row 2), has a submucous cleft palate and facial abnormalities but does not have any conotruncal heart anomalies. In contrast, BM14, a patient who has the smaller deletion (fig. 2, row 47), has all the features exhibited by BM6 (fig. 2, row 3) but, in addition, has a number of conotruncal anomalies. In this group of patients as a whole, we were unable to make any correlations between the size of the deletion and the phenotypic severity. It should be noted that 11 of the 61 patients studied did not reveal any deletions and that 1 patient MK400390 was hemizygous only for D22S425, a marker distal to D22S941 and D22S944.

Imprinting: No Role in the Etiology of VCFS

There are other human syndromes that are associated with hemizygosity for relatively large regions of individ-

ual chromosomes. Among the best studied of these is the Prader-Willi syndrome, which is associated with hemizygosity of 15q11 (Ledbetter et al. 1981). It has also been shown that parental imprinting plays an important role in the etiology of this syndrome (Knoll et al. 1989; Nichols et al. 1989). The fact that there is no preferential parental gametic origin of the deleted chromosome in VCFS has suggested that imprinting does not have a role in this syndrome. To confirm this view, we examined the familial cases of VCFS and ascertained if there is any bias in the manifestation of VCFS, depending on the parental transmission of the deleted chromosome. Results from haplotype analysis of two familial cases of VCFS are shown in figure 4*B*. In one case (fig. 4*B*) the parent, VCFS2 (referred to as "VCFS 5" in Driscoll et al. 1992*b*) has a deletion, as determined by Southern hybridization methods (Driscoll et al. 1992*b*). This individual transmitted the deleted chromosome to her daughter VCFS 3 (referred to as "VCFS 4" in Driscoll et al. 1992*b* and as "NY9" in Kelly et al. 1993), who was diagnosed as having VCFS. In contrast, patients BM15 and BM14 received their deleted chromosome from their father BM13 (fig. 4*B*). Together, these results clearly show that parental imprinting has no role to play in the etiology of VCFS. Similar conclusions have been reached for DGS (Driscoll et al. 1992*a*).

It is also noteworthy that, although BM15 and BM14 share the same deleted chromosome contributed by their father, they do not share all of the phenotypic features. Both have the typical facial features, but BM14 has several conotruncal heart anomalies not shared by his sister BM15 (fig. 2).

Discussion

VCFS and DGS are related disorders with overlapping phenotypes and are associated with hemizygosity for 22q11.2. There is substantial evidence that the sizes of the deletions among VCFS/DGS patients are variable. FISH experiments (Desmaze et al. 1993; Lindsay et al. 1993), as well as dosage studies of markers, based on Southern blot hybridization, support this view (Driscoll et al. 1992*a*, 1992*b*; Scambler et al. 1992; Kelly et al. 1993). Since several of the tissue and organ systems that are affected in these patients are derived from the neural crest, it has been suggested that a developmental defect underlies the etiology of VCFS/DGS syndromes (Oster et al. 1983; Kirby and Bockman 1984; Lodewyk et al. 1986). Therefore, it is possible to postulate that haploinsufficiency of one or a small set of genes located in 22q11 might cause the disease. Since the size of the deletions among patients seemed to be variable, identification of the endpoints of deletions in a sufficiently large number of patients might permit definition of the smallest region of DNA that is commonly deleted among patients. To facilitate such an analysis, we constructed a physical map of 22q11 in the form of a set of overlapping YACs.

The physical map contains 84 YACs, estimated to cover 5 Mb of DNA, and contains 64 markers. Therefore, the average spacing between markers is estimated to be 78 kb. This map also contains 11 highly polymorphic markers. These markers were developed by several different groups (Carey et al. 1990; Budarf et al. 1991; Collins et al. 1992; Porter et al. 1993; Gyapay et al. 1994; authors' unpublished data; Marshfield Foundation; CHLC). Since not all of these markers were used in constructing a single integrated linkage map, their relative order was unknown. The physical map that we constructed permitted us to order 10 of these markers unambiguously. Because these 11 markers span a 9-cM region, they provide an average resolution >1 cM. The map covers an estimated maximum physical distance of 5 Mb and an estimated genetic length of >9 cM. The 9-cM interval is covered by 3.5 Mb of DNA, and therefore 1 cM in this region is equivalent to 400 kb of DNA. It should be noted, however, that different genetic map lengths of chromosome 22 were deduced by different groups (44 cM by Gyapay et al. 1994, 79 cM by CHLC, etc.); and therefore establishing true relationships between genetic and physical distance must await more accurate length determinations.

An interesting feature of the physical map is that some markers are repeated in this region. For example, a marker derived from the gene gamma glutamyl transferase (GGT1) recognizes YACs at three different locations. Similarly, another marker, D22S131, recognizes YACs at two different locations. The presence of individual duplicated elements in 22q11 has been described by other investigators (Heisterkamp and Groffen 1988; Desmaze et al. 1993; Figlewicz et al. 1993; Halford et al. 1993; Lindsay et al. 1993), and our results confirm and extend these observations. The presence of these duplicated elements might provide a basis for the deletions observed in this region.

We utilized the 11 highly polymorphic markers to define the deletions in VCFS patients. Sixty-one VCFS patients were typed for each of the markers. We examined if the levels of heterozygosity for each of the markers deviate significantly from expected values. We used the values obtained from unaffected relatives, as the controls. These results (table 2) clearly show that, among the VCFS patients, the proximal markers, D22S420 and D22S427, show levels of heterozygosity that are indistinguishable from those in unaffected individuals. The frequencies of heterozygosity of the four consecutive markers D22S941, D22S944, D22S264, and D33S311 are significantly different from the expected values, suggesting that these regions are commonly deleted among VCFS patients.

A definitive way to establish that the VCFS patients

had deletions was to compare the genotypes at the polymorphic loci in patients and their unaffected parents. Haplotype analysis of 17 affected individuals in 16 families and of their parents provided valuable information about the disorder. On the basis of both the analysis of the families and the genotyping data summarized in figure 2, we conclude that the VCFS patients belong to two categories. One class, which constitutes 82% of the patients, had detectable deletions, while the rest did not. Three possible explanations can be offered to explain the cases where there are no deletions. One possibility is that VCFS has multiple etiologies involving loci at different chromosomes and that these cases represent deletions elsewhere in the genome. This is unlikely because, karyotypic analysis of a total of 15 VCFS patients, only 3 of whom had detectable chromosome 22 deletions, did not reveal deletions in other chromosomes (Driscoll et al. 1992*b*). The second possibility is that these individuals have deletions but that the polymorphic markers that we utilized are not sufficiently dense to detect such deletions. In an independent study (M. Karayiorgou, personal communication), somatic cell hybrids containing each of the two copies of chromosome 22 were generated from cells obtained from patients AP1013 and AP1016 and were analyzed for several markers contained in the physical map. This analysis revealed that none of the markers, including five in the D22S427-to-D22S264 interval, were hemizygous in these two patients (M. Karayiorgou, personal communication). Although we cannot be precise, we estimate the interval between D22S427 and D22S264 to be 2 Mb in size, and therefore the five markers provide an average resolution of 400 kb. Therefore, if those patients carry deletions, the latter must be <400 kb. The third possibility is that these patients are heterozygous for a mutation in the gene(s) critical for VCFS. Testing this possibility must await isolation of genes from the appropriate interval.

Among the patients who had deletions, the smallest deletion encompasses markers D22S941 and D22S944. Therefore, the commonly deleted region is flanked by D22S427 and D22S264. The estimated distance between these markers is 2 Mb. Additional markers in this interval are necessary to precisely define the extent of the VCFS commonly deleted region.

A marker developed by Driscoll et al. (1993), designated "N25," is being commonly used as a diagnostic probe to detect deletions in VCFS/DGS patients. Nine of the patients utilized in this study (BM6, BM34, BM11, BM45, VCFS3, BM7, VCFS2, AP1019, and AP1011) who show deletions are hemizygous for N25, while three patients (BM31, BM66, and BM75) who do not show any deletion have two signals for N25. Therefore, it is highly likely that N25 is located in the common deletion interval defined in the present report.

Another marker, cosmid sc11.1, was used to detect deletions by FISH in DGS patients (Lindsay et al. 1993). A similar study was performed on most of the patients reported here, and the results will be presented elsewhere (Lindsay et al., in press).

It is of interest to note that the proximal deletion breakpoint may be common to all patients who have deletions, whereas the distal breakpoints fall into two categories. An explanation for the distribution of breakpoints must await the cloning of the breakpoints in several VCFS patients.

Among the 15 patients who have deletions and in whom the parental origin of the deletion could be deduced, we observed that the deletion was paternal in origin in 7 and was maternal in 8. This observation, together with earlier observations of VCFS transmission through both sexes, suggests that imprinting does not play a role in the etiology of the syndrome.

Acknowledgments

This work was supported by the AECOM Human Genetics Program and NIH grant R01HD31601 (support to R.K.) and by MRC and EC (support to P.S.). B.M. is supported by a NARSAD award. The following individuals helped with patient identification and tissue collections: Drs. Alan Shanske, Susan Kirkpatrick, Martin Bialer, Wilma Krause, Diane Fountas, David Housman, and David Whiteman. Drs. Denis LePaslier and Jim Trofatter provided several chromosome 22 YACs. Two public databases, Quickmap of CEPH and the Whitehead Genome Center database, were extremely useful in assembling the YAC contig. Dr. Maria Karayiorgou provided the cell line for MK400390 and family. We are grateful to the patients and families who participated in the study. We thank Drs. Arthur Skoultchi, Kenneth Krauter, and Geoff Childs for their constant support and for reading the manuscript. Dr. Jim Trofatter provided several YACs used in this study. Ann Pulver provided some of the cell lines used in this study. We thank Drs. Antonio Baldini and Elizabeth Lindsay for providing information prior to publication and for many helpful discussions.

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