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Down Syndrome: Molecular Mapping of the Congenital Heart Disease and Duodenal Stenosis

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Summary

Down syndrome (DS) is a major cause of congenital heart and gut disease and mental retardation. DS individuals also have characteristic facies, hands, and dermatoglyphics, in addition to abnormalities of the immune system, an increased risk of leukemia, and an Alzheimer-like dementia. Although their molecular basis is unknown, recent work on patients with DS and partial duplications of chromosome 21 has suggested small chromosomal regions located in band q22 that are likely to contain the genes for some of these features. We now extend these analyses to define molecular markers for the congenital heart disease, the duodenal stenosis, and an "overlap" region for the facial and some of the skeletal features. We report the clinical, cytogenetic, and molecular analysis of two patients. The first is DUP21JS, who carries both a partial duplication of chromosome 21, including the region 21q21.1-q22.13, or proximal q22.2, and DS features including duodenal stenosis. Using quantitative Southern blot dosage analysis and 15 DNA sequences unique to chromosome 21, we have defined the molecular extent of the duplication. This includes the region defined by DNA sequences for *APP* (amyloid precursor protein), *SOD1* (CuZn superoxide dismutase), *D21S47, SF57, D21S17, D21S55, D21S3,* and *D21S15* and excludes the regions defined by DNA sequences for *D21S16, D21S46, D21S1, D21S19, BCE I* (breast cancer estrogen-inducible gene), *D21S39,* and *D21S44.* Using similar techniques, we have also defined the region duplicated in the second case occurring in a family carrying a translocation associated with DS and congenital heart disease. This region includes DNA sequences for *D21S55* and *D21S3* and excludes DNA sequences for *D21S47* and *D21S17.* The DS molecular-overlap region is defined by the three DNA sequences duplicated in both patients and includes *D21S55, D21S3,* and *D21S15.* These studies provide the molecular basis for the construction of a DS phenotypic map and focus the search for genes responsible for the physical features, congenital heart disease, and duodenal stenosis of DS.

Introduction

Down syndrome (DS) is a major cause of mental retardation and congenital heart disease, affecting over 200,000 persons in the United States (reviewed in Epstein 1986). In addition to these, other major features include a characteristic facies, skeletal anomalies, abnormalities of the immune system, an increased risk of leukemia, and an Alzheimer-like presenile dementia. Although usually caused by trisomy 21, a subset of these features, including the classical facies and mental retardation, may be caused by duplication of band q22 (Niebuhr 1974). Subsequent work based on cytogenetic analyses assigned the likely chromosomal location of these features to proximal band 21q22 (Poissonnier et al. 1976). More recent work based on molecular analyses has suggested that the facial and some of the other physical features may be associated with a duplication of the chromosomal region that includes the DNA sequence *D21S55* (Rahmani et al. 1989). Our recent work analyzing a family with DS and a chromosome 21 translocation has defined molecular markers for and has narrowed the cytogenetic region likely to contain the genes responsible for DS

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features—including the congenital heart disease, facial features, some of the hand and foot abnormalities, and a part of the dermatoglyphic features—to the region of distal band 21q22.1-q22.3 (Korenberg et al. 1990). We now present further molecular analyses of DS individual DUP21NA (patient IV-1) from this family and of another DS individual, DUP21JS, with duodenal stenosis (DST). The results define a small molecular-overlap region for the DS facial, dermatoglyphic, and other physical features and, in addition, define molecular markers for chromosomal regions that may contain the genes for the congenital heart disease and congenital DST of DS.

Material and Methods

Molecular Analysis

The DNA sequence probes used in these investigations are all present in single copy in the human genome, unique to chromosome 21. FB68L is a cDNA probe corresponding to the 3′ end of the APP locus (Tanzi et al. 1987). The cDNA probe for SOD1 was a gift from Y. Groner (Sherman et al. 1983). BCEI, a breast cancer estrogen-induced mRNA, was from ATCC (Moisan et al. 1985). *D21S1* (228C), *D21S3* (231C), and *D21S55* (518–8B), from P. Watkins, are DNA sequences subcloned in either pBR328 (200 series) or pBR322 (500 series), as described by Watkins et al. 1985*a*, 1985*b*). Probes for *D21S39, D21S44, D21S46, D21S47,* and SF57 are single-copy DNA sequences (Korenberg et al. 1987). The genomic clones for *D21S16* (pGSE9), *D21S17* (pGSH8), *D21S15* (pGSE8), and *D21S19* (B3) are from G. Stewart (Stewart et al. 1985*a*, 1985*b*). The chromosome 17 single-copy probe, HHH202 (D17S33) (Nakamura et al. 1987), was used as the reference for all experiments. Approximate map positions for these are indicated in figure 5, at the right of the ideogram. Order of sequences is as indicated by physical mapping studies (Korenberg et al. 1989; Gardiner et al. 1990; Owen et al. 1990; T. Falik-Borenstein and J. R. Korenberg, unpublished data). Brackets indicate groups of probes for which order has not been established.

Procedures for DNA isolation and digestion, agarose gel construction, Southern blotting, probe labeling, hybridization, and autoradiogram development were conducted as described by Korenberg et al. (1989). All probes were isolated as DNA fragments. DNAs for DUP21JS were obtained from a fibroblast cell line, for DUP21NA from a lymphoblastoid cell line, and for the diploid control, from peripheral blood. Approximately 3–4 μg of DNA digested with *Eco*RI (Bethesda Research Laboratories) were run per lane, in 1.2% agarose gels. As previously described, at least two independent gels were run, each with 16 lanes of alternating DNAs from patient and control. Probes were hybridized simultaneously in groups of three to five, including the chromosome 17 reference probe for each Southern blot membrane produced.

Cytogenetic Analysis

Extended chromosome preparations were made from peripheral blood lymphocyte cultures by using methotrexate synchronization (Yunis 1976). Metaphase-chromosome preparations were made from skin-fibroblast cultures by using standard procedures. The chromosomes were stained by G-banding (GTG) and R-banding (RHG).

Calculation of DNA Sequence Copy Number

DNA sequence copy number in DS patient DUP-21JS was determined from ratios representing the mean of 8–13 paired measurements of autoradiographic band densities obtained with DNAs from DUP21JS and diploid control. Autoradiograms analyzed were those generated by exposure of Kodak XAR film for the time required to bring the band signals to an approximately linear range of the film (determined using a National Bureau of Standards penetrometer). Each autoradiographic band was measured by a Helena EDC densitometer, and the area under the curve was integrated by computer. Individual paired measurements were calculated as the ratio of the chromosome 21 test sequence to the chromosome 17 reference sequence (*D17S33*) in DNA from DUP21JS, versus this same ratio in the control DNA. For DUP21JS, ratios of ~1.5 indicate three copies of the test DNA sequence versus two copies of the reference sequence, whereas ratios of ~1.0 indicate two copies of both the test DNA sequence and the reference DNA sequence. The mean ratios were shown by *t*-test to be significantly different from 1.0 ($P < .001$) or 1.5 ($P < .01$; $P < .001$). Ninety-five-percent confidence intervals (95% CI) are shown. Similar techniques were used for the analysis of DUP21NA.

Results

Phenotype and Chromosomal Analysis

Patient DUP21JS.—This individual, the product of an uncomplicated 41-wk gestation, was born to a 25-

Table 1

Clinical Features of DS Patient DUP21JS and of Four Affected Individuals

	DUP21JS[a]	DS Family[b] (total no./no. tested)
Age at evaluation (years)	2.0	.5, 2, 6.5, 34
Microcephaly	(−3 SD)	0/2
Brachycephaly		
Flat occiput	+	0/2
Hypotonia	+	−1/3
Lax ligaments	+	2/2
Poor suck at birth		2/2
Delayed milestones	Motor speech	3/3
Short stature	(−3 SD)	0/2
Failure to thrive		1/2
Dementia	−	0/3
Flat facies	+	4/4
Up-slanted palpebral fissures	+	4/4
Epicanthic folds	+	4/4
Telecanthus	+	2/2
Brushfield spots	−	
Flat nasal bridge	+	4/4
Dentition abnormal	−	1/1
Macroglossia	−	2/2
High palate	−	0/2
Open mouth	+	2/2
Ears cupped or low set	−	0/2
Ears small	−	0/2
Short neck	−	0/2
Heart anomaly	PDA	2/4
Gut anomaly	DST	0/4
Other (exstrophy of bladder)	+	0/4
Broad hands	+	2/4
Brachydactyly	Mild	
Fifth-finger clinodactyly	+	2/4
Wide space between first and second toes	+	
Finger pads		1/2
Third interdigital loops		2/2
Hypothenar patterns		2/3
Distal axial triradius (t″)		2/2
Single transverse palmar crease		1/2
Hallucal fibular loop		2/2
First interdigital loop		2/2
Sole open-field patterns		2/2

NOTE.—A plus sign (+) indicates presence of feature; a minus sign (−) indicates absence of feature; and a blank space indicates that no information was available.

[a] PDA = patent ductus arteriosus, closed spontaneously; and DST = duodenal stenosis.

[b] Data, for the DS patients from a single DS family, are adapted from Korenberg et al. (1990).

year-old G2P2 healthy mother and unrelated father. Family history was negative, and mother was adopted. Birth weight was 3 kg (−1.0 SD), length was 47.8 cm (1.7 SD), and head circumference was 33 cm (−1.5 SD). The following were noted at birth (table 1): DS features were exstrophy of the bladder and DST, both of which were surgically repaired. Laboratory results included a hematocrit of 60 on day 1 and of 52 on day 5 with a platelet count of 82,000 and a white-cell count of 17,400 (53% polymorphonucleocytes [PMN], 18% band PMNs, 25% lymphocytes, 2% monocytes, and 2% basophils). Cranial ultrasound on day 1 revealed mildly prominent lateral ventricles. There was a small patent ductus arteriosus that closed spontaneously

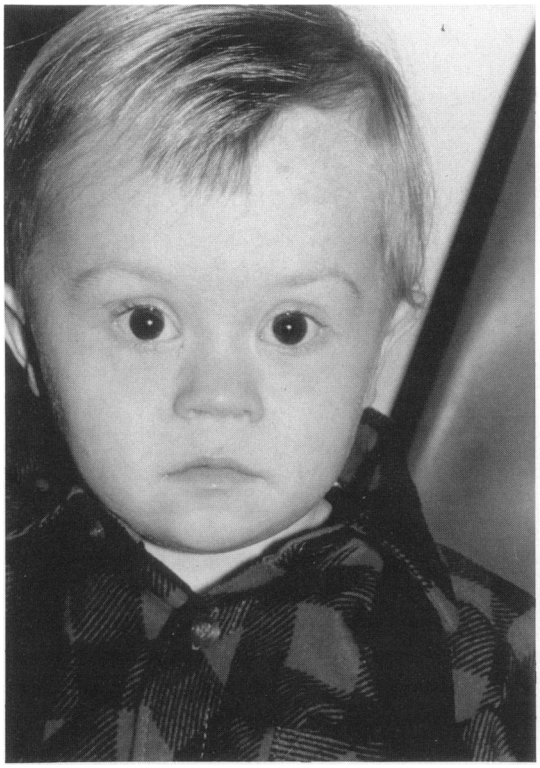

Figure 1 Photograph of DS patient DUP21JS at age 2 years.

and a normal echocardiogram. There have been no problems with infections other than occasional otitis media and urinary-tract infections secondary to his surgeries. Growth has continued since the age of about 6 mo, at below the 3% for height, weight, and head circumference. Motoric developmental delay was manifested by onset of cruising at 18 mo and walking at 23 mo. Speech was delayed, and, when the patient was 2 years of age, his vocabulary was about 12 words. No formal testing was done, and the family was not available for further evaluation. The results of the physical exam showing the features of DS at age 2 years are shown in figure 1 and table 1.

Figures 2 and 3 illustrate the results of cytogenetic analysis of DUP21JS. A total of 45 cells from the blood lymphocyte cultures and 10 cells from the skin fibroblast cultures were found to have an abnormal chromosome 21 with partial duplication of the long arm. By cytogenetic analysis, the diagnosis appears to be 46,XY,inv dups ins (21) (q22.11q22.2q21.1). This results in a duplication of the region beginning in mid 21q21.1 and extending to mid q22.2. However, we cannot rule out that the duplication is part of a more complex chromosome rearrangement. Parental chromosomes were normal.

Patient DUP21NA.—This individual and three other members of her family exhibit the features of DS listed in table 1 and described in detail by Korenberg et al. (1990). These include the facial and hand features, in addition to a part of the mental retardation and the congenital heart disease of DS. These DS individuals carry an unbalanced translocation of chromosome 21, resulting in a duplication of either 21q22.1 distal or q22.2 through qter, defined by both cytogenetic and preliminary molecular analyses (Korenberg et al. 1990).

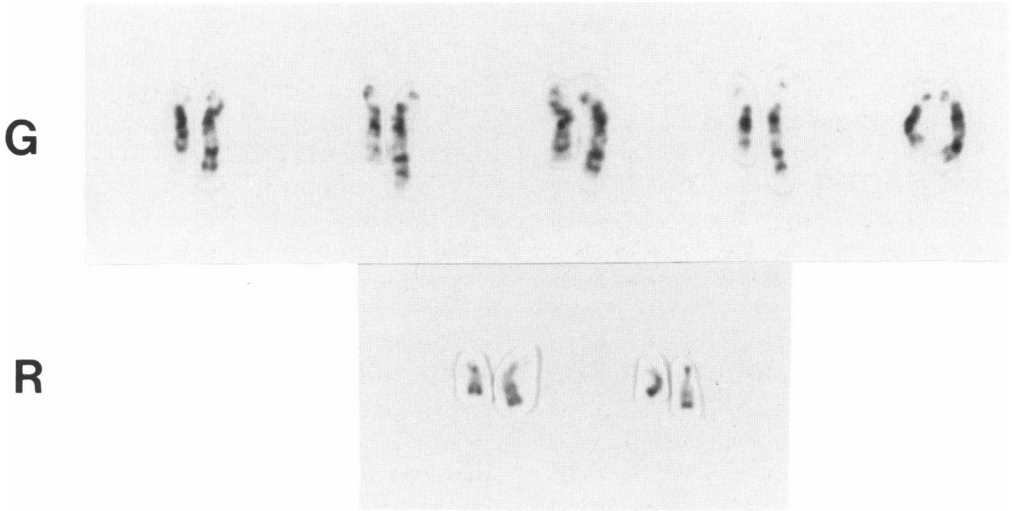

Figure 2 Cytogenetic analysis of DUP21JS. In each of the six chromosome pairs the normal 21 is shown on the left, and the duplicated 21 is shown on the right. The remainder of the karyotype was normal.

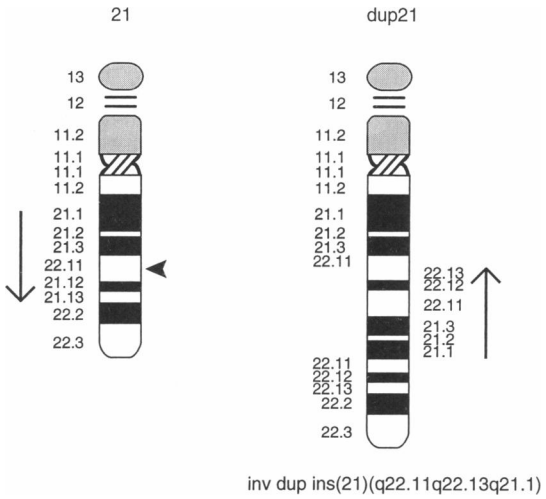

Figure 3 Ideogram of chromosomes 21 from DUP21JS that are shown in fig. 2. The arrows indicate the extent and orientation of the duplicated region. The arrowhead indicates the point of insertion.

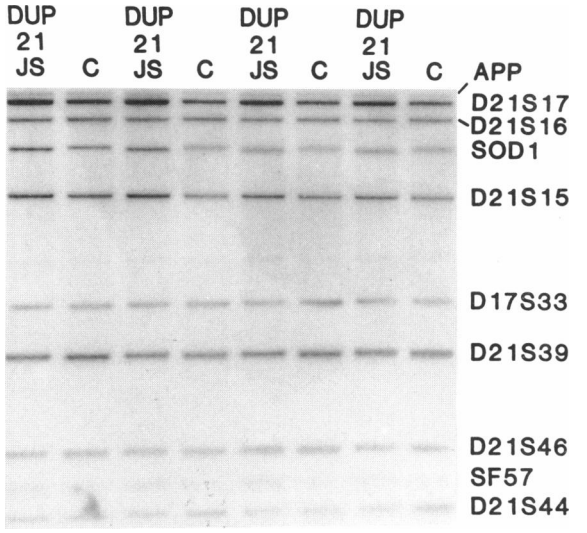

Figure 4 Representative autoradiograms from Southern blots used for analysis of DNA sequence copy number in DUP21JS and DUP21NA. Alternating lanes containing DNAs from patient DUP21JS and from the diploid control (C) are indicated. Autoradiographic bands corresponding to each DNA sequence are noted at the right of the figure. Bands corresponding to APP, *D21S17*, SOD1, and *D21S15* appear denser relative to those of the control (*D17S33*) and were present in three copies. This figure results from the superimposition of three autoradiograms, each resulting from one of three independent sequential hybridizations of the same membrane with three different sets of DNA probes. The densitometric analysis of copy number was conducted on individual autoradiograms whose bands fell in a largely linear range of film density.

Molecular Analysis

The *cytogenetic* overlap region defined by the two cases, DUP21JS and DUP21NA, is 21q22.1 (distal)-q22.2 and defines the region of chromosome 21 responsible for the shared clinical features of DS seen in these patients. To more precisely define both the extent of the regions duplicated and their molecular overlap, we made use of a physical map of this region. This physical map was constructed by using a somatic cell hybrid panel (Korenberg et al. 1990) and by quantitative Southern blot analysis in which a series of cell lines aneuploid for parts of a chromosome 21 were ordered by using the set of single-copy DNA probes specific for chromosome 21, as described in Material and Methods. We then used quantitative Southern blot dosage analysis and the DNA sequences shown in figure 5 to analyze the DNAs from DUP21JS and DUP21NA. A photograph of three superimposed representative autoradiograms used for the analysis of DUP21JS is shown in figure 4. This figure was generated as follows: A single Southern blot membrane was hybridized sequentially with three sets of probes. Autoradiograms for a given probe set were generated, and the signal was allowed to decay before the subsequent rehybridization. These individual autoradiograms were used for the densitometric analysis. However, the low background and precise band shape seen on the autoradiograms from each probe set and shown together in figure 4 permitted the superimposition of the resulting three autoradiograms. The relative band positions reflect molecular size within but not between each probe set, because of the displacement necessary to show each band clearly when superimposed. Regions that have been duplicated are present in three copies relative to the chromosome 17 reference probe (*D17S33*). Application of this technique showed that the duplication in patient DUP21JS extends from *APP* proximally through *D21S15* distally. Results of the statistical analysis for patient DUP21JS are shown in table 2 and are summarized in figure 5.

The molecular analysis for patient DUP21NA extended the previous study (Korenberg et al. 1990) and established that the duplicated region extends from *D21S55* through 21qter. The results of the statistical analysis are shown in table 2 and are summarized in figure 5. Because, on the basis of our physical map, the molecular regions of the chromosome that are present in three copies appear to be colinear in each patient, the alterations in both patients are probably simple chromosomal rearrangements.

Table 2

Copy Number in DUP21JS, for Chromosome 21-specific DNA Sequences

Probe	Ratio (95% CI)	Copy No.
D21S16	1.09** (.93, 1.27)	2
D21S46	.90** (.66, 1.23)	2
D21S1	.98*** (.88, 1.09)	2
D21S8	1.5	3
APP (FB68L)	1.54* (1.30, 1.83)	3
SOD1	1.42* (1.09, 1.56)	3
SF57	1.58* (1.37, 1.82)	3
D21S47	1.63* (1.41, 1.89)	3
D21S17	1.49* (1.38, 1.61)	3
D21S55	1.66* (1.52, 1.80)	3
D21S3	1.65* (1.38, 1.99)	3
D21S15	1.51* (1.32, 1.74)	3
D21S19	1.06*** (.95, 1.18)	2
BCE I	1.02*** (.91, 1.14)	2
D21S39	1.04** (.84, 1.28)	2
D21S44	.95** (.72, 1.26)	2

* $P < .001$ for hypothesis 1.0.
** $P < .01$ for hypothesis 1.5.
*** $P < .001$ for hypothesis 1.5.

Table 3

Copy Number in DUP21NA, for Chromosome 21-specific Sequences

Probe	Ratio (95% CI)	Copy No.
D21S47	1.16*** (1.01, 1.34)	2
D21S17	.80*** (.71, .90)	2
D21S55	1.50* (1.32, 1.70)	3
D21S3	1.62* (1.45, 1.81)	3

* $P < .001$ for hypothesis 1.0.
** $P < .01$ for hypothesis 1.5.
*** $P < .001$ for hypothesis 1.5.

Discussion

General Considerations

From a molecular analysis of patients with partial duplications of chromosome 21 and the features of DS, we have defined molecular markers for chromosomal regions likely to contain the genes for the congenital heart disease and DST seen in DS. Further, we have defined molecular markers for an overlap region

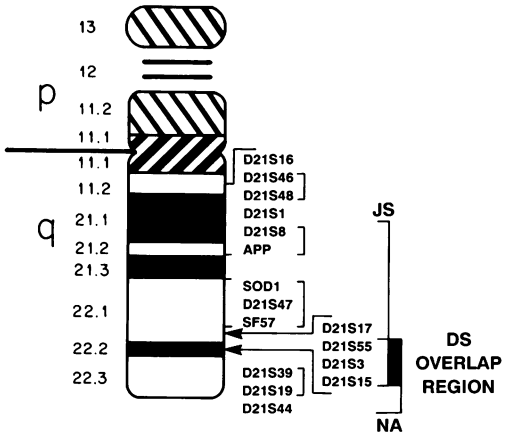

Figure 5 Chromosome 21 physical map of regions and DNA markers duplicated in DUP21JS and DUP21NA.

that may be sufficient for the development of the facial and some other of the physical features of DS. In interpreting these results, it is important to consider the following: First, the definition of chromosomal regions is limited by cytogenetic resolution, and band definition may vary depending on the technique utilized. Second, a particular feature may be mapped by its presence more than by its absence, because, with the exception of neonatal hypotonia and mental retardation, no single DS feature is present in 100% of DS individuals with trisomy 21. However, despite this variability, map position is strongly suggested when a particular feature is (*a*) *present* at about the same frequency in full trisomy 21 as it is in patients with partial duplications of a specific region but (*b*) always *absent* when this specific region is not duplicated. For example, it is significant that congenital heart disease, present in about 40%–50% of DS, has been reported in two cases of partial duplications that include the distal region of q21q22 (Miyazaki et al. 1987; Korenberg et al. 1990) but has been reported in no cases (Park et al. 1987) that do not include the region of apparent cytogenetic overlap. As expected, CHD is also absent in two cases analyzed molecularly that include this region and in six further cases that do not include the region (McCormick et al. 1989; Rahmani et al. 1989; Korenberg et al. 1990).

Second, the more specific a feature is to DS, the more likely it is that a chromosomal region may be defined that contains the genes affecting its formation. This applies both to the endocardial cushion defects (ECD) characteristic of DS and to the DS DST discussed below. For example, about 70% of all ECDs are associated with DS (Ferencz et al. 1989). In contrast, less specific features found frequently in other aneuploidies of both 21 and other chromosomes, such as mental retardation, microcephaly, or the incurved

fifth finger, may be affected by genes in more than one region of chromosome 21. Nonetheless, although the individual facial features are each less specific than ECD or DST, they appear to be more associated with duplication of the q22.2 region. Finally, from individuals with partial duplication there is little information on other important features, including the risk of leukemia, Alzheimer-like dementia, and deficits of the immune system.

Our results shown in figure 5 suggest that DNA sequences present in both patients—i.e., the overlap region—include the chromosomal sequences defined by *D21S55, D21S15,* and *D21S3,* a region of about 3.7 Mb (Gardiner et al. 1990). The phenotypic features shared by the two patients include the facial features, wide space between the first and second toes, broad short hands, incurved fifth finger, and lax ligaments. Our data are consistent with the previous reports on the molecular analysis of two patients with partial trisomy 21, suggesting that duplication of either the *D21S55* region (Rahmani et al. 1989) or the region between *D21S58* and *D21S55* (McCormick et al. 1989) was involved in generating some of the facial, hand, and foot features of DS. The region responsible for some of the DS facial features has been variably localized, by both cytogenetic and molecular techniques, to the region in or adjacent to band 21q22.2 (McCormick et al. 1989; Rahmani et al. 1989; Korenberg et al. 1990; reviewed in Park et al. 1987). The high-resolution cytogenetic analysis of the overlap region reported here for DUP21JS and DUP21NA suggests that the most likely location of this region is either band q22.2 or the adjacent region in band q22.1. The difference between this assignment and that of Rahmani et al. (1989) (to band q22.3) reflects the limited resolution of cytogenetic analyses in such small chromosomal regions. This is due in large part to differences in the banding techniques utilized and because band appearances change with chromosomal rearrangement. Therefore, definitive mapping of the DNA sequences in the overlap region awaits the development of higher-resolution mapping methods on normal chromosomes.

It is important to note that chromosomal regions defined for the congenital heart disease and DST include the overlap region and may be either located within it or entirely separate. Therefore, the current data may *not* be taken as evidence for or against the existence of a cluster of genes responsible for all of the features of DS. Rather, it is clear from previous observations of mental retardation and non-DS dysmorphic features seen in individuals with more proximal duplications of chromosome 21 (revised in Epstein 1986 and Park et al. 1987) that genes in other regions of chromosome 21 must contribute significantly to the DS phenotype.

The overlap region defined here includes 2 of the 16 known chromosome 21–specific expressed genes (Hu ets-2 and ERG, an ets-related gene), in addition to 5 of the 22 known expressed DNA sequences unique to chromosome 21 (Gardiner et al. 1990). Clearly, these are now of particular interest with respect to their effects on growth and development and, when utilized for the generation of transgenic mice, may provide interesting information on the etiology of DS.

The DS phenotype-genotype map that results from these studies is shown in figure 6. This map was constructed by placing, within the molecular region shared, the phenotypic features shared by DUP21JS and the DUP21NA family. Other features are placed in the region of respective duplication. Therefore the map illustrates the minimal regions likely to contain the genes whose duplication is sufficient to cause a given feature. It does not exclude genes located in other regions from contributing to a particular phenotypic feature. For this, more extensive data on the lack of specific features are necessary.

Congenital Heart Disease

Our findings indicate that the region likely to contain the genes for congenital heart disease in DS extends from *D21S55* to the telomere—namely, the region duplicated in DUP21NA of the DS family. This region comprises approximately 9 Mb (Gardiner et al.

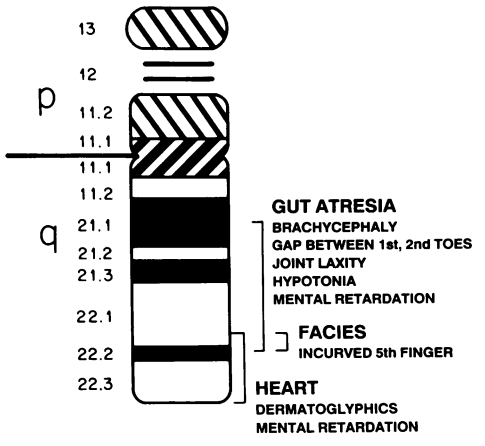

Figure 6 DS phenotypic map resulting from molecular analysis of DUP21JS and DUP21NA.

1990). Further narrowing of the region to the proximal half of this segment is suggested by combining a knowledge of the chromosome 21 physical map with data from the trisomy 16 (Ts16) mouse. This mouse has been the animal model of DS because mouse chromosome 16 (MMU 16) shares a large homologous region with human chromosome 21 (HSA 21) (reviewed in Epstein 1986). The Ts16 mice have a high incidence of congenital heart disease (Miyabara et al. 1982) similar to that seen in DS, suggesting that the genes in the MMU16 regions that are homologous to HSA 21 regions may be responsible. Preliminary data indicate that this region of homology includes only the proximal half of the region duplicated in DUP21NA and defined by *D21S55* through *MX1*, the MX influenza virus–resistance gene (Cheng et al. 1988; Reeves et al. 1989; Gardiner et al. 1990). Therefore these data, combined with ours, may narrow, to about 4–5 Mb, the region of chromosome 21 likely to contain the genes for DS congenital heart disease. This size of region may contain about 30 expressed genes, only a subset of which are expressed in the fetal heart. Therefore such studies may directly bridge the gap that allows the definition and testing of small numbers of genes responsible for heart disease.

DST

DST occurs in 1 in 10,000–40,000 live births but in 4%–7% of DS infants with full trisomy 21. Further, infants with DS constitute about 35% of infants with congenital DST (Lynn 1969). Consequently, although patients with DST and duplications of chromosome 21 are rare, definition of their duplicated regions may prove valuable in defining the genetic basis of DST. The duplicated region in DUP21JS provides a molecular definition of the regions of chromosome 21 that may be involved. This extends from *D21S8* through *D21S15*, a region of greater than 26 Mb. Although this is a large region, it is important to note that this excludes almost 40% of the known genes on chromosome 21.

The exstrophy of the bladder seen in DUP21JS is seen rarely in many chromosomal aneuploidies. It is neither specific nor common in DS and is therefore not placed on the phenotypic map.

Chromosome 21 Physical Map

Molecular analysis of the two cases presented in the present report further defines the physical map of chromosome 21. The inclusion of the DNA sequence *DS1S15* in the duplication of *DUP21JS* maps it distal to *D21S3* but proximal to *D21S39*, *D21S19*, *D21S44*, and *BCEI*, as shown in figure 4. Our cytogenetic analysis suggests that its possible map location is close to or within band 21q22.2. Further, the position of *D21S55* as distal to *D21S17* (Rahmani et al. 1989) is confirmed. The results presented in the present report begin to provide a basis for defining the molecular biology of both DS congenital heart disease and DS DST.

Acknowledgments

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