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Genetics

Down syndrome phenotypes: The consequences of chromosomal imbalance

(chromosome 21/aneuploidy)

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ABSTRACT **Down syndrome (DS) is a major cause of mental retardation and congenital heart disease. Besides a characteristic set of facial and physical features, DS is associated with congenital anomalies of the gastrointestinal tract, an increased risk of leukemia, immune system defects, and an Alzheimer-like dementia. Moreover, DS is a model for the study of human aneuploidy. Although usually caused by the presence of an extra chromosome 21, subsets of the phenotypic features of DS may be caused by the duplication of small regions of the chromosome. The physical map of chromosome 21 allows the molecular definition of the regions duplicated in these rare cases of partial trisomy. As a first step in identifying the genes responsible for individual DS features and their pathophysiology, a panel of cell lines derived from 16 such individuals has been established and the molecular break points have been determined using fluorescence *in situ* hybridization and Southern blot dosage analysis of 32 markers unique to human chromosome 21. Combining this information with detailed clinical evaluations of these patients, we have now constructed a "phenotypic map" that includes 25 features and assigns regions of 2–20 megabases as likely to contain the genes responsible. This study provides evidence for a significant contribution of genes outside the *D21S55* region to the DS phenotypes, including the facies, microcephaly, short stature, hypotonia, abnormal dermatoglyphics, and mental retardation. This strongly suggests DS is a contiguous gene syndrome and augurs against a single DS chromosomal region responsible for most of the DS phenotypic features.**

Down syndrome (DS) is a major cause of mental retardation, congenital heart disease (CHD), and congenital anomalies of the gastrointestinal tract affecting the welfare of >300,000 individuals and their families in the U.S. alone. DS is also associated with a characteristic set of facial and physical features, defects of the immune and endocrine systems, an increased risk of leukemia, and an Alzheimer-like dementia; moreover, it is the prototype for the study of human aneuploidy.

From classical studies in plant genetics, pioneered by Blakeslee (1) in *Datura*, it was known that trisomy for chromosome arms produced easily recognizable phenotypes. When human trisomies were discovered, Patau (2) immediately planned to use partial trisomics as a way of mapping the diverse symptoms of these individuals. His earliest attempt is summarized in ref. 2. The present paper continues the theme, using the much more refined methods of modern cytology and molecular genetics.

With the discovery that DS was caused by trisomy 21 (3, 4), and the subsequent proposal that chromosome 21 band q22 was "pathogenetic" for DS (5), the foundation was laid for elucidating the fundamental biochemical and morphogenetic pathways of abnormal development in this aneuploidy. There followed a series of reports of individuals with "partial trisomy 21" (for review, see ref. 6) that appeared to indicate that regions might be defined that were likely to contain genes responsible for particular features of DS. These studies provide the basis for construction of a DS phenotypic map.

By "phenotype" we mean a measurable parameter and include clinical, physical, cellular, and physiological components. By "phenotypic mapping" we mean the molecular definition of a physical region that is likely to contain the gene(s) whose overexpression is ultimately responsible in part for the phenotype. The current revolution in human molecular genetics and the development of a physical map of chromosome 21 now provide the possibility to understand the genetic basis for some of these defects and, therefore, to provide a necessary first step for their prevention, amelioration, and perhaps ultimately, their treatment.

Phenotypic maps provide the basis for clinical prognosis for individuals with partial aneuploidy for chromosome 21, and when of high resolution, the basis for the identification of the genes responsible for the phenotypes. One approach to this combines the phenotypic information from individuals with "partial trisomy" such as those described above with a molecular definition of their duplicated chromosomal regions. Once the molecular markers for a region are defined, the genes within it may then be identified, characterized, and ultimately tested for their relationship to a given phenotype. This report describes the molecular and phenotypic definition of these individuals, provides a theoretical framework, and utilizes this to construct a molecular "map" of the phenotypes associated with DS.

MATERIALS AND METHODS

Two methods are used to define the regions duplicated in patients with partial aneuploidy for chromosome 21. These

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Abbreviations: DS, Down syndrome; FISH, fluorescence *in situ* hybridization; CHD, congenital heart disease.
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are quantitative Southern blot dosage analysis and fluorescence *in situ* hybridization (FISH). Each utilizes a series of previously mapped chromosome 21 DNA markers to define the copy number and/or structural rearrangement characterizing the aneuploid chromosome.

The approximate map positions and order for each of these loci is as indicated by physical mapping studies (7–10).

Procedures for DNA isolation and digestion, agarose gel construction, Southern blot analysis, probe labeling, hybridization, and autoradiogram development were conducted as described by Korenberg *et al.* (11). Southern blots utilized 8–12 paired lanes (16–24 lanes total) of patient and control DNAs. Densitometric analyses utilized the logarithmic transformation of density measurements. All probes were isolated as DNA fragments for Southern blot procedures or as plasmids or cosmids for FISH studies. The sources and references for all probes used can be found in *Human Gene Mapping 11* (12). DNAs were obtained from peripheral blood, fibroblasts, or lymphoblastoid cell lines. FISH studies were conducted with the techniques and procedures as described in Korenberg *et al.* (13).

Extended metaphase chromosome preparations were made from peripheral blood lymphocyte cultures by using methotrexate synchronization (14) and from skin fibroblast cultures. The chromosomes were stained by GTG-banding and reverse-banding techniques.

RESULTS

As a first step in establishing a "phenotypic map," a panel of individuals with partial duplications of chromosome 21 was assembled. By the DS protocols for clinical assessment established in Epstein *et al.* (6), the clinical features of 16 individuals with partial trisomy 21 were defined. Complete data were unavailable in many cases. All data were taken from the published literature or from the original records of the examining geneticist and were confirmed by follow-up examinations or discussions with the family and patient. The clinical evaluations are summarized in Table 1.

By using the Southern blot and FISH techniques, the chromosome 21 molecular content has been determined in the 16 cell lines derived from the individuals with partial trisomy 21. The results of the molecular studies are given in Table 2 and are summarized in Fig. 1.

Table 1. Clinical features of 16 patients with partial trisomies of chromosome 21

DUP21JG	DUP21GY	TETRA21MI	DUP21WB	DUP21JS	DUP21KJ	GM 0144	DUP21DS	DUP21SOS	GM 1413	DUP21SOL	DUP21JSB	DUP21NA	DUP21NO	DUP21BA	DUP21SM	Phenotypic Feature
+	+	-	-	+	-	+	±	–		±	-	+	+		+	Short stature
+	+	+	-	+	-		+	–		+	-	-	-		-	Microcephaly
-	+	+	-	+	+	-	-	–	+	+	-	-	-		-	Brachycephaly
-	-	-	+	+	+	+		+	+	+	+	+	+	+	+	Flat facies
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Up-slant palp. fissures
-	+	-	+	+	-	-		+	+	+	+	+	+	+	+	Epicanthic folds
-	+	-	-	-	-	-		+		-	+	-	-		-	Brushfield spots
	+	-	+	+	+	+		+		-	+	+	+	+	+	Flat nasal bridge
	-		+	-	+	-	+	–		+		-	-		+	Vaulted palate
	+	-			-	+	+	–	+	-					-	Furrowed tongue
	+	+		+	+	+	+	+		+	-	+	+	+	-	Open mouth
-	-	-	-		-	-	+	–	+	+	-	-	-		+	Malpositioned ears
-	-	-	-	-	-	+	+	–	+	+	+	-	-		+	Small/Dysmorph. ears
	+	+	+	-	-	+		+	+	+	-	-	-		+	Short neck
-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	+	Cardiac anomaly
-	-	-	-	+	-	-	-	+		-	-	-	-		-	Duodenal stenosis
-	-	+	+	+	±	+	+	+	+	+	+	+	+		+	Broad short hands
-	-	+	+	+	±	+	+	–	+	+	+	+	+		-	Brachydactyly
	+	–	+	+	±	+	+	–	+	+		+			-	Clinodactyly 5th finger
	±	–		+	+	+	+	+		-	+				-	Wide gap toes 1 & 2
	-	+			-		+		+			+	+		+	Abnl. dermatoglyphics
	-	+	–		-	+	+	–	+	-		+	+		+	Palmar crease
+	±	+	+	+	-			–		-	+	-	-	+	-	Hypotonia
+	-	+	+	+	+	+		+		-	+	+	+		-	Lax ligaments
P	59	M	M	M	M	43	P	M	37	P	M	42	P	P	52	IQ or MR (Moder. or Profound)

+, Presence of a feature; −, absence of feature; ±, borderline or marginal presence of feature; blank, no information was available. MR, mental retardation; P, profound MR; M, moderate MR.

DISCUSSION

It is useful to review the phenotypic features of DS as they provide a view of the potential of this approach for understanding the development of complex phenotypes. These are detailed in recent reviews (15, 16).

There are several important issues. (*i*) Trisomy 21 is associated with a rich variety of phenotypes. (*ii*) As seen in most autosomal dominant single-gene disorders, most of the phenotypic features are variable in both prevalence and expression. Two exceptions are the existence of mental retardation and neonatal hypotonia in close to 100% of individuals with DS. (*iii*) DS phenotypes may provide significant models for understanding development even when they are variable or of low frequency. For example, duodenal stenosis is seen in 4–7% of individuals with DS, but this accounts for 30–50% of all congenital duodenal stenosis (11). Moreover, although the DS endocardial cushion defects represent only ≈50% of DS CHD, individuals with DS account for close to 70% of all endocardial cushion defects (11). For both of these features, and quite likely for more, these data suggest the existence of gene(s) on chromosome 21 that are important in the development of the heart and gut both in DS and in normal individuals (11). Similar considerations suggest the existence of genes on chromosome 21 involved in the development of megakaryocytes (acute megakaryocytic leukemia) (17), the cornea (keratoconus) (for review, see ref. 18), aging (amyloid precursor protein) (for review, see ref. 19), and the brain (for review, see ref. 20). Although this last requires the more precise definition of specific phenotypes, an intriguing first candidate may be found in the investigation of the specific abnormalities of the brain-stem auditory-evoked potentials seen in DS. Because these are measurable at all ages, it may be possible to define small molecular regions containing fewer than 10 genes. Clearly, the potential relationship of such physiological "phenotypes" to the DS clinical cognitive abnormalities of auditory–verbal processing may be of significant interest for understanding a part of the mental retardation seen in DS.

There are many well-established potential sources for the phenotypic variability seen in full trisomy 21. This includes allelic heterogeneity for chromosome 21 (trisomic) genes, epistatic interactions (of chromosome 21 genes with genes on 21 or on other chromosomes), imprinting effects (variability of gene expression associated with the parental origin of the third chromosome 21), and environmental including stochastic and other pre- and postnatal events. These sources may clearly affect single-gene traits and the considerations of mechanism are similar. However, for individuals with partial aneuploidy, the chromosome structure is altered and the potential for position effects must also be considered, particularly for genes placed in close proximity to telomeres or to centromeres. For example, as in lower organisms, it is not unreasonable to expect that the expression of genes in a trisomic region or in regions bordering a deleted region may

Table 2. Results of the molecular studies on our eight patients with partial trisomy of chromosome 21

		JG		GY		WB		KJ		DS		SOL		JSB		SM	
Probe	Locus	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#	Ratio	#
pGSE9	D21S16	1.52	3	2.19	3	0.83	2			0.90	2			0.38	2		
pGSM21	D21S13E									1.04	2						
p21-4U	D21S110					1.06	2	1.14	2	0.82	2	1.10	2				
pPW228C	D21S1	1.30	3			0.82	2			1.49	3					1.20	2
pPW236B	D21S11																
pUT-B14	D21S116					1.87	3										
pPW245D	D21S8	1.39	3			1.70	3	1.18	2	1.46	3			0.86	2	0.69	2
FB68L	APP	1.53	3	2.36	3	2.20	3			1.45	3			1.19	2		
pUT-B79	D21S121																
JG108	D21S99		3f														
pPW513-5H	D21S54	1.18	2	1.33	3	1.35	3	0.80	2					0.84	2		
JG77	D21S93	1.13	2	0.88	2	1.12	2	1.79	3			1.08	2	1.11	2		
pUT-C43	D21S129	1.17	2			0.87	2					2.00	3	0.82	2		
pSOD1	SOD1	0.90	2f	0.80	2	1.10	2	1.39	3	1.64	3	2.60	3				
pPW524-5P	D21S58					1.64	3	1.59	3								
pPW525-5H	D21S65	0.93	2	0.99	2	1.90	3	1.40	3			1.40	3				
pGSH8	D21S17	1.02	2	0.89	2	1.54	3	1.90	3	1.26	3	1.90	3	1.05	2	0.93	2
pPW518-1R	D21S55	1.07	2			1.46	3f							1.88	3	1.31	3
VS9	ERGB	0.86	2	0.82	2	1.39	3f							2.03	3		
pHO33	ETS2	0.98	2	0.93	2	0.82	2										
pPW231C	D21S3			0.96	2	0.76	2									1.68	3
pGSE8	D21S15	1.13	2	1.05	2	0.77	2	1.65	3	1.53	3	1.60	3			1.35	3
SF13a	D21S39	0.98	2	1.05	2	0.74	2			1.51	3			1.58	3	1.41	3
MX1a (1800)	MX1							1.09	2								
pS2	BCEI	0.95	2			1.08	2										
pGSB3	D21S19							1.02	2								
pGEM3	PFKL			1.15	2												
3.1.1	ITGB2			0.89	2	1.00	2										
SF50	D21S44	1.01	2	1.22	2	0.42	1			1.55	3			1.49	3		
pML1	COL6A1	0.80	2			0.58	1			1.99	3						
pML18	COL6A2											1.80	3				
pKN3	S100B																
pUT-B88	D21S123	1.00	2			0.50	1	1.07	2	1.42	3			1.65	3		

Ratio and copy number are given for Southern blot analyses. f, Independent test result by FISH study.

be decreased when rearrangement places them in regions of different chromatin structure such as centromeres, telomeres, or different bands. This apposition and the consequent potential change in expression may generate phenotypic variability unrelated to the genes in the aneuploid region. Such effects of chromatin environment on gene expression have not been demonstrated in humans but could be tested in this system.

The ultimate goal of constructing a phenotypic map is to define molecularly the chromosomal regions and ultimately the genes that are responsible for particular phenotypes. To do this, both the phenotypes and the molecular data must be well-defined. Although some individuals with small duplications exist, more often, the molecular data from many individuals must be combined to define small regions of 2–3 megabases that are suitable for molecular analysis. To combine data, one must consider the potential for both the phenotypic variability described above and for multiple sites affecting a single phenotype. When a trait is caused by the overexpression of a single gene or gene cluster, we may define the region containing that gene simply as the region of minimal molecular overlap of all individuals exhibiting the phenotype. However, if genes in more than one region contribute significantly to the phenotype, a simple overlap procedure may erroneously define the overlap region as containing the genes when, to the contrary, the gene(s) responsible are located in the nonoverlapped region. Therefore, it is important to determine which traits are caused largely by single genes or loci. To determine this, we should not ask which region is responsible, but rather, what part of the variability of a trait is contributed by the overexpression of genes in a given region. This question may be formulated in the classical genetic terms of penetrance, the probability of expressing a trait given the presence of the gene responsible, and expressivity, the variability of phenotypic expression of a trait, given that it is expressed.

The gene(s) in a single region may be largely responsible for a given phenotype when the penetrance and the expressivity of the trait are the same in individuals with full trisomy 21 and in individuals with duplications of the single region.

Then, as has been demonstrated, a simple overlap procedure may be used to create a phenotypic map. The expectation is that individuals whose duplication does not include the candidate region will not express the trait at a frequency above that seen in the normal population.

This supposition seems to hold true for the CHD seen in DS, in which the frequency of CHD in partially trisomic individuals varies with the region duplicated. In previous cytogenetic studies of individuals with duplications of regions outside of distal 21q22, 0% (0 out of 12 individuals) had CHD, whereas in 50% (9 out of 18) of those carrying duplications that included the region of distal 21q22.2–q22.3, CHD was evident (6, 11). Moreover, 33% (3 of 9 with CHD) had atrioventricular septal defects, similar to the proportion seen in full trisomy 21. Therefore, we may consider that the penetrance (percentage of DS with CHD) and expressivity (percentage of DS CHD that is atrioventricular septal defects) of CHD are similar in duplications of distal 21q22 and full trisomy 21. This suggests a single locus responsible for most of the variability of the trait.

By partitioning phenotypic variation in terms of penetrance and expressivity, our model allows for the definition of multiple-loci-affecting traits. For example, recent evidence suggests a possible role for *COL6A1* in generating DS CHDs (21). Such evidence would not necessitate a change in the map but could now be expressed as contributing a portion of the penetrance or expressivity of phenotypic variability, such as atrioventricular septal defects vs. atrial septal defects.

The DS phenotypic map, based on the 16 patients in this panel, is shown in Fig. 2. The gene candidate regions are defined and noted to reflect the possible contribution of one, two, or three and greater numbers of loci to the phenotype. This is to accommodate that, for less common traits in which the numbers of informative cases for any given phenotype become limiting, an analysis of penetrance and expressivity cannot be done. Moreover, in contrast to DS CHD, complete data for most phenotypes are lacking. Therefore, where only small numbers of informative cases exist, minimal regions are defined by the duplication observed and second sites must be considered. Finally, multiple genes must affect a trait when two nonoverlapping duplications exhibit the same phenotype. Where multiple nonoverlapping duplications are associated with a phenotype, each "island" of duplications may be analyzed separately.

Therefore, on the DS phenotypic map (Fig. 2), the following hierarchy of conventions is used:

If a single gene or cluster is responsible for most of the variability of a phenotype, the thick lines indicate its location. This minimal region is defined by the overlap of all contiguous cases manifesting the phenotype or by a single case, where only one exists. Where a single thick line is present, duplications including this minimal region generate phenotypes with the same penetrance and expressivity as seen in full trisomy 21; where there are two minimal regions, both would be necessary. However, for both single and double minimal regions, all cases with the phenotype must include at least one of these regions.

If two or more genes or clusters in a single island are responsible for a phenotype, the thin lines indicate the extent beyond the minimal region in which the two genes may be located. These are defined by the sum of the duplicated regions that have been independently associated with a phenotype. As for minimal regions, all cases with the phenotype would include at least one of these regions.

If three or more genes or clusters on chromosome 21 contribute to a phenotype, the dashed lines indicate the regions in

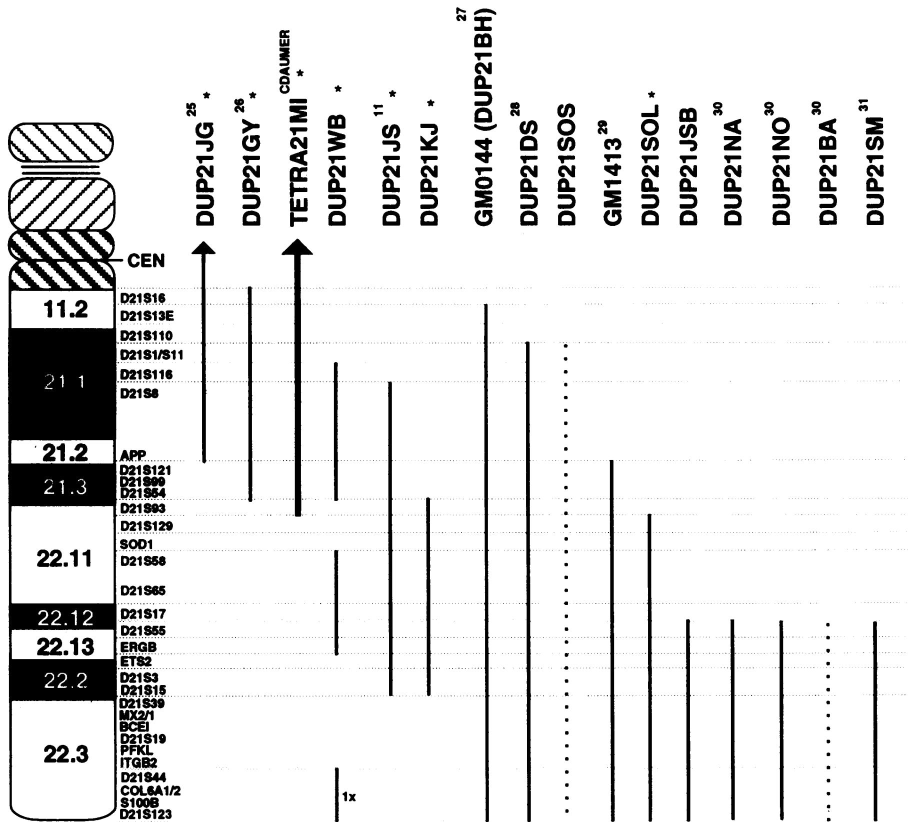

FIG. 1. Graphic summary of molecular studies on the 16 individuals used for the DS phenotypic map in Fig. 2. Solid lines, regions of duplication tested directly; dotted lines, findings inferred from family members with the same karyotype. Superscript indicates reference of published study; all other data have been collected by this laboratory except for data from TETRA21MI, which were from C.D., S.S., and J.R.K. (unpublished data). All cell lines are lymphoblastoid, except for GM 0144 and GM 1413, which are fibroblast lines obtained from the Human Genetic Mutant Cell Repository. *, Indicates aberration involving chromosome 21 only.

which the third may be located. These are defined by (usually large) duplications that have been associated with a phenotype but that include two nonoverlapping minimal or maximal regions, each of which must contain at least two genes.

The open regions have not been associated with the phenotype.

One significant conclusion from this map is that genes outside the *D21S55* region also contribute to what has been called the DS phenotype. This is based on the observations (Fig. 1 and Table 1) from three individuals with proximal trisomies that do not include this region but clearly exhibit typical DS features. It is not clear whether these features are

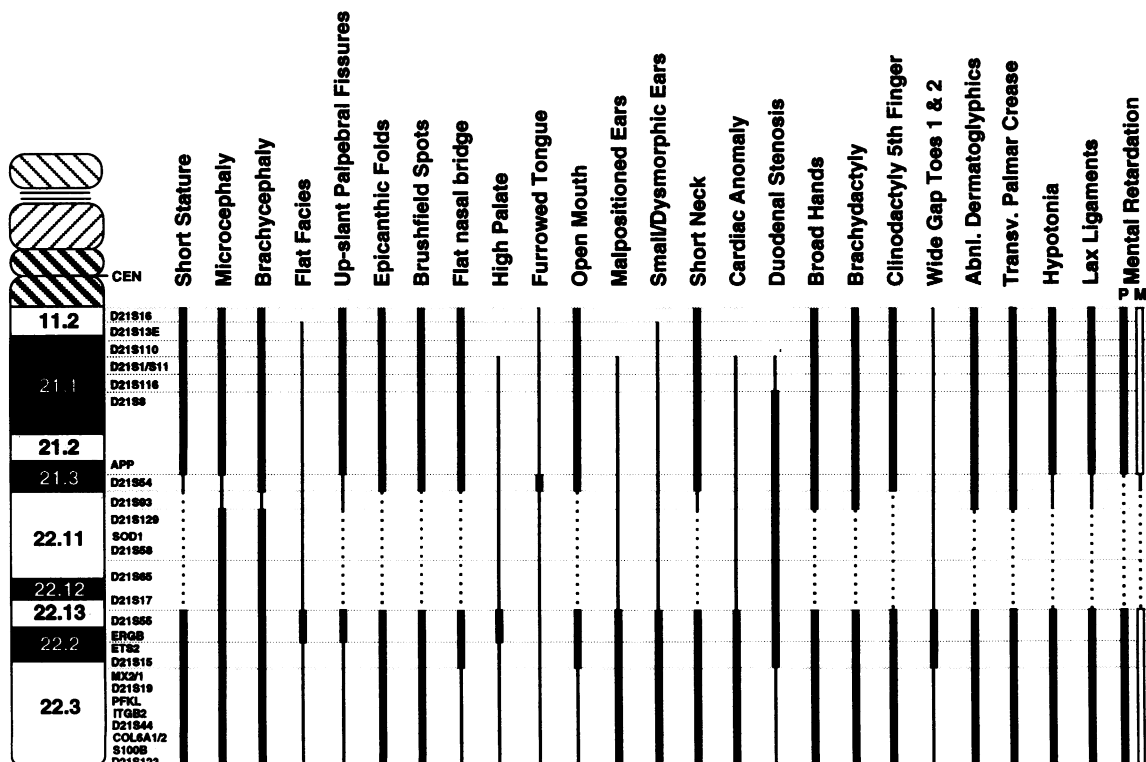

FIG. 2. Phenotypic map of 25 features associated with DS (see text for description and discussion of figure).

also affected by genes in other regions. However, the existence of second sites for many is expected in that all are somewhat nonspecific. In comparison to our model, Sinet and coworkers (22) utilize overlap exclusively to create the DS map. Because 9 of their 10 subjects include the region *D21S55*, it is difficult to conclude otherwise than this region is important in all of the features. Nonetheless, when viewed as minimal regions, their regions overlap ours for those features analyzed in common. However, the inclusion of only a single case with a non-*D21S55* duplication limits the evaluation of a DS chromosomal region and excludes the clear contribution of other regions to the DS phenotypes seen in our study.

A DS chromosomal region would imply that a single gene or gene cluster exists whose duplication is largely responsible for all DS features. From the DS phenotypic map data presented in Fig. 2, it is unlikely that such a region exists. We have shown that the duplication of regions distinct from distal 21q22 is sufficient to produce many of what have been called typical DS features. These second sites emphasize the necessity for an approach such as that detailed above for the construction of phenotypic maps. This would seem to suggest an alteration in nomenclature: away from the DS region and toward a more specific group of terms that associate particular regions with specific phenotypes, for example a DS CHD region or a DS gastrointestinal tract region.

Further indication for the role of multiple genes derives from the location of the chromosome 21 gene for amyloid precursor protein that is responsible for one type of familial Alzheimer disease, and possibly for the increased incidence of Alzheimer disease in DS. This is considerably distant from a region (*D21S55*–telomere) that is in part responsible for numerous other features including DS CHD (11). Moreover, although not yet shown, it has been suggested that the chromosome 21 gene for acute megakaryocytic leukemia associated with the 8;21 translocation (23) is also responsible for DS–leukemia risk. This gene is also found outside the region of *D21S55*–telomere. In contrast, we may still ask whether a subset of the DS phenotypes may be caused by or in some measure affected by the overexpression of a single gene or cluster. The current data from all sources are not adequate to resolve this question. In specific, there is still overlap between the regions defined for DS CHD, duodenal stenosis, and a part of the facial and other physical features, all of which could, in principle, be caused by a single gene.

Therefore, DS and its phenotypes are most accurately thought of as the result of the overexpression and subsequent interactions of a subset of the genes on chromosome 21. The DS phenotypic map thus reflects the nature of DS as a contiguous gene syndrome. Although usually reserved for syndromes caused by small deletions in which the genes are more readily defined, the term equally well represents the characteristic traits of DS.

Finally, while not directly related to DS, decreases in chromosome 21 gene copy number are under investigation (24) and may also shed light on underlying mechanisms leading to abnormal development. When the regions have been cloned in large fragment vectors such as yeast artificial chromosomes or bacterial artificial chromosomes, these reagents may be used to isolate and evaluate genes that are expressed in human (or mouse) embryonic tissues. For DS CHD, the entire region has been cloned in yeast artificial chromosomes, and cDNA libraries are being constructed from tissues obtained at this period of development.

All data suggest that we will be able to define the genetic basis of DS phenotypes and that this understanding will provide clues to understanding normal human embryonic development. Moreover, it will ultimately provide a basis for understanding and perhaps ultimately treating the associated defects, including CHD, gut disease, and some of the mental retardation.

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