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Critical role of the *D21S55* region on chromosome 21 in the pathogenesis of Down syndrome

(trisomy 21/phenotype)

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ABSTRACT **The duplication of a specific region of chromosome 21 could be responsible for the main features of Down syndrome. To define and localize this region, we analyzed at the molecular level the DNA of two patients with partial duplication of chromosome 21. These patients belong to two groups of Down syndrome patients characterized by different partial trisomies 21: (*i*) duplication of the long arm, proximal to 21q22.2, and (*ii*) duplication of the end of the chromosome, distal to 21q22.2 We assessed the copy number of five chromosome 21 sequences (*SOD1, D21S17, D21S55, ETS2,* and *D21S15*) and found that *D21S55* was duplicated in both cases. By means of pulsed-field gel analysis and with the knowledge of regional mapping of the probes *D21S17, D21S55* and *ETS2*, we estimated the size of the common duplicated region to be between 400 and 3000 kilobases. This region, localized on the proximal part of 21q22.3, is suspected to contain genes the overexpression of which is crucial in the pathogenesis of Down syndrome.**

Down syndrome (trisomy 21) is the commonest birth defect, afflicting 1 in 700 liveborn infants. It is mainly characterized by a specific phenotype and mental retardation. In most cases, it results from the presence in all cells of an extra copy of chromosome 21 (1). In rare cases, Down syndrome is associated with a partial trisomy 21. Karyotypic analyses of such cases have indicated that only the distal part of chromosome 21, band 21q22, is involved in the pathogenesis of the syndrome (2). In addition to individuals with partial trisomy for the entire band 21q22, cytogenetic studies have identified two other groups of patients characterized by either a duplication including the proximal part of the band 21q22 (3–5) or a duplication of only the distal part of 21q22 (6, 7). These observations could be explained by a duplication of only a portion of the band 21q22, adjacent to sub-band 21q22.2, that is critical for the expression of Down syndrome (8) and that is present in both groups. To test this hypothesis and to precisely define this suspected common duplicated region, we studied, at the molecular level, one patient from each group. Both patients (3, 6) have many features of Down syndrome associated with partial duplication of distinct regions of chromosome 21, respectively q11.205→q22.300 and q22.300→qter. The study of the number of copies of DNA sequences located on chromosome 21—namely *SOD1* (superoxide dismutase, soluble), *D21S17, D21S55, ETS2* (avian erythroblastosis virus E26 v-*ets* oncogene homolog 2), and *D21S15*—has shown that *D21S55* was duplicated in both cases. This finding demonstrates that the same region is duplicated in these two patients and that this region may be crucial for the pathogenesis of Down syndrome. According to the current knowledge on the linkage and regional mapping of the probes *D21S17, D21S55,* and *ETS2* on chromosome 21 (refs. 9–13; P.C.W., unpublished data), this region is located on the proximal part of 21q22.3, with a likely maximum size of 3000 kilobases (kb). Pulsed-field gel electrophoresis (PFGE) analysis indicated a minimum size of 400 kb.

MATERIALS AND METHODS

Patients. Clinical study and cytogenetic analysis of the two patients, FG (3, 14) and IG (6, 15), have been reported. Both were mentally retarded and had many of the phenotypic features of Down syndrome as summarized in Table 1. From the physical examination checklist of 25 signs proposed by Jackson *et al.* (16), patients FG and IG had, respectively, 13 and 10 signs when they were first examined. According to Jackson *et al.* individuals with 13 or more signs can confidently be diagnosed as trisomy 21, whereas there is an area of overlap between normal and Down subjects with 5–12 signs. Thus FG's score was within the range of Down syndrome patients, whereas IG's score was on the right side of the overlap range between Down syndrome and normal subjects. Signs common to both patients were flat nasal bridge, protruding tongue (macroglossia), folded ears, short and incurved fifth finger, gap between first and second toes, and muscular hypotonia. In patient FG, the initial karyotypic analysis (3) concluded that one chromosome 21 was abnormal and had a *de novo* direct duplication for 21q21→21q22.2. Recent reexamination using high-resolution R banding gave the following karyotype: 46,XY,dir dup(21)(q11.205→q22.300). In patient IG, one chromosome 21 had a *de novo* direct duplication of q22.3→qter (6, 15). Therefore the karyotype was 46,XX,dir dup(21)-(q22.300→qter). In agreement with the karyotypic analyses, erythrocyte SOD1 activity was found to be 50% higher than normal in patient FG (3, 14) and normal in patient IG (6).

Quantification of Chromosome 21 Sequences. DNA from normal individuals, patients with free trisomy 21, and these two patients was purified from blood cells by standard techniques. (Cell lines from the two patients are established and DNA is available for collaboration.) A method for the quantification of single-copy DNA sequences was used to evaluate the copy numbers of chromosome 21 sequences in patients FG

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Abbreviation: PFGE, pulsed-field gel electrophoresis; RFLP, restriction fragment length polymorphism.

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Table 1. Main clinical features characteristic of Down syndrome in patients FG and IG

Feature	FG	IG
Down's facies	Yes	Yes
IQ	50	60
Hypotonia	Moderate	Marked
Short stature	Yes	Yes
Brachycephaly	No	No
Dermatoglyphics		
Single transverse palmar crease	No	Yes (bilateral)
Palmar t″	No	Yes (bilateral)
Hypothenar ulnar loop	No	Yes (bilateral)
High Cummins index*	Yes (R, 32; L, 31)	No (R, 25; L, 19)
Brachydactyly	Yes	No
Short, incurved fifth fingers	Yes	Yes
Gap between first and second toes	Yes	Yes
Visceral abnormality	No	No
Leukemia	No	No
Alzheimer disease	(Too young)	

FG (ref. 3) and IG (ref. 6) were examined at age 7 years 8 months and 4 years, respectively.
*R, right hand; L, left hand.

and IG. This method consisted of four steps. (*i*) After denaturation with NaOH, various amounts of DNA (0.5–1.5 μg) were blotted on a Zetabind membrane by using a slot blot apparatus (Schleicher & Schuell, Minifold II). Each membrane was loaded with DNA from three sources: a normal control, a free trisomy 21 patient, and the subject to be analyzed. (*ii*) Successive hybridizations with reference probes and chromosome 21 probes were then carried out. (*iii*) Intensities of the signals on autoradiograms were quantified by densitometric scanning with a Shimadzu CS930 scanner. (*iv*) The linear correlations between reference probe signals (*x* axis) and chromosome 21 probe signals (*y* axis) were studied by graphic and statistical analysis of the data. The conclusion that the DNA from the studied subject has two or three copies for a given chromosome 21 sequence was assessed by *t*-test comparison of the slopes.

PFGE. Analysis of large DNA fragments was carried out in the vertical pulsed-field gel system by transverse alternating-field electrophoresis (17). Leukocytes from controls ($n = 8$) and patients were included in low-melting agarose as described (17) (12×10^6 cells per ml; i.e., 1.6 μg of DNA per slot). Samples were digested overnight with different restriction enzymes (*Sfi* I, *Bss*HII, *Mlu* I, *Nae* I; New England Biolabs or Pharmacia) in 100 μl of the buffer recommended by the supplier. Gels of 0.8% agarose were run in 10 mM Tris/0.5 mM EDTA, pH 8.2, first at 170 mA with a 4-sec pulse time for 30 min, then at 120 mA (195 V) with a 30- or 60-sec pulse time for 24 hr. The *Saccharomyces cerevisiae* strain YNN 281 chromosomes (245–1600 kb) used as markers were prepared as described (18).

Probes. Reference probes for the quantification of chromosome 21 sequences were a human cDNA for the proα1(I) collagen (*COL1A1*) gene located on chromosome 17 (19) and a human cDNA for the proα2(I) collagen gene (*COL1A2*) located on chromosome 7 (20). Chromosome 21 probes were the human *SOD1* (21) and *ETS2* (22, 23) cDNAs and the anonymous DNA sequences *D21S17* (24), *D21S15* (24), and *D21S55* (3.2-kb *Eco*RI fragment) (25). For all these probes, inserts were prepared by enzymatic digestion, electrophoresis, and electroelution. Inserts were labeled with [α-^{32}P]-dCTP by random priming. Prehybridization, hybridization, and washing of the membranes were carried out as recommended by the manufacturer (AMF Cuno).

RESULTS AND DISCUSSION

The previously reported methods of "3:2 gene dosage" (26–33) were hampered by various difficulties. On one hand, the gene dosage based on the relative intensities of bands corresponding to a restriction fragment length polymorphism (RFLP) requires that the subject is heterozygous at the locus of interest (30, 31). On the other hand, the measurement of the density of single autoradiographic bands after successive hybridization with chromosome 21 probes and reference probes used as a standard for intersample comparison is subject to artifacts such as partial digestion or variation in DNA transfer during Southern blotting (30, 32, 33). Such artifacts might account for the previous observations of β-amyloid gene (28) and *ETS2* (29) duplications in patients with Alzheimer disease, which have not been confirmed by other groups (30–33) or by ourselves (unpublished data on blood DNA analysis). Therefore, we designed a slot blot method for the evaluation of the copy number of different chromosome 21 sequences. This method does not require DNA digestion or Southern blotting. As a test for the validation of this method, 10 coded blood DNAs from 5 normal controls and 5 patients with free trisomy 21 were analyzed by using two reference and two chromosome 21

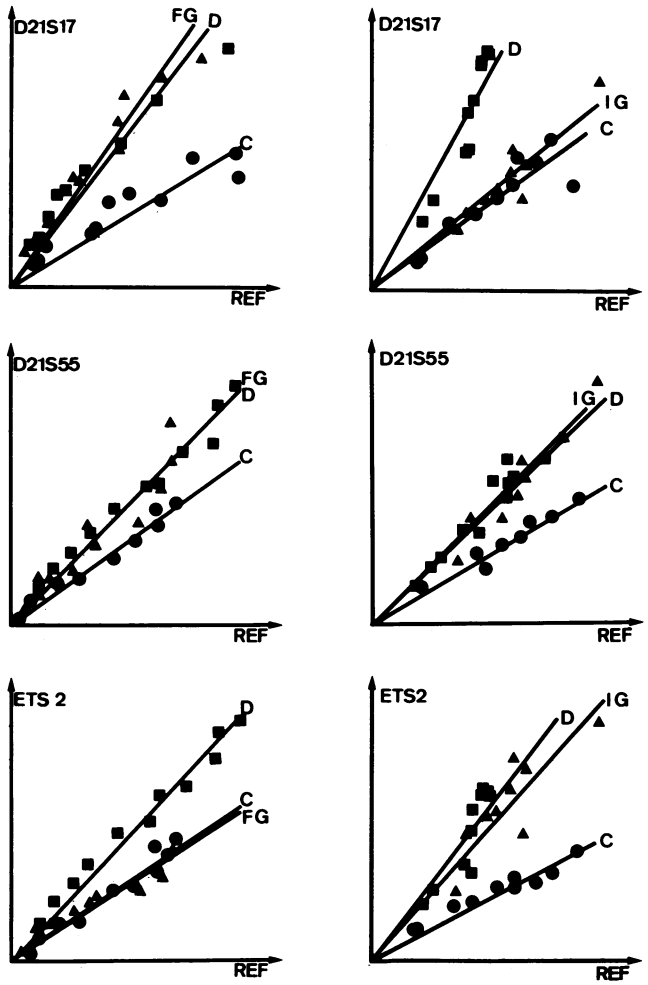

FIG. 1. Quantification of three chromosome 21 sequences by the slot blot method. Densitometric signals obtained with reference probes (either *COL1A1* or *COL1A2*) on the *x* axis are plotted against signals obtained with chromosome 21 probes (*D21S17*, *D21S55*, or *ETS2*) on the *y* axis. Linear correlation coefficients were >0.90 (the null signal for DNA amount = 0 was included in the calculation). Slot blots were loaded with DNAs from a control (C, ●), a free trisomy 21 subject (D, ■), and one of the patients (FG or IG, ▲). For each graph, scales were computed from the maximal values of *x* and *y*.

probes. Among the 10 DNAs analyzed, it was possible to diagnose normal controls and trisomic 21 individuals with 100% accuracy.

By using the same methodology with blood DNAs from patients FG and IG, we evaluated the number of copies of five DNA sequences: *SOD1*, *D21S17*, *D21S55*, *ETS2*, and *D21S15*. Fig. 1 shows typical results of slot blot assays for dosages of *D21S17*, *D21S55*, and *ETS2* in patients FG and IG. Statistical comparisons of the slopes representative of the correlation between signals from reference probe and chromosome 21 probe clearly indicated duplication of *D21S17* and *D21S55* in patient FG and of *D21S55* and *ETS2* in patient IG (Fig. 1, Table 2). Moreover, *SOD1* was duplicated in patient FG and *D21S15* in patient IG (Table 2). For all the probes, at least two slot blot analyses were performed, giving similar results (Table 2).

Table 2. Quantification of five chromosome 21 sequences in patients FG and IG

Probe	DNA	Slope (mean ± SD) Exp. 1	Exp. 2
		Analysis of FG	
SOD1	C	0.43 ± 0.01*	0.40 ± 0.01*
	FG	0.76 ± 0.06	0.56 ± 0.04
	D	0.79 ± 0.06	0.63 ± 0.02
D21S17	C	0.30 ± 0.02*	0.19 ± 0.01*
	FG	0.76 ± 0.02	0.39 ± 0.03
	D	0.70 ± 0.03	0.43 ± 0.02
D21S55	C	0.22 ± 0.01*	0.20 ± 0.01*
	FG	0.44 ± 0.02	0.29 ± 0.01
	D	0.40 ± 0.01	0.29 ± 0.01
ETS2	C	0.11 ± 0.01	0.55 ± 0.04
	FG	0.10 ± 0.01	0.64 ± 0.04
	D	0.17 ± 0.01*	1.38 ± 0.04*
D21S15	C	0.16 ± 0.01	0.15 ± 0.01
	FG	0.18 ± 0.01	0.16 ± 0.01
	D	0.33 ± 0.01*	0.31 ± 0.01*
		Analysis of IG	
SOD1	C	1.16 ± 0.03	1.27 ± 0.07
	IG	1.12 ± 0.05	1.17 ± 0.07
	D	1.91 ± 0.05*	2.04 ± 0.06*
D21S17	C	4.81 ± 0.39	3.37 ± 0.19
	IG	4.22 ± 0.35	3.81 ± 0.18
	D	9.04 ± 0.35*	8.45 ± 0.38*
D21S55	C	0.81 ± 0.03*	1.03 ± 0.03*
	IG	1.47 ± 0.06	1.66 ± 0.05
	D	1.61 ± 0.07	1.73 ± 0.05
ETS2	C	1.44 ± 0.08*	1.08 ± 0.03*
	IG	3.27 ± 0.18	2.31 ± 0.12
	D	4.01 ± 0.36	2.70 ± 0.14
D21S15	C	1.05 ± 0.05*	0.91 ± 0.03*
	IG	2.34 ± 0.24	2.34 ± 0.08
	D	2.41 ± 0.17	2.52 ± 0.13

Results are expressed as the slopes (mean ± SD) of the linear correlations between reference probe signals (x axis) and chromosome 21 probe signals (y axis). For each correlation, the number of points was 10–12 (the null signal for DNA amount = 0 was included in the calculation). Two slot blot analyses (experiments 1 and 2) are shown for each patient, with DNA from a normal control (C), a free trisomy 21 individual (D), and the patient to be analyzed (FG or IG). Slopes were compared by t test. All the comparisons of D vs. C are significant at $P < 0.001$. C or D slope values that are significantly different from patient (FG or IG) slope values are indicated by asterisks ($P < 0.001$). Other comparisons are not significant.

We also attempted to assess gene dosage by RFLP analyses on Southern blot with the *D21S17*, *D21S55*, and *ETS2* probes. The *Bgl* II/*D21S17* RFLP is diallelic, gives two bands at 18.5 and 12.3 kb (24), and is therefore appropriate for 3:2 dosage. Only IG was informative and the equal intensities of the two bands, as observed in heterozygote control subjects, confirmed that *D21S17* was not duplicated. In the absence of DNA from FG and IG's parents, multiallelic and more complex *Xba* I/*D21S55* (34) and *Msp* I/*ETS2* (35) RFLPs could not be informative.

The genetic linkage of the tested sequences has been established (9, 10), giving the following order: centromere–*SOD1*–*D21S17*, *D21S55* (same locus, P.C.W., unpublished data)–*ETS2*–*D21S15*–telomere. Regional mapping using a panel of cell hybrids containing rearranged chromosome 21 has also been reported (11–13): *D21S17*, *D21S55*, and *ETS2* are found in the proximal part of 21q22.3, since *D21S15* is on the distal part of 21q22.3. Moreover, *SOD1* has been localized at the interface of 21q21 and 21q22.1 (14, 15, 27). By taking into account this information and the results of gene quantifications, it is possible to precisely characterize the chromosomal rearrangements in patients FG and IG (Fig. 2*a*). In patient FG the duplication includes the proximal part of chromosome 21 down to a breakpoint located between *D21S55* (three copies) and *ETS2* (two copies). In patient IG, the duplication starts from a breakpoint located between *D21S17* (two copies) and *D21S55* (three copies) and extends distally towards the telomere. The *D21S55* sequence is duplicated in both cases. Therefore, there is a common duplicated region in patients FG and IG.

To evaluate the size of this common duplicated region, we performed PFGE experiments with the sequences *D21S17*, *D21S55*, and *ETS2* on DNAs from control subjects and patients FG and IG. Table 3 shows the results obtained after digestion of leukocyte DNAs from control subjects with four restriction enzymes. Within the measurable size range (100–1000 kb) no restriction fragment was found to be common to these sequences. These results are similar to those reported by Gardiner *et al.* (13). When DNA from patients FG and IG was studied, no change in the restriction patterns with *Sfi* I, *Bss*HII (Fig. 3), and *Mlu* I was observed. *Nae* I digestion was carried out only for patient FG and gave the same pattern as the controls. Therefore the 400-kb *Bss*HII fragment hybridizing with *D21S55* represents the minimum length of the duplicated region common to the two patients. The maximum size of the region has to be included in the distance between *D21S17* and *ETS2*. Regional mapping (12, 13) has indicated that these two sequences are localized on the proximal third of 21q22.3 (Fig. 2*b*). If one assumes that DNA density is homogeneous along chromosome 21, this fraction of 21q22.3 represents ≈5% of the whole chromosome, i.e., probably <3000 kb.

After review of all the published observations of partial trisomies 21 (8), it was concluded that a duplication of a small fraction of 21q22, adjacent to the sub-band 21q22.2, could be of importance in the expression of Down syndrome. Our data are consistent with these observations and preliminary reports (36, 37) on the molecular analysis of patients with partial trisomy 21 suggesting that the "Down syndrome region" includes 21q22.3 and extends proximally (37) to a border located between loci *D21S58* (5 centimorgans distal to *SOD1*; ref. 9) and *D21S55* (36). Our results strongly suggest that the duplication of the *D21S55* region is involved in the Down syndrome phenotype. Regional mapping of *D21S55* (11) has indicated that this region is in the proximal part of 21q22.3, adjacent to 21q22.2 (Fig. 2*b*).

Study of clinical scores in Down syndrome patients, such as Jackson's index (16), reveals that the phenotypic expression of trisomy 21 is variable from one individual to another. The same heterogeneity is observed when considering the

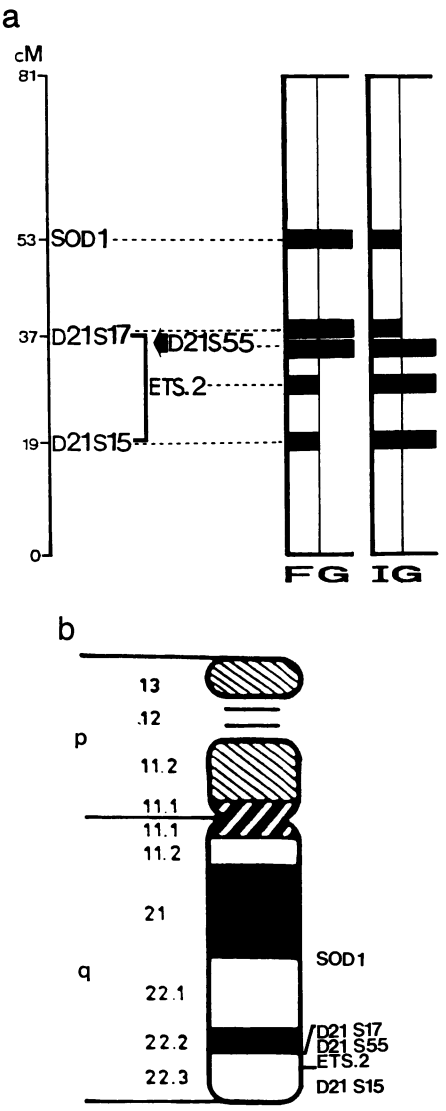

FIG. 2. (*a*) Schematic representation of the quantification of five chromosome 21 sequences in the two patients. At left is the genetic linkage map for *SOD1, D21S17, D21S15* (9), *D21S55* (P.C.W., unpublished data), and *ETS2* (10); cM, centimorgans. At right is the sequence copy number on the rearranged chromosome 21 of FG and of IG (small bar means one copy and large bar means two copies). (*b*) Regional mapping of the chromosome 21 sequences (11–15, 27). On the basis of our results *D21S55* is located between *D21S17* and *ETS2*.

intensity of mental deficiency. Patients FG and IG had phenotypic features and mental deficiency within the range of those observed in usual trisomy 21. They had, respectively, 13 and 10 signs on Jackson's checklist. Patient IG had a less marked phenotype, which is consistent with the data from

Table 3. Restriction fragments hybridizing with the *D21S17, D21S55,* and *ETS2* probes

Probe	Fragment size(s), kb			
	Sfi I	*Bss*HII	*Mlu* I	*Nae* I
D21S17	175, 300	920*	>2000	130, 600†
D21S55	115	400	>2000	300, 450‡
ETS2	100	360, 440	800	175, 230

Control and patient DNAs were digested with *Sfi* I, *Bss*HII, *Mlu* I, or *Nae* I. Fragments were resolved by PFGE and Southern blots were probed as indicated.
*Lower fragment of a series of four fragments.
†Indicates presence of a *Nae* I site in the *D21S17* probe.
‡Fragments studied only for patient FG.

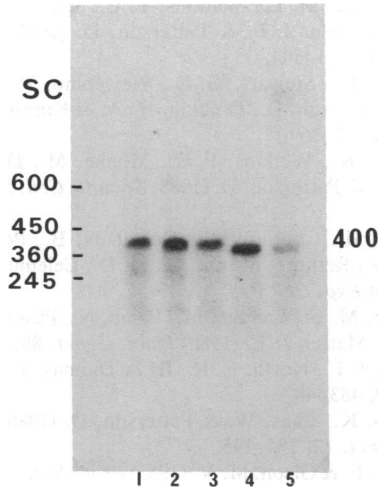

FIG. 3. PFGE analysis of DNAs digested by *Bss*HII and probed with *D21S55*. Lanes 1, 3, and 5, controls; lane 2, patient FG; lane 4, patient IG. SC, chromosomes of *S. cerevisiae* used as size (kb) markers.

other patients with partial trisomy for 21q22.2→qter (7). Both had a degree of mental retardation, language impairment, and behavior to be expected in Down syndrome. This heterogeneity suggests that, although the region around *D21S55* contains genes that, when duplicated, contribute significantly to the phenotype and the mental deficiency characteristic of trisomy 21, other genes localized outside the *D21S55* region may also play a role. Indeed, partial trisomies of chromosome 21, proximal to q22.3, have been reported with no phenotype of Down syndrome but mild (8, 38, 39) mental retardation.

Study of the genetic content of the *D21S55* region and molecular analysis of other partial trisomies 21 must undoubtedly lead to a better understanding of the pathogenesis of Down syndrome.

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