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Genetics

Human *ETS2* gene on chromosome 21 is not rearranged in Alzheimer disease

(Down syndrome/chromosomal duplications)

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ABSTRACT **The human *ETS2* gene, a member of the *ETS* gene family, with sequence homology with the retroviral *ets* sequence of the avian erythroblastosis retrovirus E26 is located on chromosome 21. Molecular genetic analysis of Down syndrome (DS) patients with partial trisomy 21 allowed us to reinforce the supposition that *ETS2* may be a gene of the minimal DS genetic region. It was originally proposed that a duplication of a portion of the DS region represents the genetic basis of Alzheimer disease, a condition associated also with DS. No evidence of either rearrangements or duplications of *ETS2* could be detected in DNA from fibroblasts and brain tissue of Alzheimer disease patients with either the sporadic or the familiar form of the disease. Thus, an altered *ETS2* gene dosage does not seem to be a genetic cause or component of Alzheimer disease.**

Two human diseases, Down syndrome (DS) and Alzheimer disease (AD), in some way interrelated (see below), represent a puzzle for geneticists. DS, is the most common inherited disease, occurring once in 1000 live births (1). The majority of DS patients present one extra chromosome 21 (2) as a result of a nondisjunction event in one of the meiotic stages. Thus, for more than 20 years, the imbalance of the entire extra set of chromosome 21 genes has been thought to be the cause of the abnormal development, the dismorphic features, and the mental retardation observed in the disease (3–6). Few DS cases present with the trisomy of just a small portion of chromosome 21. This allowed the definition that only the trisomy of the distal portion of the chromosome may be sufficient for generating the DS phenotype (7–12). In this region 20–100 genes may be present. We do not know if all or just a few of them are crucial for the appearance of the syndrome. Moreover, it is not clear whether the genes that cause the prenatal abnormal development of DS are the same genes that produce a number of postnatal abnormalities often developed by DS patients. Among these, the most commonly recorded anomalies are the high susceptibility to infections; the predisposition to leukemia in DS children; the premature aging; and the development of the neurological signs of AD (13, 14). AD, a degenerative disorder of the central nervous system, is not limited to DS individuals. AD causes profound mental and physical disability and is the fourth leading cause of death among the population in middle to late adult life (15). Two forms of AD are known: one rare familial form caused by an autosomal dominant gene defect and the most common sporadic form with unknown, possibly genetic, etiology. The occurrence of AD-like disease in the already mentally impaired DS patients originated the theory that the imbalance (trisomy?; deregulation?) of one or more genes of the DS minimal genetic region could be implicated in the origin of AD (16). The recent identification of the genetic locus causing the familial AD on chromosome 21q21 (17), far outside the DS region, would not exclude the existence of other gene(s) for the sporadic form in other sites of chromosome 21 or on other chromosomes. Actually, the recent literature is rich in reports that implicate in sporadic AD "this or that" gene on chromosome 21. Scattered microduplications of the chromosome have been proposed as an explanation of increased gene dosage for the superoxide dismutase (*SOD1*) gene (18), the protooncogene *ETS2* (19, 20), and the amyloid β-protein gene *APP* (21) as measured in the genome of AD patients. These findings prompted us to investigate this issue as far as the *ETS2* gene is concerned. The human *ETS2* gene, first identified in our laboratory (22) as a member of a family of genes related to the v-*ets* gene of the avian erythroblastosis retrovirus E26, is located on chromosome 21 (23, 24) and is highly conserved, being found from lower organisms, like *Drosophila* (25) and sea urchin (26), to man (22); this gene codes for a nuclear 56-kDa protein (27) that might play a critical role in controlling some step of the signaling transduction pathway. Thus, *ETS2*, as other genes with homology with viral oncogenes, might well be instrumental in regulating cellular growth and differentiation as well as organismal development.

MATERIALS AND METHODS

Patient Material. Fibroblasts from different patients with various chromosome aneuploidies (described in detail in Fig. 1) were analyzed. Cases T-1 and T-2 presented complete trisomy 21; C-1 (28, 29), C-2 (30), C-3 (10), and C-4 (10) presented partial trisomy 21: C-5 (30), C-6 (31), and C-7 (31) presented partial monosomy 21; M-1 (32) and M-2 (33) presented complete monosomy 21. Seven fibroblast strains were derived from AD patients. Six (A-1 to A-6) were derived from familial AD patients. A-7 was derived from a sporadic AD patient ascertained at the autopsy. All fibroblast strains, including fibroblasts from two normal individuals, were cultured in minimal essential medium (MEM) containing 10% (vol/vol) fetal calf serum. Twenty brain specimens (A-8 to A-27), all from sporadic AD patients confirmed at autopsy, were also analyzed. (The AD specimens were obtained from the Brain Bank of the Douglas Hospital Research Center, Montreal, Canada.)

Gene Dosage Analysis. Genomic DNA was extracted from cultured cells and brain specimens as described (34). Aliquots of 10 μg of genomic DNA were digested with various restriction enzymes as indicated by the supplier (Boehringer Mannheim). Agarose gel electrophoresis, Southern blotting, hybridization, and stringency of the washing conditions have been described in detail elsewhere (34). The probes used included a 1.0-kilobase (kb) genomic fragment of *ETS2* (22), a few chromosome 21 anonymous probes mapping proxi-

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Abbreviations: DS, Down syndrome; AD, Alzheimer disease; RFLP, restriction fragment length polymorphism.

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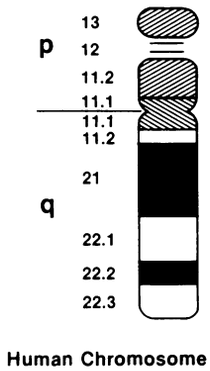

Proband	Karyotype	Chromosome 21 Defect
(C-1) Down Syndrome	46,XY, dup(21)(q22)	Partial trisomy q22>qter
(C-2) Down Syndrome	46,XX, − 6, + der(6), t(6;21)(6qter>6p25::21q11>21qter)	Partial trisomy q11>qter
(C-3) Down Syndrome Cousin of C-4	46,XX, − 10, + der(10), t(10;21)(10pter>10q26::21q2105>21qter)	Partial trisomy q21>qter
(C-4) Mentally Retarded	47,XY, + der(21), t(10;21)(21pter>21q2105::10q26>10qter)	Partial trisomy pter>q21
(C-5) Mentally Retarded	46,XY, − 21, + der(21), t(10;21)(21pter>21q22::10q24>10qter)	Partial monosomy q22>qter
(C-6) Mentally Retarded Two Down Syndrome Sisters	45,XY, − 2, − 21, + der(2), t(2;21)(2pter>2q37::21q2105>21qter)	Partial monosomy pter>q2105
(C-7) No Dismorphic Features	46,XY,r(21)(p13>q22)	Partial monosomies pter>p13; q22>qter
(M-1) Psychomotor Retardation Multiple Malformation	45,XY, − 21	Complete monosomy
(M-2) Mentally Retarded	45,XX, − 21	Complete monosomy

FIG. 1. Fibroblast strains presenting various chromosome 21 aneuploidies were derived from patients C-1 to C-7, M-1, and M-2 with the outlined karyotypic and phenotypic features.

mally or distally to *ETS2* (35, 36), and a 0.45-kb anonymous DNA fragment mapping on chromosome 22 (N.S., unpublished data).

Allelic dosage was evaluated by visual inspection of the intensity of the allelic fragments detected by the polymorphic probes within the same lane of autoradiograms of Southern blots. Quantitative densitometric analysis was performed on autoradiograms of Southern blots containing both control (diploid) and test DNAs. The ratio between the hybridization signals of the *Eco*RI 3.8-kb fragment detected by the *ETS2* probe and the *Eco*RI 3.2-kb fragment detected by the reference probe on chromosome 22 was calculated for each sample in the same Southern blot to correct for DNA loading. Southern blot filters were first hybridized to the *ETS2* probe, freed of the probe, and hybridized to the reference 0.45-kb probe. The densitometric tracing of autoradiograms were performed with a laser LKB Ultroscan densitometer. The peak areas corresponding to each hybridization signal were calculated by electronic integration.

RESULTS

First we assessed whether *ETS2* was always in three copies in the putative DS region by analyzing directly (DNA of DS cases C-1, C-2, and C-3) or indirectly (case C-6, brother of two DS patients) a few of these regions (Fig. 1). We measured the *ETS2* gene content in these patients' DNAs by using either a *Taq* I restriction fragment length polymorphism (RFLP) for a 1.0-kb *ETS2* sequence (34) or quantitative densitometry. These experiments also served to establish the best possible panel of control DNAs with one, two, or three *ETS2* copies for assessing for *ETS2* dosage in cells of AD patients.

RFLP analysis of human genomic DNA using the *Taq* I enzyme and a genomic 1.0-kb *ETS2* probe evidenced one allele (*A1*) of 4.0 kb and a second allele (*A2*) of 1.2 kb as well as a constant band of 2.0 kb (34). Thus, using this RFLP, we could easily distinguish trisomy versus disomy whenever there was heterozygosity, since by visual inspection a different ratio of one allele versus the other was evident (Figs. 2 and 3). As an example, clearly, three alleles, *A1 A2 A2*, were observed in one DS patient with partial trisomy 21 (C-1) Fig. 2, lanes 4 and 5), and only two alleles, *A1 A2*, were observed in heterozygous AD fibroblasts (patients A-2 and A-5, respectively) (Fig. 2, lanes 9 and 10). Brain DNAs from 20 patients with sporadic AD were also analyzed. Heterozygosity for *Taq* I *ETS2* polymorphism was observed in 14 cases. A typical blot is shown in Fig. 3, where one copy of each allele *A1* and *A2* was detected in heterozygous patient DNAs (lanes 1 to 4). Our observations exclude the presence of three copies of *ETS2* also in all the other 10 heterozygous patients we analyzed (data not shown). The overall RFLP analysis was indicative of (*i*) unequivocal association between three copies of *ETS2* and the extra chromosome 21 material causing DS

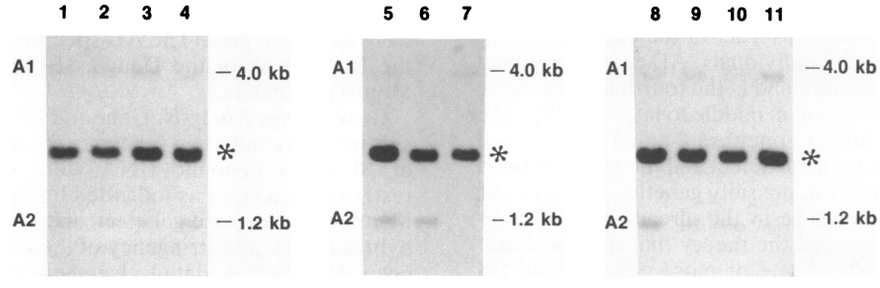

FIG. 2. RFLP analysis of human genomic DNA using the *Taq* I restriction endonuclease and a 1.0-kb *ETS2* genomic probe. Two alleles, *A1* (4.0 kb) and *A2* (1.2 kb), are identified, as well as a constant band (*) of 2.0 kb. The allelic ratio allows us to distinguish: trisomic heterozygous cells with an *A1A1A2* genotype (lanes 3 and 11) and *A1A2A2* genotype (lanes 4, 5, and 8), disomic heterozygous cells with an *A1A2* genotype (lanes 1, 2, 9, and 10), and homozygous cells with *A1A1* and *A2A2* genotypes (lanes 7 and 6, respectively). In particular, lanes 4 and 5 represent the RFLP pattern found in DNA of the DS patient with partial trisomy (C-1), thus showing the presence of three copies of the *ETS2* gene, and lanes 9 and 10 represent the RFLP pattern found in DNA of AD patients A-2 and A-5, showing the presence of two copies of the *ETS2* gene.

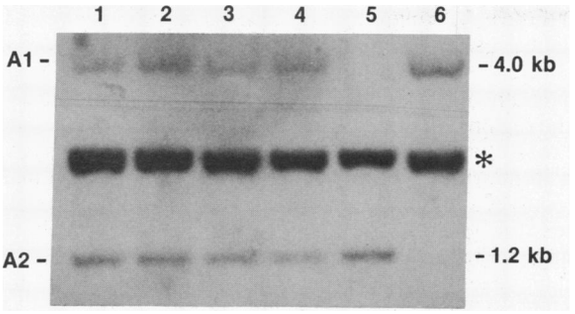

FIG. 3. *Taq* I *ETS2* RFLP of six DNA samples from the brains of autoptically ascertained AD patients presenting the sporadic form. The visual inspection of *A1* (4.0 kb) and *A2* (1.2 kb) alleles clearly shows two copies of the *ETS2* gene in DNAs in lanes 1–4. Lanes 5 and 6 contain DNA of two homozygous patients for whom RFLP analysis was not conclusive. *, Constant band of 2.0 kb.

in patient C-1 and (*ii*) presence of two copies of *ETS2* in all of the heterozygous AD patients.

We further assessed the *ETS2* dosage in all fibroblast DNAs, (including all of the chromosome 21 aneuploidies and the AD cases) by quantitative densitometric analysis of Southern blots. As a control, normal diploid fibroblast DNA was used. The ratio between the hybridization signals of an *Eco*RI 3.8-kb fragment detected by the *ETS2* probe and an *Eco*RI 3.2-kb fragment detected by the reference probe on control chromosome 22 was calculated for each test and control sample in five independent lanes in the same Southern blot to correct for DNA loading. The densitometric tracings of autoradiograms were performed with a laser LKB Ultroscan densitometer. The peak areas corresponding to each hybridization signal were calculated by electronic integration. In all cases, the test DNA was compared to the DNA from disomic cells within the same Southern blot. The results of this analysis are presented in Table 1. The means, SDs, and SEMs were calculated on the basis of the five independent ratios between *ETS2* and the reference probe obtained on each of the test DNA and control DNA. The statistical significance of the comparison of test DNA versus control DNA was calculated by the student *t* test (37). From this analysis it was possible to interpret the "probable" copy number of *ETS2* gene in each case, except for two cases, C-6 and A-4 (this is the reason for the question mark), which had *P* values of 0.0002 and 0.0015, respectively. The entire set of 36 means was further examined by analysis of variance, followed by the Student–Newman–Keuls multiple comparison test at the 0.05 probability level (37). This test identified a distinct set of three homogeneous test samples (M-1, M-2, and C-5) with mean ratios ranging from 0.524 to 0.624, which would be interpreted as having one copy of *ETS2*, and a second distinct set of five homogeneous test samples (T-1, T-2, C-1, C-2, and C-3) with mean ratios ranging from 1.407 to 1.516, which would be interpreted as having three copies of the gene (Fig. 4). The means of the remaining 10 test samples fell in a middle ground, having ratios ranging from 0.909 to 1.180, the same approximate range as those containing all of the 18 controls obtained from two individuals. In the student–Newman–Keuls test, the difference between C-6 and its control no longer remained significant, thus leading to the interpretation of two *ETS2* copies. However, the difference between A-4 and its control remained significant, although only marginally at the 0.05 probability level.

To complement the information obtained from densitometry, RFLP analysis using polymorphic chromosome 21 sequences, chosen for loci proximal or distal to *ETS2* according to the genetic map (35, 36), was also performed. In cases of heterozygosity for the alleles detected by these polymorphic probes, the analysis confirmed the disomy or the trisomy of the regions adjacent to *ETS2* (data not shown). For instance, in patient C-1 the trisomy for the region surrounding *ETS2* was reaffirmed from the observation of the allelic ratio detected by the *D21S23* probe distal to *ETS2* (35) (Fig. 5, lane 1), when compared to the heterozygous RFLP pattern of a complete trisomy 21 (lane 2) and a normal control (lane 3), respectively. In each patient the additional RFLP analysis helped to accept with confidence the conclusions relative to the *ETS2* dosage deduced from quantitative densitometry—the obligate technique whenever there was a homozygous condition for this locus.

Table 1. Quantitative densitometry of probe hybridization for cells of patients with various chromosome 21 aneuploidies and AD

Subject	Mean	SD	SEM	*P*	*ETS2*
C	1.015	0.089	0.039	0.0001	3
T-1	1.409	0.089	0.039		
C	1.015	0.066	0.029	<0.0001	3
T-2	1.407	0.076	0.034		
C	1.035	0.121	0.054	0.0003	1
M-1	0.624	0.089	0.039		
C	1.050	0.093	0.041	0.0002	1
M-2	0.575	0.027	0.012		
C	0.953	0.133	0.059	0.0001	3
C-1	1.515	0.114	0.051		
C	1.087	0.082	0.037	<0.0001	3
C-2	1.471	0.067	0.030		
C	1.076	0.110	0.049	0.0014	3
C-3	1.444	0.029	0.013		
C	1.107	0.078	0.035	0.96	2
C-4	1.105	0.080	0.035		
C	0.944	0.105	0.047	0.0001	1
C-5	0.523	0.084	0.037		
C	1.102	0.047	0.021	0.0002	2?
C-6	0.939	0.029	0.013		
C	0.922	0.033	0.014	0.88	2
C-7	0.918	0.045	0.020		
C	0.884	0.023	0.010	0.20	2
A-1	0.955	0.182	0.045		
C	1.186	0.051	0.023	0.91	2
A-2	1.179	0.119	0.053		
C	1.008	0.089	0.037	0.059	2
A-3	1.103	0.044	0.020		
C	0.927	0.099	0.044	0.0015	2?
A-4	1.166	0.053	0.023		
C	0.871	0.095	0.042	0.46	2
A-5	0.908	0.050	0.022		
C	0.975	0.146	0.065	0.087	2
A-6	1.109	0.047	0.021		
C	1.052	0.070	0.031	0.36	2
A-7	1.088	0.043	0.019		

Ratio of the densitometric readings of the *Eco*RI 3.8-kb band detected by the *ETS2* probe and the *Eco*RI 3.2-kb band of a reference probe on chromosome 22 was evaluated in five independent experiments for each test DNA and for each control (C) DNA. Test DNA (five lanes) and control DNA (five lanes) were compared in the same Southern blot. The mean, SD, and SEM were calculated for each test and control DNA on five ratio values. By using the *t* test, the probability (*P*) that the difference between the mean values of test and control samples would be due to chance was calculated and the probable number of *ETS2* gene copies was deduced in each case, with the exception of subjects C-6 and A-4 (see question marks).

DISCUSSION

The supposition that *ETS2* located at the 21q22 band (23, 24) might be an element of the putative DS genetic region (7–12) is reinforced by the present findings. *ETS2* was found in three copies in all the partial trisomies 21 associated with DS phenotype that we analyzed. In particular, *ETS2* was found

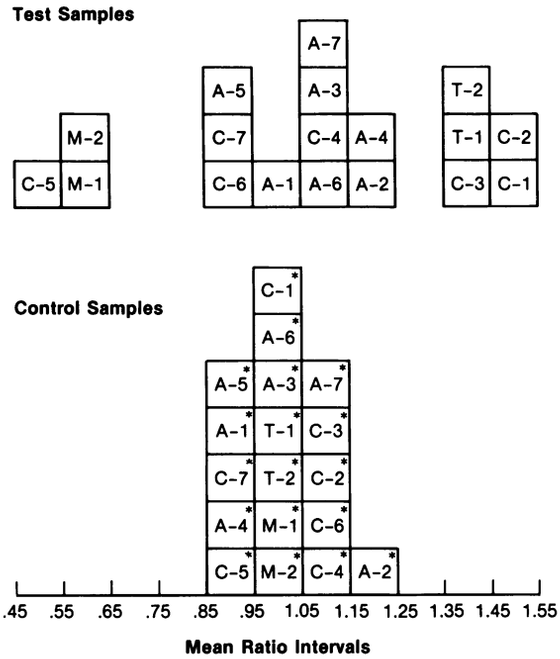

FIG. 4. The means reported in Table 1 of the 18 patients and 18 matching controls (from two normal individuals) were examined by analysis of variance followed by the Student–Newman–Keuls multiple comparison test at the probability level of 0.05. The controls are marked by an asterisk (e.g., C-1* is the control of C-1). A set of 3 homogeneous test samples (M-1, M-2, and C-5) would be interpreted as having one *ETS2* gene; a second distinct set of 5 test samples (T-1, T-2, C-1, C-2, and C-3) would be interpreted as having three copies of *ETS2*. The remaining 10 samples presented ratios in the approximate range containing all of the 18 control samples and would be interpreted as having two *ETS2* gene copies.

in triplicate when the trisomic region was barely visible at the cytogenetic analysis, as in patient C-1. Recent reports claimed that an extra copy of the *ETS2* gene was present in the DNA of AD patients. These reports, based on quantitative densitometry (19) and *in situ* hybridization (20), were in line with the proposed hypothesis that trisomy of one (or more) gene(s) in the DS region could be the cause of AD (16). On the contrary, the results of our analysis showed that two copies of *ETS2* are commonly found in both familial and sporadic forms of AD. Even if the existence of genetic heterogeneity among various AD patients cannot be excluded, the most probable reason for the difference between our results and the results of other reports (19, 20) lies in the intrinsic limitations of the techniques currently available for gene dosage studies. In contrast to RFLP analysis, which is the most reliable technique, quantitative densitometry was, in our experience, quite tricky in distinguishing between two

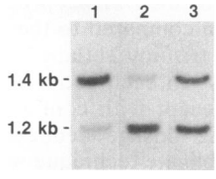

FIG. 5. Southern blot analysis of genomic DNA of fibroblasts from DS patient C-1 with dup(21)q(22) digested with *Eco*RI and probed with the pPW244D sequence for the *D21S23* locus distal to *ETS2*. The allelic ratio of C-1 DNA shows the presence of two copies of the 1.4-kb allele and one copy of the 1.2-kb allele (lane 1), an RFLP pattern of heterozygous trisomic cells with the opposite allelic ratio (lane 2), and a RFLP pattern of heterozygous disomic cells with the same allelic ratio (lane 3).

and three gene copies in some cases. This point was clear, for instance, in cases C-6 and A-4 (Table 1). In these two cases we had to rely on additional RFLP analysis for loci adjacent proximally or distally to *ETS2* to get an idea about the dosage of the region containing *ETS2*, and we could not conclude for the most "probable" *ETS2* copy number only on the basis of densitometric measurements (Table 1, see question marks). Of course, additional RFLP analysis would not be helpful to assess the dosage for very minute duplications, involving for instance, just one gene. Also, the *in situ* hybridization technique chosen by other authors (20) for gene dosage studies can have posed some limitations when used to assess the *ETS2* content. This limitation consists in the existence of a closely related *ETS2* gene, *ERG* (38), proximal to *ETS2* on chromosome 21q22. Given that in AD cells, as in other pathological conditions, the condensation status of chromosomes may differ from that of normal control cells, and assuming that a probe showing homology with both *ETS2* and *ERG* was used (20), the appearance of two hybridization peaks, one for *ERG* and one for *ETS2*, could have been interpreted as a rearrangement—i.e., duplication of *ETS2*.

The existence of duplications in AD DNAs for genes different from *ETS2* was excluded in other investigations. Duplication (trisomy) of genes on chromosome 21, like the amyloid β-protein gene *APP* (21) and the superoxide dismutase gene *SOD1* (18), have been described in some AD patients but never confirmed in several laboratories in different patients (39–41). Seemingly, even if genetic components may well be involved in the etiology of AD, they are very unlikely to be microduplications of *ETS2* and amyloid β-protein genes. This conclusion does not rule out that chromosome 21 genes (e.g., the just-mentioned amyloid β-protein gene) may in other ways contribute to the disease. AD might have a complex genetic etiology. Even now, more than one gene seems at work in AD—the familial AD gene distinct (42) from the amyloid β-protein gene and the τ-protein gene (43).

The search for other genes that may be implicated in AD should not be restricted only to chromosome 21. It has been proposed (44) that recently evolved genes responsible for the most elaborated regions of human brain may be the ones that when mutated or deregulated confer AD. According to this hypothesis, AD, which does not find a naturally occurring analog in animals (45) and has a predilection for the association neocortices, some parts of the hyppocampal formations, the nucleus basalis of Meynert, and the amygadala (44) is a "phylogenetic" disease. In view of this attractive hypothesis, it is more likely that genes generated through recent molecular evolution should contribute to AD. On this ground, *ETS2* having features of a highly conserved gene should be extraneous to this pathology. Indeed, *ETS2* is found from invertebrates (25, 26) to humans (22) and shares sequence homology even with some yeast cell-cycle genes (46). On the other hand, the considerations that make improbable a relationship between *ETS2* and AD may support *ETS2* as a possible genetic element of an "ontogenetic" anomaly like DS. *ETS2*, as proposed for other oncogenes, might be critical for normal developmental processes. In this context, an overproduction of otherwise normal *ETS2* proteins (27) could alter the delicate balance of biochemical pathways or could create interferences with cell–cell interaction processes necessary for a proper fetal development, thus causing some of the multiple abnormalities of DS. This hypothesis can be partially tested by introducing *ETS2* in transgenic mice. Comparative human and murine genetic maps showed that *ETS2* belongs to the group of genes on chromosome 21 that are present on mouse chromosome 16 (23, 47). Interestingly, a few DS features are present in mice with trisomy 16 or mosaics of trisomy 16 (48–50). Recently, it has been shown that transgenic mice carrying an extra copy

of individual chromosome 21 genes can be constructed (51). These transgenic mice can be used to engineer mice carrying other human chromosome 21 genes, individually or in sets of two or more. From these animal models we should learn whether, indeed, an extra dosage of *ETS2* is responsible for some of the major dismorphic features of DS, or whether it is simply predisposing to postnatal DS conditions like the increased susceptibility to leukemia (52).

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