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Molecular Definition of the Chromosome 7 Deletion in Williams Syndrome and Parent-of-Origin Effects on Growth

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Summary

Williams syndrome (WS) is a developmental disorder with variable phenotypic expression associated, in most cases, with a hemizygous deletion of part of chromosomal band 7q11.23 that includes the elastin gene (ELN). We have investigated the frequency and size of the deletions, determined the parental origin, and correlated the molecular results with the clinical findings in 65 WS patients. Hemizygosity at the ELN locus was established by typing of two intragenic polymorphisms, quantitative Southern analysis, and/or FISH. Polymorphic markers covering the deletion and flanking regions were ordered by a combination of genetic and physical mapping. Genotyping of WS patients and available parents for 13 polymorphisms revealed that of 65 clinically defined WS patients, 61 (94%) had a deletion of the ELN locus and were also hemizygous (or noninformative) at loci D7S489B, D7S2476, D7S613, D7S2472, and D7S1870. None of the four patients without ELN deletion was hemizygous at any of the polymorphic loci studied. All patients were heterozygous (or noninformative) for centromeric (D7S1816, D7S1483, and D7S653) and telomeric (D7S489A, D7S675, and D7S669) flanking loci. The genetic distance between the most-centromeric deleted locus, D7S489B, and the most-telomeric one, D7S1870, is 2 cM. The breakpoints cluster at ~1 cM to either side of ELN. In 39 families informative for parental origin, all deletions were de novo, and 18 were paternally and 21 maternally derived. Comparison of clinical data, collected in a standardized quantifiable format, revealed significantly more severe growth retardation and microcephaly in the maternal deletion group. An imprinted locus, silent on the paternal chromosome and contributing to statural growth, may be affected by the deletion.

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Introduction

Williams syndrome (WS) is a neurodevelopmental disorder characterized by distinctive facial features, mental disability with unique cognitive and personality profiles, vascular stenoses, growth retardation, connective tissue abnormalities, and, occasionally, infantile hypercalcemia (Martin et al. 1984; Burn 1986; Morris et al. 1988; Udwin 1990). The disorder was described independently by Williams et al. (1961) and Beuren et al. (1962) as a syndrome involving unusual facial appearance, supravalvar aortic stenosis and mental retardation. It is usually a sporadic occurrence with an estimated incidence of 1 in 20,000 live births (Martin et al. 1984; Grimm and Wesselhoeft 1980), although a few reports of affected parents and children are consistent with autosomal dominant inheritance (Morris et al. 1993; Sadler et al. 1993). When it was found that elastin (ELN) gene disruptions are causative in the autosomal dominant disorder supravalvar aortic stenosis (SVAS) (Curran et al. 1993; Ewart et al. 1994; Olson et al. 1995), hemizygous deletions of ELN were described in the majority of WS patients (Ewart et al. 1993; Borg et al. 1995; Kotzot et al. 1995*a*; Lowery et al. 1995; Mari et al. 1995; Nickerson et al. 1995;). The more complex phenotype in WS as compared to SVAS suggests a microdeletion with haploinsufficiency at ELN being responsible only for the cardiovascular features and possibly the connective tissue abnormalities, while haploinsufficiency for other genes in the deletion causes the remaining WS features. The deletion was found to be "submicroscopic" (Ewart et al. 1993; Nickerson et al. 1995) and had not been characterized at the molecular level with respect to heterogeneity of size and location of the deletion breakpoints until the recent report of Robinson et al. (1996)

Despite the consistency of the overall clinical pattern, the spectrum of anomalies in WS patients is very wide, and there is significant phenotypic variability among patients (Lopez-Rangel et al. 1992). Even the cardiovascular features are present only in a subset of patients with ELN hemizygosity (Kotzot et al. 1995*a*; Lowery et al. 1995; Mari et al. 1995; Nickerson et al. 1995). The

variability in the phenotype could be due to differences in the size of the deletions, but as yet there is no evidence that different genomic regions or genes are associated with particular phenotypic features. The phenotype could also be modified by variation in gene content or gene activity of the hemizygous alleles on the nondeleted chromosome. Gametic imprinting, defined as the total or partial inactivation of an allele as a consequence of having passed through gametogenesis in one parent, does not appear to play a major role in the causation of WS, because there are established cases of maternal and paternal transmission of WS and deletions in de novo WS cases have been reported to occur on either parent's chromosome 7 (Ewart et al. 1993; Gilbert-Dussardier et al. 1995; Kotzot et al. 1995*a*; Nickerson et al. 1995). The clinical phenotypes, however, have not been analyzed in detail with respect to the parental origin of the deletion.

In this study, we investigated the frequency of 7q11.23 deletions in 65 WS patients. By genotyping 13 polymorphic loci from the 7q11-q21 region, we determined the approximate size of the deletions and the parental origin of the deleted chromosomes. We then attempted to correlate the molecular findings with the clinical phenotype of the patients in order to determine whether there is a molecular basis for the phenotypic heterogeneity in the disease.

Patients and Methods

Patients and Phenotypic Evaluation

Sixty-five patients with a clinical diagnosis of WS were ascertained through clinical geneticists at the Lucile Salter Packard Children's Hospital, Stanford (n = 10), the Children's Hospital of Philadelphia (n = 32), the University Hospital Nijmegen (n = 9), the Children's Hospital at Denver (n = 2), and the Williams Syndrome Association (n = 12). All patients were nonfamilial except for two sets of second cousins, both reported elsewhere (White et al. 1977; Sujansky et al. 1995), and a set of monozygotic twins. None of the parents had features of the WS, indicating that all cases were de novo presentations. The patients represented various ethnic groups (e.g., Caucasians, Hispanics, Asians, and African-Americans). To collect uniform clinical data, a score sheet/questionnaire containing historical, clinical, and anthropometric parameters was prepared on the basis of the description of the phenotype in the literature. Fifty-three patients were clinically examined, and/or their medical records were reviewed by several of the authors. Each patient underwent a full genetic and dysmorphology evaluation with careful attention to documenting critical features of WS, as well as complete cardiac and ophthalmologic examinations. Echocardiograms were performed when warranted by physical findings.

The frequencies of the clinical features in our WS population are summarized in table 1. Anthropometric measurements were expressed as standard deviation scores (SDS) for age and sex, according to the U.S. standards of Tanner and Davies (1985). Correction for parental heights was performed when available (Tanner et al. 1970). Statural data were also compared to the standards of the WS population established by Pankau et al. (1992). Twelve additional patients who had carried a possible diagnosis of WS were considered not to meet clinical criteria and were excluded from the study. Most patients had received routine cytogenetic analyses prior to entering the study. High-resolution prometaphase chromosome analysis (~850 bands) was performed on five patients in the Clinical Cytogenetics Laboratory at Stanford.

In order to determine whether a difference was detectable between groups of patients with different molecular results, the following clinical features were analyzed: parental age at conception; duration of gestation; birth weight and length; history of irritability or feeding problems in infancy; hypercalcemia before and after 2 years of age; height, weight, and head circumference at the time of last evaluation; degree of cardiovascular involvement; degree of mental retardation; behavior; skeletal and connective tissue anomalies (joint elasticity, joint contractures, vertebral deformities, and inguinal/umbilical hernias); esotropia-strabismus; hoarse voice; and hyperacusis. Cardiovascular disease was classified into severe (requiring therapeutic intervention such as surgery or continued medication), moderate (significant cardiovascular anomaly), mild (borderline vascular stenoses by echocardiogram without hemodynamic significance or borderline high blood pressure or mitral valve prolapse), and absent. Mental retardation was classified into severe (IQ < 40), borderline (IQ > 75), and mild/moderate (IQ between 40 and 75). Patients in whom no IQ testing was performed were considered mild/moderately retarded on the basis of clinical impression.

FISH

Blood samples were obtained with informed consent from all the WS patients and from parents of 39 probands on the basis of protocols approved by the Human Investigation Committees of each participating institution. Chromosome spreads were prepared from short-term phytohemagglutinin-stimulated lymphocyte cultures. Two-color FISH was performed on 12 patients. A P1 phage clone containing the 5′ of the elastin gene and a P1 clone containing the calcitonin-receptor gene, mapped to band 7q21.3 outside the WS deletion, were

Table 1

Frequencies of Clinical Features in our WS Patients

Feature	Frequency (%)
Time of delivery (at term)	37/43 (86.0)
Time of delivery (preterm)	2/43 (4.6)
Time of delivery (postterm)	4/43 (9.3)
Birth weight <3%	8/44 (18.2)
Birth length <3%	4/28 (14.3)
Irritability/FTT in infancy	33/41 (80.5)
Hypercalcemia before 2 years	5/13 (38.5)
Hypercalcemia after 2 years	3/33 (9.1)
Height at examination <3%	13/42 (30.9)
Weight at examination <3%	19/42 (45.2)
OFC at examination <3%	11/34 (32.3)
Craniofacial:	
Dolichocephaly	16/28 (57.1)
Bitemporal narrowing	26/32 (81.2)
Medial eyebrow flare	22/33 (66.7)
Periorbital fullness	42/44 (95.5)
Epicanthal folds	27/38 (71.0)
Stellate irides	30/45 (66.7)
Flat nasal bridge	37/41 (90.2)
Short, upturned nose	37/41 (90.2)
Flat malar region, full cheeks	43/43 (100.0)
Long smooth philtrum	35/42 (83.3)
Full lips	42/43 (97.7)
Dental abn/malocclusion	25/31 (80.6)
Cardiovascular disease:	
SVAS	24/48 (50.0)
Peripheral pulmonary stenosis	13/48 (27.0)
Mitral valve prolapse	5/48 (11.4)
High blood pressure	5/25 (20.0)
Other (atrial or ventral septum defect)	2/48 (4.5)
Mental retardation:	
Severe (IQ <49)	6/46 (13.0)
Mild/moderate	36/46 (78.3)
Borderline (IQ >75)	4/46 (8.7)
Behavior/Personality traits:	
Outgoing/gregarious	38/42 (90.5)
Relative language strength	28/37 (75.6)
Hyperactivity—ADD[a]	18/42 (42.3)
Skeletal and connective tissue:	
Joint hyperelasticity	21/43 (48.8)
Joint contractures	13/45 (28.9)
Kyphosis	3/45 (6.7)
Lordosis	10/45 (22.2)
Scoliosis	4/45 (8.9)
Radioulnar synostosis	3/45 (6.7)
Extra sacral crease	3/21 (14.3)
Inguinal/umbilical hernia	13/46 (28.3)
Genitourinary tract:	
Congenital malformation	2/46 (4.3)
Enuresis/bladder dysfunction	17/25 (68.0)
Nephrocalcinosis	2/46 (4.3)
Esotropia	15/45 (33.3)
Hoarse voice	31/41 (75.6)
Hyperacusis	28/33 (84.8)

[a] Attention deficit disorder.

labeled with digoxigenin-11-dUTP and biotin-16-dUTP, respectively, by nick-translation, and hybridized simultaneously to denatured metaphase spreads as reported by Pérez Jurado et al. (1995).

ELN Gene Dosage Analysis

For gene dosage analysis by Southern blotting, genomic DNA from 59 patient samples was digested with *Eco*RI (New England Biolabs), fractionated by electrophoresis through 0.8% agarose gels, and transferred to Hybond N membranes (Amersham). Blots were hybridized simultaneously with a 660-bp PCR-amplified probe containing exons 20 and 21 of the ELN gene (Tromp et al. 1991) and with a 550-bp PCR-amplified product containing exons 2 and 3 of the growth hormone–releasing hormone gene (GHRH) on chromosome 20q as reference probe (Pérez Jurado et al. 1994). Hybridization signals were compared by densitometric analysis using the ImageQuant software (Molecular Dynamics). Hemizygosity was defined as a mean signal strength ratio <0.6 after two evaluations.

Polymorphic Marker Typing

For genotyping, 13 polymorphisms from the region were analyzed by PCR. The exon 20–intron 20 region of the ELN gene containing two RFLPs were PCR amplified as described by Tromp et al. (1991), and the products were digested with *Bst*NI (New England Biolabs), separated on a 5% agarose gel, and detected by ethidium bromide staining. A tandem repeat sequence within an intron of the ELN locus (originally published as intron 17 and renumbered intron 18 by Bashir et al. [1989]) was also utilized for genotype analysis (Foster et al. 1993), as well as marker AFMa060xc9 (locus D7S1870), which was reported to be deleted in some WS patients (Gilbert-Dussardier et al. 1995). Other polymorphic microsatellite loci mapping near the critical region were identified through the Genome Data Base (GDB) and/or the public data releases from the CEPH and the Whitehead Institute Center for Genome Research (Dib et al. 1996; Hudson et al. 1995). PCR reactions were carried out in mixtures containing 40 ng of genomic DNA, 150 μM of each dNTP, 10 mM Tris-HCl pH 8.9, 50 mM KCl, 1.5 mM MgCl2, 1 μCi 32P dCTP or 1.5 μCi 35S dATP, and 0.5 units of *Taq* polymerase in a total volume of 12.5 μl. Samples were processed through 32 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) and a final extension step of 7 min at 72°C. The PCR products were visualized by autoradiography following electrophoresis in 6% denaturing polyacrylamide gels.

Genetic and Physical Mapping of 7q11-q21 Polymorphic Markers

Eight reference families of the CEPH panel (Gyapay et al. 1994) were genotyped for the polymorphic loci

D7S489 (AFM136xe3), ELN *Bst*NI RFLP, and D7S1870 (AFMa060xc9). The results were compared with those of other markers previously assigned to 7q by using version 5.1 of the LINKAGE software package (Lathrop et al. 1984). Recombination frequencies were assumed to be equal in males and females. YAC clones containing the loci D7S489 and D7S1870 were kindly provided by Stephen Scherer and Lap-Chee Tsui, University of Toronto, from a chromosome 7–specific YAC library (Kunz et al. 1994). Other YACs containing D7S489 loci, as well as the overlapping YACs, were identified on the basis of sequence-tagged-site (STS) content information available through data releases of the Whitehead Institute—M.I.T Human Genome Mapping Project (Hudson et al. 1995) and were obtained from the German Genome Project Resource Center at the Max Planck Institut Berlin-Dahlem. PCR-amplified products with primers specific for AFM136xe3 were cloned into the TA cloning vector PCRTMII (Invitrogen) and sequenced using the Sequenase 2.0 reagents (Stratagene).

Statistical Analysis

For statistical analysis, Student's *t*-test or Kruskal-Wallis test and the χ^2 test were applied as appropriate. A level of confidence of $P = .05$ was used to assume a statistically significant difference.

Results

Detection of ELN Deletions in WS Patients by Dosage Analysis and FISH

Quantitation of Southern blot hybridization signals revealed an ELN dosage reduced by ~50%, consistent with hemizygosity at the ELN locus, in 56 (94%) of the 59 patients analyzed. Figure 1 includes two patients' samples (1244 and 1260) with apparently normal ELN gene copy numbers (intensity values > 0.9), who were later determined not to have WS. FISH generated an ELN-specific signal on only one chromosome 7 in 10 of 12 WS patients studied. In contrast, the calcitonin-receptor gene-containing P1 clone consistently hybridized to both chromosomes 7 in all patients tested (not shown). Cells from two WS patients who had normal ELN gene dosage revealed ELN-specific FISH signals on both copies of chromosome 7, as did controls. Altogether, hemizygosity at the ELN locus was found in 61 (94%) of the 65 patients analyzed by either one or both of the methods described above, while 4 (6%) WS patients were not deleted at ELN.

Physical and Genetic Mapping of Markers Flanking the ELN Gene

PCR amplification with primers for the microsatellite marker AFM136xe3 detected two major polymorphic

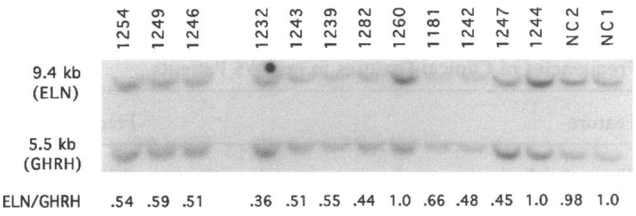

Figure 1 Elastin gene dosage analysis by Southern blotting. A membrane with *Eco*RI-digested DNAs from several WS patients and controls (NC) was simultaneously hybridized with two probes, one specific for elastin ELN and the other for growth hormone-releasing hormone (GHRH). ELN/GHRH signal intensity ratios derived from densitometric analysis are given below each lane. NC1 was taken as the standard by assigning it a ratio value of 1.0.

loci in normal individuals, named "D7S489A" and "-B," that are distinguished on the basis of size differences. Alleles at D7S489A range between 138 and 146 bp and at D7S489B between 168 and 180 bp. Cloning and sequencing of PCR products in the respective size ranges revealed very high sequence identity (97%) with the expected changes in the length of the $(CA)_n$ repeat and minor differences in the flanking regions (not shown). The observed heterozygosity in 80 unrelated normal individuals was 60% at D7S489B and 35% at D7S489A. PCR amplification of a panel of somatic cell hybrids (Francke et al. 1986) verified that both D7S489 loci map to the long arm of chromosome 7. Linkage analysis using a partial CEPH panel revealed that D7S489A and -B, ELN, and D7S1870 belong to a linkage group on chromosome 7 with the most likely order of loci determined by multipoint analysis as shown in figure 2. In family 1413, a crossover event between D7S2476 and ELN/D7S613 was found on the maternal chromosome of individual 1413-04. In family 1362, a crossover was observed between ELN and D7S1870 on the paternal chromosome (fig. 2). D7S489A was not informative in these families.

Therefore, two YAC clones containing D7S489A were identified, both of which were also positive for D7S1870, indicating that D7S489A is telomeric to ELN. One of these YACs also contained the locus D7S2472. One independent YAC was found to contain D7S489B, as well as the low heterozygosity marker AFM116yg5 (locus D7S1778). D7S1778 allowed us to physically link this clone with other YAC clones containing the highly polymorphic STS loci D7S1816, D7S1483, and D7S653 (fig. 2). All the YACs from this region are highly unstable, and different insert sizes were obtained in different isolations. Thus, no precise estimate of the physical distance between markers can be obtained from this YAC contig.

Molecular Definition of the Deleted Region

The results of genotyping multiple polymorphic markers in WS patients are summarized in figure 3. A failure

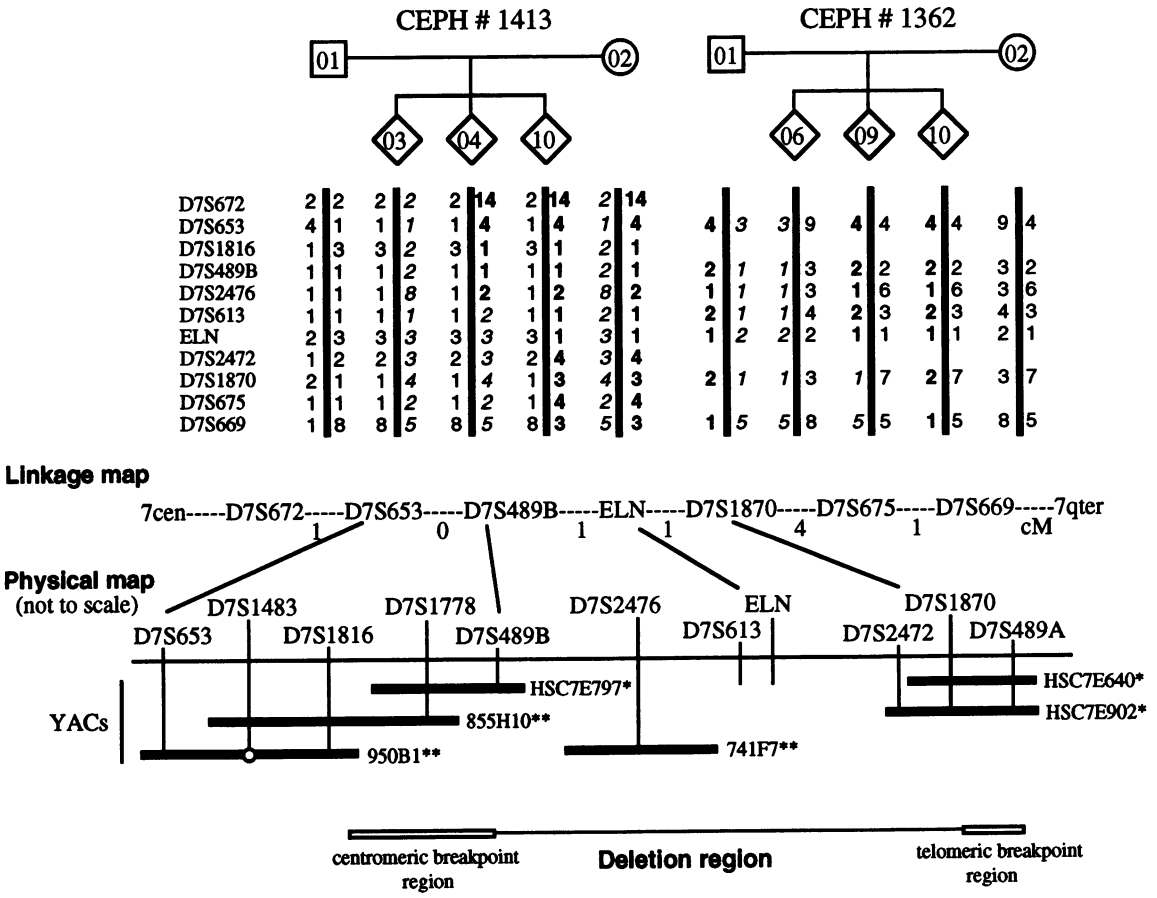

Figure 2 Genetic and physical mapping of the deletion region. *Top,* Haplotypes of five representative members each of CEPH families 1413 and 1362 typed for multiple polymorphic markers from 7q11.23. Haplotypes from the nonrecombining parent are in Roman font, while from the recombining parent one haplotype is shown in bold, and the other in italics. A crossover event between D7S2476 and D7S613/ELN on the maternal chromosome of individual 1413-04 that positioned D7S489B/D7S1816 and D7S2472 to either side of the crossover. In family 1362, recombination between ELN and D7S1870 is evident on the paternal chromosome of 1362-09. Data on nonrecombinant siblings with the parental haplotypes are shown as reference. *Middle,* Most-likely order of loci, as determined by multipoint linkage analysis. Genetic distance between the markers is in centimorgans. *Bottom,* Partial physical map with contigs of YAC clones, aligned on the basis of STS content, that encompass the deletion breakpoints. The deleted interval with the location of the common breakpoints is shown below. Single asterisks (*) represent YAC clones from the chromosome 7–specific YAC library (Kunz et al. 1994). Double asterisks (**) represent YAC clones from the CEPH MegaYAC library (Hudson et al. 1995).

of parental inheritance could be detected at locus D7S489B in 15 (38.5%) of 39 patients, ELN(CA)$_n$18 in 11 (29.7%) of 37, ELNBstNI RFLP in 13 (33.3%) of 39, D7S613 in 9 (47.4%) of 19, D7S2472 in 23 (59%) 39, D7S2476 in 9 (33.3%) of 27, and D7S1870 in 24 (63.1%) of 38. Examples of genotyping results at the seven intradeletion markers are shown in figure 4. Lack of inheritance of a parental allele involved the maternal (21 cases) or the paternal (18 cases) allele and was consistent at all informative loci. No patient hemizygous at ELN was heterozygous at any of these loci, suggesting that there is a commonly deleted interval from D7S489B through D7S1870. Normal codominant inheritance was found for the following polymorphic loci (marker names in parentheses): D7S645 (AFM238zc9), D7S672 (AF-M289ve9), D7S653 (AFM259zc1), D7S1483 (UT5719), D7S1816 (GATA21D12), D7S675 (AF-M295yg9), and D7S669 (AFM286xf9), which are, therefore, outside of the critical deleted region.

It is interesting that, while one of the loci detected with the AFM136xe3 primers (D7S489B) revealed hemizygosity in all informative patients, the related locus D7S489A did not. Heterozygosity at D7S489A was 35% in patients, similar to that reported for control individuals (GDB), indicating that this locus is outside the deletion in most WS patients. The common telomeric breakpoint, therefore, is located between D7S1870 and D7S489A, a region covered by the YAC clones HSC7E640 and HSC7S902 (see fig. 2). In light of the low informativity of locus D7S1778, we used other

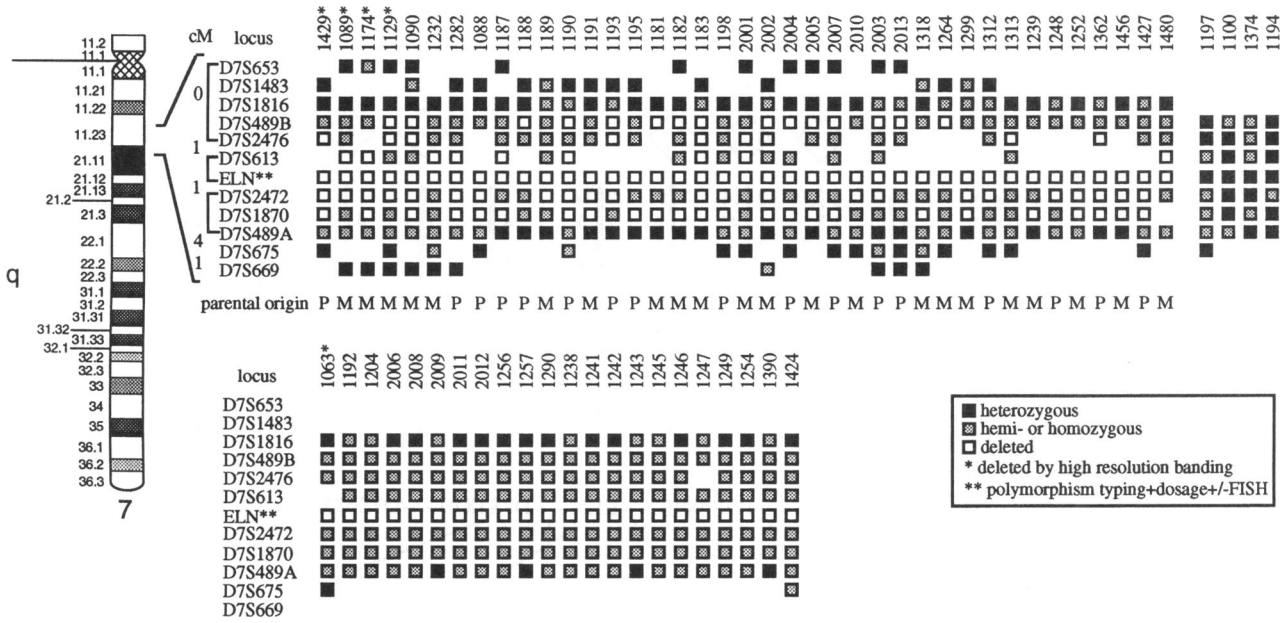

Figure 3 Composite results of genotyping multiple polymorphic loci in WS patients. In the *top* row are patients for whom parental samples were available, and the maternal (M) or paternal (P) origins of the deletions are indicated. To the right are the four patients in whom no deletion was detected. The *bottom* row contains results on patients with no parental samples available. Markers are ordered centromere to telomere. The loci from D7S489B through D7S1870 are deleted in all informative patients and, therefore, define the common WS deletion interval.

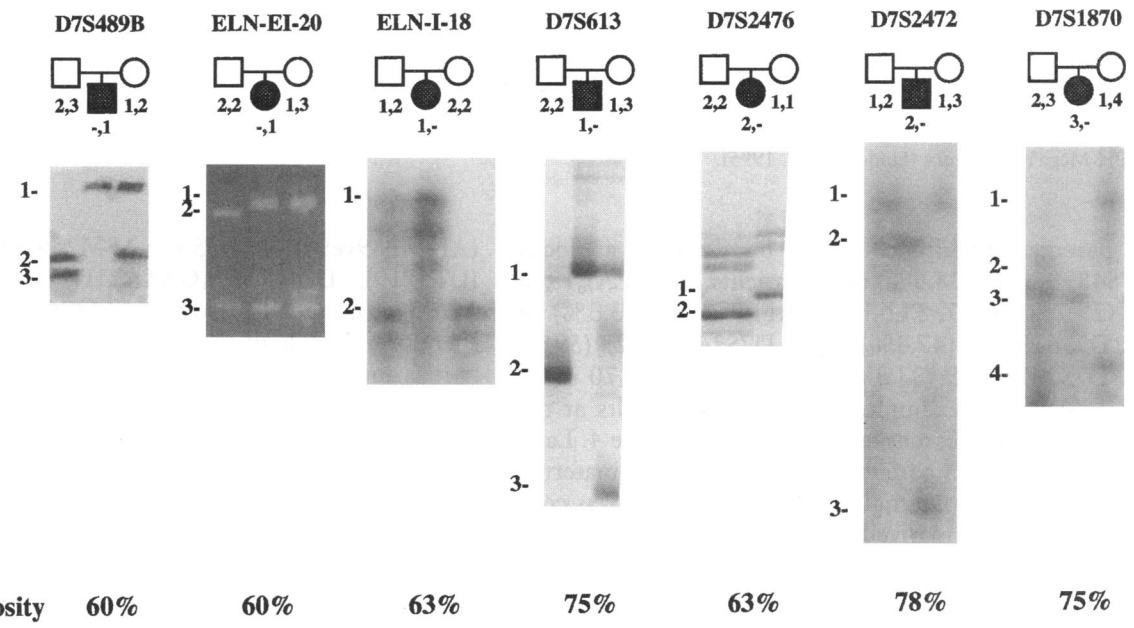

Figure 4 Genotyping of the seven intradeletion markers in WS families. Failure of parental inheritance of one allele, represented by a minus sign (−), due to de novo deletions, is observed in all cases.

markers contained in overlapping YACs to define the centromeric extension of the deletion. High levels of heterozygosity at the centromeric flanking loci D7S1816 (40 (66%) of 61), D7S1483 (12 (75%) of 16), and D7S653 (11 (92%) of 12) indicate that these loci are consistently outside the deletion and that the common centromeric breakpoint lies between D7S1816/D7S1483 and D7S489B, within the partial YAC contig.

As loci flanking ELN at a distance of 1 cM to either side are within the deletion, a deletion size approaching 2 mbp is suggested. Thus, we hypothesized that the deletion may be detectable microscopically. We retrospectively examined high-resolution (~650–750 bands) karyotypes from five WS patients and found subtle deletions of one chromosome 7 at band q11.23 in the majority of cells. No band is missing, but a reduction in the size of band q11.23 causes the grey band q11.22 to be located in the middle of q11.2 instead of its normal position closer to q11.1 as indicated on the ideogram of the high-resolution banding pattern of normal chromosome 7 (Francke 1994). While this deletion is clearly at the limit of detection by light microscopy, our findings support the notion of a ~2-mbp deletion size, as determined by the genetic and physical mapping studies and the genotyping results in WS patients.

Familial WS Due to Independent Mutational Events

We have studied two sets of second cousins with a clinical diagnosis of WS. In both families, no other member had signs of WS, and the mothers were related as first cousins. Deletion studies by dosage determination and/or FISH analysis demonstrated hemizygosity at the ELN locus in each of the four probands. Normal results on all of the seven available parents confirmed the de novo deletions in the probands. Pedigree analysis suggested the possibility of a chromosome 7 region, identical by descent, that might predispose to the 7q11.23 deletions. Genotyping of multiple intradeletion polymorphisms revealed, however, that the deletions had occurred on the paternally inherited chromosome 7 in three of the four patients (fig. 5). Although the father in family 2 was deceased, the available data are consistent with a paternal origin of this deletion, as well. Since the deletions affected the chromosome 7 contributed by the nonconsanguineous parents, each of the four deletions must represent an independent mutational event, and the occurrence of two cases within the same families is likely due to chance.

Clinical and Molecular Evaluation of Patients with No Demonstrable ELN Deletions

All four patients with normal dosage at the ELN locus had biparental inheritance at all informative loci tested. Thus, no molecular defect, such as a smaller deletion, could be detected. Clinical reevaluation of these four patients was consistent with a diagnosis of WS based on the presence, during some period of development, of characteristic facial features, mental retardation ,and strongly suggestive cognitive and personality profiles. Three of them were at or above the 50th percentile for height and head circumference (on normal growth charts). None of them had hypercalcemia or any vascular stenoses; the only cardiac anomaly was a defect in the atrial septum secundum in one of them. Their features are included in table 1.

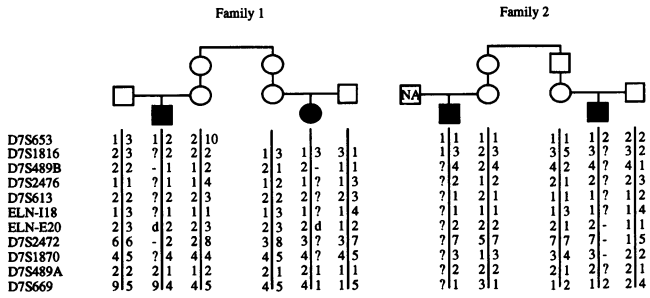

Figure 5 Haplotypes of 7q11.23 loci in two kindreds with familial Williams syndrome. The letter d indicates deletion by dosage studies, a minus sign (−) indicates deletion of a parental allele, and a question mark (?) indicates hemi- or homozygozity. The deleted chromosome is paternally derived in the three fully informative cases. In the one case with paternal sample not available (NA), the data are consistent with paternal origin of the deletion. These results rule out a maternally inherited, identical-by-descent 7q region that is uniquely predisposed to de novo deletion formation.

Genotype/Phenotype Correlations in WS Patients with a 7q11.23 Deletion

No variability in the size of the deletion could be detected between WS patients by genotyping of polymorphic markers, suggesting that the chromosomal breakpoints may consistently fall into narrowly defined physical regions. To search for other parameters that could provide possible correlations with clinical features, we tested for an amino acid polymorphism in the single remaining ELN gene and also determined the parental origin of the deletions.

The ELN *Bst*NI polymorphism represents a protein variant with either serine or glycine at amino acid position 422, with allele frequencies of 42% and 58%, respectively, in the general population (Tromp et al. 1991). This amino acid variant could convey a subtle difference in function of the protein or could be in disequilibrium with another allelic variant with functional significance. Thirty-three patients (56%) had a Ser422 in the nondeleted allele, while 26 (44%) retained an ELN allele with Gly422. No significant difference was observed between the two groups with respect to their

physical, cognitive, connective tissue, or cardiovascular manifestations ($P > .2$, in all cases).

Search for Parent-of-Origin Effects on the Phenotype in WS Patients with Deletions

The parental origin of the deleted allele could be unequivocally determined in 39 cases. Statistical analysis of the clinical variables (table 2) revealed a significant correlation between the degree of growth retardation and parental origin. In patients with a maternally derived deletion, the mean height was −1.5 SDS (± 0.94), and in paternal deletion patients it was −0.5 SDS (± 1.06) ($P = .015$). Because WS girls are slightly more growth retarded than boys and have earlier pubertal development, the observed statural differences could be biased because of sex and/or age differences between the two groups. To correct for such a bias, we also compared the height of each patient with the standards for age and sex of the WS population (Pankau et al. 1992). The correlation was still evident, with maternal deletion patients having a mean height of −0.58 SDS (± 0.67) and paternal deletion patients having a height of 0.28 SDS (± 0.86) ($P = .007$). Similar correlations were observed for weight (maternal deletion: mean weight −1.48 SDS± 1.80; paternal deletion: mean weight −0.65 ± 0.96) ($P = .017$) and head circumference (OFC) (maternal deletion: mean OFC −1.71 ± 0.52; paternal deletion: mean OFC −0.71 ± 1.02) ($P = .0007$). Birth parameters did not show a significant variation between the two groups of patients, suggesting a more severe growth retardation of postnatal onset in the group of WS patients with a maternal deletion. No significant difference was observed between the two groups with regard to the presence and/or severity of other phenotypic features (table 2). No parental age effect was apparent in either group.

Discussion

The 7q11.23 Deletion Is Common and Consistent in Size

Most patients with a clinical diagnosis of WS (61 (94%) of 65) were found to have a heterozygous deletion at chromosomal band 7q11.23 as detected by quantitative Southern blotting or FISH studies with ELN gene probes. This fraction is similar to that reported by others (Kotzot et al. 1995*a*; Lowery et al. 1995; Mari et al. 1995; Nickerson et al. 1995). To determine whether the deletions are heterogeneous in size, we first established the locus order for multiple genetic markers in the 7q11.23 region by using a combination of meiotic and physical mapping strategies. When all 65 WS patients and available parents were typed for these polymorphic markers, a commonly deleted interval could be delineated. More complex rearrangements, such as noncontiguous deletions, were not detected. In a report that appeared after our paper was submitted, Robinson et al. (1996) identified two cases with hemizygosity at D7S489A (called "D7S489L" in their paper) and seven who were heterozygous at this marker. In our series, 21 of 61 cases were heterozygous and the rest were either hemi- or homozygous. Thus, it is possible that some heterogeneity exists with respect to the location of the distal breakpoint (although Robinson et al. placed D7S489L proximal to ELN, we have convincing evidence from our physical mapping studies that it is in the distal flanking region).

The size of this common deletion can be estimated by the fact that we were able to detect it on high-resolution banded chromosomes. The reason why cytogenetic deletions at 7q11.23 have not generally been reported in WS patients may be due to their location within a single large R-band (7q11.23) that could contain ≤8 mbp of DNA. Only with careful attention to the relative position of band 7q11.22 within the entire 7q11.2 region on elongated chromosomes can a reduction in the size of band 7q11.23 be appreciated in the majority of cells. The deletion size estimate of 1.5–2.5 mbp is consistent with the 2% recombination between intradeletion genetic markers.

The Deletion Breakpoints Occur at Duplicated Regions of Sequence Homology

WS occurs sporadically in >99% of cases with a mutation rate close to 5×10^{-5}. The frequency of WS deletions is the same in maternally and paternally inherited chromosomes, and no parental age difference is apparent between the two groups. Primers for AFM136xe3 amplify two or more products that are chromosome 7–specific and highly polymorphic between individuals. At least two of these D7S489 loci, with >97% sequence identity, are located close to the common deletion breakpoints: D7S489B is near the centromeric breakpoint and is included in the deletion, while D7S489A is outside the deletion but close to the telomeric breakpoint. Other sequences within this region in the vicinity of the breakpoints are also duplicated (Pérez Jurado et al. 1996). The repetitive nature of these sequences and their clustering within a relatively small chromosomal interval provides a hypothetical mechanism for aberrant recombination or replication events, either by unequal crossover or intrachromosomal rearrangement, that may lead to the high frequency of de novo deletions in these regions. Similarly, low-copy-number repeat sequences are present within other deletion-prone chromosomal regions, such as the Kallmann syndrome region at Xp22.3, the DiGeorge/velo-cardiofacial syndrome region at 22q11, the Prader-Willi/

Table 2

Comparison of Clinical Findings between Williams Syndrome Patients with a Deletion on the Maternally and the Paternally Inherited Chromosome 7

	MATERNAL			PATERNAL			
FEATURE	Mean or %	SD	*n*	Mean or %	SD	*n*	*P* (α = .05)
Male/Female	35/65	...	20	50/50	...	18	...
Age at clinical evaluation	6.95	6.93	16	10.16	6.60	15	*P*(1) = .198
Paternal age (years)	30.0	7.35	16	30.43	4.60	14	*P*(1) = .850
Maternal age (years)	28.06	6.06	16	28.73	4.22	16	*P*(1) = .723
Time of delivery (at term: 37–42 wk)	75	...	16	86.7	...	15	*P*(3) = .187
Time of delivery (preterm/postterm)	12.5/12.5	...	16	0/13.3	...	15	
Birth weight (SDS)	−1.45	0.95	16	−1.01	.97	15	*P*(1) = .216
Birth length (SDS)	−1.12	1.24	9	−.74	.82	9	*P*(1) = .456
Irritability/Failure to thrive in infancy	75	...	16	78.6	...	14	*P*(3) = .893
Hypercalcemia before 2 years	50	...	4	50	...	4	*P*(3) = 1.0
Hypercalcemia after 2 years	15.3	...	13	7.7	...	13	*P*(3) = .587
Anthropometric data (SDS):							
Height (vs. WS population)	−.58	.67	16	.28	.86	13	**P(1) = .007**
Height (vs. general population)	−1.50	.94	16	−.50	1.06	13	**P(1) = .015**
Weight (vs. general population)	−1.48	1.80	16	−.65	.96	13	**P(2) = .017**
OFC (vs. general population)	−1.71	.52	12	−.71	1.02	10	**P(2) = .0007**
Cardiovascular disease/stenoses:							
Severe	22.2	...	18	20	...	15	*P*(3) = .682
Moderate	5.6	...	18	13.3	...	15	
Mild	44.4	...	18	53.3	...	15	
No	27.8	...	18	13.4	...	15	
Mental retardation:							
Severe (IQ <40)	12.5	...	16	6.7	...	15	*P*(3) = .860
Mild/moderate	75	...	16	80	...	15	
Borderline (IQ >75)	12.5	...	16	13.3	...	15	
Behavior (>3 years of age):							
Outgoing/gregarious	93.3	...	15	92.8	...	14	*P*(3) = .936
Hyperactivity—ADD	46.6	...	15	42.8	...	14	*P*(3) = .887
Skeletal and connective tissue:							
Joint hyperelasticity	56.2	...	16	53.3	...	15	*P*(3) = .913
Joint contractures	31.2	...	16	23	...	13	*P*(3) = .624
Vertebral deformities	37.5	...	16	46.2	...	13	*P*(3) = .638
Inguinal/umbilical hernia	37.5	...	18	20	...	15	*P*(3) = .283
Esotropia	31.2	...	16	33.3	...	15	*P*(3) = .901
Hoarse voice	80	...	15	66.7	...	12	*P*(3) = .432
Hyperacusis	100	...	12	88.9	...	9	*P*(3) = .308

NOTE.—Information on each trait was not available for all patients; SDS = standard deviation score, OFC = occipital-frontal circumference, ADD = (attention deficit disorder); *P*(1) = Student's *t*-test; *P*(2) = Kruskal-Wallis test; *P*(3) = χ^2 test. *P*-values in bold are statistically significant.

Angelman syndrome region 15q11-q13, the spinal muscular atrophy region at 5q13, and the 21-hydroxylase genes on 6p (Sinnott et al. 1990; Yen et al. 1990; Buiting et al. 1992; Halford et al. 1993; Theodosiou et al. 1994).

To explore the possibility of an inherited predisposition to deletion formation, we studied two kindreds in which a set of second cousins had been diagnosed with WS. All four probands had de novo deletions on the chromosomes inherited from the nonconsanguineous parents. Each of the probands, therefore, has WS caused by an independent mutational event, and the familial clustering is likely due to chance, in light of the high mutation rate of WS. These observations are relevant for studies of other diseases in which distantly affected relatives are often assumed to harbor abnormal alleles that are identical by descent.

Phenotype/Genotype Correlations

The overall clinical spectrum of anomalies and the phenotypic variability among individuals in our WS population are similar to previous reports (Kotzot et al. 1995*a*; Lowery et al. 1995). Cardiovascular manifesta-

tions are present in ~75%, with SVAS the most common (in 50%), followed by peripheral pulmonic stenosis (in 27%). Since disruption of the ELN locus by translocation or deletion causes cardiovascular manifestations identical to those of WS patients (Curran et al. 1993; Ewart et al. 1994; Olson et al. 1995), haploinsufficiency for ELN must be responsible for the cardiovascular manifestations of the syndrome. Whether the ELN gene deletion alone accounts for other connective tissue manifestations—the periorbital fullness and thick lips, inguinal hernias, skin and joint changes, diverticula of bladder and intestine—remains to be investigated.

The four patients with clinically defined WS without detectable deletion had some of the characteristic facial features and cognitive defects, but three of them were within the normal range for growth, and none had cardiovascular stenoses. Neither did the three nondeleted patients reported by Nickerson et al. (1995) and the two by Borg et al. (1995). No other cytogenetic abnormalities were present. These patients may have more subtle 7q11.23 deletions or point mutations in genes that map within the deletion or elsewhere, because there are several reports of WS cases with abnormalities involving chromosomes 4, 6, 13, and 18 (Jefferson et al. 1986; Bzduch and Lukacova 1989; Colley et al. 1992).

The cardiovascular abnormalities are not fully penetrant in the presence of a deletion, and the clinical severity is quite variable among patients. This nonconcordance between ELN deletions and cardiovascular phenotype may be due to allelic variation associated with differential ELN expression or function, modifier genes at other loci, or stochastic processes. We excluded a possible correlation between a protein polymorphism, either serine or glycine at amino acid position 422 of tropoelastin present on the retained ELN allele, and cardiovascular abnormalities.

Parent-of-Origin Effects

Although it is well established that ELN deletions in WS patients are present on the paternal or the maternal chromosome at about equal frequency (Ewart et al. 1993; Gilbert-Dussardier et al. 1995; Kotzot et al. 1995*b*; Nickerson et al. 1995), possible parent-of-origin effects on individual phenotypic traits have not been examined. When we searched systematically for phenotypic differences between the groups of patients with paternally (n = 18) and maternally derived (n = 21) deletions, we found a significant correlation between more severe growth retardation and maternal deletion. To correct for differences in sex-ratio and/or mean age differences between the two groups, statural data were compared with the standards for the age- and sex-matched WS population (Pankau et al. 1992). That the significant difference in height measurements is not just a statistical artifact is supported by significant differences in weight and head circumference, as well. Since weight and length at birth were not different, the more severe growth deficiency associated with maternal deletions must be of postnatal onset. Two possible reasons for this clinical-molecular correlation have to be considered: (1) maternal deletions might be slightly larger in size than paternal deletions affecting additional gene(s) involved in growth control, or (2) one or several genes altered by the deletion might be imprinted with diminished or abolished expression of the paternal allele; deletion of the maternal homologue(s) would lead to complete or almost-complete gene silencing causing growth retardation. Although the first possibility cannot be completely ruled out, since the size estimate of the deletion is still rough, we favor the second hypothesis, the existence of paternal gametic imprinting in the WS region. This tentative conclusion needs to be evaluated by studies of a larger sample. No differences in other phenotypic features dependent on the parental origin of the deletion were found, indicating that gametic imprinting does not play a role in the variability of those features.

There is evidence for at least one maternally imprinted gene on chromosome 7 that controls intrauterine and postnatal growth, but not for paternally imprinted genes. Maternal uniparental disomy (UPD) is associated with primordial growth retardation (Kotzot et al. 1995*b*), beyond homozygosity for recessive mutations in some cases (Spence et al. 1988; Voss et al. 1989). A single patient with paternal UPD 7, detected on molecular investigation of congenital chloride diarrhea, did not reveal growth retardation (Höglund et al. 1994). We have recently identified a gene that is hemizygous in WS patients, RFC2, encoding subunit 2 of the replication factor C (Peoples et al. 1996). The product of this gene is an essential part of the enzyme complex responsible for DNA strand elongation during replication. Expression studies of RFC2 have not revealed any parent-of-origin effects. Mouse breeding experiments have not demonstrated any imprinting effects for chromosomes 5 where the murine elastin gene (Eln) is located (Beechey and Cattanach 1996).

Diagnostic Considerations

The diagnosis of WS based on clinical evaluation alone may be difficult because of the variability of the manifestations, but early diagnosis is desirable to assure parental counseling, adequate special education, and evaluation for, and treatment of, the associated medical problems. Molecular diagnosis can be accomplished by ELN dosage blotting or FISH. The availability of multiple PCR-based polymorphic DNA markers that map consistently within the deleted interval allows for easy

detection of hemizygosity and for identification of the parental origin of the deleted chromosome. Even in the absence of parental samples, in light of the high composite heterozygosity of these markers (99.97%), failure to detect heterozygosity is indicative of allelic loss and can be used as a diagnostic tool. The expected false positive rate is 0.3 per 1,000, in the absence of parental consanguinity.

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