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Characterization of a COL1A1 Splicing Defect in a Case of Ehlers-Danlos Syndrome Type VII: Further Evidence of Molecular Homogeneity

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Summary

A child affected by the type VII form of Ehlers-Danlos syndrome (EDS VII) was shown to have a heterozygous structural defect in the amino-terminus of pro-α1(I) collagen. As a result, type I procollagen trimers containing defective subunits are not converted to mature collagen molecules. To identify the cause of the protein abnormality, specifically primed cDNAs and genomic DNA were PCR amplified and sequenced. This analysis disclosed that the protein structural defect is caused by a single base substitution (A for G) at position −1 of the splice donor site of intron 6 of the pro-α1(I) collagen gene (COL1A1). The affected allele produces (*a*) transcripts lacking exon 6 sequences and (*b*), in lesser amount, normally spliced transcripts. Furthermore, the rate of exon 6 skipping is temperature dependent, for it appears to decrease substantially when the patient's fibroblasts are incubated at 31°C. These findings are similar to those we previously reported for other unrelated EDS VII cases and, therefore, reemphasize the molecular homogeneity of this rare connective tissue disorder.

Introduction

Extreme joint laxity and joint dislocations, soft but nonfragile skin displaying limited bruising, and minimal hyperextensibility are the cardinal features of the type VII form of Ehlers-Danlos syndrome (EDS VII), also known as arthrochalasis multiplex congenita (Byers et al. 1983). Often, affected infants have congenital dislocation of both hips and marked hypotonia; short stature has been occasionally observed, but bone density is always normal (Byers et al. 1983).

When first recognized, this rare connective tissue disorder was thought to be caused by a deficiency of procollagen N-proteinase, the enzyme that normally cleaves the N-propeptide of type I procollagen (Lichtenstein et al. 1973). Subsequently, it was shown that defective conversion of procollagen to collagen was associated with structurally abnormal type I procollagen chains in four unrelated EDS VII patients (Steinmann et al. 1980; Eyre et al. 1985; Cole et al; 1986; Wirtz et al. 1987). On the basis of these findings, an international committee subdivided this condition into (*a*) EDS VIIA, structurally defective pro-α1(I), and (*b*) EDS VIIB, structurally defective pro-α2(I) (Beighton et al. 1988). Recently, we demonstrated that the defect in the aforementioned patients resulted from splicing mutations leading to skipping of exon 6 sequences during pre-mRNA processing (Weil et al. 1988, 1989*a*, 1989*b*, 1990). As the manuscript of the present paper was being submitted for publication, Vasan et al. (1991) reported a splicing mutation identical to one we previously described (Weil et al. 1990) in an EDS VIIB patient.

In the present paper, we have characterized another sporadic case of EDS VIIA. As in previous cases, we

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found that this individual is affected by a splicing mutation which results in an interstitial in-frame deletion of 24 amino acids around the N-proteinase cleavage site of pro-α1(I) collagen.

Subject and Methods

Case Report

The patient (fig. 1) was the 3.33-kg female product of a term pregnancy and normal delivery to a phenotypically normal 25-year-old mother and an unrelated, clinically normal 28-year-old father; four previous siblings were normal. Fetal movements were noted to be mildly reduced during pregnancy. Birth length was 49.5 cm (50%), and head circumference was 35 cm (60%). Bilateral dislocations of hips and knees, with camptodactyly of fingers 2 and 3, were found at birth. The hip dislocations were treated conservatively, but at age 4 years open reduction and capsular plication of the right hip was required. At 18 mo of age, the patient was 78.5 cm (25%) in height with a head circumference of 49 cm (80%). The anterior fontanel was widely patent (2 × 2.5 cm). The facies was unusual, with frontal bossing; broad, flat nasal bridge; and midfacial hypoplasia. Marked generalized large- and small-joint laxity and a left hip click were present. Camptodactyly of left fingers 2 and 3 and of right fingers 2–4 was noted. There was bilateral hallux valgus and pronated pes planus. A 5-cm umbilical hernia was present. The skin was doughy, moderately hyperextensible with moderately increased bruisability, but lacking unusual scars. The remainder of the physical examination was unremarkable. Subsequent history has been benign. Frequent bruising and lacerations at one time led to the suspicion of child abuse. One humeral fracture required open reduction; the surgical scars from this procedure are unremarkable. Continued joint hypermobility reportedly impairs physical activity as compared with that of peers. The subject is now 7 years of age and has completed the first grade.

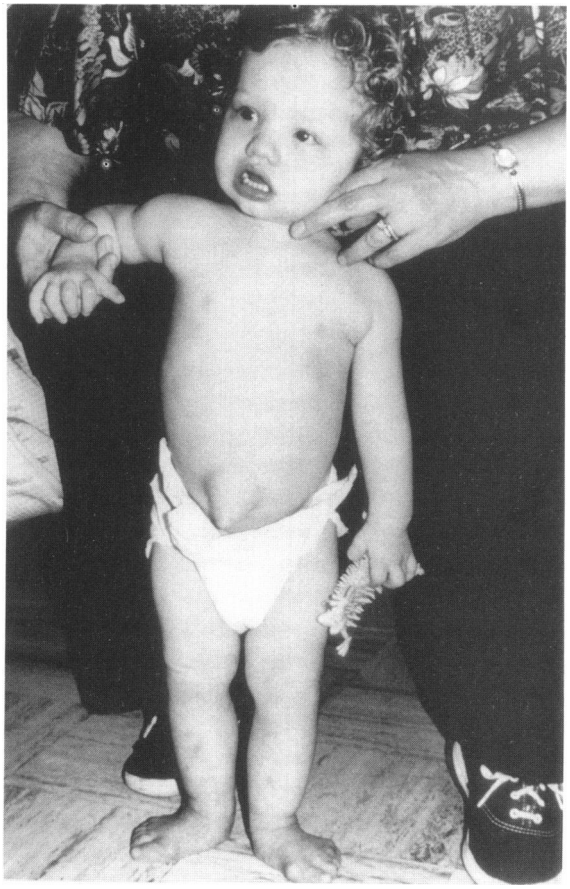

Figure 1 Affected EDS VII patient studied in present paper.

Patient Material

A 3-mm punch biopsy of skin was obtained from the deltoid region, resulting in a gaping 1-cm wound with herniation of subcutaneous fat and requiring three sutures to close. Dermal fibroblast cultures were established and maintained in Dulbecco's modified Eagle media (DMEM) containing antibiotics and 8% FCS as previously described (Wirtz et al. 1987).

Collagen Analysis

Confluent patient and control fibroblasts were radiolabeled with ^{3}H-proline in the presence of 100 μg ascorbic acid/ml for 16 h. Supernatant and cell layer were harvested separately, and the procollagens were precipitated with 30% ethanol at 0°C (Wirtz et al. 1987). Procollagen was converted to collagen by digestion with 100 μg porcine pepsin/ml in 1 M acetic acid at 4°C for 6 h. Aliquots of media and cell-layer collagens were subjected to discontinuous SDS-PAGE. Gels were processed for fluorography as previously described (Wirtz et al. 1987).

Western Blotting

Unlabeled procollagen was prepared using roller bottles of cultured control and EDS fibroblasts (Wirtz et al. 1987). The procollagens were cleaved with cyanogen bromide (CNBr), separated by SDS-PAGE,

electrophoretically transferred to nitrocellulose, and probed with antisera to the α1(I) N-propeptide as described previously (Wirtz et al. 1990).

DNA Amplification and DNA Sequencing

Collagen-specific cDNA cloning was accomplished as previously described (Weil et al. 1989*a*) using the synthetic primer 5′-GGGACCTTGGAAGCCTTGGGGACC-3′, which is complementary to the mRNA sequences encoded by the 5′ portion of exon 8 of COL1A1 (D'Alessio et al. 1988). Amplification of cDNA molecules by the PCR technique utilized the exon 8 oligonucleotide and a primer (5′-GGAATTCGAAACCACCGGCGTCGAAGGA-3′) corresponding to the junction sequence between exons 3 and 4 (D'Alessio et al. 1988). For genomic amplification, the oligonucleotide primers were an exon 5 14mer (5′-GGACCCGCAGGCCC-3′) and an exon 7 14mer (5′-GGGGGGCCAGGGAG-3′) (D'Alessio et al. 1988). Conditions for PCR amplification and analysis of the resulting products were as previously described (Lee et al. 1991). Different PCR products, eluted from gels and subcloned into the pUC18 plasmid vector, were sequenced by the chain-termination method on double-stranded DNA (Zagursky et al. 1986) by using universal primers and COL1A1-specific oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer. The sequences presented in the present paper were derived from sequencing at least three times both DNA strands.

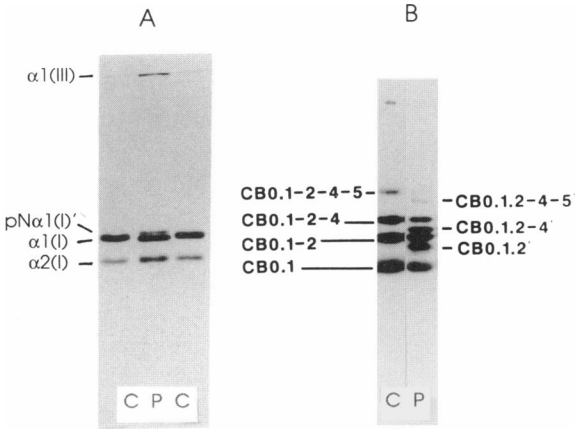

Figure 2 Electrophoretic analysis of procollagens. *A*, Pepsin-digested procollagens produced by patient (lanes P) and control (lane C) cells in culture were analyzed by SDS–5% PAGE followed by fluorography. The patient's cells yielded the expected α1(I) and α2(I) chains of type I collagen, and an additional collagenous band (designated pN α1(I)′) was present, migrating on electrophoresis just above the α1(I) chain. *B*, Western blot of CNBr-digested control (lane C) and patient (lane P) procollagens subjected to SDS-PAGE and probed with rabbit antisera to the α1(I) N-propeptide. The identities of the normal fully and incompletely cleaved CNBr peptides are given on the left; on the right, only the fully and incompletely cleaved peptides derived from the deleted mutant pro α1(I) chain are given. By convention, individual CNBr peptides are designated by number, and peptides normally lacking intervening methionine residues are separated by a period (e.g., CB 1.0), whereas peptides with uncleaved methionine residues are linked by a dash (e.g., CB0.1–2). It is apparent that the patient's CNBr peptide profile contains both a normal set of peptides and an additional set, of greater mobility, identified as either the deleted CB 0.1.2′ peptide or larger uncleaved peptides harboring this peptide.

Results

To establish the nature of the molecular lesion responsible for the EDS VII phenotype in the subject of this study, biochemical analyses of her cultured dermal fibroblasts were initially employed. Results of electrophoretic analysis of the patient's media and cell-layer procollagens were similar to those of controls, and the relative ratio of the chains of types I and III appeared to be normal. In contrast, analysis of the collagens generated by limited pepsin digestion revealed the consistent presence of an additional band. The mobility of this band was similar to that of a partially processed procollagen retaining the N-propeptide (fig. 2*A*), and its apparent amount varied inversely with the extent of pepsin digestion (not shown). Both the α1(I) and α2(I) chains were normal in mobility and apparent amount (fig. 2*A*). The additional band seen in the proband was considered to be a mutant pNα1(I) chain and was designated pNα1(I)′.

Western blots of the patient's CNBr peptide profile (fig. 2*B*) showed a set of fully cleaved and partially cleaved small CNBr peptides derived from the amino-terminal end of the mutant collagen chain and exhibiting higher electrophoretic mobility than did their normal counterparts. Specifically, the fully cleaved mutant peptide CB 0.1.2′, harboring the deletion and missing a methionine residue, displays a higher electrophoretic mobility than does the normal CB 0.1–2 partial cleavage product (fig. 2). The patient's CNBr peptide profile, containing both the normal set of small peptides and an additional set with greater electrophoretic mobility as compared with that of the control, confirmed the protein deletion in the mutant collagen chain. These findings were identical to those

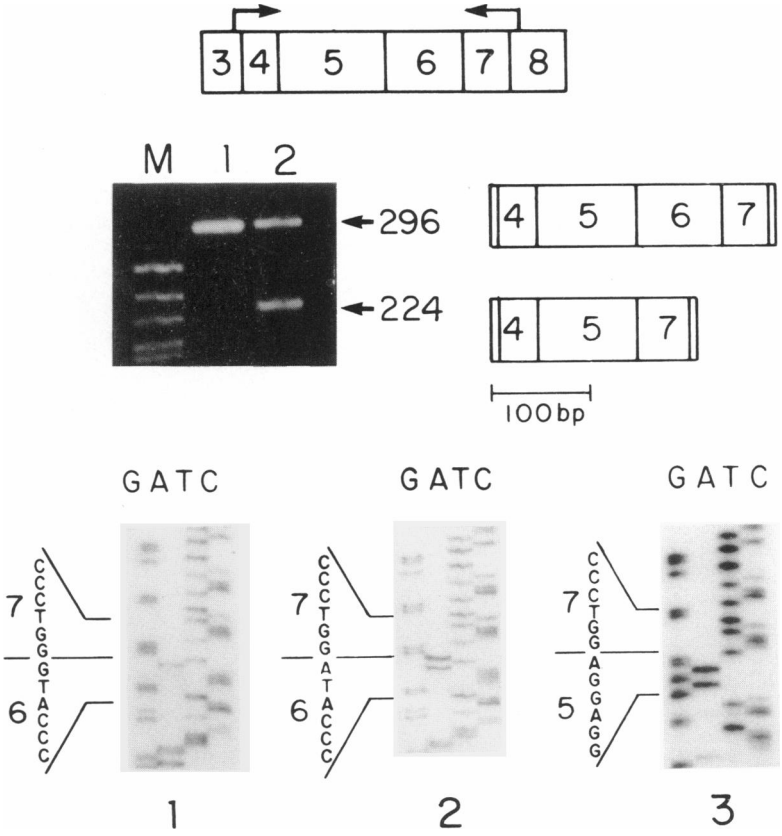

Figure 3 PCR amplification and DNA sequences of proband's cDNAs. *Top,* Schematic representation of cDNA amplification strategy, with arrows highlighting relative positions of primers. *Middle,* Ethidium bromide–stained agarose gel showing DNA size markers (lane M) and aliquots of control (lane 1) and patient (lane 2) RT-PCR products. Sizes of bands (in bp) are indicated. *Bottom,* Nucleotide sequences of patient's cDNAs. Panel 1, Sequence of normal transcript. Panel 2, Sequence of normally spliced transcript of affected allele. Panel 3, Sequence of abnormally spliced transcript.

previously found for another EDS VIIA patient shown to have a deletion of the 24 amino acids constituting the N-telopeptide of pro-α1(I) chain (Cole et al. 1986), thus strongly suggesting that the present patient harbored a similar deletion.

To test this hypothesis, the patient pro-α1(I) mRNA was reverse transcribed using a specific oligonucleotide primer and was amplified by the PCR technique (RT-PCR) (Kawasaki and Wang 1989). Analysis of the resulting RT-PCR products documented the presence of two major pro-α1(I) mRNA species in the patient's dermal fibroblasts, a normal one and one approximately 100 bp shorter (fig. 3). Sequencing of two sets of five independent subclones derived from each of the two RT-PCR species demonstrated the absence of the 72 bp of exon 6 in the shorter product (fig. 3).

To determine the nature of the defect at the genomic level, a defined segment of the patient's COL1A1 encompassing exon 6 was PCR amplified. Sizing of the genomic amplification products documented the presence of a single band, effectively ruling out a genomic deletion (fig. 4). Sequencing of 10 independent PCR subclones revealed in three of them a single base difference, notably a G-to-A transition in the last nucleotide of exon 6 (position − 1 of the splice donor site of intron 6) (fig. 4). The substitution is identical to one we previously reported in an unrelated EDS VIIA patient (Weil et al. 1989*a*). In that study, we demonstrated that the exonic change causes alternative splicing of the procollagen transcripts. In addition, we noted that the base change eliminates an *Nco*I restriction site in the cDNA synthesized from the normally

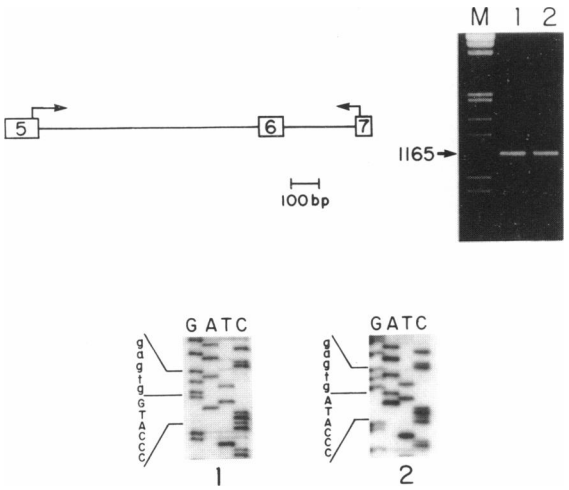

Figure 4 PCR amplification and DNA sequences of proband's COL1A1 alleles. *Top,* Schematic representation of genomic DNA amplification strategy, with arrows highlighting approximate positions of primers. On the right is an ethidium bromide–stained agarose gel showing DNA size markers (lane M) and aliquots of control (lane 1) and patient (lane 2) genomic PCR products. Size of bands (in bp) is indicated. *Bottom,* Autoradiograms of exon 6/intron 6 sequences of patient's COL3A1 alleles. Exon and intron sequences are denoted by uppercase and lowercase letters, respectively.

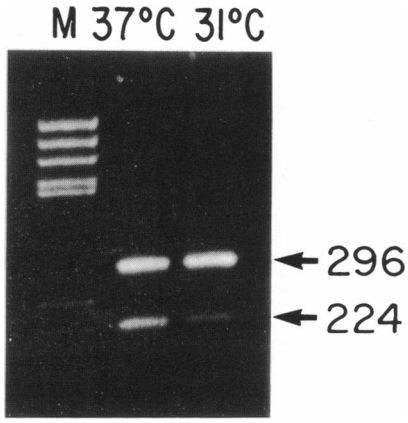

Figure 5 Temperature-dependent expression of proband's exon skipping. Ethidium bromide staining of agarose gel showed DNA size markers (lane M) and aliquots of the patient's RT-PCR-amplified products from fibroblasts grown at 37°C and 31°C. Sizes of bands (in bp) are indicated.

spliced product of the affected allele (Weil et al. 1989*a*). To demonstrate that also in the patient in the present paper the mutation leads to alternative splicing, 30 subclones of the patient's normal size RT-PCR products were scored for the presence of the *Nco*I site. This identified four recombinants lacking the *Nco*I site (data not shown). Sequence analysis confirmed that these molecules represent the normally spliced product of the affected allele, since they harbor the A-for-G change in the last nucleotide of exon 6 (fig. 3).

To confirm that the abnormal transcript is alternatively spliced, we examined the extent of correct versus incorrect splicing in the patient's fibroblasts cultured at 37°C and 31°C. The premise of this analysis stemmed from the observation that an identical −1 substitution (A for G) in COL1A2 displays temperature-dependent alternative splicing *in cellula* (Weil et al. 1989*b*). Hence, RNAs from fibroblasts of this EDS VIIA cultured at 37°C and 31°C were used as templates for RT-PCR amplification. Comparative analysis of the resulting RT-PCR products confirmed that, also in this case, temperature changed the rate of expression of the shortened product relative to that in the normal size transcript (fig. 5).

Discussion

In this paper we describe the characterization of an exon substitution causing abnormal pre-mRNA splicing and ultimately leading to the production of structurally defective pro-α1(I) collagen in a sporadic case of EDS VIIA. This study presents the sixth case of aberrant splicing in EDS VII—and the first recurrence of the same mutation in COL1A1 (Weil et al. 1988, 1989*a*, 1989*b*, 1990; Vasan et al. 1991). In all of the patients characterized to date, exon skipping is caused by single base substitutions at the junction sequence of exon 6/intron 6. Two of the changes (G to A and T to C) alter the highly conserved GT dinucleotide (positions +1 and +2) of the splice donor site of COL1A2 (Weil et al. 1988, 1990; Vasan et al. 1991). The other two substitutions are the same base change (A for G) in the last nucleotide of exon 6 (position −1) of either COL1A1 or COL1A2 (Weil et al. 1989*a*, 1989*b*). Mutations at positions +1 and +2 lead only to exon skipping, while those at position −1 result in alternative splicing of exon 6 sequences (Weil et al. 1988, 1989*a*, 1989*b*, 1990). The differential expression of defective splicing in the two groups of mutations does not, however, seem to influence the phenotypic outcome of the disorder. More generally, these findings are in agreement with the notion that individual nucleotides of the splice sites contribute differently to pre-mRNA processing (Krainer and Maniatis 1988). In this respect and like other collagen splicing mutations at moderately conserved nucleotides, the −1 substitutions exhibit temperature-dependent exon

skipping *in cellula* (Weil et al. 1989*b*; Bonadio et al. 1990; Lee et al. 1991*b*).

From our studies it is increasingly apparent that EDS VII is homogeneous not only on a clinical level but also on a molecular one. Indeed, we are aware of at least five additional EDS VII cases affected by splicing defects that alter the normal processing of exon 6 sequences. In this respect, the EDS VII homogeneity greatly contrasts with the heterogeneity of other collagenopathies—namely, osteogenesis imperfecta, EDS IV, and the chondrodysplasias (reviewed in Lee et al. 1991*a*).

While the mechanism responsible for the procollagen abnormality in EDS VII is well understood, the pathogenesis of the disease is still the subject of speculation. There is the possibility that this rare condition might also arise from structural alterations in the peptide encoded by exon 6. Alterations either block excision of the amino propeptide or remove the cross-link precursor lysine, thus distinguishing between defective fibrillogenesis (and secondary defects in cross-linking) and a primary defect in cross-linking.

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