COL5A1 Exon 14 Splice Acceptor Mutation Causes a Functional Null Allele, Haploinsufficiency of α1(V) and Abnormal Heterotypic Interstitial Fibrils in Ehlers-Danlos Syndrome II* 

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We studied four affected individuals from a family of three generations with Ehlers-Danlos Syndrome II. Type V collagen transcripts of affected individuals were screened by reverse transcriptase-polymerase chain reaction. Amplification of the exon 9–28 region of α1(V) yielded normal and larger products from the proband. Sequencing of cDNA revealed a 100-base pair insertion from the 3′-end of intron 13 between exons 13 and 14 in one allele. The genomic defect was identified as an A→G substitution at the exon 14 splice acceptor site. A cryptic acceptor site –100 nucleotide within intron 13 is used instead of the mutant splice site. The insertion shifts the reading frame +1 and results in a stop codon within exon 17. The mutant transcript was much less abundant than normal allele product in untreated cultured fibroblasts but was approximately equimolar in cycloheximide-treated cells, suggesting that the mutation causes nonsense-mediated decay of mRNA. By RNase protection experiments, the level of mutant transcript was determined to be 8% that of the normal transcript in untreated proband fibroblasts. Relative to type I collagen, proband fibroblasts secreted only 65% of the amount of type V collagen secreted by normal controls. Selective salt precipitation of proband secreted collagen provided supportive evidence that the α chain composition of type V collagen remains α1(V)α2(V) even in the context of α1(V) haploinsufficiency. Type V collagen incorporates into type I collagen fibrils in the extracellular matrix and is thought to regulate fibril diameter. Transmission electron micrographs of type I collagen fibrils in a proband dermal biopsy showed greater heterogeneity in fibril diameter than in a matched control. The proband had a greater proportion of both larger and smaller fibrils and occasional fibrils with a cauliflower configuration. Unlike the genotype/phenotype relationship seen for type I collagen defects and osteogenesis imperfecta, the null allele in this family appears to cause clinical features similar to those seen in cases with structural alterations in type V collagen.

Ehlers-Danlos Syndrome (EDS) is a heterogeneous group of connective tissue disorders (1). Clinical manifestations in the milder forms of the disorder occur predominantly in the dermis (loose skin, abnormal scars, easy bruising) and joints (hyperextensibility), whereas the vascular (aneurysms) and visceral (ruptures, pneumothoraces) symptoms are found in the severe type IV form of EDS. A corresponding heterogeneous range of gene defects causes the broad range of EDS forms. These forms have involved the genes for type I (EDS VII) or III (EDS IV) collagen or for enzymes involved in the collagen metabolic pathway (EDS VI and VII) (2). Recently, a noncollagenous defect in tenasin-X has also been demonstrated in EDS (3). Furthermore, there must be a number of additional genes in which defective forms cause EDS, because many EDS patients do not have mutations in the genes already described.

In the last 4 years, type V collagen joined the growing list of matrix molecules associated with Ehlers-Danlos Syndrome. Type V collagen was a prime candidate for such a role because it is present as a minor component of the extracellular matrix in tissues in which type I collagen is the predominant structural molecule, especially skin and tendon (4). Type V collagen is a fibrillar collagen in which the central helical region is the same length as that of type I collagen. It occurs as two forms of heterotrimers or α1(V)3 homotrimer. In the dermis, tendon, and bone, α1(V)3α2(V) is the usual trimer composition (5–7).

The role of this type V collagen in the matrix appears to involve the formation of heterotypic fibrils with type I collagen and a direct effect on regulating the diameter of those fibrils. The formation of heterotypic fibrils by types I and V collagen was the first codistribution of different collagen types into fibrils to be demonstrated (8). Double-labeled immunoelectron microscopy with colloidal gold-tagged monoclonal antibodies was used to simultaneously localize types I and V collagen in chick corneal stroma fibrils and demonstrate that type V epitopes were blocked unless fibrils were partially dissociated. Because fibrils are relatively thin in corneal stroma and type V is more abundant than in other type I collagen predominant tissues, a regulatory role for type V was postulated in limiting the diameter of heterotypic fibrils. This was confirmed by in vitro studies forming copolymers of types I and V collagen. Increasing the proportion of type V collagen resulted in a progressive decrease of fibril diameter (9). The retained N-terminal portion of type V a chains was essential to the regulatory role. This regulatory model involves the localization of type V trimers at the periphery of type I fibrils with the helical regions of type V buried and the N-terminal extensions of type V projecting onto the surface and limiting fibril growth by steric hindrance (10). This role was supported by gene targeting of collagen; RT-PCR, reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; nt, nucleotide; bp, base pair(s).

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The abbreviations used are: EDS, Ehlers-Danlos Syndrome; COL,
experiments of COL5A2 in mice. Homozygous mice producing structurally abnormal α2(V) lacking the N-telopeptide have reduced tissue strength and fibrils that are heterogeneous in size (11).

Both structural defects and null alleles of type V collagen have been delineated in patients with EDS. Clinically, these patients have had EDS I or II, the gravis and mitis forms in the older Berlin nosology (12), now also described collectively as the classical type in the more recent Villefranche nosology (13). Of the structural defects in type V collagen, seven were in the α1(V) chain, and three were in the α2(V) chain (14–20). Most cause splicing defects, two cause amino acid substitutions, and one results from a chromosome translocation. Transmission electron micrographs of heterotypic fibrils in four cases are characterized by the irregularity of fibril shape and variation in diameter. More recently, null alleles of COL5A1 have been recognized as a cause of a significant proportion of classical EDS (21, 22). Transmission electron micrographs in one patient showed larger and more irregular fibrils (22).

In the present study, we have characterized molecularly and biochemically a type V collagen mutation causing a straightforward null allele. Steady-state secretion of type V collagen is decreased. Also, both steady-state-secreted collagen and selective salt precipitation of proband secreted collagen show maintenance of the α1(V)α2(V) chain composition of type V collagen even in the context of α1(V) haplinsufficiency. Heterotypic dermal fibrils have greater heterogeneity of diameter than in the control. The implications of these data for understanding the genotype/phenotype paradigm in EDS are discussed.

MATERIALS AND METHODS

Cell Culture—Dermal fibroblast cultures were established from skin punch biopsies of individuals III-3, III-10, and IV-5 (Fig. 1). Cells were grown in Dulbecco’s modified Eagle’s medium, enriched with 10% calf serum plus 2 mM glutamine in the presence of 5% CO2.

RT-PCR Screening—Total RNA was isolated from cultured fibroblasts using TRI Reagent (Molecular Research Center, Inc., Cincinnati, Ohio), according to manufacturer protocol (23). Patient and control total RNA (0.5–1 μg) were reverse-transcribed using murine leukemia virus reverse transcriptase, oligo(dT), and an RT PCR core kit (PerkinElmer Life Sci.) for 1 h at 42 °C. After cDNA synthesis, regions along both α1(V) and α2(V) transcripts were amplified using Taq polymerase (Life Technologies, Inc.) (24). PCR reactions contained 2 mM MgCl2, 300 μM each dNTP, 200 μg/ml of β-mercaptoethanol, and 2.5 units of Taq polymerase. The PCR reaction amplifying the region of α1(V) cDNA from exons 9 to 28 used the forward primer E9s (5′-GGGAGAACCAGC-3′) and the reverse primer in exon 13, E13s (5′-GGGAGAACCAGC-3′), complementary to nt 2396–2413 (25). Cycling conditions were 94 °C for 1 min followed by 35 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72°C for 7 min. Products were electrophoresed on a 1% agarose gel and isolated using QIAquick Gel Extraction Kit (QIAGEN, Inc., Valencia, CA). They were then subcloned using the TA cloning system (Invitrogen, Carlsbad, CA).

RT-PCR of Proband Total RNA from Cycloheximide-Treated Cells—Confluent fibroblasts in growth medium were treated with 100 μg/ml cycloheximide for 4 h (27). Total RNA was isolated as described above. RT-PCR used primer pair E9s and E28as and the same cycling conditions used for screening.

RNA Protection Analysis—The mutant and normal cDNA fragment spanning exons 13–28 was re-isolated from the TA cloning vector and subcloned into pGEMZf(+) plasmid (Stratagene, La Jolla, CA). Uniformly labeled antisense riboprobe was synthesized as described previously (28) using linearized plasmid carrying the mutant form of exons 13–28, TT RNA polymerase (Promega), and [α-32P]CTP. Targets for RNA protection comprised patient and control fibroblast total RNA (5 μg) or normal and mutant sense synthetic RNA transcript spanning exons 13–28 (50 ng). T7 or SP6 RNA polymerase (Promega) was used to transcribe normal or mutant sense target RNA, respectively. Target RNAs were mixed with 2 × 106 cpn of antisense probe, denatured at 85 °C for 5 min, and then hybridized overnight at 50 °C. The hybrids were digested with RNase A (8 μg/ml Type X, Sigma) as described previously (28). Digestion products were electrophoresed on 5% polyacrylamide–7 M urea gels.

Protein Analysis—Fibroblasts from III-5 (P–10) and the control cells (GMO 3349, P–8–15) were plated at a density of 2.5 × 105 cells/35-mm culture dish and were allowed to attach overnight at 37 °C. The cells were pre-incubated with ascorbate for 2 h and then labeled with medium containing 50 μM ascorbate and 325 μCi of [1-2,3-4,5-3H]proline/well (Amersham Pharmacia Biotech) for about 16 h. The medium and cell lysates were harvested separately as described previously (29). Samples were precipitated with EtOH (half-volume of 95% ethanol) and then centrifuged at 37,000 × g for 40 min. The pellets were resuspended in 0.2 M NaCl, 50 mM Tris, pH 7.5. Collagens were prepared by digestion of procollagen with pepsin (75 μg/ml), electrophoresed on a 5.5% polyacrylamide–2 M urea–SDS gel, and visualized by autoradiography.

For selective salt precipitation, proband fibroblasts were stimulated with ascorbate and labeled with [14C]proline (250 μCi/mmole, Amer sham Pharmacia Biotech). Medium was acidified and digested with pepsin (1:10 weight ratio to collagen as measured by the Sirol assay). The pepsinized sample was concentrated by Centricon 30 and then successively precipitated with 0.9, 1.2, and 2.0 M NaCl using a modified procedure from Gimenez et al. (6) and Rhodes and Miller (30). Samples were electrophoresed on a 5% polyacrylamide–2 M urea–SDS gel.

Transmission Electron Microscopy of Proband Dermis—A dermal punch biopsy was obtained from the upper arm of proband IV-5, and a control matched for age, gender, and race. The sample was fixed in 2.5% glutaraldehyde and then treated with 1% osmium tetroxide followed by en bloc staining with 2% uranyl acetate. After dehydration, the tissue was infiltrated with Spurr’s plastic resin. 600–800 A sections were examined in a Zeiss EM10 CA transmission electron microscope, and representative areas were photographed (JFE Enterprises, College Park, MD).

RESULTS

The Affected Members of an Extended Pedigree Have Symptoms of Mild EDS II—Affected individuals were ascertained at two different medical centers from two branches of a five-generation pedigree and referred independently to the NIH Clinical Center (Fig. 1). Unless otherwise specified, the molec-
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null and biochemical data were obtained from proband A and her affected sons.

III-3 is a 58-year-old woman (proband A). Her height is 153 cm (5% for adult women, 50% for 12-year-old girls), and her span is 160 cm. She has soft skin on her face and arms. The skin is moderately loose around the neck and elbows, and moderate bruising was noted. Lacerations have healed with cigarette paper scars. She reports that her hip and knee joints were hyperextensible as a child but this has decreased with age. She carried four pregnancies including a twin pregnancy to term without premature rupture of membranes or other complications. At age 49, she had a hysterectomy for uterine prolapse.

IV-5 is the 18-year-old son of III-3. He weighed 10 lb, 1 oz at birth. He was first evaluated for EDS at age 3.5 years when he was noted to have thin skin, easy bruising, and a pectus excavatum. At that time, his height was 97.3 cm (50% for age). The skin around face, neck, elbows, and knees is moderately hyperextensible. Multiple bruises were noted on arms and legs. He has several cigarette paper scars. Large joints are not hyperextensible. Pes planus was noted.

IV-3 is the 24-year-old son of III-3. He weighed 9 lb, 12 oz at birth. At age 10 years, he had a height of 124 cm (<5% for age, 50% for 7.5-year-old boys). From infancy, he was noted to have moderately loose skin, easy bruising, and thin scars. He does not have hyperextensibility of large or small joints.

III-10 is a 46-year-old woman (proband B). Her height is 159 cm (25% for adult women, 50% for 14-year-old girls). She has moderately loose skin around the elbows and knees, but facial and neck skin are not extensible. Her thumbs are hyperextensible, both elbows and knees have mild recurvatum. Superficial lacerations have healed normally with the exception of one cigarette paper scar on her leg. There is no evidence of bruising. Her surviving son reportedly is mildly affected but declined examination. Two additional pregnancies were premature rupture of membranes at 27–28 weeks. The babies died of complications of prematurity at 14–16 days. No tissues were collected.

RT-PCR of Proband a1(V) RNA Yields both Normal and Larger Size Products—The type V collagen mRNAs of a series of patients with EDS types I and II were screened for structural alterations by RT-PCR of overlapping regions along both a1(V) and a2(V) transcripts. Amplification of the region of a1(V) cDNA encompassing exons 9–28 (17) from patient III-3 yielded two products, the 1049-bp fragment expected from normal cDNA structure and a unique minor product ~100 bp larger than the normal fragment (Fig. 2A). Digestion of the amplification products with HhaI and NcoI localized the structural alteration to the region spanning exons 13–23 (Fig. 2, B–C).

Mutant cDNA Contains a 100-bp Intron Insertion between Exons 13 and 14—To define the structure of the abnormal cDNA, the exon 13–28 region of a1(V) cDNA was amplified by RT-PCR, yielding an 804-bp fragment expected from the normal cDNA and a fragment ~100 bp larger (data not shown). Both products were subcloned and sequenced (Fig. 3, A and B). The 804-bp product contained normal cDNA sequence for this region (25). The unique larger fragment contained a 100-bp insertion between exons 13 and 14 derived from the 3′-end of intron 13. Its sequence exactly matches the known intronic sequence (31) except for an A→G change within the exon 14 splice acceptor site. Inspection of intron 13 sequences near the 5′-end of the insertion reveals a cryptic splice site located 100 bp upstream of exon 14, which is used as the alternative acceptor site.

To determine whether alternative acceptor sites were used, RT-PCR was performed using an antisense primer complementary to the 3′-end of intron 13 paired with sense primers in exons 9 or 13. Only the product containing the 100-bp insertion was detected.

The Gene Level Defect Is a Point Mutation in the Exon 14 Splice Acceptor Site—The region of the a1(V) gene extending from about 300 bp at the 3′-end of intron 13 into exon 14 was amplified from the leukocyte DNA of patient III-3, subcloned, and sequenced (Fig. 4). One set of subclones contained normal intron 13 sequences. A second set contained the same A→G transversion in the exon 14 splice acceptor site noted in the mutant cDNA.

The mutation generates a novel AcI I site. AcI digestion of a genomic PCR product containing the mutation was used to confirm heterozygosity in affected family members (III-3, III-10, IV-3, and IV-5) and the absence of the mutation in an unaffected member (IV-2) (Fig. 5).

Use of a Cryptic Splice Acceptor Site Shifts the Collagen

Fig. 1. Pedigree of family with EDS type II. IV-5 received attention because of easy bruising in childhood and III-10 was noted to have abnormal skin during an obstetric admission. Individuals III-3, III-10, IV-1, IV-3, IV-4, and IV-5 were examined by J. C. M. Remainder of pedigree was obtained by report.
FIG. 2. Screening of cDNA by RT-PCR amplification and restriction enzyme digestion. A, analysis of cDNA by RT-PCR. Selected EDS patients were screened by RT-PCR along both chains of type V collagen. Amplification of total RNA from patient III-3 (lane 4) for the exon 9–28 region of α1(V) revealed a second band approximately 100 bp larger than the expected 1049-bp fragment from normal cDNA. Lanes 1–3, RT-PCR product from other EDS patients; lane 4, RT-PCR product from III-3; lane 5, RT-PCR product from control cell line. B, digestion of RT-PCR products in panel A with HhaI and NcoI. Lanes 1 and 3, digestion of unrelated EDS patients; lane 4, digestion of III-3; lane 5, control cell line PCR fragment. C, a schematic presentation of cDNA amplified by RT-PCR and digested by HhaI and NcoI. Diagram shows exons, restriction enzyme digestion sites, and sizes of expected cleavage products.

FIG. 3. Sequence of mutant and normal cDNA. A, the sequence of the cDNA with the 100-bp insertion is shown. B, a schematic diagram for splicing of normal and mutant cDNA. Exonic and intronic sequence are shown in capital and lowercase letters, respectively. The cag cryptic acceptor site is underlined, and the bases involved in the transversion (a → g) are in bold. C, a diagram of α1(V) null allele caused by 100-bp intronic insertion. The insertion causes a shift of +1 in the reading frame and the occurrence of a premature termination codon (UGA) downstream in exon 17. The triple helical domain begins in exon 14.
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The 100-bp intronic insertion in the \( \alpha_1(V) \) transcript resulted in a \( \alpha_1 \) shift of the collagen reading frame. In the new reading frame, exons 14–16 are translated into a noncollagenous protein chain, and there is a premature termination codon (UGA) at the sixth amino acid of exon 17 (Fig. 3) (25). We compared the stability of mutant transcript in proband total RNA isolated from fibroblasts with and without cycloheximide treatment (27). The exon 9–28 region was amplified from both RNAs (Fig. 6). In the RT-PCR using RNA from treated cells, the normal and mutant products were approximately equimolar, whereas in the amplification from untreated cells, the mutant product was ~7-fold less abundant than the normal product. The decreased stability of the mutant transcript was presumably due to nonsense-mediated decay.

To more accurately quantitate the relative amounts of normal and mutant \( \alpha_1(V) \) transcript in untreated cells, RNase protection (28) was performed (Fig. 7). The uniformly labeled antisense probe for this assay was transcribed from a subclone of proband mutant cDNA encompassing exons 13–28. Mutant mRNA would protect a 914-nt probe fragment, whereas normal transcript would protect fragments of 760 and 54 nt. Protection of the expected probe fragments was verified using synthetic sense transcripts of the normal and mutant cDNAs. When the probe was hybridized with proband total RNA, probe protection by mutant transcript appeared to be less than 10% of that protected by the normal transcript. Excision, solubilization, and scintillation counting of the protected fragments demonstrated that the level of mutant transcript was 8% that of the normal transcript.

Proband Fibroblasts Secrete Reduced Amounts of Type V Collagen with Normal \( \alpha \) Chain Composition—Procollagen was precipitated from both the medium and cell layer of fibroblasts from proband III-3 and control (GMO 3349). Collagens were derived from these samples by pepsin digestion (Fig. 8A). In the cell layer sample, the type V \( \alpha \) chains appear to have an approximately equimolar ratio. To normalize the collagen secreted into the medium, lanes were loaded to achieve equal intensity of \( \alpha_1(I) \). Longer exposure of the media samples revealed that proband secretion of \( \alpha_1(V) \) was ~65% that of control cells, supporting the haploinsufficiency detected at the RNA level. A prominent band was noted on several preparations migrating between \( \alpha_1(I) \) and \( \alpha_2(I) \). CNBr peptide analysis (Fig. 8B) revealed that in the control sample this was a mixture of incompletely pepsin-digested \( \alpha_2(I) \) chain and some slightly truncated \( \alpha_1(I) \) chain. For the proband, the band consisted of an incompletely digested \( \alpha_2(I) \) chain. The intensity of this band

Reading Frame Resulting in the Use of a Premature Termination Codon and a Decreased Stability of the Mutant Transcript—The 100-bp intronic insertion in the \( \alpha_1(V) \) transcript resulted in a \( +1 \) shift of the collagen reading frame. In the new reading frame, exons 14–16 are translated into a noncollagenous protein chain, and there is a premature termination codon (UGA) at the sixth amino acid of exon 17 (Fig. 3C) (25).

We compared the stability of mutant transcript in proband total RNA isolated from fibroblasts with and without cycloheximide treatment (27). The exon 9–28 region was amplified from both RNAs (Fig. 6). In the RT-PCR using RNA from treated cells, the normal and mutant products were approximately equimolar, whereas in the amplification from untreated cells, the mutant product was ~7-fold less abundant than the normal product. The decreased stability of the mutant transcript was presumably due to nonsense-mediated decay.

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was variable from preparation to preparation. It had no apparent significance for type I/type V collagen interactions in EDS.

The composition of the type V collagen secreted by the proband fibroblasts was not altered by the haploinsufficiency of $\alpha_{1(V)}$ chain. The type V collagen appeared to have an $\alpha_{1(V)}\alpha_{2(V)}$ composition in the steady-state sample (Fig. 8). In addition, a sample of secreted collagen was obtained from ascorbate-stimulated proband fibroblasts and subjected to serial selective salt precipitation (Fig. 9). All of the type V collagen appeared in the 1.2 M NaCl pellet as expected. This sample also had the expected 2:1 ratio of $\alpha_1(V)$ and $\alpha_2(V)$ chains.

**Fibrils**—The collagen fibrils from a dermal punch biopsy of individual IV-5 were examined by scanning electron microscopy (Fig. 10) in comparison to the fibrils of a control matched for age, gender, and race. On the longitudinal section, a predominantly normal fibril size and arrangement were noted with occasional wide fibrils (Fig. 10, panel A). On cross-section, heterogeneity in fibril diameter was noted in the proband sample as were occasional fibrils with a cauliflower configuration (Fig. 10, panels B–D). The diameters of 500 fibrils each from proband and control were measured (Fig. 10, panels B and E). Although the median fibril diameter is about the same for the proband and control, the proband has more fibrils with both a larger and smaller diameter.

**Fig. 7. Quantitation of normal and mutant $\alpha_1(V)$ transcripts by RNase protection.** A, RNase A protection of in vitro transcripts and total fibroblast RNA. To confirm that this antisense probe would protect the predicted fragments with equal efficiency, it was hybridized with synthetic sense transcripts from the same region of normal and mutant cDNA (lanes N and M). Hybridization with total RNA (lanes C and ED) yielded the normal 760-nt product from both proband and control cells. Only proband RNA protection also contains a minor amount of the full probe protection product from mutant transcript (arrow). Lane df, $\phi X$ HaeIII marker; lane P, probe. B, diagram of mutant RNA antisense probe and the expected protection fragments from hybridization with normal and mutant transcripts. Hybridization of probe to normal RNA does not protect the intronic insertion present in mutant probe and yields a 760-nt protected fragment.

**Fig. 8. Collagen synthesis by fibroblasts of proband III-3 (EDS) and control (C) cells.** A, secreted and cell layer procollagen from cultured fibroblasts was digested with pepsin and analyzed by SDS-urea-polyacrylamide gel electrophoresis. B, CNBr digestion of band migrating between $\alpha_1(I)$ and $\alpha_2(I)$ in proband and control samples.
DISCUSSION

This report describes a type V collagen splicing defect in a pedigree with type II of Ehlers-Danlos Syndrome. Because collagen exons encode an exact multiple of the characteristic Gly-X-Y helical amino acid sequence (32), splicing defects that result in the straightforward splicing-out of an exon would leave the reading frame intact and cause the production of a structurally abnormal \( \alpha \) chain. Instead, the \( \text{A}^{-2} \rightarrow \text{G} \) defect we delineated in an \( \alpha_1(\text{V}) \) exon 14 acceptor site resulted in the use of a cryptic acceptor site at -100 nt in intron 13. The cryptic acceptor had the sequence cagt rather than the usual cagg of \( \alpha_1(\text{V}) \) exon 14 and most other fibrillar collagen splice acceptor sites. In addition, there were a handful of alternative cagg sites in the 3'-end of intron 13 in which the position ranged from -57 to -378 nt. Careful examination of the mutant transcript by RT-PCR with an antisense primer at the extreme 3’-end of intron 13 paired with sense primers in exon 9 or 13 revealed no additional alternative splicing products. The exclusive use of the -100 site probably resulted from the presence of a strong adjacent (T/C) run containing 17/19 T or C in the upstream sequence. The cagg site at -64 with 9/14 adjacent T or C nt was the next best candidate and would have been detected in our assay.

No functional \( \alpha_1(\text{V}) \) chain should be produced from this aberrantly spliced transcript with a 100-nt insertion. First, the 100-nt insertion shifts the collagen reading frame +1 and causes the occurrence of a premature stop codon in exon 17. Second, any truncated \( \alpha \) chain translated from the mutant transcript cannot be incorporated into COL V heterotrimer. Trimer incorporation requires the selection and alignment region in the C-terminal extension of \( \alpha \) chains (33). In the \( \alpha_1(\text{V}) \) chain, exon 14 contains the junction between the N-propeptide and the helical region (31). Thus, any truncated transcripts will contain neither \( \alpha \) helical sequences nor a C-propeptide and

![Figure 9](null) Fractionation of proband secreted collagen by sequential salt precipitation. The NaCl concentration at which the pellet was obtained is indicated below in each lane. Samples were electrophoresed on a 5% polyacrylamide-2M urea-SDS gel.

![Figure 10](null) Transmission electron micrographs of type I collagen fibrils from a dermal biopsy of patient IV-5 and matched control sample. A, longitudinal section of proband collagen fibrils. Occasional wide fibrils are indicated by small circles. B–D, cross-sections of proband collagen fibrils. Fibrils with a smaller diameter than normal are indicated by arrowheads, and occasional cauliflower configurations are indicated by arrows. E, cross-section of matched control collagen fibrils. Magnifications: A, 75,000; B, 48,000; C, 75,000; D, 94,500; and E, 48,000. Bar graph shows the distribution of fibril diameters in proband and control samples.
would be degraded intracellularly. Third, only a minimal amount of even the nonfunctional truncated chain can be produced because the mutant transcript is subject to nonsense-mediated decay and is present in the fibroblasts at only 8% the level of normal transcript. These features combine to produce a functionally null allele at the transcriptional level. This is reflected in the quantity of type V collagen secreted by the fibroblasts of the proband. When the gel loading of secreted collagen is adjusted to equal intensities of type I collagen (measured as a1(I) chain), the pro-2086 2086 2086 b 2086 erate secretes about half as much (65%) type V collagen as does the control. This confirms that the heterotopic fibrils of the proband must be formed with a reduced type V/type I collagen ratio. Selective salt precipitation was done to explore the question of whether the type V trimers secreted in the context of a1(V) haploinsufficiency still has the same ratio of a1(V) and a2(V) chains. There have been reports demonstrating that the chain ratio was a function of the tissue extracted. In articular cartilage, only a1(V) was detected (20). In placenta (30), synovial membrane, and skin (34), the two chains were present in a 1:1 ratio. In bone, the trimers was composed of a 1.1:1 ratio of a1(V), a2(V) and the cartilage a1(XI) chain (7). Furthermore, the recently described a3(V) chain is transcribed in fibroblasts (35). Thus, haploinsufficiency of a1(V) might alter the molecular configuration of the type V trimers. In the collagen secreted by our proband, all the type V collagen forms were precipitated at the expected 1.2 M NaCl, and the a1(V) and a2(V) chains were present in a 2:1 ratio. This is strongly suggestive that trimer composition is unchanged, although full proof will await tissue extractions from an animal model. Because the type V collagen in our proband is secreted in a reduced amount and with the usual fibroblast α chain composition, the consequences of this mutant for matrix and for phenotype must result from COL V haploinsufficiency rather than a dominant negative effect.

Haploinsufficiency is apparently a general mechanism for a sizable proportion of EDS I and II cases, of which this is the first case reported in molecular, biochemical, and ultrastructural detail. Schwarz et al. (22) examined a1(V) RNA from 16 cases of EDS I or II. In seven cases, they were able to identify either complex splicing abnormalities or small insertions or deletions leading to premature stop codons. Wenstrup et al. (21) reported the screening of 53 EDS patients for loss of polymorphism heterozygosity. Twenty-seven patients were heterozygous for at least one restriction fragment length polymorphism, and eight of them had only one allele expressed in cDNA. The study of these two sets of patients suggests that at least one-third of EDS I or II patients have mutations in a1(V), which results in functional haploinsufficiency. Furthermore, most of these mutations apparently result in substantial degradation of mutant transcript by nonsense-mediated decay.

Nonsense-mediated decay is one type of mRNA surveillance during which transcripts with premature termination codons are degraded by a mechanism involving rapid decapping while still fully adenylated (36). Premature termination codons are known to have a greater effect on mRNA decay if located early in the transcript (37), and the termination codon in this pedigree fills that expectation. However, other elements must also be involved, because most of the other cases of EDS with haploinsufficiency are located relatively 3' in the transcript. The sequence context of the termination codon is also known to be crucial. Downstream cis-acting elements promote mRNA decay. Specifically, the presence of one or more ATG codons enhances destabilization, possibly by promoting translational re-initiation (38). ATG codons are located 14 and 135 nt downstream of the premature stop codon in the mutant transcript described here.

Comparison of this null mutation with COL V structural defects provides new insight on genotype/phenotype relationships of COL V mutations. Seven structural abnormalities of a1(V) and three structural abnormalities of a2(V) have been reported previously (14–20). Six abnormalities are single exonsplicing defects, and the others are amino acid substitutions or more complex insertions and a chromosomal translocation. The phenotype of these patients with COL V structural abnormalities covers the range of classical EDS I and/or II. The family reported here has mild to moderate EDS II. The cases in the screening series reported by Schwarze et al. (22) and Wenstrup et al. (21) apparently cover the EDS gravis to mitis range. Thus, type V haploinsufficiency appears to have a different general effect on phenotype than does a null allele of type I collagen. For type I collagen, null alleles of a1(I) are responsible for the mildest form of osteogenesis imperfecta (type I), whereas structural defects of type I collagen cause the more severe to lethal types II, III, and IV of osteogenesis imperfecta. In EDS, the consequences of haploinsufficiency appear to be similar to those of dominant negative structural defects.

This distinction between the genotype/phenotype relationships of types I and V collagen may reside in their different roles in heterotypic fibrils. For type I collagen null alleles, a diminished quantity of fibrils does not have a major impact on skeletal tissue and causes mild osteogenesis imperfecta. In contrast, because of the regulatory role of type V collagen in fibril formation, both quantitative and structural defects of type V collagen cause abnormalities of fibril diameter, which are crucial to the integrity of the skin and tendon. The four cases of EDS with COL V structural defects for which transcription electron microscopy of fibrils has been published include splicing defects of a2(V) exon 27, a1(V) exon 33, a1(V) exon 49, and an a1(V) C1181S substitution (16–19). All show the same irregularity of fibril shape and variation in diameter. It is interesting that this is also the fibril pattern seen in this report in which the COL 5 defect causes haploinsufficiency, because a generalized increase in fibril diameter might have been predicted. This finding suggests that the regulation of heterotypic fibril formation in vivo is not simply based on the ratio of types V and I collagen. Instead, there is apparently a difference in the distribution of type V collagen among fibrils with most fibrils being rather normal in size and shape. In view of the prevalence of null mutations in COL 5 and these intriguing fibril patterns, a murine model for COL 5 haploinsufficiency could provide important insight into the mechanisms of heteropolymerization.

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COL5A1 Exon 14 Splice Acceptor Mutation Causes a Functional Null Allele, Haploinsufficiency of \(\alpha 1(V)\) and Abnormal Heterotypic Interstitial Fibrils in Ehlers-Danlos Syndrome II

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