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A Mutation in the Proα2(I) Gene (COL1A2) for Type I Procollagen in Ehlers-Danlos Syndrome Type VII: Evidence Suggesting That Skipping of Exon 6 in RNA Splicing May Be a Common Cause of the Phenotype

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Summary

Fibroblasts from a proband with Ehlers-Danlos syndrome type VII synthesized approximately equal amounts of normal and shortened proα2(I) chains of type I procollagen. Nuclease S1 probe protection experiments with mRNA demonstrated that the proα2(I) chains were shortened because of a deletion of most or all of the 54 nucleotides in exon 6, the exon that contains codons for the cleavage site for procollagen N-proteinase. Sequencing of genomic clones revealed a single-base mutation that converted the first nucleotide of intron 6 from G to A. Therefore, the mutation was a change, in the -GT- consensus splice site, that produced efficient exon skipping. Allele-specific oligonucleotide hybridizations demonstrated that the proband's mother, father, and brother did not have the mutation. Therefore, the mutation was a sporadic one. Analysis of potential 5′ splice sites in the 5′ end of intron 6 indicated that none had favorable values by the two commonly employed techniques for evaluating such sites. The proband is the fourth reported proband with Ehlers-Danlos syndrome VII with a single-base mutation that causes skipping of exon 6 in the splicing of RNA from either the COL1A1 gene or COL1A2 gene. No other mutations in the two type I procollagen genes have been found in the syndrome. Therefore, such mutations may be a common cause of the phenotype. The primers developed should be useful in screening for the same or similar mutations causing the disease.

Introduction

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of heritable disorders of connective tissue that are characterized by varying degrees of joint hypermobility, joint dislocations, skin hyperextensibility, easy bruisability, abnormal scarring, and other manifestations (Beighton 1970; McKusick 1972, 1983, pp. 151–153, 695–697, 1009; Byers 1983; Shamban and Uitto 1989). More than 11 types of EDS have been defined by the clinical, biochemical, and genetic changes (McKusick 1983, pp. 151–153, 695–697, 1009). EDS type VII, originally known as the arthrocalasis multiplex form, produces extreme joint laxity, multiple joint dislocations, skin hyperextensibility, abnormal scarring, and easy bruisability (Byers 1983; McKusick 1983, pp. 151–153, 695–697, 1009). In all the probands investigated to date, there was a deficiency in processing the N-propeptide of type I procollagen (Lichtenstein et al. 1973; Minor et al. 1986; Bateman and Golub 1990). Three subtypes of EDS VII were defined on the basis of the molecular defects (Beighton et al. 1988): subtype A, mutations in the COL1A1 gene that change the primary structure of

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the proα1(I) chain so as to make the N-propeptide resistant to cleavage by procollagen N-proteinase (Cole et al. 1986; Weil et al. 1989*a*); subtype B, mutations similar to those described for subtype A but in the COL1A2 gene (Wirtz et al. 1987; Weil et al. 1988, 1989*b*); and subtype C, mutations that decrease processing of the N-propeptide possibly because of a deficiency in procollagen N-proteinase (Lichtenstein et al. 1973; Minor et al. 1986). Gene analysis in one proband with EDS type VIIA (Weil et al. 1989*a*) and in two probands with type VIIB (Weil et al. 1988, 1989*b*) demonstrated three single-base mutations, at the 5′ end of intron 6 or at the 3′ end of exon 6, that caused exon skipping during processing of RNA from either the COL1A1 or COL1A2 gene. The exon skipping produced in-frame deletions of either the 24 amino acids in the proα1(I) chain or the 18 amino acids in the proα2(I) chain that included the site at which procollagen N-proteinase cleaves the chain (Chu et al. 1984; Dickson et al. 1985; de Wet et al. 1987; D'Alessio et al. 1988; Kuivaniemi et al. 1988*b*; Tromp et al. 1988). Here we define a mutation in a proband with EDS VII whose fibroblasts were previously shown to synthesize shortened proα2(I) chains (Minor et al. 1986).

Material and Methods

The Proband and Her Family

The proband (fig. 1) was a female with easy bruisability and recurrent dislocations of shoulder and knee joints. She was followed by one of us (R.W.) while she was between the ages of 10 and 16 years. The past medical history revealed that at birth her head circumference was 36 cm, chest circumference 30.5 cm, length 48 cm, and weight 2,270 g. She had a broad biparietal diameter. Other findings at birth included broad forehead, flat nose, depressed nasal bridge, slightly low-set ears, micrognathia, long cylindrical fingers, bilateral club feet, and generalized hypotonia together with dislocations of hips, knees, ankles, elbows, shoulders, wrists, and fingers (fig. 1, *top*). There was no evidence of cleft palate, micromelia, or heart murmur. Shortly after birth she was diagnosed as having Larsen syndrome.

In her first year she was treated with a plaster spica for both her hips and knees. She required open reduction of both hips as well as a left quadricepsplasty. She was also noted to have an equino valgus deformity and loose ligaments. In her second year she had recurrent hip dislocations which were treated with abduction casting. At the age of 5 years she required braces for the extreme laxity of ligaments in her ankles, and

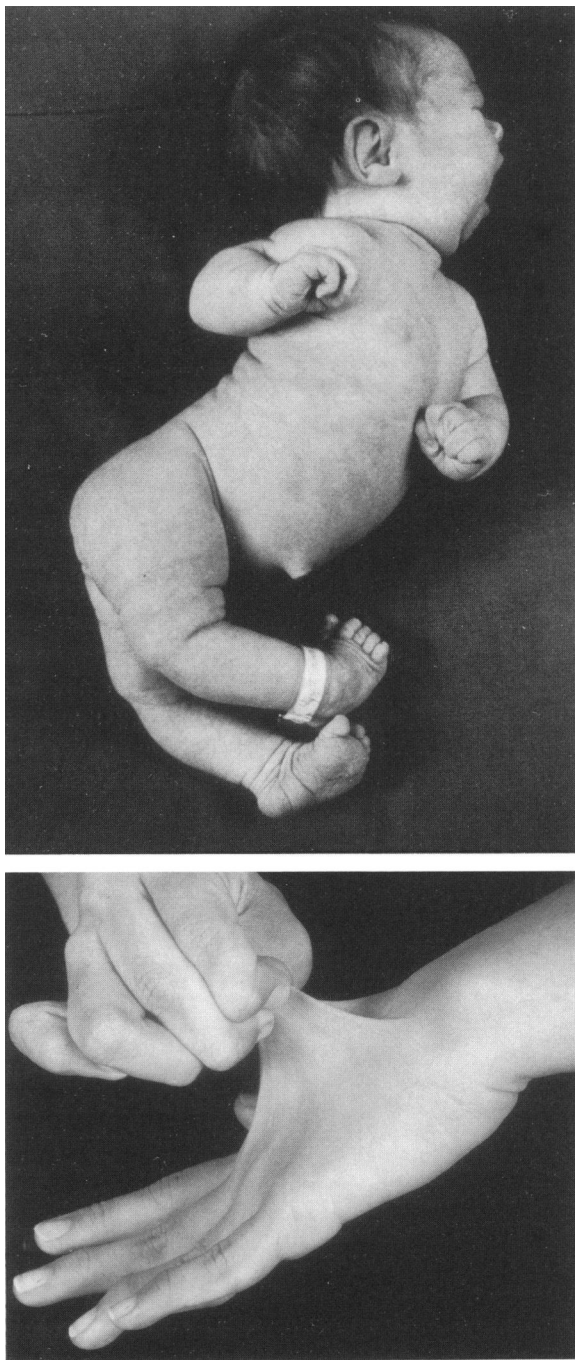

Figure 1 Clinical features of proband. *Top,* Multiple joint dislocations evident at birth. Note the anterior (forward) dislocation of the knee joint. *Bottom,* hyperelasticity of the skin at the age of 10 years.

she had a right quadricepsplasty and medical plication. At the age of 9 years she required a patellectomy on the right, with imbrication of the right-quadriceps mechanism. She also had subluxation of the first metatarsal-phalangeal joints. She was placed in a Boston bucket for scoliosis. She continued to have intermittent lateral dislocations of her left knee. Her first metatarsal joints showed severe hallux valgus deformity. At the age of 13 years she required a posterior spinal fusion from T4-5 to L3-4, with suprastability instrumentation. At the age of 15 years she developed paraesthesiae in both lower limbs, with occasional calf pain. At the age of 16 years she still had marked joint hypermobility and was able to voluntarily dislocate her right kneecap, shoulders, and hips. She was 4 feet 10 inches tall. Her head circumference was 56 cm, and she weighed 110 lbs. Her intellectual development was normal.

The proband also had a history of bruising easily, with unusual swelling of the bruised area. Her skin was soft and velvety and showed marked elasticity (fig. 1, *bottom*). She experienced some skin splitting with minor injury and developed cigarette-paper scars after injury. Because of her skin involvement, the diagnosis of Larsen syndrome was changed to EDS VII. The parents were phenotypically normal, and the mother had an unaffected son from a second marriage.

Analysis of Labeled Medium and Cell-Layer Proteins

Skin fibroblasts from the proband and from three control cell lines were cultured under standard conditions (Minor et al. 1986). The fibroblast cultures were incubated with [^{3}H]proline for 4 h. The medium proteins were precipitated with ammonium sulfate, and they were dissolved in 0.2 M NaCl and 1 mM EDTA in 50 mM Tris-HCl buffer (pH 7.4). The sample was digested with pepsin (Boehringer-Mannheim) in a 0.5 M acetic acid (pH 2.6) at 4°C for 6 h. The sample was then assayed by PAGE in SDS without reduction, followed by fluorography. Cell-layer proteins were digested with pepsin, as described previously except that 200 μg of pepsin/ml was used (Steinmann et al. 1980). Digestion was stopped with the addition of a 20-fold molar excess of Pepstatin (Sigma), and the collagens were precipitated with the addition of 5 M NaCl in 0.5 M acetic acid to give a final concentration of 2 M NaCl. Precipitation was continued overnight at 4°C with slow stirring, and samples were centrifuged at 40,000 *g* for 1 h at 4°C. The pellets were washed twice with ice-cold 75% ethanol and were lyophilized and stored at −70°C.

For the analysis by reverse-phase high-performance liquid chromatography (HPLC), pepsin-digested medium and cell-layer proteins were used. Lyophilized pellets were extracted with 500–700 μl of 0.01 M trifluoroacetic acid (TFA; Sigma) for 3 h at 4°C and were centrifuged at 40,000 *g* for 20 min at 4°C. The supernatant was filtered with a 0.2-μm Spartan-3 nylon filter (Schleicher and Schuell) and was heated for 10 min at 60°C prior to being injected into the column. Reverse-phase chromatography was performed on a BioRad Model 700 titanium system, using a 10-cm μBondapak C_{18} cartridge (Waters) in an RCM-100 radial compression module (Waters) that was maintained at 40°C. Separation of collagen chains was achieved in 30 min with a gradient of 18.2%–33.6% acetonitrile in 0.01 M TFA with a flow rate of 1 ml/min. Absorbance was monitored at 206 nm with an LKB Uvicord S column monitor with an 8-μl flow cell. Radioactivity in the column effluent was continuously monitored with a Radiomatic model IC/DS Flo-One monitor controlled by a Compaq portable computer. For detection of ^{3}H, a 1/10th split of the flow stream was mixed with 500 μl of liquid scintillant/min (Liquiscint, National Diagnostics), and detection was in a 500-μl cell. The remaining 9/10ths split of the flow stream was collected in microfuge tubes, in 1-min fractions.

Lyophilized aliquots of peak fractions from the reverse-phase chromatography were dissolved in 10–20 μl of 1 × gel sample buffer (Laemmli 1970; Minor et al. 1986), with or without 2% β-mercaptoethanol. The samples were run on a 6% acrylamide separating gel in a Mini-Protean II apparatus (BioRad). Fluorography was performed according to a method described elsewhere (Minor et al. 1986).

S1 Nuclease Probe Protection

The S1 nuclease probe protection experiments were carried out using three different single-stranded DNA probes. The 856-nucleotide (nt) and 900-nt probes were prepared according to a method described elsewhere (Kuivaniemi et al. 1988*a*). The third single-stranded DNA probe, of about 470 nt, was obtained by digesting a full-length cDNA clone (Kuivaniemi et al. 1988*b*) with *Eco*RI and *Pst*I. The *Eco*RI/*Pst*I fragment extended from bp 53 to bp 522 of the cDNA clone and, therefore, contained 83 bp of 5′ untranslated region and coding sequences that began with the start of translation and ended with the ninth nucleotide of exon 9. The fragment was subcloned into *Eco*RI/*Pst*I-digested M13mp18. Antisense DNA

probes with the 956-, 900-, and 470-bp inserts in M13 were prepared according to a method described elsewhere (Kuivaniemi et al. 1988*a*).

To isolate RNA, fibroblasts were cultured according to a method described elsewhere (Minor et al. 1986). RNA was extracted with guanidinium isothiocyanate and was pelleted by centrifugation through cesium chloride (Maniatis et al. 1982). Poly(A)$^+$ RNA was isolated from the total RNA by chromatography on a column of oligo-dT cellulose. To form the RNA-DNA hybrids, about 50,000 cpm (about 0.5 femtomoles) of the probe were hybridized to 1 μg of poly(A)$^+$ RNA in 80% formamide, 0.25 mM EDTA, and 300 mM NaCl in 25 mM Hepes (pH 7.0) at 56°C for 3 h. The sample was then digested for 30 min with 1–4 units of S1 nuclease (Seikagaku) at 40°C in 300 μl of 50 mM NaCl and 1 mM of $ZnSO_4$ in 30 mM NaOAc buffer (pH 5.3). The products were precipitated with ethanol and were separated on a 5% DNA-sequencing gel (Sanger et al. 1977). The size of the fragments was estimated by comparison with 5′ end–labeled fragments from an *Hae*III digest of ΦX174 DNA and a 123-bp DNA ladder (BRL).

Isolation and Sequencing of Genomic DNA

To isolate the proband's genomic DNA, skin fibroblasts were cultured under standard conditions (Minor et al. 1986), and the DNA from six 175-cm^2 flasks was extracted by a procedure involving treatment with SDS and digestion with proteinase K (Maniatis et al. 1982). The DNA was digested with *Eco*RI, and a small aliquot of it was used for a Southern blot experiment (Maniatis et al. 1982) to establish that the proband had the *Eco*RI polymorphism in her proα2(I) collagen gene (Myers et al. 1983; Tsipouras et al. 1983; Borresen et al. 1985). Results of the Southern blot experiments on total genomic DNA indicated that the proband's DNA generated two *Eco*RI fragments, one each of 10 and 14 kb, for the region of interest. The remainder of the DNA digested with *Eco*RI was used for sucrose density gradient (10%–40% w/v) (Maniatis et al. 1982), and fragments of about 10 and 14 kb were cloned into the bacteriophage vector lambda-DASH (Stratagene). The fragments were ligated in a 1:2 ratio of insert fragments to vector and were packaged with a commercial packaging extract for lambda bacteriophage (Promega). About 7×10^6 individual clones were generated. About 2.7×10^6 clones were screened with a 2.0-kb *Hin*dIII/*Hin*dIII fragment from NJ-3, a genomic clone for the proα2(I) gene (Myers et al. 1983). DNA from 17 positive clones was purified and used for *Bam*HI and *Hin*dIII digestion. The digests were run on agarose gels and were blotted on nitrocellulose (Maniatis et al. 1982). Because the size of the fragments was not totally consistent with the previously published restriction map of the gene (de Wet et al. 1987), Southern blotting experiments were performed to identify the fragment containing exon 6. The probe was an exon 6–specific primer (Kuivaniemi et al. 1988*b*). A *Hin*dIII/*Hin*dIII fragment of 1.35 kb was shown to contain exon 6 sequences. The 1.35-kb *Hin*dIII/*Hin*dIII fragment from 10 positive clones was subcloned into M13mp19 for nucleotide sequencing (Sanger et al. 1977). The subclones were sequenced using a universal primer, primers specific for sequences in the exon 6 (Kuivaniemi et al. 1988*b*), and then with primers specific for selected regions of the introns.

PCR using Genomic DNA as a Template

All genomic clones in lambda-DASH vector were derived from the allele that gave a 14-kb fragment after *Eco*RI digestion, and all the 10 clones sequenced were normal. Therefore, the PCR (Saiki et al. 1985) was carried out on genomic DNA in order to obtain sequences from the other allele. Two oligonucleotide primers were designed, one for intron 5 (5′ CGCGGATCCTGGGAGTGGAGACACCGAGTT 3′) and one for intron 6 (5′ CGGCGAATTCCCTAAGTATTGAGTGTTAACTTA 3′), according to the sequences obtained from the normal allele. The primers were used for the PCR under the following conditions: denaturation at 94°C for 1.5 min, annealing at 64°C for 1 min, and extension at 74°C for 1 min, for a total of 40 cycles. The PCR products were analyzed on 2% agarose gel, were digested with *Eco*RI and *Bam*HI, were cloned into *Eco*RI/*Bam*HI-digested M13mp18 and mp19, and were sequenced.

PCR Using cDNA as a Template

RNA was isolated from cultured skin fibroblasts (Maniatis et al. 1982), and first-strand cDNA synthesis was done according to the method of Gubler and Hoffman (1983), after which the RNA template was hydrolyzed by alkaline treatment. The sample was then extracted with phenol:chloroform and was applied to an S-500 spun column. A 1/40 volume of the cDNA was used as a template in PCR (Saiki et al. 1985). The 5′ primer (5′ CGGCGGATCCTTATGGCTAGCAACATGC 3′) in the amplification reaction was identical to sequences in exon 1, and the 3′ primer (5′ CGCGAATTCGGGCCTCTAGGTCCCATTAAG

3′) was complementary to sequences in exon 7 in proα2(I) collagen cDNA (Kuivaniemi et al. 1988*b*). The PCR conditions were 94°C for 1.5 min, 54°C for 1 min, and 74°C for 1 min, for a total of 40 cycles. To analyze the PCR products, a 4% NuSieve agarose gel (FMC BioProducts) in 1 × TBE buffer was used.

The cDNA was also used as a template in a PCR in which one of the primers was radioactively end-labeled by polynucleotide kinase and ^{32}P-ATP. After 20 cycles the PCR products were precipitated and run on 6% DNA sequencing gel. End-labeled ΦX174 *Hae*III fragments were used as markers (New England Biolabs).

Analysis of Family Members for the Mutation

To determine the presence or absence of the mutation in members of the family, the PCR was performed on genomic DNA isolated from cultured skin fibroblasts of the proband's mother, father, and half-brother. The PCR products were analyzed by slot-blot hybridization (Studencki and Wallace 1984; Kontusaari et al. 1990). Each of the duplicate filters was hybridized with ^{32}P-labeled 19-mer oligonucleotides corresponding to either the normal allele (5′ ATAAG-CATACCATTGGTCC 3′) or the mutant allele (5′ GGACCAATGATATGCTTAT 3′). The filters were washed first with 2 × SSC for 30 min at room temperature and then with 5 × SSC for 10 min either at 55°C (for the normal oligonucleotide) or at 56°C (for the mutant oligonucleotide). The calculated theoretical melting temperatures (Meinkoth and Wahl 1984) were 60°C and 62°C, respectively.

All the slot-blot experiments with the DNA isolated from cultured skin fibroblasts from the different family members were repeated twice, using two or three different DNA isolations and DNA from three different PCRs. Water blanks were consistently negative.

Results

Characterization of the Mutation at the Protein Level

Previous studies had indicated that the processing of procollagen to collagen was very slow in cultured skin fibroblasts from the proband (Minor et al. 1986). It also had been noted that in polyacrylamide gels the proα2(I) chains synthesized by cells from the proband migrated slightly faster than control proα2(I) chains (Minor et al. 1986). The increased migration was observed when the procollagen chains were hydroxylated, as well as when the cells were labeled in the presence of the iron chelator and hydroxylation inhibitor α,α′-dipyridyl. The results suggested, therefore, that the proband's procollagen contained a shortened proα2(I) chain (Minor et al. 1986).

To investigate the mutation further, the proband's fibroblasts were incubated with [^{3}H]proline for 4 h, the medium and cell layer proteins were digested with pepsin, and the pepsin-resistant type I collagen chains were examined by PAGE (fig. 2). The results indicated that there was an additional band migrating between the normal α1(I) and α2(I) collagen chains. The ratio of the normal α1(I) collagen chain to the normal α2(I) collagen chain in the proband's cell layer and medium samples was about 4:1, instead of the normal 2:1 ratio. To identify the additional band seen on the gel, the pepsin-digested medium proteins from control and the proband's fibroblasts were separated by reverse-phase HPLC. As shown in figure 3*A*, the α1(I) chains of type I collagen were eluted from the column in fractions 4–9, and α2(I) chains were eluted in fractions 10–13 from the control cell line. Analysis of the proband's protein demonstrated (fig. 3*B*) that an additional chain was eluted from the reverse-phase HPLC together with the normal α2(I) chains. Cyanogen bromide peptide maps and amino acid analysis on α1(I) and α2(I) chains (not shown) confirmed that the additional chain was indeed derived from an α2(I) chain. Therefore, this chain was named "α2(I)′" (fig. 3*B*).

Characterization of the Mutation by S1 Nuclease Probe Protection Experiments.

To locate the mutation at the mRNA level, three overlapping cDNA clones were used in S1 nuclease

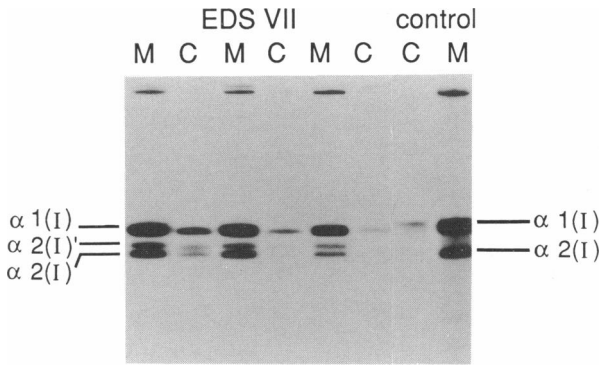

Figure 2 SDS-PAGE of labeled proteins from pepsin-digested medium (M) and cell layer (C). An additional peptide band is evident between the α1(I) and α2(I) bands of both cell layer and medium of the proband's fibroblasts. The results of three different labelings of the proband's cells are shown.

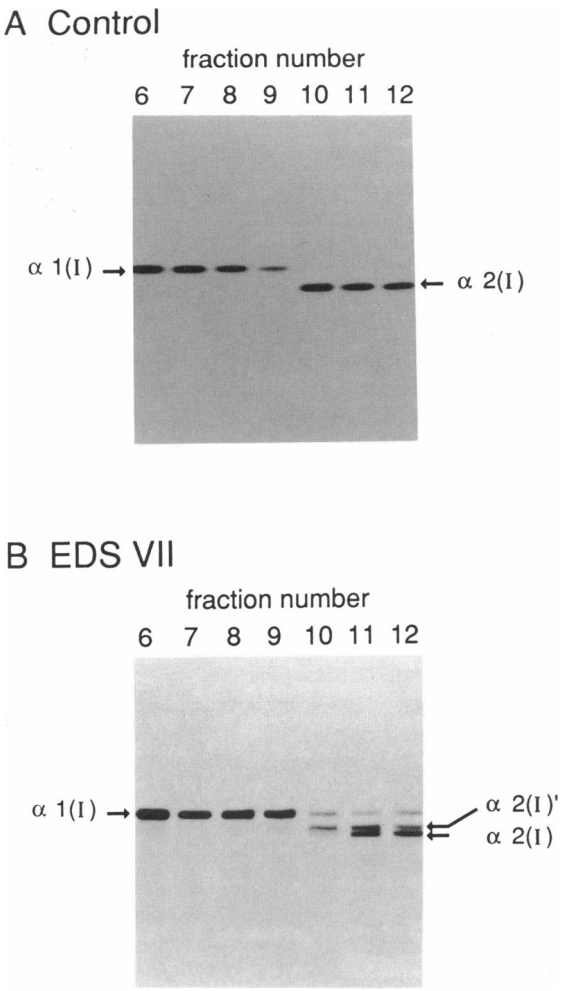

Figure 3 SDS-PAGE of pepsin-digested medium proteins from cultures of control (*A*) and of proband's fibroblast (*B*) that were separated by reverse-phase chromatography. In *B*, note the additional peptide band moving slightly slower than the normal α2(I) band. The identification of α-chains was confirmed with cyanogen bromide peptide maps and amino acid analysis.

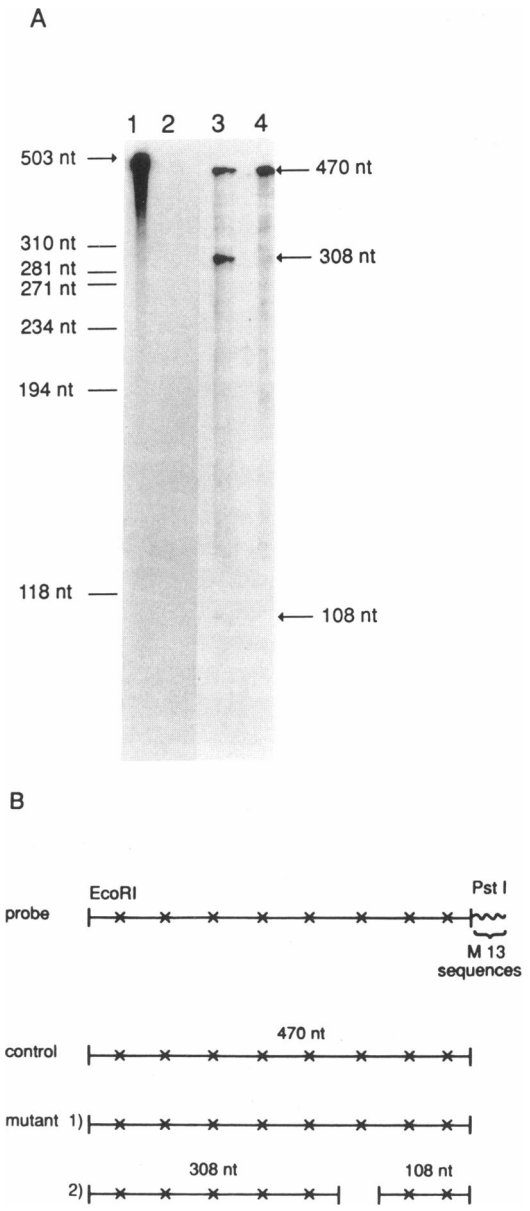

Figure 4 S1 nuclease probe protection experiment using single-stranded cDNA probe extending from exon 1 to exon 9. *A*, Radiograph of electrophoretic gel. Lane 1, Probe without S1 nuclease digestion. Lane 2, S1 nuclease digestion of probe without RNA. Lane 3, RNA from proband's fibroblasts; digestion with 2 units of S1 nuclease. Lane 4, Control RNA; digestion with 2 units of S1 nuclease. *B*, Schematic presentation of probe used for experiments, and sizes of protected fragments expected with control RNA and with RNA from proband.

probe protection experiments. The probes covered nt 53 to nt 1327 and extended from exon 1 to exon 21 of the proα2(I) collagen cDNA. They therefore included codons for amino acids from the beginning of the translation to amino acid 307 of the triple-helical domain (Kuivaniemi et al. 1988*b*). The 956-nt probe gave two protected fragments with the proband's RNA: a fully protected 956-nt fragment and fragment of about 900 nt (not shown). The 900-nt probe gave three protected fragments, one each of 900, 700, and 150 nt (not shown). The third 470-nt probe gave three protected fragments, one each of 470, 308, and 108 nt (fig. 4). The S1 nuclease probe protection experiments with the three different overlapping probes therefore indicated that the proband's mRNA had two species: normal size proα2(I) collagen mRNA and proα2(I)

collagen mRNA that was shortened because most or all of the codons of exon 6 were missing.

Sequencing of Genomic DNA

Since the S1 probe protection experiments indicated that the mutation in the proband's type I procollagen gene involved exon 6 of the proα2(I) gene and because of a lack of sequence information around exon 6, the structure of the normal human proα2(I) gene in the region around exon 6 was defined here at this point. The proband's DNA was digested with *Eco*RI and was cloned into lambda-DASH. Seventeen clones were isolated using as a probe a 2-kb *Hin*dIII/*Hin*dIII fragment from the 5′ end of the gene (Myers et al. 1983). The lambda insert was digested with *Hin*dIII and was analyzed by Southern blotting with an exon 6–specific oligonucleotide used as a probe. A 1.35-kb *Hin*dIII/*Hin*dIII fragment that hybridized with the probe was subcloned into M13. The fragments from 10 lambda-DASH clones were partially sequenced. The top panel of figure 5 shows 730 nt of the sequence: 318 nt from the 3′ end of intron 5, the 54 nt of exon 6, and 358 nt from the 5′ end of intron 6. The 3′ and 5′ splice sites were 79% (11/14) and 63% (5/8) identical with the consensus sequences (Shapiro and Senapathy 1987; Jacob and Gallinaro 1989). The sequence for exon 6 was identical to that previously published for the human proα2(I) collagen cDNA sequence in exon 6 (Kuivaniemi et al. 1988*b*). However, all 10 clones sequenced were from the allele containing the 14-kb *Eco*RI fragment.

In order to obtain the sequences around exon 6 in the proα2(I) collagen gene from the other allele of the proband, a PCR was carried out using two primers based on the sequences described above. The PCR product was cloned into M13mp18 and mp19 and was sequenced. Seven clones had the normal sequence, and three clones had a single-base mutation that converted the first nucleotide in intron 6 from G to A (fig. 5, *bottom*).

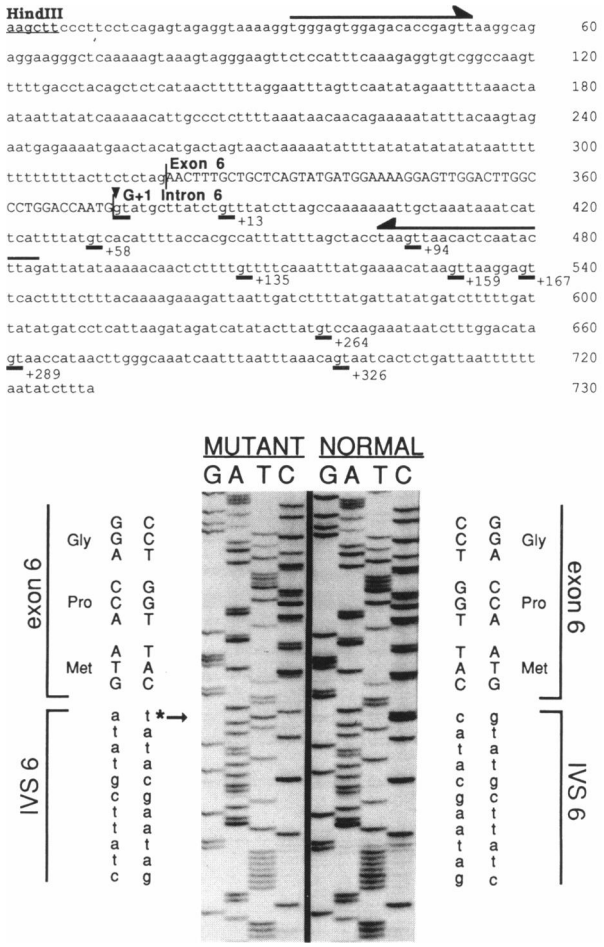

Figure 5 *Top,* Nucleotide sequence of 730 bp of normal proα2(I) collagen gene extending from last 318 bp of intervening sequence 5 to first 358 bp of intervening sequence 6. Horizontal arrows denote primers used to amplify genomic DNA to obtain sequences from both alleles; vertical arrowhead denotes site of mutation (see bottom panel); *Hin*dIII denotes cleavage site for restriction endonuclease *Hin*dIII (5′ cloning site). *Bottom,* Nucleotide sequence of genomic DNA clones of normal and mutant allele from proband. The sequences are in the antisense orientation. The arrow and asterisk indicate the G-to-A mutation of the first nucleotide of intervening sequence 6.

Demonstrating That Most of the mRNA from the Mutated Allele Undergoes Exon Skipping

To define the extent of exon skipping caused by the mutation, two PCR experiments were carried out. In the first experiment, primers spanning from exon 1 to exon 7 were used for the PCR. As indicated in the top panel of figure 6, cDNA from normal fibroblasts gave a single band of the expected 274-bp size. In contrast, cDNA from the proband gave two bands, a normal band of 274 bp and a shortened band of 220 bp. The two bands were equal in intensity. Also, no other bands were seen. Therefore, the results were consistent with the conclusion that one allele was normally spliced and that the other allele underwent efficient exon skipping.

Because the experiment in the top panel of figure 6 may have failed to detect minor RNA splicing products, additional PCRs were carried out using labeled primers. As indicated in the bottom panel of figure 6, the PCR using normal cDNA as template gave the expected

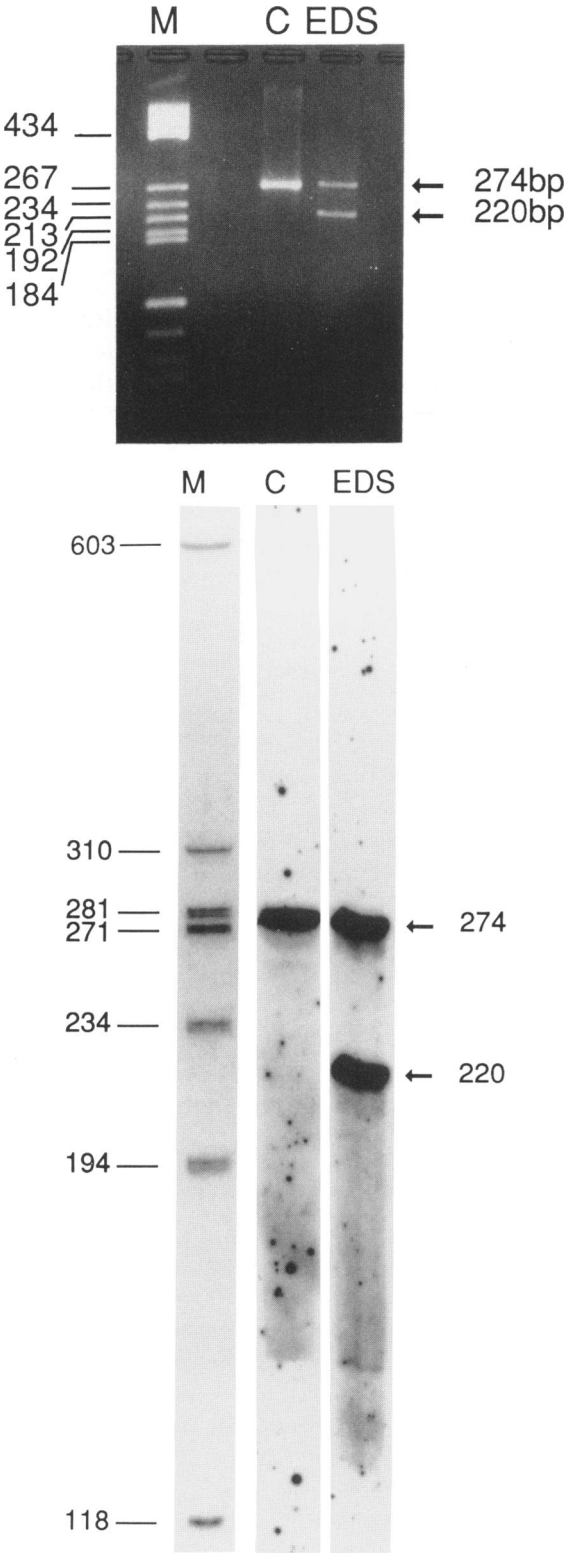

Figure 6 Analysis of PCR products synthesized with cDNA as template. *Top,* Agarose gel and ethidium bromide staining for PCR products using control (C) and proband's (EDS) cDNA as template. The 5′ primer was identical to sequences in exon 1, and the 3′ primer was complementary to sequences in exon 7. *Bottom,* Polyacrylamide gel of radioactively labeled PCR products using control (C) and proband's (EDS) cDNA as a template.

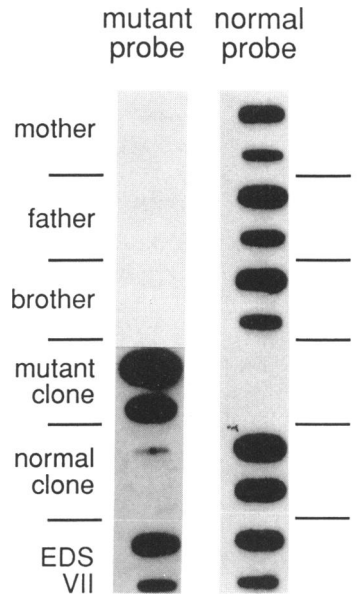

Figure 7 Allele-specific oligonucleotide hybridization of PCR products synthesized using genomic DNA or M13 clones as template.

274-bp band. PCR using template from the proband's cDNA gave two bands, one that was the normal 274 bp and one that was a shortened band of 220 bp. Again, the bands were of equal intensity, and no other bands were seen. The results, therefore, were consistent with the conclusion that the mutation caused highly efficient skipping of exon 6.

Confirmation of the Mutation in the Proband's Genomic DNA and Absence in the Proband's Family

To confirm the presence of the mutation in the proband, the proband's genomic DNA was used as template for the PCR using primers that spanned from intron 5 to intron 6 (see fig. 5, *top*). PCR products were then used for slot-blot hybridization with allele-specific oligonucleotides. As indicated in figure 7, DNA from the proband hybridized both with the normal oligonucleotide and with the mutated oligonucleotide. In contrast, only the normal oligonucleotide hybridized with DNA from the mother, father, and half-brother.

Evaluation of Potential 5′ Splice Sites in Intron 6

Because of the highly efficient exon skipping produced by the mutation, an attempt was made to evaluate potential 5′ splice sites in intron 6. Two methods were employed. One was a modified form (Kuivaniemi et al. 1990) of the statistical evaluation technique described by Shapiro and Senapathy (1987). The other was a calculation of the free-energy change for binding to U1 RNA (Freier et al. 1986). Analysis of the sequences in intron 6 (fig. 5, *top*) indicated that there were nine -GT- sequences that could serve as potential 5′ splice sites. All nine gave unfavorable scores by both analytical techniques (table 1). In the numerical scoring system, eight of the potential 5′ splice sites (nucleotides +13, +58, +94, +135, +159, +167, +264, and +326) had values of 46 or less. The first six of these sites also had unfavorable free-energy changes and values of about −0.2 kcal/mol at 37°C. A site at +289 had a numerical score of 50 and a free-energy-change value of −2.4 kcal/mol. The site at +326 had a similar free-energy-change value, −2.5 kcal/mol. The normally used splice site in the intron had a numerical value of 61 and a free-energy-change value of −4.7 kcal/mol. In contrast, there were in intron 6 of the proα1(I) gene several potential 5′ splice sites that had high values with both methods (table 1).

Discussion

EDS type VII is characterized by extreme laxity of joints that give rise to multiple dislocations (Beighton 1970; McKusick 1972, 1983, pp. 151–153, 695–697, 1009; Byers 1983; Shamban and Uitto 1989). The common feature of all probands studied to date is decreased processing of the N-propeptide of type I procollagen. The decreased processing of the N-propeptide can be caused by either a mutation that changes the primary structure of the proα1(I) chain (subtype A) (Cole et al. 1986; Weil et al. 1989*a*), a mutation that changes the primary structure of the proα2(I) chain (subtype B) (Wirtz et al. 1987; Weil et al. 1988, 1989*b*; present study), or possibly a mutation that decreases the activity of procollagen N-proteinase (subtype C) (Lichtenstein et al. 1973; Minor et al. 1986). Persistence of the N-propeptide on the molecule probably interferes with normal assembly of collagen into fibrils (Hulmes et al. 1989) and probably generates the thin and highly distorted fibrils of the

Table 1

Evaluation of Normal and Potential 5′ Splice Sites in Intron 6 of COL1A2 and in Intron 6 of COL1A1

Site	Numerical Score[a] (%)	ΔG°_{37} value[b] (kcal/mol)
COL1A2		
Normal 5′ site (+1)	61	−4.7
+58	46	0
+94	46	−0.2
+289	50	−2.4
+326	36	−2.5
COL1A1		
Normal 5′ site (+1)	73	−6.8
+43	38	−5.4
+56	69	−5.4
+73	53	−4.7
+91	50	−5.0
+146	68	−6.8
+149	36	−3.3

[a] Values calculated according to the method of Shapiro and Senapathy (1987). All potential 5′ splice sites with numerical scores of greater than 30 are shown. For COL1A2, only the first 358 nt of intron 6 were analyzed.

[b] Values for binding to U1 RNA, calculated with free-energy parameters of Freier et al. (1986). Calculations were carried out without allowance for internal bulges, but G-U mismatches and internal loops by two or three nucleotides were allowed. Values include increments for terminal mismatches.

Table 2

Comparison of Effects of Four Different Single-Base Mutations in Splicing Event in Four EDS VII Patients

	Splice site[a] Sequence	Location	Splicing Pattern	References
Normal	ATG gt	COL1A1 COL1A2		
Proband 1.....	ATG gc	COL1A2	Exon skipping	Wirtz et al. 1987; Weil et al. 1988
Proband 2.....	ATA gt	COL1A1	Exon skipping; low amounts of normal splicing	Cole et al. 1986; Weil et al. 1989*a*
Proband 3.....	ATA gt	COL1A2	Exon skipping, normal splicing at 31°C	Steinmann et al. 1980; Weil et al. 1989*b*
Proband 4.....	ATG at	COL1A2	Exon skipping	Minor et al. 1986; present study

[a] For both the proα1(I) collagen gene and the proα2(I) collagen gene, the last three nucleotides of exon 6 are -ATG- (Kuivaniemi et al. 1988*b*; Tromp et al. 1988). The underlining indicates the mutated base.

kind seen in animal cognates of the same disease (Minor et al. 1987; Uitto and Minor 1987). As reported recently (Wirtz et al. 1990), all three chains of the N-propeptide may remain associated with the collagen molecule after cleavage of two normal chains.

Initial studies of three probands with EDS type VII suggested that deficiencies in procollagen N-proteinase are common in the disease (Lichtenstein et al. 1973). Also, deficiencies of the enzyme together with recessive inheritance of fragile skin were found in cattle (O'Hara et al. 1970; Lenaers et al. 1971) and sheep (Fjolstad and Helle 1974)—and probably in dog (Hegreberg et al. 1970) and cat (Counts et al. 1980). The enzyme, however, is difficult to assay either in tissues or in cultured cells, apparently because it is membrane bound and not readily extracted (Hojima et al. 1989). However, the only probands with EDS type VII in whom the molecular defect was definitively established were three probands with mutations in either the gene for the proα1(I) (Weil et al. 1989*a*) or the gene for the proα2(I) (Weil et al. 1988, 1989*b*) chain of type I procollagen (table 2). The three probands, like the one examined here, had single-base mutations that produced exon skipping. One mutation was in the proα2(I) chain and converted the consensus sequence of -GT- at the 5′ end of intron 6 to -GC- (Weil et al. 1988). The other two were both G-to-A mutations in the last nucleotide of exon 6, one in the proα1(I) chain (Weil et al. 1989*a*) and the other in the proα2(I) chain (Weil et al. 1989*b*). Because the codons of exon 6 are in-frame codons, the reading frame of the mRNA was maintained after exon skipping. One of the mutations (proband 3 in table 2) produced variable RNA splicing, in that normal splicing was observed at 31°C (Weil et al. 1989*b*).

The G-to-A mutation defined here destroyed the -GT- dinucleotide sequence at the 5′ end of intron 6. The -GT- dinucleotide is found at the 5′ end of at least 99.4% of some 3,000 introns that have been analyzed (Shapiro and Senapathy 1987; Jacob and Gallinaro 1989). A series of mutations that change the -GT- dinucleotide sequence at the 5′ end of introns were shown to markedly alter RNA splicing (Mitchell et al. 1986; Weil et al. 1988; Grandchamp et al. 1989; Kuivaniemi et al. 1990). However, not all produced efficient exon skipping (Kuivaniemi et al. 1990). The efficient skipping of exon 6 of the proα2(I) collagen gene, a skipping seen both here and in two other probands with EDS type VII, may well be explained by the absence in intron 6 of any potential 5′ splice sites that have favorable values as judged by the two commonly employed techniques for evaluating cryptic splice sites. Efficient skipping of exon 6 in the proα1(I) collagen gene, however, cannot be explained on this basis (table 2). As discussed elsewhere, the efficiency of exon skipping versus the use of cryptic splice sites may well be dependent on the normal sequence in which introns in a multiexon gene are spliced (Lang and Spritz 1987; Kuivaniemi et al. 1990).

The results from both the proband studied here and the three probands previously reported suggest that single-base mutations that cause skipping of exon 6 may be a common cause of the EDS type VII phenotype. The primers here for analysis of exon 6 in the proα2(I) collagen chain should be useful in screening for the same or similar mutations causing the disease, by using the current technology to detect single-base mutations (see Cotton 1989; Gibbs et al. 1989; Ganguly and Prockop 1990; Higuchi et al. 1990).

Note added in proof: While the present work was in

press, Weil et al. (1990) published a study on an EDS VII patient. The defect found in the patient they described was identical to the defect found in the unrelated patient reported here.

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References

Bateman JF, Golub SB (1990) Assessment of procollagen processing defects by fibroblasts cultured in the presence of dextran sulphate. Biochem J 267:573–577

Beighton P (1970) The Ehlers-Danlos syndrome. William Heinemann, London

Beighton P, de Paepe A, Danks D, Finidori G, Gedde-Dahl T, Goodman R, Hall JG, et al (1988) International nosology of heritable disorders of connective tissue, Berlin, 1986. Am J Med Genet 29:581–594

Borresen A-L, Berg K, Tsipouras P, Dickson LA, Prockop DJ, Ramirez F (1985) DNA polymorphisms in collagen genes: potential use in the study of disease. In: Berg K (ed) Medical genetics: past, present, future. Alan R Liss, New York, pp 37–51

Byers PH (1983) Inherited disorders of collagen biosynthesis: Ehlers-Danlos syndrome, the Marfan syndrome, and osteogenesis imperfecta. In: Spittel JA Jr (ed) Clinical medicine. Harper & Row, Philadelphia, pp 1–41

Chu M-L, de Wet W, Bernard M, Ding J-F, Morabito M, Myers J, Williams C, et al (1984) Human proα1(I) collagen gene structure reveals evolutionary conservation of a pattern of introns and exons. Nature 310:337–340

Cole WG, Chan D, Chambers GW, Walker ID, Bateman JF (1986) Deletion of 24 amino acids from the pro-α1(I) chain of type I procollagen in a patient with the Ehlers-Danlos syndrome type VII. J Biol Chem 261:5496–5503

Cotton RGH (1989) Detection of single base changes in nucleic acids. Biochem J 263:1–10

Counts DF, Byers PH, Holbrook KA, Hegreberg GA (1980) Dermatosparaxis in a Himalayan cat. I. Biochemical studies of dermal collagen. J Invest Dermatol 74:96–99

D'Alessio M, Bernard M, Pretorius PJ, de Wet W, Ramirez F (1988) Complete nucleotide sequence of the region encompassing the first twenty-five exons of the human pro-alpha1(I) collagen gene (COL1A1). Gene 67:105–115

de Wet W, Bernard M, Benson-Chanda V, Chu M-L, Dickson L, Weil D, Ramirez F (1987) Organization of the human pro-α2(I) collagen gene. J Biol Chem 262:16032–16036

Dickson LA, de Wet W, Di Liberto M, Weil D, Ramirez F (1985) Analysis of the promoter region and the N-propeptide domain of the human proα2(I) collagen gene. Nucleic Acids Res 13:3427–3438

Fjolstad M, Helle O (1974) A hereditary dysplasia of collagen tissues in sheep. J Pathol 112:183–188

Freier SM, Kierzek R, Jaeger JA, Sugimoto N, Caruthers MH, Neilson T, Turner DH (1986) Improved free-energy parameters for predictions of RNA duplex stability. Proc Natl Acad Sci USA 83:9373–9377

Ganguly A, Prockop DJ (1990) Detection of single-base mutations by reaction of DNA heteroduplexes with a water-soluble carbodiimide followed by primer extension: application to products from the polymerase chain reaction. Nucleic Acids Res 18:3933–3939

Gibbs RA, Nguyen P-N, McBride LJ, Koepf SM, Caskey CT (1989) Identification of mutations leading to the Lesch-Nyhan syndrome by automated direct DNA sequencing of in vitro amplified cDNA. Proc Natl Acad Sci USA 86:1919–1923

Grandchamp B, Picat C, Mignotte V, Wilson JHP, Te Velde K, Sandkuyl L, Romeo PH, et al (1989) Tissue-specific splicing mutation in acute intermittent porphyria. Proc Natl Acad Sci USA 86:661–664

Gubler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25:263–269

Hegreberg GA, Padgett GA, Henson JB (1970) Connective tissue disease of dogs resembling Ehlers-Danlos syndrome of men. III. Histopathologic changes of skin. Arch Pathol 90:159–166

Higuchi M, Wong C, Kochhan L, Olek K, Aronis S, Kasper CK, Kazazian HH Jr, et al (1990) Characterization of mutations in the factor VIII gene by direct sequencing of amplified genomic DNA. Genomics 6:65–71

Hojima Y, McKenzie JA, van der Rest M, Prockop DJ (1989) Type I procollagen N-proteinase from chick embryo tendons: purification of a new 500-kDa form of the enzyme and identification of the catalytically active polypeptides. J Biol Chem 264:11336–11345

Hulmes DJS, Kadler KE, Mould AP, Hojima Y, Holmes DF, Cummings C, Chapman JA, et al (1989) Pleomorphism in type I collagen fibrils produced by persistence of the procollagen N-propeptide. J Mol Biol 210:337–345

Jacob M, Gallinaro H (1989) The 5′ splice site: phylogenetic evolution and variable geometry of association with U1RNA. Nucleic Acids Res 17:2159–2180

Kontusaari S, Tromp G, Kuivaniemi H, Ladda RL, Prockop DJ (1990) Inheritance of an RNA splicing mutation ($G^{+1\ IVS20}$) in the type III procollagen gene (COL3A1) in a family having aortic aneurysms and easy bruisability: phenotypic overlap between familial arterial aneurysms and Ehlers-Danlos syndrome type IV. Am J Hum Genet 47:112–120

Kuivaniemi H, Kontusaari S, Tromp G, Zhao M, Sabol C, Prockop DJ (1990) Identical G^{+1} to A mutations in three different introns of the type III procollagen gene (COL-

3A1) produce different patterns of RNA splicing in three variants of Ehlers-Danlos syndrome IV: an explanation for exon skipping with some mutations and not others. J Biol Chem 265:12067–12074

Kuivaniemi H, Sabol C, Tromp G, Sippola-Thiele M, Prockop DJ (1988*a*) A 19-base pair deletion in the proα2(I) gene of type I procollagen that causes in-frame RNA splicing from exon 10 to exon 12 in a proband with atypical osteogenesis imperfecta and in his asymptomatic mother. J Biol Chem 263:11407–11413

Kuivaniemi H, Tromp G, Chu M-L, Prockop DJ (1988*b*) Structure of a full-length cDNA clone for the preproα2(I) chain of human type I collagen: comparison with the chicken gene confirms unusual patterns of gene conservation. Biochem J 252:633–640

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lang KM, Spritz RA (1987) In vitro splicing pathways of pre-mRNAs containing multiple intervening sequences. Mol Cell Biol 7:3428–3437

Lenaers A, Ansay M, Nusgens BV, Lapiere CM (1971) Collagen made of extended α-chains, procollagen, in genetically-defective dermatosparaxic calves. Eur J Biochem 23:533–543

Lichtenstein JR, Martin GR, Kohn LD, Byers PH, McKusick VA (1973) Defect in conversion of procollagen to collagen in a form of Ehlers-Danlos syndrome. Science 182:298–300

McKusick VA (1972) The Ehlers-Danlos syndrome. In: Heritable disorders of connective tissue, 4th ed. CV Mosby, St. Louis, pp 292–371

——— (1983) Mendelian inheritance in man: catalogs of autosomal dominant, autosomal recessive, and X-linked phenotypes, 6th ed. The Johns Hopkins University Press, Baltimore

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Meinkoth J, Wahl G (1984) Hybridization of nucleic acids immobilized on solid supports. Anal Biochem 138:267–284

Minor RR, Sippola-Thiele M, McKeon J, Berger J, Prockop DJ (1986) Defects in the processing of procollagen to collagen are demonstrable in cultured fibroblasts from patients with the Ehlers-Danlos and osteogenesis imperfecta syndromes. J Biol Chem 261:10006–10014

Minor RR, Wootton JAM, Patterson DF, Uitto J, Bartel D (1987) Genetic diseases of collagen in animals. In: Uitto J, Perejda AJ (eds) Connective tissue disease: molecular pathology of the extracellular matrix. Marcel Dekker, New York, pp 293–319

Mitchell PJ, Urlaub G, Chasin L (1986) Spontaneous splicing mutations at the dihydrofolate reductase locus in Chinese hamster ovary cells. Mol Cell Biol 6:1926–1935

Myers JC, Dickson LA, de Wet WJ, Bernard MP, Chu M-L, DiLiberto M, Pepe G, et al (1983) Analysis of the 3′ end of the human proα2(I) collagen gene. J Biol Chem 258: 10128–10135

O'Hara PJ, Read WK, Romane WM, Bridges CH (1970) A collagenous tissue dysplasia of calves. Lab Invest 23:307–314

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Shamban A, Uitto J (1989) Hereditary diseases of connective tissue. In: Ruiz-Maldonado R, Parish LC, Beare JM (eds) Textbook of pediatric dermatology. Grune & Stratton, Philadelphia, pp 117–141

Shapiro MB, Senapathy P (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res 15:7155–7174

Steinmann B, Tuderman L, Peltonen L, Martin GR, McKusick VA, Prockop DJ (1980) Evidence for a structural mutation of procollagen type I in a patient with the Ehlers-Danlos syndrome type VII. J Biol Chem 255: 8887–8893

Studencki AB, Wallace RB (1984) Allele-specific hybridization using oligonucleotide probes of very high specific activity: Discrimination of the human β^A- and β^S-globin genes. DNA 3:7–15

Tromp G, Kuivaniemi H, Stacey A, Shikata H, Baldwin CT, Jaenisch R, Prockop DJ (1988) Structure of a full-length cDNA clone for the preproα1(I) chain of human type I procollagen. Biochem J 253:919–922

Tsipouras P, Myers JC, Ramirez F, Prockop DJ (1983) Restriction fragment length polymorphism associated with the proα2(I) gene of human type I procollagen. J Clin Invest 72:1262–1267

Uitto J, Minor RR (1987) Animal models of the Ehlers-Danlos syndrome. In: Maibach HI, Lowe NJ (eds) Models in dermatology, vol 3. S Karger, Basel, pp 148–158

Weil D, Bernard M, Combates N, Wirtz MK, Hollister DW, Steinmann B, Ramirez F (1988) Identification of a mutation that causes exon skipping during collagen pre-mRNA splicing in an Ehlers-Danlos syndrome variant. J Biol Chem 263:8561–8564

Weil D, D'Alessio M, Ramirez F, de Wet W, Cole WG, Chan D, Bateman JF (1989*a*) A base substitution in the exon of a collagen gene causes alternative splicing and generates a structurally abnormal polypeptide in a patient with Ehlers-Danlos syndrome type VII. EMBO J 8:1705–1710

Weil D, D'Alessio M, Ramirez F, Eyre DR (1990) Structural and functional characterization of a splicing mutation in the pro-α2(I) collagen gene of an Ehler-Danlos type VII patient. J Biol Chem 265:16007–16011

Weil D, D'Alessio M, Ramirez F, Steinmann B, Wirtz MK,

Glanville RW, Hollister DW (1989*b*) Temperature-dependent expression of a collagen splicing defect in the fibroblasts of a patient with Ehlers-Danlos syndrome type VII. J Biol Chem 264:16804–16809

Wirtz MK, Glanville RW, Steinmann B, Rao VH, Hollister DW (1987) Ehlers-Danlos syndrome type VIIB: deletion of 18 amino acids comprising the N-telopeptide region of a pro-α2(I) chain. J Biol Chem 262:16376–16385

Wirtz MK, Keene DR, Hori H, Glanville RW, Steinmann B, Rao VH, Hollister DW (1990) In vivo and in vitro noncovalent association of excised α1(I) amino-terminal propeptides with mutant pNα2(I) collagen chains in native mutant collagen in a case of Ehlers-Danlos syndrome, type VII. J Biol Chem 265:6312–6317