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Temperature-dependent Expression of a Collagen Splicing Defect in the Fibroblasts of a Patient with Ehlers-Danlos Syndrome Type VII*

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In this article we report the characterization of the molecular lesion in a patient with Ehlers-Danlos syndrome Type VII and provide evidence that a *de novo* substitution of the last nucleotide of exon 6 in one allele of the pro-α2(I) collagen gene produces normally spliced mRNA and transcripts from which exon 6 sequences have been outspliced as well. Unexpectedly, the expression of the alternative splicing was found to be temperature-dependent, for missplicing *in cellula* is effectively abolished at 31 °C and gradually increases to 100% at 39 °C. In contrast, in a similar patient harboring a substitution in the obligatory GT dinucleotide of the 5′ splice site of intron 6, complete outsplicing of exon 6 sequences was found at all temperatures.

Ehlers-Danlos syndrome (EDS)[1] is a clinically heterogeneous group of inherited disorders of connective tissue characterized by cutaneous hyperextensibility, joint hypermobility, and easy bruisability (1). Variations in these diagnostic manifestations, the presence of distinctive associated features, and differing modes of inheritance serve to distinguish the 10 types of EDS presently recognized (2). Among them is the Type VII form of EDS (EDS VII), which is characterized by extreme joint hypermobility associated with dislocations (usually bilaterally dislocated hips at birth) and minimal skin fragility, in addition to the typical features (1). Early studies of such patients demonstrated partially processed Type I procollagen retaining the N-propeptide (pN-collagen) in directly extracted tissue samples (3). To account for these findings, a deficiency of procollagen N-proteinase was postulated (3) and analogy drawn to a clinically dissimilar, recessively inherited condition in animals (dermatosparaxis, literally "torn skin") in which this enzyme deficiency had been demonstrated (4). Restudy of one of the original patients, however, failed to confirm the enzyme deficiency and suggested heterozygosity for structurally abnormal pro-α2(I) chains, one of the two subunits of Type I procollagen (5). This individual (patient S. N.) is the subject of the present study.

Subsequently, three additional EDS VII patients have been examined in more detail and shown to harbor interstitial, in frame deletions of 24 or 18 amino acid residues in pro-α1(I) or pro-α2(I) chains, respectively (6–9). The deleted peptides correspond precisely to the sixth exon of the Type I procollagen genes, COL1A1 and COL1A2 (10, 11), which encodes the short N-telopeptide region of the procollagen chain (12). Based on these findings, EDS VII is now subclassified into EDS VIIA, structural mutations of pro-α1)I), and EDS VIIB, structural mutations of pro-α2(I) (2).

Published (13, 14) and unpublished data[2] indicate that the protein deletions are the result of distinct mutations that lead to outsplicing of exon 6 sequences during pre-mRNA processing. Exon skipping in an EDS VIIB patient has been shown to be caused by a GT to GC transition at the 5′ splice site of the sixth intron of COL1A2 (13), while alternative splicing in an EDS VIIA patient results from a substitution of A for G in the last nucleotide of exon 6 of COL1A1 (14). The EDS VIIB patient harboring the intron mutation (patient W. A.) is compared with the case discussed in this report.

In the Type I procollagen genes, the loss of exon 6 sequences eliminates two important structural elements; the N-proteinase cleavage site, and the cross-link precursor lysine in the N-telopeptide (12). Loss of the former results in *in vivo* retention of the N-propeptide which is thought to interfere with normal fibrillogenesis, whereas loss of the latter is predicted to diminish the biomechanical integrity of the fibrils (15),[3] The relative role of each of these abnormalities in the pathogenesis of EDS VII is presently unclear. However, the recent finding of an interstitial deletion removing an α2(I)

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05049.

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[1] The abbreviations used are: EDS, Ehlers-Danlos syndrome; CB, designation of cyanogen bromide-derived peptides; COL1A1, pro-α1(I) collagen gene; COL1A2, pro-α2(I) collagen gene; HPLC, high pressure liquid chromatography; N-propeptide, amino-terminal propeptide; N-proteinase, the specific proteinase that cleaves the procollagen N-propeptide; N-telopeptide, the short junction segment that connects the N-propeptide to the triple helical domain; PCR, polymerase chain reaction; pN-collagen, partially processed procollagen retaining the N-propeptide; RFLP, restriction fragment length polymorphism; RP, reverse-phase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[2] F. Ramirez, unpublished data.

[3] M. K. Wirtz and D. W. Hollister, manuscript in preparation.

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helical cross-link precursor in an atypical osteogenesis imperfecta patient with overlapping EDS VII phenotype indirectly suggests that defective cross-linking may be an important feature (16).

In order to extend the characterization of this group of disorders, we have now elucidated the molecular lesion in the EDS VIIB patient believed to be heterozygous for an unidentified structural abnormality of pro-α2(I) collagen (patient S. N.) (5, 8). Like the recently studied EDS VIIA case (14), this individual exhibits a *de novo* single-base substitution in the last codon of exon 6 (A for G) which leads to alternative splicing and consequently to the production of two protein products, one of which harbors an 18-residue deletion and the other a single amino acid substitution. Surprisingly, the expression of the splicing defect is temperature-dependent, for missplicing is effectively suppressed *in cellula* at 31 °C or made essentially complete at 39 °C.

MATERIALS AND METHODS

Purification, Analysis, and Sequencing of Collagen Proteins—Large scale dermal fibroblast culture, procollagen production, conversion of procollagen to collagen by limited pepsin digestion, HPLC purification of native collagen, and fractionation of native normal and mutant Type I collagens by DEAE batch chromatography have been described in detail (9) and the conditions for DEAE chromatography are given in the legend to Fig. 1. Similarly, the purification of normal and mutant α2(I) chains by C_{18} RP-HPLC, cleavage with CNBr, fractionation of the resultant peptides by sieve HPLC, and further resolution of the amino-terminal peptides by C_{18} RP-HPLC have been described (9). For α2(I) chains purified from Pool 1 of the DEAE column (see Fig. 1), this protocol yielded the normal amino-terminal CB-1 peptide plus an abnormal peptide designated CB-1.0′. For normal α2(I) chains isolated from DEAE Pool 2, the CB-1 peptide was absent and replaced by an abnormal amino-terminal peptide, designated CB-X, as previously observed for patient W. A. (9).

To generate sequence information, the purified CB-1.0′ and CB-X peptides were dissolved in 0.2 M Tris-HCl, pH 7.8, containing 1 mM $CaCl_2$ and digested with trypsin (Sigma, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) at an enzyme:substrate ratio of 1:50 overnight at 37 °C. The resultant tryptic peptides were resolved by C_{18} RP-HPLC and the amino acid compositions and sequences determined as described. To determine the carboxyl-terminal sequence of CB-X, 1500 pmol of peptide was dissolved in 50 mM ammonium acetate, pH 6.5, containing 8.3 mM of L-α-amino-*n*-butyric acid as internal standard and 0.1 mg of carboxypeptidase Y (Boehringer Mannheim) was added. Times aliquots were removed after incubation at 37 °C for 0, 0.5, 1, 2, 4, and 8 h, and the liberated amino acids determined.

To examine the temperature-dependence of the splicing defect, confluent fibroblast cultures were labeled with [^{3}H]proline for 20 h at 31, 33, 35, 37, and 39 °C (±0.3 °C), and media and cell layer procollagens harvested separately in the presence of proteolytic inhibitors. Following conversion to collagen by limited pepsin digestion, the labeled proteins were resolved by SDS-5% PAGE containing 0.5 M urea and detected by fluorography using pre-flashed Kodak X-Omat film.

Cloning Experiments and DNA Sequence Analysis—Cloning of pro-α2(I) cDNAs was accomplished by priming the proband's fibroblast poly(A)$^+$ RNA with the synthetic exon 8 oligomer 5′-CAGGTCCTTGGAAACCTTGA-3′. Conditions for the generation of the cDNA library and the phage vector λgt10 were essentially as in the standard protocol (17). Screening of the library was carried out by probing phage plaques in parallel with the exon 8 20-mer and the exon 6 17-mer, 5′-CCATCATACTGAGCAGC-3′, under conditions previously detailed (13). Likewise, the strategy employed for the generation and screening of the *Eco*RI size-fractionated genomic library in the Charon 4 phage vector has been described (13).

For sequence analysis appropriate DNA fragments were subcloned into pUC18 or 19 vectors and both strands were sequenced by the chain terminator method on double-stranded DNA (18). Oligonucleotides were synthesized on an Applied Biosystems Model 380A synthesizer.

DNA Amplification—The oligonucleotides employed for the cDNA amplification were: 5′-TTTACAAGAGGAAACTGTAAGA-3′, which corresponds to the coding sequences encompassing exons 2 and 3, and the exon 8-specific 20-mer described above. The latter oligomer was used to prime 20 μg of total RNA purified from each of the fibroblast lines seeded and grown at either 31 or 37 °C. Subsequently, 50% of the resulting double-stranded cDNA was amplified for 25 cycles with 2.5 units of Taq polymerse (Perkin-Elmer) and 200 ng of each primer in a 100-μl volume (19). Following this first round of amplification, one-tenth of the PCR product was further amplified for an additional 25 cycles. In both amplifications denaturation was at 94 °C for 1.5 min, annealling was at 52 °C for 2.5 min, and extension was at 72 °C for 3.5 min. Amplified PCR products were visualized by ethidium bromide staining after electrophoresis in a 1.6% NuSieve (FMC Bioproducts) agarose gel. Identity of the two PCR products from patient S. N. fibroblasts grown at 37 °C was confirmed by DNA sequencing, after elution and subcloning of the fragments.

RESULTS

Characterization of the Protein Abnormality—To characterize the structural mutation, fibroblast collagen from patient S. N. was analyzed and the results compared with similar data on patient W. A. Electrophoresis of patient S. N.'s pepsin-digested collagen yielded bands comigrating with the α1(I) and α2(I) chains of control fibroblast Type I collagen and an additional band (not found in control) migrating between these chains and resembling the mobility of a pN-α2(I) chain (for example, see Fig. 5). The relative amounts of the pN-α2(I)-like (designated α2(I)′) and α2(I) chains varied somewhat between different preparations but generally appeared to be approximately 1:1.5–2. These data confirmed the production of structurally abnormal pro-α2(I) chains by the proband's fibroblasts, and are qualitatively similar to findings in patient W. A., known to produce pro-α2(I) collagen chains with an interstitial deletion of 18 amino acids comprising the N-telopeptide (9). The apparent paradox of a deletion giving rise to a larger collagen chain is explained by the removal of a pepsin cleavage site in the N-telopeptide which results in the retention of the 58-residue N-propeptide.

To explore further the expression of the mutant collagen, patient S. N.'s native fibroblast collagen was purified, pepsin digested, fractionated by DEAE batch chromatography, and compared to similarly obtained data from patient W. A. (Fig. 1). From this comparison, it was apparent that the non-bound fraction (Pool 1) of patient S. N. contained relatively more, and the bound fraction (Pool 2) relatively less collagenous material than those of patient W. A. Furthermore, SDS-PAGE indicated that Pool 1 contained only the normally migrating chains of Types I and III collagen, whereas Pool 2 contained exclusively mutant collagen with normally migrating α1(I) chains and pN-α2(I)-like chains. This fractionation of native normal and mutant collagens was identical to that found previously for patient W. A. (9). Unlike the latter, however, the relative amount of mutant collagen was noted to be decreased, suggesting a different level of expression of the affected allele in patient S. N.

To characterize the mutation in the pN-α2(I)-like chain, this protein was purified, cleaved with CNBr, and the CB peptides resolved by sieve chromatography, resulting in the isolation of the amino-terminal peptide CB-X. The apparent M_r and amino acid composition was consistent with an α2(I) N-propeptide containing a deletion. Amino acid sequencing confirmed this identity and demonstrated the loss of 18 amino acids comprising the N-telopeptide region (Fig. 2*A*). The deleted segment corresponds precisely to the residues encoded by the sixth exon of COL1A2 (11) and is identical to the deletion previously described in patient W. A. (9).

Detection of the Alternative Splicing—To complement the protein studies and provide further evidence of the interstitial deletion, fibroblast RNA was used to generate an exon 8-

primed cDNA library. Ten exon 8 hybridizing clones were randomly selected and probed with an exon 6 oligomer. Three clones yielded no signal and sequencing disclosed the precise deletion of the exon 6 sequences (Figs. 2*A* and 3*A*). Five of the exon 6 hybridizing cDNAs were similarly sequenced and, surprisingly, in only two was the normal collagen sequence identified (Figs. 2*B* and 3*B*). The other three clones were in fact found to contain a single base substitution in the last codon of exon 6 which converts the normal Met (ATG) codon to Ile (ATA) (Figs. 2*C* and 3*C*). These data strongly suggested that transcripts of one of the COL1A2 alleles undergo alternative splicing to yield two different protein products. To confirm this postulate at the protein level, we examined the amino acid sequences of the normal sized α2(I) chains.

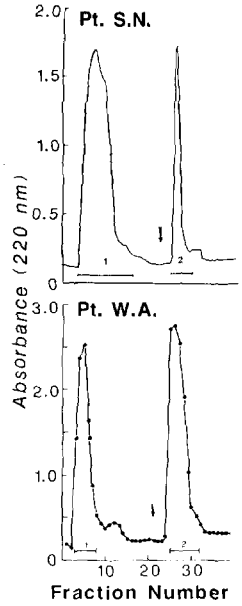

FIG. 1. **DEAE batch chromatography of pepsin-digested fibroblast collagens from patient S. N. (*top panel*) and patient W. A. (*bottom panel*).** Between 20 and 40 mg of collagen were dissolved in 40 mM Tris-HCl, 2 M urea, pH 7.2, at 1 mg/ml and applied to a DEAE-Tris acryl M (2.5 × 5.0 cm) column equilibrated with the same buffer at 4 °C. Pool 1 represents the non-bound fraction eluting with equilibration buffer. The retained fraction (Pool 2) was eluted with 125 mM Tris-HCl, 2 M urea, pH 7.2; *arrows* indicate initiation of this elution buffer.

Characterization of the Variant Protein Product—Electrophoretically normal α2(I) chains were purified from the DEAE Pool 1 fraction, cleaved with CNBr, and the resultant peptides resolved by sieve chromatography. The observed chromatographic profile was identical to normal control and no abnormal peaks were observed. Reverse-phase chromatography of the most retarded sieve peak revealed the expected normal amino-terminal peptide, CB-1; however, an abnormal additional peptide (designated CB-1.0′) was also found. Amino acid composition of this latter peptide was identical to CB-1 except for 1 additional residue of Gly and Leu and the presence of 1 residue of Ile. This composition matched that expected for a normal amino-terminal peptide missing a single CNBr cleavage site (Fig. 2*C*). Attempts to directly sequence CB-1.0′ yielded no residues, presumably because the amino terminus was blocked. However, trypsin digestion produced an unblocked fragment which yielded a sequence identical to normal, except for the substitution of Met by Ile in the last residue encoded by exon 6 (Fig. 2*C*). Thus, the observed amino acid sequence precisely reflected the predicted sequence from mRNA studies, and identified the normally spliced, but structurally variant translation product of the COL1A2 allele.

Identification of the Splicing Mutation—In order to determine the molecular basis of the alternative splicing, we cloned and partially sequenced the proband's COL1A2 alleles. To obtain the relevant regions of both alleles, we made use of the patient's heterozygosity for a polymorphic *Eco*RI site which lies within intron 12 of COL1A2 and gives rise to 14 (−) or 10.5 kilobase (+) allelic fragments (11) (Fig. 4*A*). Selective sequencing of the segments comprising exons 5, 6, and 7, the 5′-GT/3′-AG splice sites, and the putative lariat branch point signals revealed only one difference between the two alleles, namely the presence of the ATA codon in the sixth exon of the RFLP (+)-allele (Fig. 4*B*).

To gain further information regarding the nature of the substitution, family studies were undertaken. Accordingly, the DNA of the proband's parents was genotyped for the *Eco*RI

FIG. 2. **Complementary DNA nucleotide and amino acid sequences of CB-X (*A*), normal CB-1 (*B*), and mutant CB-1.0′ (*C*) encoded by exons 5, 6, and 7.** The first line indicates the determined nucleotide sequence and the second line the derived amino acid sequence. On the third and fourth lines, the ¬ denotes identity with the known amino acid sequence, and ⌐ indicates sequences deduced from carboxypeptidase Y analyses. *Hyp*, hydroxyproline. Exons are numbered at the right and exons transitions are indicated by (▼); *arrows* indicate the N-proteinase cleavage site; the cross-link precursor lysine is *underlined*; the *vertical* (|) signifies the end of the N-telopeptide and beginning of the triple helical domain. Note that in *A*, the nucleotide and protein sequences of exons 5 and 7 are juxtaposed with deletion of exon 6 sequences (compare with the normal shown in *B*). In *C*, the A for G substitution at the terminal codon of exon 6 results in a Met to Ile substitution confirmed by protein sequencing.

A.

```
CCC ACA GGC CCT CCT GGT CCA CCT GGT CCT CCT GGC CCC CCT GGT CTC GGT GGG▾  5
Pro Thr Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Leu Gly Gly
 ¬   ¬   ¬   ¬  Hyp  ¬   ¬  Hyp  ¬   ¬  Hyp  ¬   ¬  Hyp  ¬   ¬   ¬   ¬
                                                                    (¬)

GGC TTA ATG GGA CCT AGA GGC CCA CCT GGT GCA GCT GGA GCC CCA▾              7
Gly Leu Met Gly Pro Arg Gly Pro Pro Gly Ala Ala Gly Ala Pro
(¬) (¬)
 ⌐   ⌐   ⌐
```

B.

```
CCC ACA GGC CCT CCT GGT CCA CCT GGT CCT CCT GGC CCC CCT GGT CTC GGT GGG▾  5
Pro Thr Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Leu Gly Gly

AAC TTT GCT GCT CAG TAT GAT GGA AAA GGA GTT GGA CTT GGC CCT GGA CCA ATG▾  6
Asn Phe Ala Ala↓Gln Tyr Asp Gly Lys Gly Val Gly Leu Gly Pro|Gly Pro Met

GGC TTA ATG GGA CCT AGA GGC CCA CCT GGT GCA GCT GGA GCC CCA▾              7
Gly Leu Met Gly Pro Arg Gly Pro Pro Gly Ala Ala Gly Ala Pro
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C.

```
                                                    A
CAG TAT GAT GGA AAA GGA GTT GGA CTT GGC CCT GGA CCA ATG▾GGC TTA ATG      6/7
↓Gln Tyr Asp Gly Lys Gly Val Gly Leu Gly Pro|Gly Pro Met Gly Leu Met
                                          ¬   ¬   ¬   ¬   ¬   ¬   ¬   ¬  Ile  ¬   ¬
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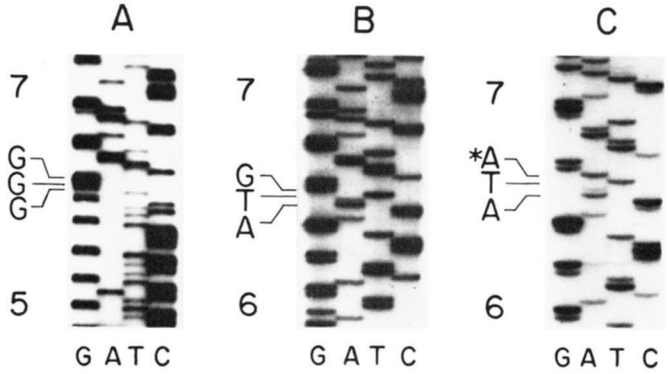

FIG. 3. **Nucleotide sequences of the proband's cDNAs.** *Panel A*, sequence of the outspliced transcript; *panel B*, sequence of the normal transcript; *panel C*, sequence of the correctly spliced variant transcript. For comparison to the sequences shown in Fig. 2, codons of transition between different exons (*numbers* on the left of each *panel*) are indicated. The *asterisk* highlights the A for G substitution in the normally spliced variant transcript.

RFLP. This demonstrated inheritance of the relevant RFLP (+)-allele from the father since the mother was found to be (−/−) and the father (+/−) (Fig. 4*A*). The father's RFLP (+)-allele was therefore cloned and, upon sequence inspection, found to contain the normal ATG codon in exon 5 (Fig. 4*B*). Since the father is phenotypically normal and his skin contains only normal collagen chains (5), we concluded that a *de novo* substitution of A for G in the nucleotide immediately preceding the 5′ splice site of intron 6 is indeed responsible for the splicing abnormality.

Relative Quantitation of the Alternatively Spliced Products—To estimate the extent of correct *versus* incorrect splicing of the mutant transcripts, large amounts of procollagen were produced at 37 °C, converted to collagen by limited pepsin digestion, and fractionated on the DEAE column. Under the assumptions that both alleles are equally transcribed and translated, and that 10% of total fibroblast collagen synthesis is Type III collagen, we then calculated that 39–51% of the mutant allele is correctly spliced based on integration of the DEAE absorbance profiles (similar to Fig. 1) from two independent preparations. Similar calculations, based on recovered dry weights from these columns, yielded 44–56% correct splicing of the mutant allele. These estimates suggested that the alternative splicing pathways are approximately equal at 37 °C, as judged by the secreted media collagen.

Temperature-dependent Expression of the Alternative Splicing—As previously stated, during the course of these investigations occasional but consistent variations in the relative production of shortened pro-α2(I) chains were noted among different preparations. In light of the evidence of alternative splicing, we investigated whether or not identifiable changes in cell culture conditions might be influencing the expression of the mutation. Hence, the relative ratio of α2(I) and α2(I)′ chains was assessed according to variable parameters of cell density, number of cell passages, and temperature. While the first two factors did not appear to affect the relative production of α2(I)′ chain, temperature was found to have a surprisingly significant effect.

Briefly, confluent fibroblasts were radiolabeled at varying temperatures from 31 to 39 °C in 2-degree increments and the resultant media and cell layer collagens analyzed by SDS-PAGE. For comparison, fibroblasts from patient W. A. and normal control were similarly labeled and collagens analyzed in parallel. The results showed that patient S. N. media collagen produced at 31 °C contains relatively little of the

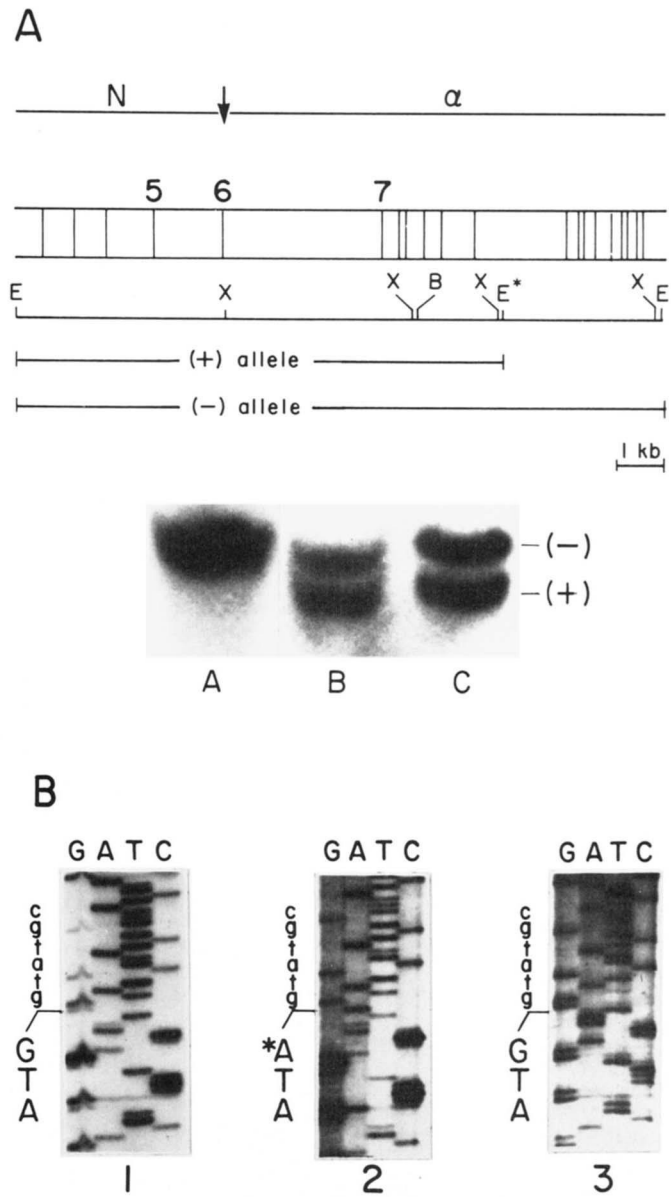

FIG. 4. **Identification of the splicing mutation.** *Panel A*, the map at the top represents the overall organization of the region of COL1A2 that codes for most of the N-propeptide (*N*) and the amino terminus of the α chain (α). This region is comprised within the two allelic *Eco*RI fragments (*E* and *E**) whose relative positions with respect to the organization of the exons (*vertical bars*) are shown. Additional enzymes are *Bam*HI (*B*) and *Xba*I (*X*). Below are the *Eco*RI RFLP patterns of the mother (*A*), father (*B*), and proband (*C*). *Panel B*, nucleotide sequences at the exon 6/intron 6 junction of the proband's (−)- and (+)-alleles (*lanes 1* and *2*, respectively) as well as the father's (+)-allele (*lane 3*). *Lower case letters* represent intervening sequences, *capital letters*, coding sequences. The *asterisk* indicates the single point mutation in the sixth exon of the proband's *Eco*RI (+)-allele.

larger α2(I)′ chain but that the relative amount of this marker of missplicing increases progressively with increasing temperature (Fig. 5). At 39 °C, there appears to be approximately equivalent amounts of α2(I) and α2(I)′ chains, suggesting that the mutant transcript is virtually completely misspliced. In contrast, the relative amounts of α2(I) and α2(I)′ chains in patient W. A. collagen did not significantly vary at any temperature (Fig. 5). Analysis of cell layer collagens from the patient yielded comparable data, and specifically eliminated the possibility of temperature-dependent differential secre-

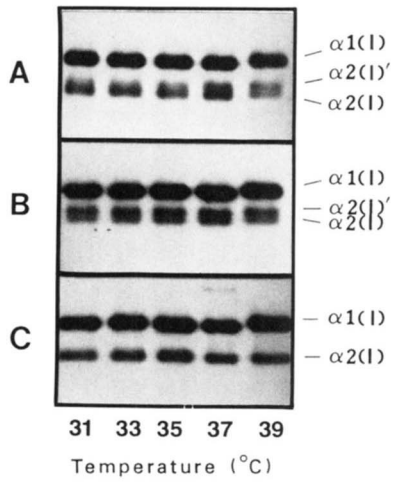

FIG. 5. **Demonstration of temperature-dependent expression of the collagen missplicing by protein analysis.** The relative abundance of the misspliced α2(I)′ chain was compared with α2(I) chains in media collagen produced at various temperatures by patient S. N. (*A*), patient W. A. (*B*), and control (*C*) fibroblasts. The temperatures at which the fibroblasts were grown and radiolabeled peptide produced are shown below the electrophoretic lanes. For both patients, the α2-derived chains appear as a poorly resolved doublet and the chain identities are indicated. Compare the relative amounts of α2(I) and α2(I)′ chains at different temperatures for patient S. N. (*A*) and patient W. A. (*B*).

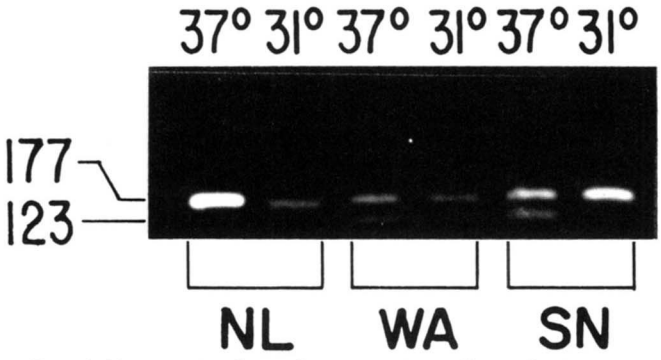

FIG. 6. **Demonstration of temperature-dependent expression of the collagen missplicing by the PCR amplification.** The temperatures at which the fibroblasts from control (*NL*), patient W. A. (*WA*), and patient S. N. (*SN*) were grown are shown above the electrophoretic lanes. The *numbers* on the left represent lengths in nucleotides of the PCR products corresponding to the normal (*177*), and the misspliced (*123*) mRNAs.

tion of mutant collagen (data not shown).

The protein data were independently verified by parallel mRNA studies. Accordingly, RNA was extracted from each of the three fibroblast lines grown at 31 and 37 °C and used to generate cDNAs which were, in turn, PCR amplified (19). Analysis of the PCR products showed that patient S. N. fibroblasts grown at 37 °C produced normally spliced and misspliced mRNAs, while only correctly spliced products were detectable when the cells were cultured at 31 °C (Fig. 6). In contrast, patient W. A. fibroblasts displayed no appreciable change in exon 6 outsplicing at both temperatures (Fig. 6). Together, therefore, the protein and RNA data conclusively demonstrated that expression of the splicing abnormality in the fibroblasts of this particular EDS VII patient displays temperature-dependence.

DISCUSSION

In this study, we used a combination of approaches to elucidate the molecular nature of the collagen defect in a heterozygous EDS VIIB patient. Both protein and cDNA sequencing demonstrated the presence of three distinct COL1A2 products. These include the normal sequence, and two sequences, one of which deletes exon 6 and the 18 amino acids that it encodes, and the other of which leads to the production of a normal sized, variant pro-α2(I) chain with a Met (ATG) to Ile (ATA) substitution in the last residue encoded by exon 6. The last two sequences are the products of the mutant allele and arise by alternative splicing. Estimates based on the protein products of the mutant allele produced at 37 °C suggest that alternative splicing is approximately equal at physiologic temperature. The finding of G to A substitution in the sixth exon of one of the proband's alleles but not in the relevant gene of the unaffected father provides compelling evidence that the *de novo* change is the mutation responsible for the alternative splicing. This notion is indirectly supported by the recent identification of the same mutation in the sixth exon of COL1A1 in one of the three EDS VII patients previously characterized at the biochemical level (7, 14). Interestingly, this individual also produces alternative spliced RNA but with a relative rate of outsplicing (30%) clearly different from that seen in patient S. N. (50%) (14).

Deletion of the exon 6 sequences does not alter the coding frame of the mRNA, the ability of the shortened pro-α2(I) chains to assemble into trimers, or the secretion of mutant molecules. In the present heterozygous case, about 25% of the total Type I collagen produced at 37 °C harbors the abnormal pro-α2(I) chain. Since the deleted segment encompasses the procollagen N-proteinase cleavage site, such mutant molecules cannot undergo physiologic excision of the N-propeptide, as indicated by the *in vivo* accumulation of the abnormal pN-α2(I) chain in the patient's skin (5). Moreover, present evidence indicates that the cognate α1(I) N-propeptides, although cleaved from the α1(I) helical domains, remain in noncovalent association with the abnormal pN-α2(I) chain via the small triple-helical domain of the N-propeptide[3], the functional result is the retention of a modified form of pN-collagen within tissue. Retention of the N-propeptide may interfere with normal fibrillogenesis and cause defective cross-linking for steric reasons. Alternatively, deletion of the N-telopeptide lysine cross-link precursor may also contribute to the increased solubility of dermal collagen previously documented (5). We do not know if the correctly spliced, but substituted pro-α2(I) chain is normally functional. Conceivably, the Met-to-Ile substitution could disrupt the secondary structure of the α2(I) N-telopeptide, impair the subsequent cross-linking of this domain, and therefore participate in the pathogenesis of this phenotype. Alternatively, and by analogy to the transgenic mouse in which as little as 10% of abnormal collagen results in prenatal death (20), the production of only 25% shortened pro-α2(I) chains may be sufficient to eventuate the dominant phenotype in this patient.

Although earlier data suggested that coding sequences do not play a major role in splice-site selection (for reviews, see Refs. 21 and 22), more recent investigations have shown that splicing patterns are also significantly affected by mutations within exons (23–25). In the most comprehensive of these studies, Reed and Maniatis (25), using an *in vitro* cis-competition assay, were in fact able to demonstrate that splice-site selection is highly dependent on the relative strength of the whole sequence context (including exon sequence) of individual splicing signals, as well as on the proximity between the strongest pair of 5′ and 3′ splice sites. A corollary to such a postulate explains why splicing mutations in multi-exon genes seldom activate weaker, cryptic splice sites but rather result

in the utilization of immediately proximal, wild-type signals with the consequent phenomenon of exon skipping (13, 14, 24, 26–31).

The findings in our naturally occurring collagen variants are consistent with the aforementioned conclusions, since both intron and exon mutations at the exon 6/intron 6 junction inactivate the selection of this splice site and result in the utilization of the most proximal donor site, namely that of intron 5. The exon mutation, however, is not sufficient to completely inhibit splice-site selection, for both mutated and proximal, wild-type donor sites appear to compete for splice-site recognition at a nearly equal rate. This is conceivably the result of the loss of an important G:C interaction in the base pairing between 5′ splice site and U1 snRNA (32). The failure to observe alternative splicing in the GT intron mutation may, on the other hand, reflect the unique contribution of this highly conserved element to other steps of the splicing reaction (33).

The proposed balance between strength and proximity of 5′ and 3′ splice sites (25) could be also invoked to reconcile the difference seen in the relative rate of alternative splicing in this and the recently studied EDS VIIA patient bearing the same base substitution in the sixth exon of COL1A1 (14). Accordingly it could be argued that, since in COL1A2 introns 5 and 6 are substantially larger than in COL1A1 (11, 34), the rate of exon 6 skipping in the two mutants may correlate with different kinetics of substrate folding during the formation of the splicing complex (25). Alternatively, the difference may simply reflect the distinct effect of the same mutation in eliciting the competing strength of the wild-type, proximal splicing signal in the two collagen transcripts.

In their *in vitro* experiments Reed and Maniatis (25) have also observed that the pattern of splice-site selection can sometimes be affected by dilutions of the splicing extract. Similar findings have been obtained by varying ionic conditions in the *in vitro* splicing reaction (35). Hence, it has been suggested that alternative splicing *in cellula* could be similarly modulated by subtle cell-specific variations. Here, evidence is presented that the expression of a splicing defect can be effectively and specifically suppressed *in cellula* by a simple physiological parameter, namely temperature. Although the mechanism(s) underlying the temperature effect are unknown and conceivably are complex, our finding raises the intriguing possibility that this factor may participate in regulating normal patterns of alternative splicing in different tissues, such as skin *versus* internal organs.

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