We identified a splicing mutation in a patient with Ehlers-Danlos syndrome type IV, a heritable connective tissue disorder associated with dysfunctions of type III collagen. The mutation was first localized in the patient’s type III procollagen mRNA by amplifying the reverse transcribed product in several overlapping fragments using the polymerase chain reaction. Amplified products spanning exon 24–26 sequences displayed two distinct fragments, one of normal size and the other lacking the 99 base pairs of exon 25. Sequencing of amplified genomic products identified a G to T transversion at position +5 of the splice donor site in intron 25 in one of the patient’s procollagen III genes. Expression of allelic minigene constructs correlated the T for G substitution with skipping of exon 25 sequences. Like previously characterized splicing mutations in other collagen genes, lowering the temperature at which the patient’s fibroblasts were incubated nearly abolished exon skipping. As a part of this study, we also identified a highly polymorphic, intronic DNA sequence whose different allelic forms can be detected easily by the polymerase chain reaction technique.

The type IV form of Ehlers-Danlos syndrome (EDS IV) is a dominantly inherited condition characterized by severe fragility of blood vessels, skin, and viscera (1). The disease often has a fatal outcome because of the spontaneous rupture of large arteries and hollow organs (1). Early biochemical studies on several EDS IV patients have indicated a deficiency of mature type III collagen in tissues and a defective metabolism of precursor type III procollagen in cultured fibroblasts (2–7). Since fibroblasts normally produce relatively small amounts of type III collagen, the biochemical approach did not elucidate the exact nature of the defect in these patients. Consequently, the pathological spectrum of type III collagen mutations in EDS IV still remains largely unknown. Recently, however, sequencing of full size pro-α1(III) cDNAs and partial characterization of the corresponding gene (COL3A1) have provided structural information and suitable reagents for the molecular analysis of this group of genetic disorders (8–11). As a result, multiexon deletions (6),2 single point mutations (12, 19), and splicing defects (14–16) have been identified in a relatively small group of EDS IV patients.

In the present study, we characterized the mutation in a sporadic case of EDS IV. Dermal cultured fibroblasts from this individual were previously shown to retain increased amounts of type III procollagen in the cell layer, conceivably as a result of a defective triple helical structure (5). We used in vitro amplification of cDNA and genomic DNA by the polymerase chain reaction technique (17), in conjunction with expression of minigene constructs in COS cells, to demonstrate that a G to T transversion at the +5 position of the donor splice site of intron 25 (IVS 25) causes skipping of the preceding exon during pre-mRNA processing.

**MATERIALS AND METHODS**

**The Patient**—The patient was born to healthy, unrelated parents. Five brothers and three sisters were unaffected. Birth weight was 2,650 g; bilateral clubfoot was present at birth. Easy bruising was noticed since boyhood, and he had several episodes of hemorrhage, occurring spontaneously or after trivial trauma, including bleeding into a joint (at age 7 years), the thigh (13 years), the subdural space and right pleural cavity (17 years), the epigastrium (22 years), the thigh and both pleural cavities (27 years), and again the right pleural cavity (31 years). On physical examination at age 31 years, he presented a thin, delicate skin with hemosiderotic, atrophic scars as well as numerous keloids, easily visible superficial veins, and flexion contractures of the thumb and the third finger of the right hand. He also had partial right bundle branch block and pulmonary stenosis (confirmed by angiography at age 19 years). He died a few years later after falling from a bar stool.

Two skin biopsies were obtained from this patient with informal consent and submitted to the American Type Culture Collection (ATCC CRL-1299 and CRL-1409). Fibroblasts from this patient have been utilized in numerous studies on EDS IV (2, 6, 7, 18, 19). Histologic examination of a skin fragment revealed marked fragmentation and distortion of collagen fibers in the dermis, consistent with the Ehlers-Danlos syndrome; skin biopsies from both parents were

1 This work was supported by Grant AR-38648 from the National Institutes of Health, Grant 1-1196 from the March of Dimes, Birth Defect Foundation, and Grants 3-8601.066 and 32-27884.89 from the Swiss National Science Foundation. This is article 40 from the Brookdale Center for Molecular Biology at Mt. Sinai School of Medicine, New York. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 Vissing, H., D’Alessio, M., Lee, B., Ramirez, F., Byers, P. H., Steinmann, B., and Superti-Furga, A. (1991) J. Biol. Chem. 266, 5244–5248.

3 Lee, B., D’Alessio, M., Vissing, H., Ramirez, F., Steinmann, B., and Superti-Furga, A. (1991) Am. J. Hum. Genet. 48, in press.
normal. Immunohistochemistry showed that type III collagen was present but in reduced amounts.4

Nucleic Acid Purification, cDNA Synthesis, and Polymerase Chain Reaction Amplification—Total RNA and genomic DNA were purified from cultured dermal fibroblasts using the guanidinium isothiocyanate method and the proteinase K treatment, respectively (20). A commercial kit (Amersham) was employed for the random primed synthesis of double-stranded cDNA on 5 μg of template RNA, according to the manufacturer's recommendations. An aliquot of the reaction was amplified with COL3A1 specific oligonucleotide primers in a programmable heat block (Cetus) following the published conditions (21). The same protocol was utilized for the polymerase chain reaction-amplification of the IVS 184 (exon II1) chains, they did not locate the defect within the triple helical domain of the procollagen chain (5). To this end, we scanned the entire length of the pro-nl(II1) collagen mRNA that the patient's fibroblasts produce structurally abnormal α1(III) chains, they did not locate the defect within the triple helical domain of the procollagen chain (5). To this end, we scanned the entire length of the pro-nl(II1) collagen mRNA by amplifying the reverse-transcribed product in several overlapping fragments using the polymerase chain reaction technique (RT-polymerase chain reaction). Using various combinations of primers, this approach documented the patient's heterozygosity for a small deletion located in the mRNA sequences that correspond to exons 25 to 29 (data not shown). The identity of the deleted sequence was eventually revealed by comparing the migration patterns of patient and control RT-polymerase chain reaction products amplified with exon 24 and 26 primers (Fig. 1). Sequencing of the subcloned RT-polymerase chain reaction products confirmed that the faster migrating fragment of the patient's polymerase chain reaction product lacks the 99-bp encoding residues 388–420 of the triple helical domain (Fig. 1) (11). The deleted sequence corresponds precisely to the sequence encoded by exon 25.3

RESULTS AND DISCUSSION

Although previous biochemical studies strongly suggested that the patient's fibroblasts produce structurally abnormal α1(III) chains, they did not locate the defect within the triple helical domain of the procollagen chain (5). To this end, we scanned the entire length of the pro-α1(III) collagen mRNA by amplifying the reverse-transcribed product in several overlapping fragments using the polymerase chain reaction technique (RT-polymerase chain reaction). Using various combinations of primers, this approach documented the patient's heterozygosity for a small deletion located in the mRNA sequences that correspond to exons 25 to 29 (data not shown). The identity of the deleted sequence was eventually revealed by comparing the migration patterns of patient and control RT-polymerase chain reaction products amplified with exon 24 and 26 primers (Fig. 1). Sequencing of the subcloned RT-polymerase chain reaction products confirmed that the faster migrating fragment of the patient's polymerase chain reaction product lacks the 99-bp encoding residues 388–420 of the triple helical domain (Fig. 1) (11). The deleted sequence corresponds precisely to the sequence encoded by exon 25.3

4 K. von der Mark and B. Steinmann, unpublished data.

Fig. 1. Nucleotide sequences of the proband type III collagen cDNAs. Panel A, ethidium bromide staining of a 1.5% agarose gel showing the amplified products of control (lane 1) and EDS IV (lane 2) RT-polymerase chain reactions using primers for exons 24 and 26. Sizes of bands are indicated in base pairs on the right. Panel B, sequences of the abnormal cDNA.

In COL3A1, exon 24 and exon 26 are 953 bp apart.3 Amplification of the patient genomic DNA using exon 24 and 26 primers yielded a single polymerase chain reaction product, thus suggesting that the mRNA deletion is caused by defective splicing of pre-mRNA. To elucidate the cause of the defective splicing, the patient polymerase chain reaction products were subcloned and sequenced. In two of the five subclones analyzed, a single base change (T for G) was noted at position +5 of the splice donor site of IVS 25 (Fig. 2). To exclude possible artifacts, the polymerase chain reaction products of the patient were also sequenced directly without subcloning. This revealed the presence of two bands of nearly identical intensity in the T and G tracks at position +5 of IVS 25 in the proband (autoradiogram 3) but not in the control DNA (autoradiogram 4).

Fig. 2. Nucleotide sequence of the polyA's COL3A1 alleles. Intron and exon sequences are denoted by lowercase and capital letters, respectively. Sequences of the normal and affected alleles shown in autoradiograms 1 and 2, respectively, were generated from sequencing polymerase chain reaction products subcloned into the Smal site of pUC18. Autoradiograms 3 and 4 show the same sequences derived from direct sequencing of in vitro-amplified DNA. Note the two bands of nearly identical intensity in the T and G tracks at position +5 of IVS 25 in the proband (autoradiogram 3) but not in the control DNA (autoradiogram 4).
Above the sequences are the differences found in the affected COL3A1 letters, whereas intron sequences are indicated by lowercase letters. 

Underlined are the polymorphic sequences in Table I.

In a recent study, familial inheritance of a phenotypic overlap between EDS IV and arterial aneurysm has been confirmed that the +5 base change in IVS 25 interferes with normal splicing. 

In a related study on a homozygous lethal perinatal OI variant, a G to T transition at position +5 of the donor splice site of IVS 14 of COL1A1 was shown to reduce the efficiency of pre-mRNA processing (26). In this OI variant, exon 14 was spliced alternatively with an estimated efficiency of 50% (24). Moreover, the extent of exon skipping was reduced to approximately 30% in cells grown at 31 °C (26). This observation was also consistent with the characterization of two additional collagen variants (29). In these individuals, point mutations at the moderately conserved position -1 of the splice donor site of IVS 6 of COL1A1 and COL1A2 similarly led to temperature-dependent alternative splicing (29).

Based on these data, we tested whether temperature had the same effect on the relative rate of exon skipping in the COL3A1 mutant. Accordingly, RNA purified from fibroblasts grown at 37 °C and 31 °C were subjected to RT-polymerase

3 M. D'Alessio, F. Ramirez, B. D. Blumberg, M. K. Wirtz, and D. W. Hollister, manuscript in preparation.
chain reaction amplification using primers for exons 8 and 27. The resulting products were analyzed by Southern blot hybridization to a COL3A1 cDNA (10). Like the previously described cases, temperature appeared to modulate the rate of exon skipping in *cellula*, since the patient's faster moving band nearly disappeared in cells grown at 31 °C (Fig. 6). Lacking suitable markers, we do not know if any normally spliced transcript is produced by the affected COL3A1 allele at 37 °C, nor can we negate the possibility of alternative splicing based solely on the pattern seen in the COS cell expression system. This functional assay has in fact replicated the defective splicing of a different collagen mutation leading exclusively to exon skipping (27). In contrast, minigene constructs derived from patients affected by mutations resulting in alternative splicing have consistently expressed only the pattern of exon skipping in these cells (25, 26).

Because of the peculiar organization of the fibrillar collagen genes (30), exon skipping does not alter the translational frame of the mature mRNA. This results in the production of structurally abnormal precursor procollagen chains capable, however, of trimer assembly (31). The shortened procollagens conceivably affect the rate of trimer assembly and decrease secretion of the procollagen trimers, by exposing unassembled chains to overmodification (30). In this and in an unrelated EDS IV patient, we have previously shown that the amount of retained type III procollagen in the cell layer can be decreased by lowering the temperature at which cells are cultured to 32 °C (5). This result was interpreted as suggesting that temperature, by stabilizing the defective protein molecules, leads to more efficient secretion (5). We now argue that the increase in the relative pool of structurally normal proα1(III) collagen chains in this patient's cell layer at 31 °C is mostly caused by the effect that temperature exerts on splice-site selection. Parenthetically, this implies that the postulated stabilization of defective protein molecules in the other EDS IV patient previously analyzed biochemically (5) may also reflect the partial effect of a temperature-dependent splicing mutation. Thus, incubation of fibroblasts at temperatures below 37 °C may be applied as a relatively simple means to score for mutations leading to unstable trimers and/or alternative splicing in collagenopathies. More generally, our data on this and other collagen variants (25, 26, 29), as well as data from other naturally occurring mutants (32, 33) indicate that mutations at the moderately conserved nucleotides of splice donor sites alter significantly but do not eliminate completely splice-site selection.

Finally, recent reports of G to A substitutions in the +1 position of IVS 16, 20, 41, and 42 have correlated single exon skipping events and cryptic splicing with severe EDS IV phenotypes (14–16). These mutations and the present report of a novel +5 G to T substitution associated with single exon skipping and severe EDS IV collectively argue that these types of mutations produce generally severe phenotypes with no specific relation to their locations in the triple helical domain. Interestingly, the phenotypic severity exhibited by single exon skipping mutations in the homotrimeric type III collagen parallels observations of these types of defects in type I collagen associated with osteogenesis imperfecta (31).

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