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Null Alleles of the *COL5A1* Gene of Type V Collagen Are a Cause of the Classical Forms of Ehlers-Danlos Syndrome (Types I and II)

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Ehlers-Danlos syndrome (EDS) types I and II, which comprise the classical variety, are well characterized from the clinical perspective, but it has been difficult to identify the molecular basis of the disorder in the majority of affected individuals. Several explanations for this failure to detect mutations have been proposed, including genetic heterogeneity, failure of allele expression, and technical difficulties. Genetic heterogeneity has been confirmed as an explanation for such failure, since causative mutations have been identified in the *COL5A1*, *COL5A2*, and tenascin X genes and since they have been inferred in the *COL1A2* gene. Nonetheless, in the majority of families with autosomal dominant inheritance of EDS, there appears to be linkage to loci that contain the *COL5A1* or *COL5A2* genes. To determine whether allele-product instability could explain failure to identify some mutations, we analyzed polymorphic variants in the *COL5A1* gene in 16 individuals, and we examined mRNA for the expression of both alleles and for alterations in splicing. We found a splice-site mutation in a single individual, and we determined that, in six individuals, the mRNA from one *COL5A1* allele either was not expressed or was very unstable. We identified small insertions or deletions in five of these cell strains, but we could not identify the mutation in the sixth individual. Thus, although as many as one-half of the mutations that give rise to EDS types I and II are likely to lie in the *COL5A1* gene, a significant portion of them result in very low levels of mRNA from the mutant allele, as a consequence of nonsense-mediated mRNA decay.

Introduction

The classical forms of Ehlers-Danlos syndrome (EDS type I [MIM 130000] and EDS type II [MIM 130010]) are characterized by very soft and hyperextensible skin, easy bruising, "cigarette-paper" scars, and marked small- and large-joint laxity (Beighton 1993; Steinmann et al. 1993; Beighton et al. 1998). These forms of EDS are inherited in an autosomal dominant fashion, but new mutations are common. The altered mechanical properties of skin and ligaments made alterations in the constitutive "collagen" a strong candidate(s) for the location of the basic defect (Grahame and Beighton 1969). The results of morphological studies of skin from affected individuals demonstrated that collagen fibrils in the dermis (which comprise largely type I collagen) were larger than normal and that there was a population of aggregated fibrils (Vogel et al. 1979; Hausser and Anton-Lamprecht 1994). The findings of biochemical studies of cultured fibroblasts from affected individuals were remarkably unrevealing, with the exception of the identification of rare individuals who appeared to be homozygous for "null" *COL1A2* alleles (Sasaki et al. 1987; Hata et al. 1988). In those families, the phenotype appeared to be the consequence of a recessive mutation. Thus, type I collagen genes were not good candidates for mutations in the dominantly inherited phenotypes of EDS. The results of directed linkage studies in families with autosomal dominant inheritance of EDS type I then excluded type I collagen genes as well as several other genes that encode extracellular matrix proteins (Wordsworth et al. 1985, 1991; Sokolov et al. 1991).

Studies done using polymorphic markers in the *COL5A1* gene, which is located at chromosome 9q34 and which encodes the proα1(V) chains of type V collagen, provided evidence that EDS type II (Loughlin et al. 1995) and mixed EDS type I/II in the same family (Burrows et al. 1996; Burrows et al. 1997) were linked to that locus. In some families, linkage to *COL5A1* (Greenspan et al. 1995) or to *COL5A1* and *COL5A2* (Wenstrup et al. 1996), the latter of which encodes the proα2(V) chains of type V collagen, could be excluded. The findings from biochemical analysis of type V collagen molecules synthesized

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by cultured fibroblasts supported the concept that abnormalities of these proteins contributed to the phenotype of EDS type I or II (Nicholls et al. 1994). In one child with EDS type I and an X/9 chromosomal translocation, the breakpoint occurred within intron 24 of the *COL5A1* gene at chromosome 9q34 (Toriello et al. 1996). No protein product of the fusion gene was identified. Deletions that resulted from exon-skipping mutations in either the region of the *COL5A1* gene that encodes the carboxyl-terminal propeptide (Wenstrup et al. 1996) or the triple-helical domain (Nicholls et al. 1996; De Paepe et al. 1997; Burrows et al. 1998), or a point mutation that changed a cysteine to serine in the carboxyl-terminal propeptide each resulted in the EDS type I or II phenotype (De Paepe et al. 1997). More recently, point mutations in the *COL5A2* gene that produced single amino-acid substitutions (G934R) (Richards et al. 1998) or exon skipping (Michalickova et al. 1998) in the triple-helical domain were identified in individuals with EDS type I or II.

The coding sequences of both *COL5A1* and *COL5A2* have been searched extensively, but the yield of identified mutations has been surprisingly small. A study of 28 affected individuals identified only four mutations in the two genes (two mutations were found in each gene) (Michalickova et al. 1998). The possibility that other genes might be involved was raised by linkage studies that appeared to exclude both genes (Wenstrup et al. 1996) and by the identification of a deletion of one allele of tenascin X, adjacent to the 21-hydroxylase gene (Burch et al. 1997), in an adolescent with EDS and 21-hydroxylase deficiency. Most analyses of *COL5A1* and *COL5A2* coding sequences have been done with the use of mature mRNA obtained from cultured fibroblasts—a strategy that will identify mutations in stable and abundant mRNA species.

Although missense and splice-site mutations in both type V collagen genes can cause the classical EDS type I and II phenotypes, it is plausible that other mutations in the genes also play a role. The chromosomal translocation resulted in failure to synthesize a detectable protein product from the affected *COL5A1* allele, which, even if expressed, would lack the chain-assembly region of the carboxyl-terminal propeptide (Toriello et al. 1996). The mutation that resulted in deletion of the sequence encoded by exon 65 of the *COL5A1* gene removed a domain that is predicted to function in chain-chain interaction during molecular assembly (Michalickova et al. 1998). Together, these examples suggest that other mutations that diminish the amount of proα1(V) should result in the EDS type I or II phenotypes.

To test this hypothesis, we selected cells from 16 individuals with histories and clinical findings characteristic of EDS type I or II, examined mRNA for the expression of both alleles of *COL5A1,* and, in 5 of the 6 individuals whose cells expressed only one allele, identified the causative mutations. On the basis of the results of the present study, it appears that mutations that cause premature-termination codons in the *COL5A1* transcript and therefore lead to "nonsense-mediated decay" (Maquat 1995; Frischmeyer and Dietz 1999) of the altered allele are common causes of the classical forms of EDS.

Material and Methods

Clinical Data and Criteria for Inclusion

The clinical records of individuals whose cells had been provided for analysis of collagen abnormalities and who had a diagnosis of EDS were reviewed. Of these individuals, 16 were selected for screening because they had clinical findings compatible with the diagnosis of EDS type I or II (the classical types in the revised nomenclature) (Beighton et al. 1998). The clinical findings are summarized in table 1. All samples were obtained with appropriate consent.

Cell Culture and Isolation of RNA and DNA

Cells were cultured as previously described, and the procollagen and collagen molecules synthesized by these cells were analyzed after labeling with [^{3}H]-proline and SDS-PAGE separation of proteins in the medium and the cell layer (Bonadio et al. 1985). All cells synthesized normal amounts of procollagen types I and III, which had normal electrophoretic mobilities and were secreted normally. The chains of type V collagen were apparent on the gels, but no specific alterations could be identified. RNA and DNA were isolated from the cells by standard methods (Sambrook et al. 1989).

Analysis of cDNA from COL5A1 *and* COL5A2 *and of Genomic DNA Fragments of* COL5A1

Complementary DNA was synthesized from the total cellular RNA, and primers from the coding sequences of *COL5A1* (Greenspan et al. 1991) and *COL5A2* (Emanuel et al. 1985; Weil et al. 1987) were used to amplify six and five overlapping fragments, respectively, of the full length of each product. The primers that were used are shown in table 2. The fragments were separated on 6% polyacrylamide gels.

Genomic DNA from each patient was used as the substrate to amplify individual exons (with the exception of exon 1) from the *COL5A1* gene. The *COL5A1* gene comprises 66 exons, of which 65 are distributed over ~150 kb; the first intron is >500 kb in length (Takahara et al. 1995). Each fragment was analyzed by separation on mutation-detection-en-

Table 1

Clinical Features of Patients with EDS Types I and II

Patient and (Sex/Age [years][b])	Clinical Features[a]						
	Family History	Premature Birth	Skin Soft, Velvety, and Hyperextensible	Scars Wide and Atrophic	Joint Hypermobility	Easy Bruising	Referral Diagnosis[c]
P1 (M/17)	+	NA	+	NA	+	+	I (?)
P2 (M/13)	+	NA	+	NA	+	NA	I (?)
P3 (M/39)	−	−	+	−	+	+	II
P4 (F/32)	NA	NA	+	+	+	+	II
P5 (F/NA)	NA	NA	NA	NA	NA	NA	II
P6 (F/4)	NA	NA	+	+	+	+	I
P7 (F/36)	NA	NA	+	NA	+	−	EDS
P8 (F/4)	+	(+) 32 wk	+	+	+	+	I
P9 (F/2)	+	NA	NA	+	+	+	EDS
P10 (M/25)	NA	NA	+	NA	+	+	II
P11 (M/14)	−	+	(+) Friable	+	(+) Small	+	I (?)
P12 (M/6)	−	−	+	NA	+	+	II (?)
P13 (F/14)	NA	NA	+	+	+	+	II
P14 (M/5)	Adopted	NA	+	+	+	+	I
P15 (F/14)	+	−	+	+	+	+	I (?)
P16 (M/57)	NA	NA	+	+	+	+	I/II

Note.—NA = not available.

[a] Plus sign (+) denotes that the trait was present; minus sign (−), the trait was absent.

[b] Age at clinical diagnosis of classical EDS.

[c] EDS type.

hancement (MDE) gels, to identify heteroduplexes. The sequences of the intron primers used are contained in table A1 in the Appendix, which appears only in the electronic version of the *Journal*. The full genomic structure of the *COL5A2* gene has not been published, but the results of analysis of the 5′ coding region suggest that the gene is similar to other fibrillar collagen genes (*COL1A1, COL1A2,* and *COL3A1*) and, thus, should have 50–52 exons (Truter et al. 1993).

Four polymorphic sites in the *COL5A1* gene were analyzed in genomic DNA from each affected individual, by use of intron-based genomic primers. Fragments were amplified, were cleaved with the appropriate restriction enzyme, and were separated by means of PAGE. The polymorphic variants used were a *Pst*I site in exon 5 (Cappa et al. 1995), a *Dpn*II site in exon 66, and a *Bst*UI site in exon 66 (Greenspan and Pasquinelli 1994). A single-nucleotide polymorphism in exon 58 (nucleotide 4482C/G; U. Schwarze, unpublished data) was assayed as a *Bsl*I site, with the use of a mismatch primer to create the site. *Bsl*I cleaves the sequence 5′-CCN$_5$N$_2$GG-3′ after N$_5$. The mismatch primer sequence used was 5′-CAGGCCTGATCGGGCTCATCCGT-3′, in which C is usually G. In the product 5′-CAGGCCTGATCGGGCTCATCCGTCCTCCGG.....-3′, the enzyme *Bsl*I cleaved when the penultimate nucleotide (position 4482 of the coding mRNA) was **G**. The antisense primer used in cDNA was exon 62A (table 2), and the genomic primer was in intron 58 (see table A1 in the Appendix, which appears only in the electronic version of the *Journal*).

DNA-Sequence Determination

Aberrant bands identified by analysis of cDNAs from the *COL5A1* gene were cut from polyacrylamide gels, were reamplified, and were purified (with QIA quick-spin columns) for sequence determination on the ABI 310 genetic analyzer using Big Dye Terminator Cycle Sequencing Ready Reaction Kit chemistry (PE Biosystems). Products of genomic amplification that had heteroduplexes identified by MDE gel electrophoresis were purified by means of column chromatography (with QIA quick-spin columns) and were sequenced on the ABI 310 genetic analyzer (PE Biosystems).

Electron Microscopy

A skin sample available from one patient (patient P10) was prepared for electron microscopy as previously described and then was viewed on the Phillips 420 scope in transmission mode (Smith et al. 1992). Fibril diameter and spacing were analyzed after the images were scanned.

Table 2

Primers in cDNA Used to Amplify Large Fragments of the *COL5A1* and *COL5A2* Coding Regions

Primer[a]	Primer Location[b]	Primer Sequence (5′→3′)	Size (bp)
COL5A1:			
Exon:			
1S	7	GTCCATACCCGCTGGAAAGCGCGCA	1,026
7A	1032	CTCACTGGGCACGTAGTCATAGTCC	
5S	703	GATTACTGTGAGCACTACAGCCCTG	1,167
17A	1869	TTGTCCAGGCATTCCTCTGGCTCCA	
13S	1639	CAAGCCATTCTCCAGCAGGCCA	1,009
31A	2647	CCTTTGGTCCTTGTCTTCCTGGAT	
27S	2361	CTACCCAGGTCCTCGAGGAGTCAA	1,483
48A	3843	CACTGCACCAGGGTTTCCTATTCC	
46S	3631	CAGAAAGGTGATGAAGGTCCCAGA	1,183
62A	4813	CGTAGTCCACGTAGTTCTCGCCATT	
60S	4620	TCCAAAAGGTGCTAAGGGCTCCT	943
3′-UTR-A	5562	TGCTGAGGTACGAGGTTGCTCTC	
COL5A2:			
A	−78	CAGCTGACTTCATGGTGCTA	1,001
B	923	TCAAGACCTTTGTGTCCTCGG	
C	793	GAAGATGGTGAACCTGGCAGA	1,108
D	1900	CTCCAGGATTTCCTGCTTCTC	
E	1721	ATCCTGGTGTTCAAGGTCCTG	1,193
F	2913	ATCTTCTCCTGGGTCCCCTTT	
G	2677	AAAGGTGGTCGAGGAACCCAA	1,101
H	3777	GTTTTTGTCATCAGGAGCCGC	
I	3485	GTGCTGGAATCCCTGGACCAT	947
J	4431	CACAGGAGCAAGATCTATGAT	

[a] The primer sets 13–31, 27–48, 46–62, and 60–3′-UTR were previously published by Nicholls et al. (1996).

[b] For *COL5A1* and *COL5A2,* location is in reference to the A of the initiator methionyl ATG as nucleotide +1.

Results

A Single, Stable COL5A1 *Allele in Cells from Six Patients with EDS Type I or II*

Samples of genomic DNA from 16 individuals with EDS type I or II were analyzed for heterozygosity of expressed *COL5A1* markers. All but one individual were heterozygous for at least one marker, and six individuals were hemizygous in cDNA. Patients P8 and P11 were heterozygous for a *Pst*I polymorphic marker in exon 5 (fig. 1*A*). When cDNA amplified from exon 1 to exon 7—the interval that contained the single polymorphic *Pst*I site—was digested with the enzyme, in the sample from patient P8, the abundant species was the (+) allele, although there was a very small amount of the (−) allele present (fig. 1*A*). The cDNA sample from patient P11 contained only the (−) allele. Patients P4 and P6 were heterozygous for the *Dpn*II polymorphic site in exon 66 (fig. 1*B*). When cDNA amplified using primers in exon 65 and 66 was digested with the enzyme, only the (−) allele was stable from both patients (fig. 1*B*). Patients P1 and P10 were heterozygous for the *Bst*UI polymorphic site in exon 66 (fig. 1*C*). When cDNA amplified with the use of primers in exon 65 and 66 was digested with the enzyme, only the (−) allele was stable from both patients (fig. 1*C*).

Exon 15 Skip in a Rare Product in One Patient with EDS Type I

RNA isolated from all 16 individuals was used to synthesize cDNA, which was then amplified in six and five overlapping fragments for the *COL5A1* and the *COL5A2* coding sequences, respectively, and the products were separated on 6% polyacrylamide gels. This strategy can identify heteroduplexes formed when even very small amounts of mRNA with insertions or deletions are present (Schwarze et al. 1999). The product of the second and third fragments (exons 5–17 and 13–31, respectively) in the *COL5A1* mRNA from one patient (patient P16) had a small amount of an additional fragment that ran faster than the normal fragment (fig. 2). The sequence of the smaller fragment indicated that it was missing all of exon 15, and the genomic sequence showed an IVS14−11T→A transversion (cagacatta**a**cacaccatgtctccctagGGT, in which the boldfaced **a** at −21 is a potential branch point and the t at −11 is changed

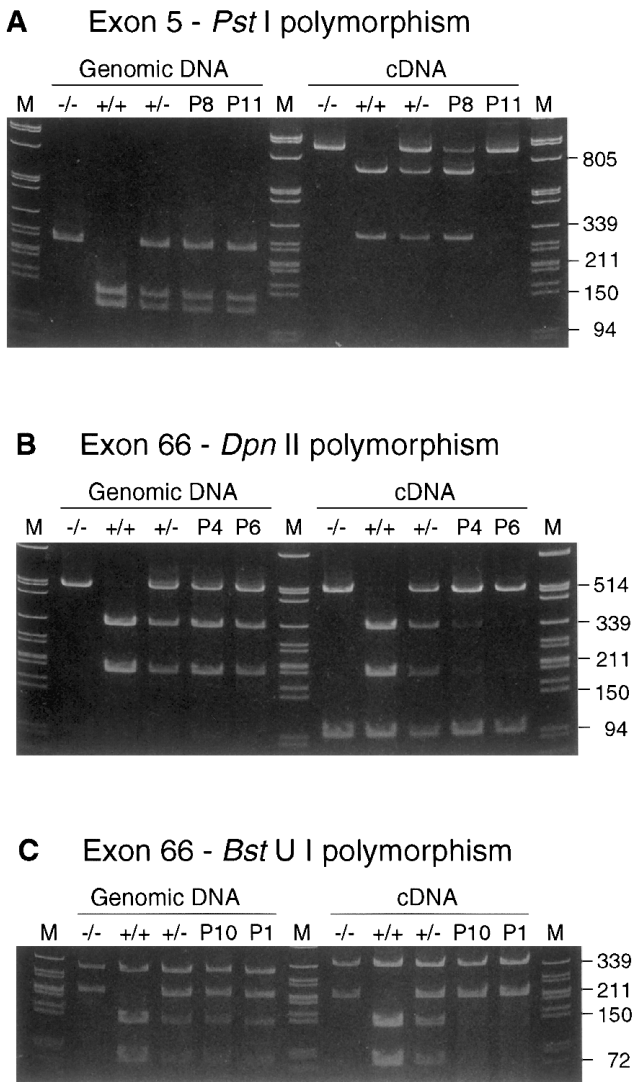

Figure 1 Demonstration of loss of heterozygosity, for one allele of the *COL5A1* gene, in fibroblasts from six patients with EDS type I or II. *A,* Heterozygosity for the exon 5 *Pst*I polymorphic site in the genomic DNA (*left*) of patients P8 and P11. In the cDNA from patient P8, a small amount of the (−) allele is apparent and is probably enhanced by heteroduplex formation. The cDNA from patient P11 contained only the (−) allele. *B,* Heterozygosity for the exon 66 *Dpn*II polymorphic site in the genomic DNA (*left*) of patients P4 and P6. In the cDNA (*right*) from patient P4, a small amount of the (+) allele was apparent, but none was apparent in the sample from patient P6. *C,* Heterozygosity for the exon 66 *Bst*UI polymorphic site in the genomic DNA (*left*) of patients P10 and P1. When cDNA amplified with the use of primers in exons 65 and 66 was digested with the enzyme, only the (−) allele was stable from both patients.

to a). This mutation does two things. First, it creates a run of three consecutive purines in the middle of the polypyrimidine tract of an already pyrimidine-poor stretch; this should decrease splicing efficiency. Second, it creates a new acceptor sequence that could be used in conjunction with a compatible branch point ~20 nucleotides upstream. If used, this acceptor site would add 9 nucleotides to the mRNA, in which the third codon would be TAG, which is a termination codon. We searched for this product in nuclear RNA, including RNA isolated after 6-h incubation with cycloheximide to inhibit nonsense-mediated decay; however, we were unable to identify it. Patient P16 was homozygous at all expressed polymorphic loci, so we could not determine (1) whether the low amount of product indicated that a second, highly unstable product was also formed or (2) whether the product of the mutant allele was usually normal and the exon-skipped product was in low abundance.

Identification of Sequence Variants That Led to Unstable mRNA

Because analysis of the cDNA in all 15 of the other patients failed to demonstrate heteroduplexes, we then analyzed genomic DNA from those individuals whose mature mRNA contained only one expressed allele. Exons 2–66 of the *COL5A1* gene were amplified from genomic DNA obtained from each of the patients in whom loss of heterozygosity could be demonstrated. Each fragment was screened using the MDE gel system, to detect heteroduplexes indicative of sequence variation between the two alleles. The products from each fragment that demonstrated variants were sequenced with use of the ABI 310 automated sequencer (PE Biosystems). There were five to eight variants per patient (data not shown). A frameshift mutation was identified in five of the six individuals in whom we could demonstrate loss of heterozygosity at the mRNA level. In each instance, a single-nucleotide insertion or deletion produced a shift in the reading frame and resulted in a downstream stop codon separated by eight or more introns from the constitutive termination codon (fig. 3). When cDNA was sequenced in these individuals, through the region of the

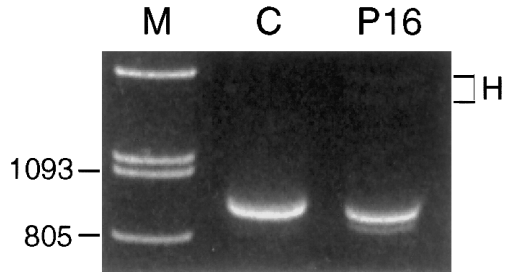

Figure 2 Amplification of the coding regions encompassed by exons 13–31 of the *COL5A1* gene, from a control individual and from one patient (patient P16). In the cDNA from patient P16, a lighter band is seen that migrates faster than the normal band; in addition, a faint heteroduplex (designated by "H") is also apparent.

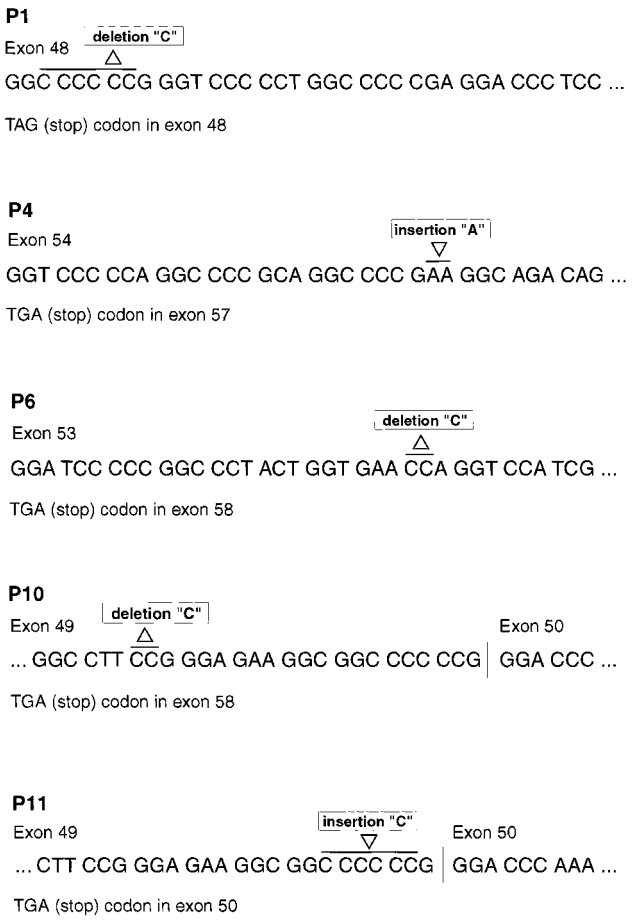

Figure 3 Mutations identified in genomic DNA from five patients in whom one *COL5A1* allele was not stable in mRNA.

genomic insertion or deletion, only the normal allele was identified (not shown).

In the genomic DNA from the sixth individual (patient P8), we found a single G→A transition at position +20, in intron 49 of one allele of the *COL5A1* gene. Although this transition changes a G in a GGG triplet that could function as an intron-splicing enhancer (McCullough and Berget 1997), we found the same change in one other individual who expressed both copies of the *COL5A1* gene in equal amounts. If the mutation changed the specificity of the splicing and permitted use of a downstream donor site, the use of the donor site embedded within the constitutive donor site would add four nucleotides to the mRNA and would result in a downstream stop codon. We searched for evidence of this product in nuclear and cytoplasmic RNA, both with and without the addition of cycloheximide to stabilize the abnormal product. We could not find any evidence of the product, and we observed no heteroduplexes after amplification of the cDNA in this region and separation of the products on polyacrylamide gels—a sensitive test for the presence of small amounts of product. Thus, we remain uncertain about the molecular basis for the absence of one allelic product in the sixth individual.

Alteration of Collagen-Fibril Architecture in Skin from an Individual with a COL5A1 *Null Mutation*

A skin-biopsy specimen from patient P10 was fixed in glutaraldehyde, embedded in Epon, sectioned, and examined by transmission electron microscopy. Fibrils were larger and less compact, and composite fibrils were apparent in the skin (fig. 4). These findings are characteristic of findings in the skin of individuals with EDS

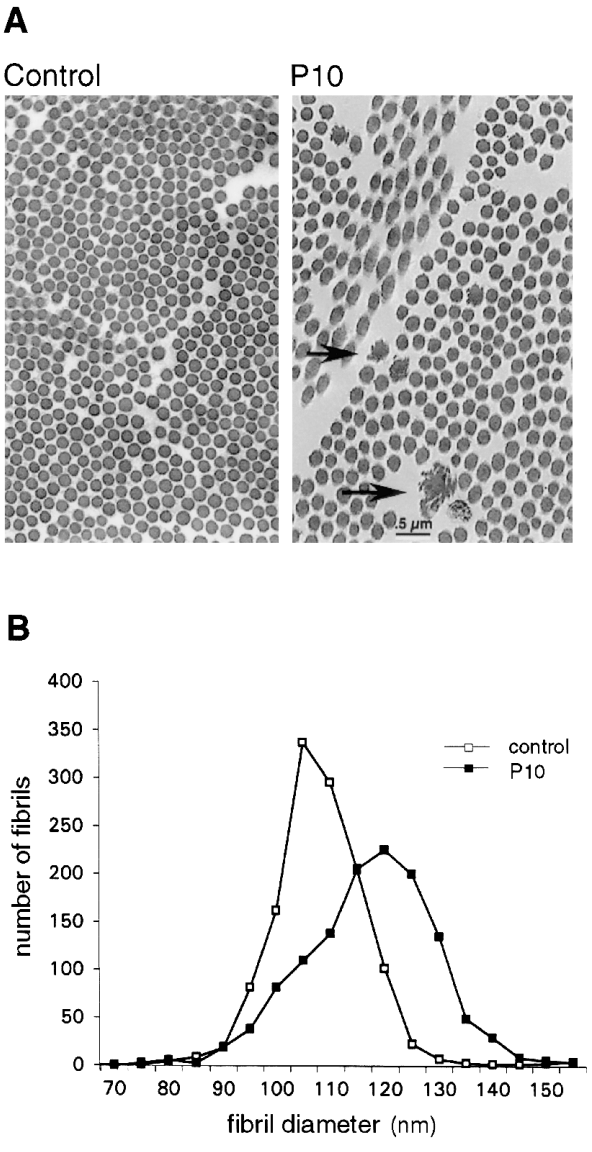

Figure 4 *A*, Electron micrograph of collagen fibrils in skin from patient P10 (*A*), compared with fibril diameters of a sample from a control individual (*B*). The fibrils from the patient are larger and more irregular. In addition, there are composite fibrils (*arrows*).

types I and II (Vogel et al. 1979; Holbrook and Byers 1989; Hausser and Anton-Lamprecht 1994). Similar structures have been identified in skin from individuals with EDS type I or II and exon-skipping mutations in *COL5A1* (Burrows et al. 1998) and *COL5A2* (Nicholls et al. 1996; Michalickova et al. 1998), or a mutation in the carboxyl-terminal propeptide of the proα1(I) chain encoded by *COL5A1* (De Paepe et al. 1997).

Discussion

Mutation analysis in individuals with EDS types I and II has been frustratingly unproductive, despite the finding that many families appear likely to have mutations in either the *COL5A1* or *COL5A2* gene. Analysis of the expressed coding sequences of these genes has identified mutations in the minority of cases—that is, in probably <20% of patients, overall, from several studies. Until now, it has not been clear whether failed detection was a consequence of analysis of the wrong gene, imperfect technique, or failure of allele expression or stability. In the present study, we have demonstrated that a significant proportion of individuals with the EDS type I and II phenotypes have premature-termination codons within the coding sequence of the *COL5A1* gene. These mutations lead to mRNA instability, presumably by means of the mechanism of nonsense-mediated mRNA decay (Maquat 1995; Frischmeyer and Dietz 1999).

We identified mutations in genomic DNA from five of the six cell strains in which we could demonstrate that the product of one *COL5A1* allele was absent from mRNA. Despite amplification of and a search for variants in all the exons of patient P8, we were able to find only one possibly relevant variation: a G→A transition in intron 49 in the first of several GGG motifs downstream from the donor site. Although mutations in such motifs can lead to aberrant splicing (McCullough and Berget 1997; McCarthy and Phillips 1998), this did not appear to occur in this instance, since we identified the same alteration in another individual who expressed both *COL5A1* alleles in equal abundance.

It is possible that promoter mutations in *COL5A1* could alter allele expression, although such mutations are uncommon causes of human disorders. We have not completed screening of the coding sequences of the *COL5A1* and *COL5A2* genes, nor have we identified, in *COL5A2,* useful polymorphic variants with which to assess allele expression and stability. Although the majority of causative mutations appear to be in these two genes, we cannot yet define the precise proportion that could be in other genes. It is clear, nonetheless, that *COL5A1* null alleles significantly contribute to the molecular etiology of EDS types I and II.

Throughout vertebrate tissues, type V collagen is widely distributed in the form of an $\alpha1(V)_2\alpha2(V)$ heterotrimeric molecule. Other reported forms of type V collagen include an $\alpha1(V)_3$ homotrimer that is produced by a line of hamster cells (Haralson et al. 1980) and that may also exist in normal tissues (Kumamoto and Fessler 1980; Moradi-Ameli et al. 1994) and an $\alpha1(V)/\alpha2(V)/\alpha3(V)$ heterotrimer that is isolated from human placenta and that contains the poorly characterized $\alpha3(V)$ chain (Rhodes and Miller 1981; Niyibizi et al. 1984). The $\alpha1(V)_2\alpha2(V)$ heterotrimer is synthesized by cultured dermal fibroblasts at relatively low levels (compared with levels for collagen types I and III), as a precursor molecule containing two pro$\alpha1(V)$ chains and a single pro$\alpha2(V)$ chain, which are encoded by *COL5A1* and *COL5A2,* respectively. The pro$\alpha1(V)$ and pro$\alpha2(V)$ chains have amino- and carboxyl-terminal propeptides that are proteolytically processed to yield the mature $\alpha1(V)_2\alpha2(V)$ molecule. While it is uncertain whether fibrils composed solely of type V molecules are formed in vertebrate tissues (Fessler and Fessler 1987), it is clear that $\alpha1(V)_2\alpha2(V)$ molecules can combine with the much more abundant type I collagen to form heterotypic fibrils (Birk et al. 1988; Linsenmayer et al. 1993)—that is, fibrils that contain more than one type of collagen, typical of those in skin. The results of in vitro fibrillogenesis experiments, in which the amount of type V collagen is varied, show an inverse correlation between the diameter of heterotypic fibrils of type I and V collagens and the relative amount of type V collagen. In other words, the greater the relative amount of type V collagen, compared with that of type I collagen, the smaller the fibril (Linsenmayer et al. 1993). The results of such experiments have led to the suggestion that at least one function of $\alpha1(V)_2\alpha2(V)$ heterotrimers is to regulate the size and shape of heterotypic fibrils.

In contrast to the mature chains of collagen types I and III, the mature $\alpha1(V)$ chain of the $\alpha1(V)_2\alpha2(V)$ collagen molecule retains a significant portion—several hundred amino acids in length—of the large amino-terminal peptide of the pro$\alpha1(V)$ precursor (Linsenmayer et al. 1993). The results of analyses of heterotypic fibrils indicate that type V collagen is located throughout the fibril (Birk et al. 1988). However, the amino-terminal–extended structure of the $\alpha1(V)$ chain appears to protrude beyond the surface of the heterotypic fibrils, where it has been proposed to regulate the diameter of the fibril by hindering further addition of type I collagen monomers (Linsenmayer et al. 1993). The latter effect may be the result of steric hindrance, or it may involve the highly charged nature of the residual $\alpha1(V)$ amino-terminal sequence, which contains a number of sulfated tyrosines (Fessler and Fessler 1987). The $\alpha1(V)$ amino-terminal sequence, which projects onto the surface of fibrils, may also normally serve to prevent the aggregation of adjacent fibrils into the types of composite

fibrils that are seen in cases of classical EDS and that are shown here for patient P10 (see fig. 4).

On the basis of the postulated role of the α1(V) chain in the control of the diameter and the shape of heterotypic fibrils, the mode whereby functional haploinsufficiency for *COL5A1* leads to the outsized and aggregated dermal collagen fibrils in patients with EDS is conceptually simple. However, mutant α1(V) and α2(V) chains that have either exon skipping or glycine substitutions have dominant-negative effects that result in abnormal dermal collagen fibrils and classical EDS, by means of less-apparent mechanisms (De Paepe et al. 1997; Burrows et al. 1998; Michalickova et al. 1998; Richards et al. 1998). In either case, the presence of abnormal heterotypic fibrils correlates with marked changes in the mechanical properties of many tissues in which type V collagen genes are expressed. It is unclear whether these altered mechanical characteristics reflect changes in intermolecular cross-links or more global alterations that are a response to variations in the amount or structure of the relatively minor extracellular component, type V collagen. The results presented in this study indicate that more than one-third of cases of classical EDS are likely to result from *COL5A1* null alleles.

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