Am. J. Hum. Genet. 66:1766–1776, 2000

COL5A1 Haploinsufficiency Is a Common Molecular Mechanism Underlying the Classical Form of EDS

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We have identified haploinsufficiency of the COL5A1 gene that encodes the proα1(V) chain of type V collagen in the classical form of the Ehlers-Danlos syndrome (EDS), a heritable connective-tissue disorder that severely alters the collagen-fibrillar structure of the dermis, joints, eyes, and blood vessels. Eight of 28 probands with classical EDS who were heterozygous for expressed polymorphisms in COL5A1 showed complete or nearly complete loss of expression of one COL5A1 allele. Reduced levels of proα1(V) mRNA relative to the levels of another type V collagen mRNA, proα2(V), were also observed in the cultured fibroblasts from EDS probands. Products of the two COL5A1 alleles were approximately equal after the addition of cycloheximide to the fibroblast cultures. After harvesting of mRNAs from cycloheximide-treated cultured fibroblasts, heteroduplex analysis of overlapping reverse transcriptase–PCR segments spanning the complete proα1(V) cDNA showed anomalies in four of the eight probands that led to identification of causative mutations, and, in the remaining four probands, targeting of CGA→TGA mutations in genomic DNA revealed a premature stop at codon in one of them. We estimate that approximately one-third of individuals with classical EDS have mutations of COL5A1 that result in haploinsufficiency. These findings indicate that the normal formation of the heterotypic collagen fibrils that contain types I, III, and V collagen requires the expression of both COL5A1 alleles.

Introduction

The Ehlers-Danlos syndromes (EDSs) are a group of inherited disorders with common characteristics of joint laxity and varying degrees of dermal fragility (Barabas 1967; Beighton 1992; Steinmann et al. 1993; Byers 1995). The classical form of Ehlers-Danlos syndrome (types I [MIM 130000] and II [130010]) is characterized by joint laxity, fragile and hyperextensible skin, poor wound healing, and autosomal dominant inheritance. Dermal scars after trauma are thinned and atrophic and may stretch considerably after primary repair. Approximately half the individuals affected with classical EDS have a history of premature birth due to rupture of fetal membranes.

Ultrastructural studies of the skin in patients with classical EDS show evidence of abnormal fibrillogenesis of the heterotypic collagen fibrils that contain types I, III, and V collagens (Vogel et al. 1979). In these studies, the collagen fibrils have a mean diameter increased by 13%–40% and show a higher degree of variability in width and shape than is seen in control collagen fibrils. Approximately 5% of the fibrils, referred to as "cauliflowers," are five to six times wider than normal and are highly irregular in shape (Hausser and Anton-Lamprecht 1994).

Linkage studies have excluded type I collagen genes in several pedigrees with classical EDS (Sokolov et al. 1991; Wordsworth et al. 1991). However, mutations resulting in classical EDS have been identified in two of the genes that code for type V collagen—COL5A1 (Toriello et al. 1996; Nicholls et al. 1996; Wenstrup et al. 1996; De Paepe et al. 1997; Burrows et al. 1998; Giunta and Steinmann 2000) and COL5A2 (Michalickova et al. 1998; Richards et al. 1998)—in a few families. Deletion of exon 6 from the mouse col5a2 gene, which removes the N-proteinase cleavage site, also produces a phenotype that resembles classical EDS (Andrikopoulos et al. 1995). The COL5A1 gene encodes the proα1(V) chain, and the COL5A2 gene encodes the proα2(V) chain of type V collagen. The COL5A3 gene, which, to date, has not been linked to any disease phenotype (D. Greenspan, personal communication) encodes the proα3(V) collagen gene (Imamura et al. 2000).

Type V collagen was first identified in human placenta but subsequently was found in virtually all tissues where type I collagen is expressed. There are several *isoforms* of type V collagen that differ in the type and ratios of

Received February 17, 2000; accepted for publication April 10, 2000; electronically published April 24, 2000.

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0002-9297/2000/6606-0006$02.00

constituent chains. The most abundant and most widely distributed isoform is $\alpha1(V)_2\alpha2(V)$ (Sage et al. 1981); an $\alpha1(V)_3$ homotrimer has been reported and may be most abundant in fetal tissues (Moradi-Ameli et al. 1994). Type V collagens containing $\alpha3(V)$ chains—$\alpha1(V)\alpha2(V)\alpha3(V)$—are present in uterine (Abedin et al. 1981), placental (Rhodes and Miller 1981), skin (Brown et al. 1978; Brown and Weiss 1979; Woodley et al. 1987), and synovial tissue (Brown and Weiss 1979; Sage and Bornstein 1979; Ashhurst et al. 1991) but not in bone (Niyibizi and Eyre 1989), placental membranes (Rhodes and Miller 1981) or corneal fibroblasts (Poschl and von der Mark 1980). Type V collagen chains also form heterotypic molecules with type XI–collagen chains. Trimers with chain composition $\alpha1(XI)_2\alpha2(V)$ have been identified in bovine vitreous humor (Mayne et al. 1993), $\alpha1(XI)$ and $\alpha2(V)$ chains are coexpressed in noncartilagenous tissues of developing mice (Andrikopoulos et al. 1992; Lui et al. 1995; Yoshioka et al. 1995*a*, 1995*b;* Sponseller et al. 1997; Moursi et al. 1997), and measurements of individual chain ratios indicate that the highly homologous $\alpha1(V)$ and $\alpha1(XI)$ chains may coform an $\alpha1(V)\alpha1(XI)\alpha2(V)$ trimer in bone and cartilage (Niyibizi and Eyre 1989). Thus, $\alpha1(V)$ collagen chains are present in a complex pattern of stoichiometric ratios in several tissues, each of which may have differing capacities for dosage compensation when the number of available pro$\alpha1(V)$ chains is reduced.

Several studies have reported a low yield of COL5A1 and COL5A2 mutations in families and individuals with classical EDS (De Paepe and Nuytinck 1998; Michalickova et al. 1998). One reason for the latter observation is that another locus for classical EDS is likely to be present in families that are discordant for linkage to COL5A1 and COL5A2 (Greenspan et al. 1995; Wenstrup et al. 1996). Another reason is that haploinsufficiency of COL5A1 or COL5A2 may be present but not detectable by the cDNA-based mutation-detection methods used in the studies reported thus far. Several lines of evidence suggest that haploinsufficiency of COL5A1 can produce classical EDS. First, gross interruption of the COL5A1 gene, reported in one case, produced classical EDS (Toriello et al. 1996). Second, the reported primary structure changes in the carboxyl-terminal propeptide of the pro-$\alpha1(V)$ chain in several families with classical EDS were likely to prevent the mutant chains from being included in trimeric type V collagen molecules (Wenstrup et al. 1996; De Paepe et al. 1997).

In the present study, we show that haploinsufficiency of COL5A1 is a common cause of classical EDS. Approximately 30% of individuals who were informative for polymorphisms in the 3′-untranslated region (3′ UTR) had only one of the two COL5A1 alleles represented in cDNA prepared from their cultured dermal fibroblasts. The amount of mRNA derived from the underrepresented allele was significantly elevated by the addition of cycloheximide to the cultures. The cycloheximide treatment enabled mutation analyses to be undertaken on amplified cDNA products that contained approximately equal amounts of the two allelic products. Analyses of cDNA and genomic DNA identified five individuals who were heterozygous for COL5A1 null mutations.

Subjects, Material, and Methods

Clinical Material

The study population of 59 probands fulfilled the clinical criteria either for EDS types I or II, according to the 1988 Berlin Classification (Beighton et al. 1988), or for the classical form, according to the 1997 Villefranche Nosology conference (Beighton et al. 1998). In brief, all individuals had generalized joint hypermobility, hyperextensible dermis, widened and atrophic scars, and a family history consistent with autosomal dominant transmission or new dominant mutation. Included in the clinical material were seven pedigrees that contained at least five matings. Cultured dermal fibroblasts were obtained from at least one affected individual, and genomic DNA was obtained from other family members.

Null-Allele Detection

Expression of the COL5A1 alleles was determined by use of the 3′ UTR *Bst*UI and *Dpn*II polymorphisms and methods described by Greenspan and Pasquinelli (1994). Passaged fibroblasts (12,700 cells/cm^2) were grown to confluence in Dulbecco's modified Eagle Medium containing 10% fetal calf serum, 2 mM L-glutamine (BDH Chemicals), 1 × antibiotic/mycotic (Gibco BRL), and 50 μg ascorbate/ml. Total cellular RNA was extracted and cDNA was prepared by standard methods, except that first-strand cDNA synthesis was performed in the presence of 10% dimethyl sulfoxide.

RFLP analysis with the endonucleases *Bst*UI and *Dpn*II was performed as described by Greenspan and Pasquinelli (1994). The analysis was performed initially on genomic DNA, to determine the genotype of each patient with EDS, and subsequently on cDNA. The digestion products were resolved on nondenaturing 10% PAGE (Bio-rad) gels or 3% agarose (Nusieve) gels, visualized by ethidium-bromide staining and photographed by means of Polaroid Polapan 667 film.

When one COL5A1 transcript was absent or substantially reduced, cycloheximide (Sigma Chemical) was added to fibroblast cultures at 1 mg/ml of medium for 4 h, to stabilize mRNA (Carter et al. 1995). Total cellular RNA was extracted, and *Bst*UI and *Dpn*II RFLPs were determined on the basis of reverse transcriptase–PCR (RT-PCR) products, by the methods described above.

Quantitation of mRNA

Quantitation of the relative amounts of the proα1(V) and proα2(V) mRNA produced by cultured dermal fibroblasts was undertaken by the ribonuclease-protection assay (RPA) (RPA III; Ambion Scientific). Cycloheximide was not added to these cultures. RPA probes were originated by PCR amplification of first-strand cDNA, and in vitro transcription of these amplified PCR fragments incorporated, at its 3′ end, the sequence of the T7 phage polymerase promoter. GADPH mRNA was used as an internal reference. The primer pairs for the probes were α1(V), (5′-GAATGGCGAGAACTACGTGGAC-3′) and (5′-CGCCTAATACGACTCACTATAGGGAGGGAGCATCCTTGGTTAG-3′); α2(V), (5′-CGGACCCAGGGGTTCATGC-3′) and (5′-GGATGCTAATACGACTCACTATAGGGAGGCGTGGTACACTGGATGGG-3′); and GAPDH, (5′-GGTCGTATTGGGCGCCTGGTCACCAGGGCT-3′) and (5′-CCCAGTGATGGCATGGATGTGGTCATGAG-3′).

Amplified DNA either was used directly in the transcription reaction or was concentrated before use by ethanol precipitation and gel purification. Amplified DNA (40–50 ng) was transcribed by T7 RNA polymerase in the presence of ^{32}[P]-UTP, by reagents for in vitro transcription (Ambion Scientific). Total cellular RNA (2.5 or 5 μg) and 2 fmol of each labeled probe were used for each assay, according to the manufacturer's instructions. Hybridization was performed at 56°C for 18 h and was followed by RNase digestion, using a 1:100 dilution of RNase A/T1, at 37°C for 30 min. Protected fragments were separated on a 5% polyacrylamide/8 M urea gel and were detected by exposure either to X-omat (Kodak) for 18 h at −80°C with an intensifying screen or to a phosphor screen (Molecular Dynamics). Films were scanned and evaluated by the Pharmacia GSXL system. Phosphor images were scanned in the STORM820 system and were evaluated by ImageQuant software.

Mutation Analysis

Heteroduplex analyses of overlapping proα1(V) cDNA PCR products prepared from cycloheximide-supplemented cultures were performed (Korkko et al. 1998). Targeted analyses of genomic DNA, for point mutations of Arg (CGA) codons to premature-termination codons (TGA) and for deletions or insertions in CCCCCT sequences, also was performed.

Seven overlapping PCR products spanning the α1(V) cDNA were amplified from patient and control cDNAs prepared from cycloheximide-treated fibroblast cultures (fig. 4*A*). The sequences of the primers were selected on the basis of the published α1(V) cDNA sequence (Greenspan et al. 1991) (GenBank accession number M76729). The PCR products (5 μl) were analyzed for size variants and heteroduplexes, by electrophoresis on a nondenaturing 5% polyacrylamide gel (Bio-Rad). The products were visualized by ethidium bromide staining and were photographed by means of Polaroid Polapan 667 film.

Mutations involving conversion of the Arg codon CGA in COL5A1 exons 2, 16, 27, 33, 40, 43, 48, and 55 to the premature-translational termination codon TGA were sought after genomic PCR amplification using primers corresponding to published exon-flanking sequences (Takahara et al. 1995). Point mutations of CGA to TGA were sought in exons 40, 43, and 48 after digestion of the purified PCR products with *Dde*I, which recognizes the CTNAG sequence. The Arg mutation was sought in exons 16, 27, and 55 after digestion with *Taq*I, which recognizes the TCGA sequence. The digestion products were resolved on nondenaturing 10% PAGE. Exons containing abnormal digestion products were sequenced. Direct sequencing of exons 2 and 33 was undertaken to detect the mutation, since these exons lacked suitable restriction sites.

Single-base insertions or deletions in CCCCCT sequences also were targeted. Forward-and-reverse primer pairs were designed to amplify exons 12, 15, 30, 36, 39, 41, 43, 46, 48, 50, 52, and 59 which contained the CCCCCT sequence, and PCR amplification was performed as described above. Mutation analysis of the targeted CCCCCT sequences was performed by sequencing of the gel-purified PCR products.

DNA Sequencing

The polymorphic regions of the 3′ UTR region of 1(V) cDNA and COL5A1 were sequenced after fibroblasts were cultured in the absence and presence of cycloheximide. The 1(V) cDNA PCR products showing size variants, as well as their corresponding COL5A1 exons and exon/intron junctions, were sequenced in patients EDS8, EDS43, EDS56, and EDS82. Similarly, exon 27, which showed an abnormal *Taq*I restriction pattern, consistent with a CGA→TGA mutation, was sequenced in patient EDS53. The primers were designed on the basis of the published DNA sequence (Greenspan et al. 1991; Takahara et al. 1991, 1995). Gel-purified cDNA (150 ng) or genomic DNA (200 ng) PCR products were used for fluorescent-dye terminator cycle sequencing with a Thermo Sequenase Cy 5 Dye Terminator Kit and a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Results

Null COL5A1 Alleles

Of 59 probands with classical EDS, 30 (51%) were heterozygous for the *Bst*UI polymorphism and 24 (40%) were heterozygous for the *Dpn*II polymorphism (fig. 1). Overall, 61% of individuals were heterozygous for one

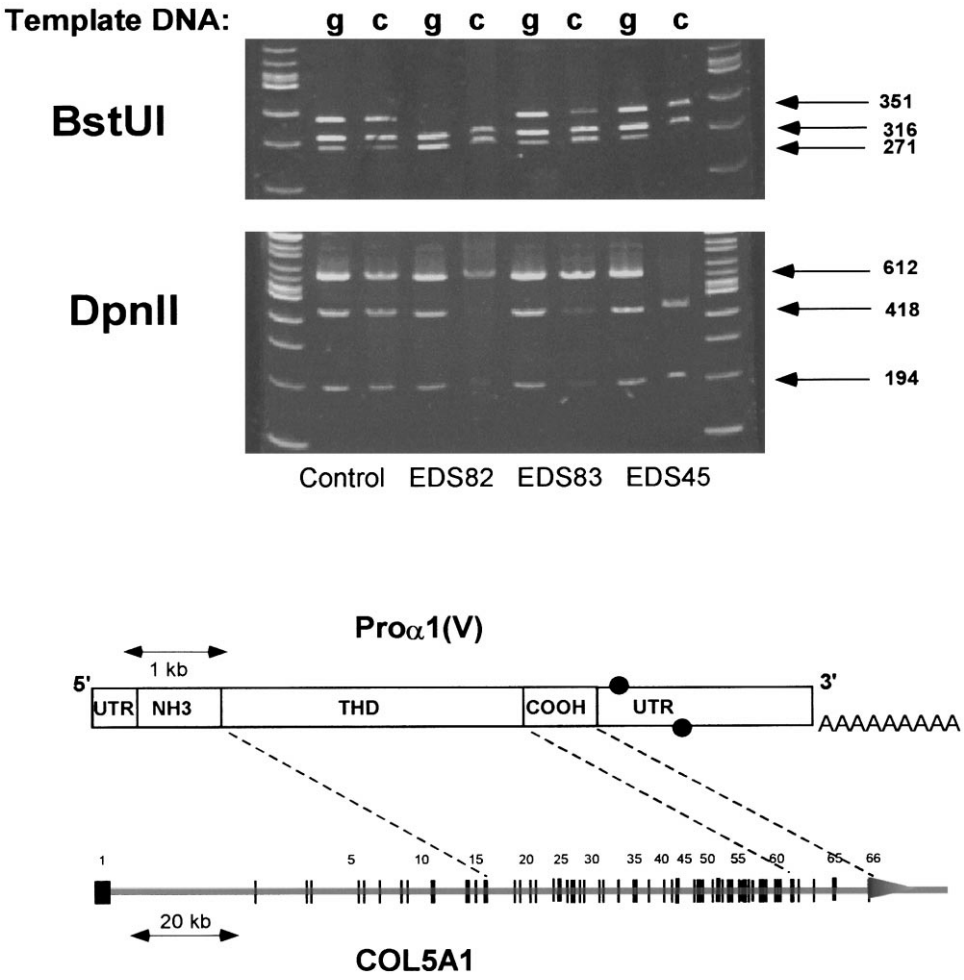

Figure 1 Detection of COL5A1 haploinsufficiency. *Top*,Representative *Bst*UI (*upper gel*) and *Dpn*II (*lower gel*) digestions of PCR-amplified genomic (lanes g) or cDNA (lanes c) segment from the 3′ UTR of COL5A1 in a control and three probands with classical EDS. Numbers on the right show the sizes of the predicted restriction fragments. EDS82 is +/+ for the *Bst*UI site, resulting in 316-bp and 271-bp fragments after *Bst*UI digestion, and is +/− for the *Dpn*II polymorphism; there is loss of the *Dpn*II+ allele in amplified cDNA, with loss of the 418-bp and 194-bp fragments. EDS83 is −/− for the *Bst*UI site and +/− for the *Dpn*II site, with loss of the *Dpn*II+ allele. EDS45 is *Bst*UI +/− and *Dpn*II +/− and shows loss of the *Bst*UI+/*Dpn*II− allele. *Bottom,* cDNA and genomic map of COL5A1, showing locations of PCR primers (*blackened circles*) in the 3′ UTR that bracket the *Dpn*II and *Bst*UI polymorphic sites.

or both polymorphisms, in agreement with the heterozygosity frequencies reported by Greenspan and Pasquenelli (1994). Of 28 heterozygous cell strains for which both genomic DNA and cultured cells were available, 8 (EDS8, EDS39, EDS45, EDS53, EDS56, EDS81, EDS82, and EDS83) showed complete or nearly complete loss of an *Bst*UI and/or *Dpn*II allele (fig. 1). In each case, the RFLP band missing after *Bst*UI or *Dpn*II digestion of RT-PCR products was restored after incubation of fibroblasts with cycloheximide (fig. 2).

The proα1(V):proα2(V) mRNA ratios were measured by RPA, to confirm the reduction of proα1(V) mRNA levels in EDS fibroblasts showing complete or near-complete loss of one COL5A1 allelic product. The ratios were determined in 12 control fibroblast cell lines, 7 classical EDS cell lines with RFLP evidence of haploinsufficiency, 14 classical EDS cell lines informative for either RFLP but without haploinsufficiency, and 12 classical EDS cell lines homozygous for both RFLPs and therefore uninformative for loss of a COL5A1 allele (fig. 3). As a group, control cells and classical EDS cell strains in which haploinsufficiency was excluded had a similar range of proα1(V):proα2(V) ratios (0.59–1.11). EDS cells in which RT-PCR analysis showed haploinsufficiency had proα1(V):proα2(V) ratios of 0.32–0.53. A group of EDS cell lines that were uninformative for the *Bst*UI and *Dpn*II polymorphisms, which were likely to be heterogeneous with respect to COL5A1 haploinsufficiency, exhibited a range of proα1(V):proα2(V) ratios that spanned the values of the known COL5A1-deficient

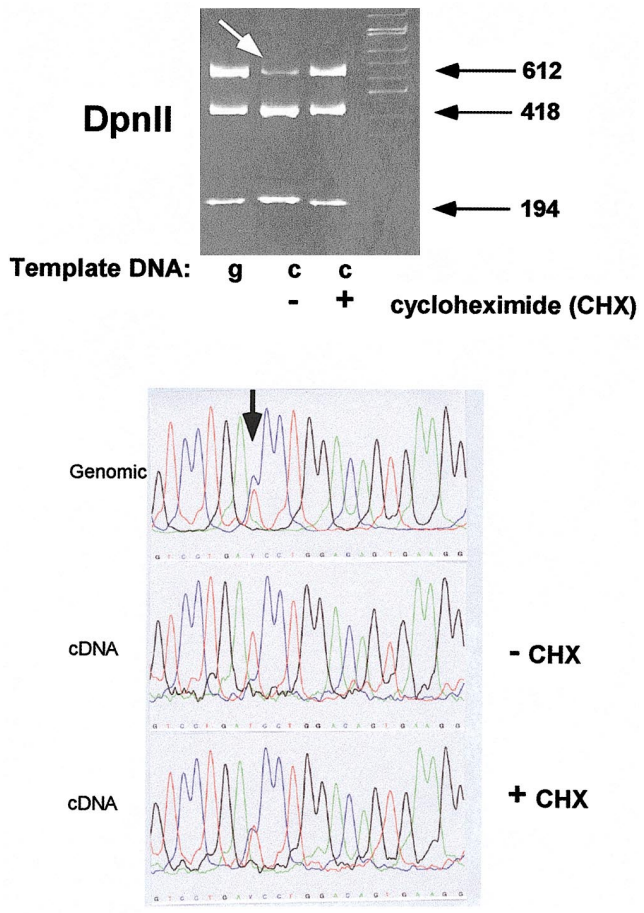

Figure 2 Restoration of mRNAs from mutant COL5A1 alleles, after incubation of cultured classical EDS fibroblasts with cycloheximide (CHX). *Top, Dpn*II digestion of PCR-amplified genomic (lane g) DNA and RT-PCR (lanes c) products, with (+) and without (−) CHX from proband EDS43. The *Dpn*II− allele is significantly reduced in the CHX− lane (*white arrow*) and is restored in the CHX+ lane. *Bottom,* DNA sequence of *Dpn*II site in 3′ UTR from proband EDS43. The arrow points to the polymorphic C/T site present in genomic DNA of proband EDS43; only T is present in the CHX− RT-PCR products, and C/T heterozygosity is restored in CHX+ cells.

and -nondeficient cell lines (0.38–0.97). Measured proα1(V):proα2(V) ratios generated by RPA do not necessarily reflect molar ratios of proα1(V):proα2(V) mRNAs, which depend on the efficiency of labeling for each probe and on the efficiency with which each probe protects the complementary RNA segment.

Characterization of Mutations Leading to Haploinsufficiency

Mutation analysis was undertaken in eight classical EDS cell strains with apparent loss of one COL5A1 allelic product and reduced proα1(V):proα2(V) mRNA ratios. The cultures were treated with 1 mg cycloheximide/ml, for 4 h prior to harvest. All eight EDS cell lines with apparent haploinsufficiency in the absence of cycloheximide showed the two COL5A1 alleles in approximately equal amounts after cycloheximide treatment (fig. 2). The proα1(V) mRNAs stabilized by cycloheximide were utilized for mutation detection.

Seven overlapping PCR-amplified cDNA fragments, spanning the entire 5,514-bp coding sequence of proα1(V) cDNA, were examined for apparent alterations in molecular weight after PAGE was performed fig. 4*A*). Abnormally sized PCR products were observed in four patients (fig. 4*B*). In proband EDS82, a $G^{+1}\rightarrow T$ transversion resulted in an in-frame exclusion of all 168 bp encoded by exon 2 (table 1). The abnormal 580-bp PCR product shown in figure 4*B* lacks the 168 bp encoded by exon 2. In proband EDS43, a $G^{+1}\rightarrow A$ transition in intron 28 resulted in the inclusion of the 5′ 178 bp of intron 28, which caused a frameshift and premature stop codon. The 178-bp sequence immediately preceded a GT dinucleotide that provided an alternative splice-donor site in intron 28. The abnormal cDNA PCR product of 500 bp shown in figure 4*B* was found, by sequence analysis, to contain the 178-bp insertion. In proband EDS8, a deletion of a GA dinucleotide in exon 47 (nucleotides [nt] 3957–3958 or 3959–3960 of the cDNA sequence) resulted in a frameshift and a premature stop codon. In proband EDS56, an insertion of a TG dinucleotide in exon 52 resulted in a frameshift and a premature stop codon. Electrophoretic analysis of the cDNA segments containing splice-junction mutations (EDS82 and EDS43) contain higher-molecular-weight fragments (30 kb and 1.7 kb, respectively) that are pre-

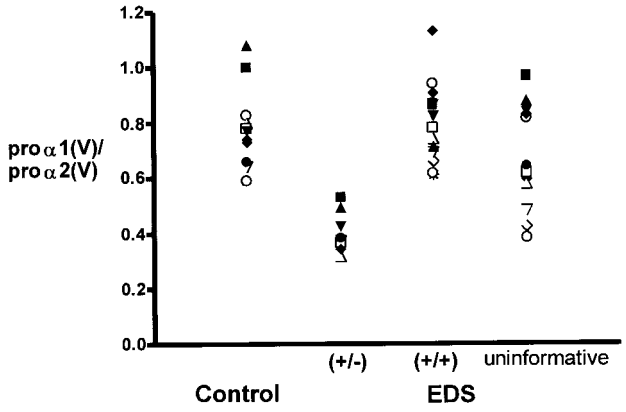

Figure 3 Histogram showing scanned proα1(V):proα2(V) ratios in 12 control fibroblast cell lines; in 7 classical EDS fibroblast cell lines with evidence of haploinsufficiency (+/−); in 14 classical EDS fibroblast cell lines informative for either *Bst*UI or *Dpn*II, without loss of a COL5A1 allele (+/+); and in 12 uninformative classical EDS fibroblast cell lines.

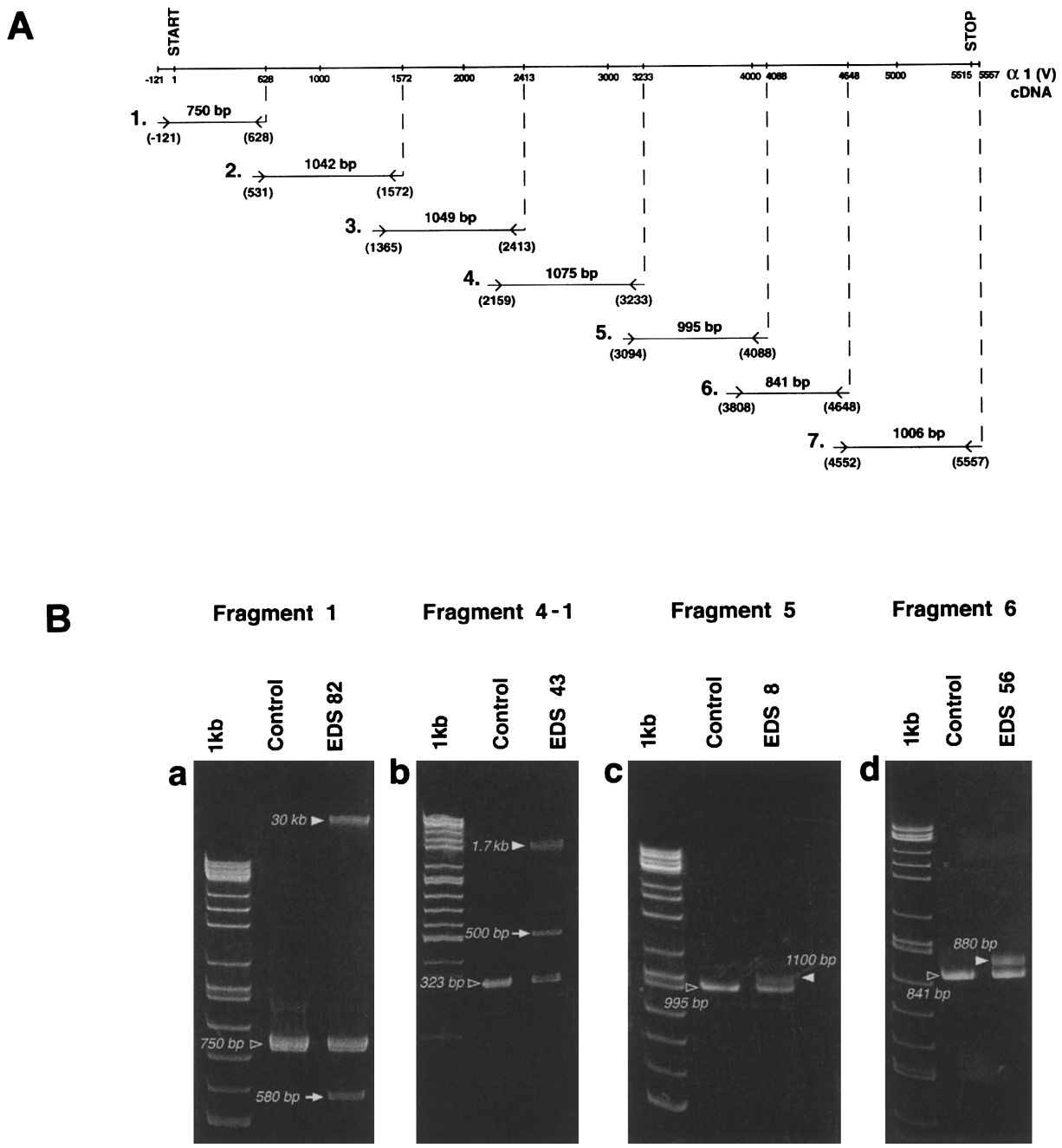

Figure 4 Heteroduplex analysis of proα1(V) cDNA. *A,* Seven overlapping fragments, labeled "1"–" 7," that span the entire proαa1(V) cDNA were amplified from patient cDNA by means of the corresponding seven sets of primers. Nucleotide positioning of primers was according to Greenspan et al. (1991). Fragment 1, 5′ UTR exon 4 (signal peptide to N propeptide.); fragment 2, exons 4–15 (N propeptide to helix); fragment 3, exons 9–28 (helix); fragment 4, exons 23–41 (helix.); fragment 5, exons 39–52 (helix); fragment 6, exons 48–60 (helix); fragment 7, exon 66 3′ UTR (helix to stop). *B,* PAGE of molecular-weight variants and heteroduplexes, after PCR of α1(V) cDNA fragments prepared from cultured fibroblasts exposed to cycloheximide. Each panel includes a GeneRuler 1-kb DNA ladder (MBI Fermantas), as well as control and patient PCR products, after 5% PAGE and ethidium bromide staining. Panel a, Patient EDS82. In addition to the expected, 750-bp PCR product, there were migrating fragments that had apparent sizes of 30 kb and 580 bp. Panel b, Patient EDS43. Nested PCR products of fragment 4 showed products that had apparent sizes of 1.7 kb and 500 bp, in addition to the expected, 323-bp fragment size. Panel c, Patient EDS8. This patient had both the expected, 995-bp fragment size and another fragment with an apparent size of 1,100 bp. Panel d, Patient EDS56. This patient had both the expected, 841-bp product and an unexpected product with an apparent size of 880 bp.

sumed to be heteroduplexes that contain one strand containing normal sequence and another strand containing the abnormal splice form. Sequence analysis of the products of multiple cDNA PCR reactions did not provide evidence of other splicing products in either proband EDS43 or proband EDS82.

In the eight cell strains with genetic evidence of haploinsufficiency, CGA codons in exons 2, 16, 27, 33, 40, 43, 48, and 55 were examined for C→T transitions that lead to Arg→premature-stop-codon mutations. In proband EDS53, a *Taq*I restriction site was lost because of a C→T transition, in exon 27, that created a stop at codon 792 (not shown). The exons containing the CCCCCT sequence (exons 12, 15, 30, 36, 39, 41, 43, 46, 48, 50, 52, and 59), which is reported to predispose to single-nucleotide insertions or deletions in the homologous COL1A1 and COL2A1 genes, were amplified and sequenced. No mutations of this type were identified.

Of the eight probands with classical EDS with complete or nearly complete haploinsufficiency of COL5A1, the mutations were localized and sequenced in only five cases and were not localized or sequenced in the remaining three cases. Two probands (EDS43 and EDS56) were members of pedigrees in which classical EDS segregated as an autosomal dominant trait (fig. 5) The G^{+1}→A transition identified in proband EDS43 also was identified in the eight other affected family members. Proband EDS56 is a member of a pedigree in which classical EDS has been shown to cosegregate with one COL5A1 allele (Wenstrup et al. 1996). The GT-dinucleotide insertion in exon 52 was identified in two other affected family members and was absent in an unaffected family member.

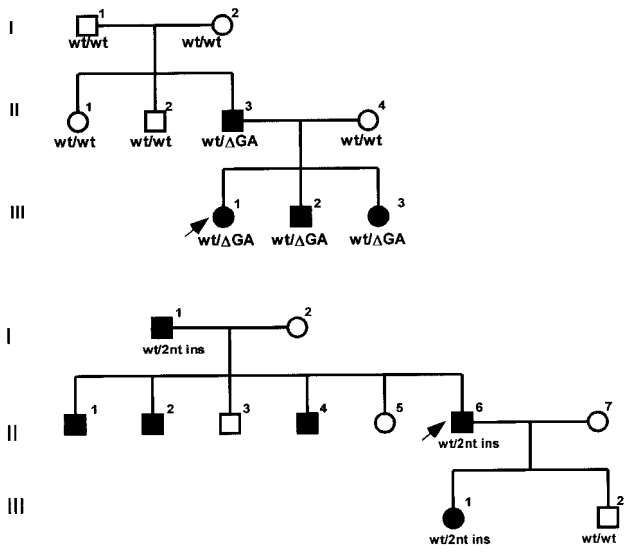

Figure 5 Cosegregation of COL5A1 mutations and EDS in pedigrees. *Top,* G^{+1}→A substitution found in EDS43 (*arrow*), which is present in affected individuals but not in unaffected individuals. *Bottom,* 2-nt insertion at position 4332, found in patient EDS56 (*arrow*) and also present in I-1 and III-1 but absent in III-2.

Discussion

This report has summarized investigations into the prevalence of COL5A1 haploinsufficiency in a series of 59 probands with classical EDS. Classical EDS represents a combination of the gravis (EDS I) and the mitis (EDS II) forms of EDS, from earlier classifications, forms that were grouped together because they can be allelic disorders and because both phenotypes can occur as a range of phenotypic variation within a single family (Beighton et al. 1988; Burrows et al. 1996).

Approximately 30% of individuals who were heterozygous for either of two RFLPs expressed in the 3′ UTR of COL5A1 had markedly reduced or absent expression from one COL5A1 allele. Quantitation of proα1(V) mRNAs by RPA confirmed that cells with genetic evidence of haploinsufficiency had diminished expression of COL5A1, compared with controls and classical EDS

Table 1

Patients with EDS Type I with *COL5A1* Mutations

	Change in		
Patient	Genomic DNA[a]	cDNA[b]	Protein[c]
EDS8	Exon 47 deletion of GA at nt 38 or 40[c]	nt 3957 or nt 3959 deletion of GA	Frameshift and a premature stop codon at amino acid 1265
EDS43	Intron 28 G^{+1}→A	nt 2660 insertion of 178 nt from intron 28	Frameshift and a premature stop codon at amino acid 840
EDS56	Exon 52 insertion of TG at nt 35[c]	nt 4332 insertion of TG	Frameshift and a premature stop codon at amino acid 1488
EDS82	Intron 2 G^{+1}→T	nt 339 deletion of 168 nt	Deletion of amino acids 38–92
EDS53	Exon 27 C43T[c]	C2603T	R792X

[a] Nucleotides are numbered from 5′ nt of exon.
[b] Nucleotides are numbered according to Greenspan et al. (1991).
[c] Amino acids are numbered from the first methionine of the signal peptide.

cells in which COL5A1 haploinsufficiency was excluded. Nonsense-mediated mRNA decay is the likely mechanism that would account for the loss of the abnormal allelic product observed in the four probands (EDS8, EDS43, EDS56, and EDS53) with mutations that would be expected to yield stop codons (Leeds et al. 1992; Pulak and Anderson 1993; Belgrader et al. 1994). Similar types of mutations had been reported to be common causes of haploinsufficiency in the COL1A1 and COL2A1 genes in osteogenesis imperfecta and Stickler syndrome, respectively (Willing et al. 1993, 1994). Cycloheximide, a potent inhibitor of protein synthesis, also stabilizes labile RNAs, through a serine/threonine phosphorylation-dependent action, on the 3′ UTR, that is independent of its action as a protein-synthesis inhibitor (Rao and Mufson 1993; Ohh and Takei 1995), making it an ideal component in the identification of mutations underlying null alleles.

The splice-donor mutation of intron 2 in proband EDS82 yielded an in-frame spliced product that lacked the exon 2 encoded sequence. We detected no other spliced products—and, in particular, no product that would be expected to yield a stop codon. Similar splicing mutations in COL5A1, COL5A2, and other fibrillar-collagen genes usually have resulted in expression of the mutant allele. The reason for the instability of the incorrectly spliced allele in proband EDS82 was not determined.

The observed increase in COL5A1 mRNA from the mutant allele that was produced by cycloheximide treatment of cultured fibroblasts should enable any of the standard cDNA-based mutation-detection systems to be used. Heteroduplex analysis was found to be a simple method for detection of abnormal PCR fragments in patients shown to have insertions or deletions, but this method probably is too insensitive to detect point mutations. For example, no heteroduplexes were detected in proband EDS53, in whose helical coding region there was a point mutation leading to a stop codon. Analysis of overlapping cDNA PCR products by the protein-truncation test may provide a useful alternate mutation-screening method, since premature stop codons resulted from the COL5A1 mutations in four of the five probands in whom mutations were determined. Alternatively, complete cDNA sequence analysis may be an efficient screening method if high-throughput systems are available, in cases in which the mutant message is stabilized by cycloheximide.

This report represents the first attempt to determine the prevalence of COL5A1 haploinsufficiency in a large series of patients with classical EDS. Evidence of COL5A1 haploinsufficiency was observed in ~30% of the individuals who were informative for the 3′ UTR polymorphisms used in this study. The addition of other expressed polymorphisms, such as the *Pst*I site in exon 5, should increase the overall heterozygosity rates, from 60% to as high as 90%. Consequently, these RFLPs should enable COL5A1 haploinsufficiency to be identified efficiently in patients with classical EDS.

In our study population of 59 cases of classical EDS, 8 were shown, in the present study, to have haploinsufficiency of COL5A1. In previous studies, one case was shown to have an exon 65–skipping mutation of COL5A1, and two cases had skipping mutations of either exon 27 or exon 28 of COL5A2. Assuming that the 30% prevalence of COL5A1 haploinsufficiency observed in the subset of the patients who were informative for the 3′ UTR polymorphisms applies to the complete group, we predict that ~19 of the 59 cases will be haploinsufficient. Overall, we predict that, at a minimum, 22 (37%) of the 59 cases have mutations of either COL5A1 or COL5A2. We did not determine whether haploinsufficiency of COL5A2 was present in the study population.

Although additional studies are in progress to determine the prevalence of all types of mutations in COL5A1 and COL5A2 in classical EDS, it is likely that up to half of the cases may be due to mutations in other gene loci. Phenocopies clearly occur, because segregation with COL5A1 and COL5A2 has been excluded in several families with classical EDS (Greenspan et al. 1995; Wenstrup et al. 1996). Some of these families still may have mutations in the COL5A3 gene or in genes that code for other proteins that interact with or are involved in the formation of the type I collagen–rich fibrils. For example, deficiencies of decorin (Danielson et al. 1997), thrombospondin 2 (Kyriakides et al. 1998), and fibromodulin (Svensson et al. 1999) produce a fragile-skin phenotype in mice. Also, a heritable deficiency of tenascin X in humans results in an EDS-like phenotype with recessive inheritance (Burch et al. 1997*a*, 1997*b*).

The finding described here—that is, that the classical EDS phenotype is commonly due to 50% reduction of proα1(V) chains—indicates that biomechanical integrity of connective tissues, particularly that of healing dermis, is exquisitely sensitive to type V–collagen content. The precise role of type V collagen in the maintenance of connective-tissue strength is not completely known. Type V collagen has been postulated to be a negative regulator of collagen-fibril diameter (Marchant et al. 1996), and dermal collagen fibrils in patients with EDS are, on average, larger than normal (Vogel et al. 1979; Hausser and Anton-Lamprecht 1994). Yet, larger collagen fibrils are correlated with *increased* structural and material properties of tissues (Parry and Craig 1988)—rather than with the apparent decrease in those properties that is found in most connective tissues of patients with EDS. A possible pathogenetic mechanism underlying loss of tissue integrity in EDS may be inter-

ference with a putative role of type V collagen in nucleation of type I collagen–rich fibrils, a role consistent with the localization of type V–collagen triple-helical epitopes in the interior—rather than on the surface—of collagen fibrils (Birk et al. 1988). Abnormal nucleation of collagen fibrils in dermis may account for the appearance of disorganized fibrils in dermis of some patients with EDS (Hausser and Anton-Lamprecht 1994). Alternately, loss of connective tissue integrity in EDS may be related to a reduced capacity of type V-deficient collagen fibrils for binding to noncollagenous matrix components, including heparin sulfate proteoglycan (Delacoux et al. 1998), decorin (Fleischmajer et al. 1991), osteonectin (Xie and Long 1996), and chondroitin sulfate proteoglycan (Fleischmajer et al. 1991). Heparin sulfate, in particular, exhibits higher binding to type V than to type I collagen; binding to type V collagen varies by molecular isoform and is directly proportional to the $\alpha1(V)$ chain content: $\alpha1(V)_3 > \alpha1(V)_2\alpha2(V) > \alpha1(V)\alpha2(V)\alpha3(V)$ (Delacoux et al. 1998). Morphologic, biochemical, and cellular analyses of COL5A1- haploinsufficient cells and tissues from patients with classical EDS may elucidate the function of type V collagen in the regulation of tissue biomechanical properties.

Acknowledgments

We thank those clinicians who provided material from patients under their care. We thank Dr. D. Greenspan for providing additional COL5A1 intronic sequence. This work was supported by grants from the Medical Research Council of Canada (to W.G.C), the Canadian Arthritis Network Centre of Excellence (to W.G.C), the Markey Foundation and the Trustees of the Children's Hospital Research Foundation (to R.J.W.), and by the Swiss National Science Foundation (grant 32-59455.99, to B.S.).

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for human type V collagen genes COL5A1 [M76729], COL5A2 [NM000393], and COL5A3 [AF177941)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for EDS types I [MIM 130000] and II [MIM 130010])

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