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Human Ehlers-Danlos Syndrome Type VII C and Bovine Dermatosparaxis Are Caused by Mutations in the Procollagen I N-Proteinase Gene

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Summary

Ehlers-Danlos syndrome (EDS) type VIIC is a recessively inherited connective-tissue disorder, characterized by extreme skin fragility, characteristic facies, joint laxity, droopy skin, umbilical hernia, and blue sclera. Like the animal model dermatosparaxis, EDS type VIIC results from the absence of activity of procollagen I N-proteinase (pNPI), the enzyme that excises the N-propeptide of type I and type II procollagens. The pNPI enzyme is a metalloproteinase containing properdin repeats and a cysteine-rich domain with similarities to the disintegrin domain of reprolysins. We used bovine cDNA to isolate human pNPI. The human enzyme exists in two forms: a long version similar to the bovine enzyme and a short version that contains the Zn^{++}-binding catalytic site but lacks the entire C-terminal domain in which the properdin repeats are located. We have identified the mutations that cause EDS type VIIC in the six known affected human individuals and also in one strain of dermatosparactic calf. Five of the individuals with EDS type VIIC were homozygous for a C→T transition that results in a premature termination codon, Q225X. Four of these five patients were homozygous at three downstream polymorphic sites. The sixth patient was homozygous for a different transition that results in a premature termination codon, W795X. In the dermatosparactic calf, the mutation is a 17-bp deletion that changes the reading frame of the message. These data provide direct evidence that EDS type VIIC and dermatosparaxis result from mutations in the pNPI gene.

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Introduction

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inherited connective-tissue disorders characterized by altered mechanical properties of skin, joints, blood vessels, and ligaments. At least 10 types and subtypes of this syndrome have been defined, on the basis of clinical, genetic, and biochemical criteria (Steinmann et al. 1993). A simplified classification of EDS into six major types has been proposed recently (Beighton et al. 1998). Mutations in the genes encoding $\alpha1(I)$, $\alpha2(I)$, $\alpha1(III)$, $\alpha1(V)$, and $\alpha2(V)$ chains of procollagens, lysyl hydroxylase, and tenascin-X have been found in different forms of EDS (Levinson et al. 1993; Steinmann et al. 1993; Toriello et al. 1996; Wenstrup et al. 1996; Burch et al. 1997; De Paepe et al. 1997).

EDS type VIIC (MIM 225410) (Nusgens et al. 1992; Smith et al. 1992; Reardon et al. 1995; Fujimoto et al. 1997) and the related animal disease, dermatosparaxis (Hanset and Ansay 1967), are recessively inherited connective-tissue disorders. They result from a defect in the processing of type I procollagen to collagen, with accumulation in most tissues of molecules that retain the amino-terminal propeptide (Lenaers et al. 1971). These precursor molecules self-assemble into abnormal ribbonlike fibrils that fail to provide normal tensile strength to tissues (Piérard and Lapière 1976; Nusgens et al. 1992; Smith et al. 1992). The defect in the processing of type I reflects the absence of activity of procollagen I N-proteinase (pNPI), the enzyme that normally removes the aminopropeptide of type I and type II procollagen (Lapière et al. 1971; Colige et al. 1995). The bovine pNPI enzyme is a member of the metzincin superfamily of metalloproteinases (Colige et al. 1997). In addition to the zinc-binding catalytic site, pNPI contains four domains homologous to properdin repeats and a cysteine-rich region with some similarity to the disin-

Table 1

PCR Primer Pairs Used for Amplification of Overlapping RT-PCR Fragments from Human and Bovine pNPI Transcripts and Portions of Human pNPI Gene

	Amplicon Length (bp)	Primer (5′→3′) Forward	Primer (5′→3′) Reverse
RT-PCR products:			
1	330	CGCTTGGTGTCCCACGTGGTGT	CCAGCCCATCGCAGTTGCTGAG
2	1,034	TTTGGCCGAGACCTGCACCTGC	AGGTAGCGGCTCAGCTCCTGCT
3	1,110	GCATGCAAGGCTATGCTCCTGTCA	GGATCTCAAACATCTTGATGTAACCA
4	1,226	CCGGGGAGGTGGTGTCCATGAA	CCTACAGAATATTGACTTGTCGCCTT[a]
5	1,124	ACAACACCACCCGCTCCGTGCA	CTGCTTAGCAACTTGGGGCCTATT[b]
6[c]	339	CCTGACAACCCCTACTTTTGCAAG	GACTGGAAGCAGCGACGCGATCTT
7	191	CTACAAGGACGCCTTCAGCCTCT	GGATCTCAAACATCTTGATGTAACCA
8[c]	217	TTGGGAGGCCAAGGTAGGTGGAT	GACTGGAAGCAGCGACGCGATCTT
PCR products:			
A	197	GGATGCCAGTTCCAAAACCTTCATT	GGCCACGCAGAGTGTCACCTGAA[d]
B	±1,900	CAAGGCCGTGTGCATGTGGTGTAT	CGGCTGAGGCTGTCCAGGCTGT
C	101[e]	CACCCGCGTGGAGCCCCTGCT	CCAGCCCATCGCAGTTGCTGAG

[a] CCTACAGAACACTGACTTGTCACCTT for the amplification of bovine mRNA.
[b] CTGCTCAGAAACTTGGGGCAAAGG for the amplification of bovine mRNA.
[c] Primer pair allowing the amplification of exon B for spNPI only (see figs. 1 and 3).
[d] Primer specific for intron sequence situated upstream of the mutation. Other primers were designed from the cDNA sequence.
[e] In dermatosparactic calves, the amplicon length is 84 bp.

tegrin domain of ADAMs (a disintegrin and metalloproteinase).

In this study, we report the cDNA and the amino acid sequences of human pNPI, compare the human and the bovine enzymes, and describe the mutations that cause EDS type VIIC in six unrelated patients and that cause dermatosparaxis in the Belgian strain of cattle in which the disease was first identified (Hanset and Ansay 1967).

Patients, Material, and Methods

Patients and Biological Material

Total RNA (Chirgwin et al. 1979) and genomic DNA (QIAamp Blood kit; Qiagen) were purified from fresh tissues or from cultured skin fibroblasts derived from human and bovine nonaffected controls, from five previously described patients with EDS type VIIC (Nusgens et al. 1992; Smith et al. 1992; Wertelecki et al. 1992; Petty et al. 1993; Reardon et al. 1995; Fujimoto et al. 1997), from one additional patient, and from a dermatosparactic calf (Hanset and Ansay 1967). All cell strains were used at low passage level, had the characteristic fibroblastic appearance, and had a normal rate of proliferation in culture.

Clinical Summary of the New Patient

The sixth known infant with EDS type VIIC was a male infant delivered at 31 wk gestation, because of fetal distress. Both parents were of Ashkenazi Jewish background but could not identify common ancestors. He remained in the intensive-care unit for 2 mo, because of multiple problems. He was noted to be dysmorphic and had a large umbilical hernia. The diagnosis of EDS type VIIC was made when examination of skin by electron microscopy demonstrated characteristic changes in the collagen fibrils. The diagnosis was confirmed by demonstration that cultured dermal fibroblasts did not convert type I procollagen to collagen. At the age 8 mo he was below the 5th percentile in height and weight, had a gastrostomy tube placed for caloric supplementation, and was delayed in motor development. His physical appearance was similar to that of other infants with EDS type VIIC, in that he had multiple redundant skin folds at the wrists, elbows, axillae, knees, and groin; was mildly hirsute; and had grey sclerae, a small chin, and a slightly widened scar from the removal of the large umbilical hernia.

Isolation of cDNAs for Human N-Proteinase

Total RNA was isolated from human skin fibroblasts (CRL1262; American Type Culture Collection) and was reverse transcribed with oligo-dT and random primers to cDNA (cDNA synthesis kit; Pharmacia). The cDNA was then inserted into lambda phage vector (ZapII; Stratagene) to make the cDNA library. The cDNA library was screened with a [^{32}P]-labeled bovine N-proteinase cDNA probe (Colige et al. 1997). Nine cDNA inserts of 2–5 kb were isolated and sequenced with an automated

instrument (model 377; ABI). Overlapping sequences were used to define the structures of the full-length cDNA.

Northern Blot Analysis

Seven micrograms of mRNA were purified from normal human fibroblasts in culture (PolyATtract mRNA Isolation System III; Promega), were separated by electrophoresis in 0.9% agarose/formaldehyde gel, and were transferred to a nylon membrane (Hybond N; Amersham). The filter was prehybridized at 52°C for 16 h in 5 × SSPE (1 × SSPE = 150 mM NaCl; 1 mM EDTA; 10 mM NaH_2PO_4, pH 7.4), 40% deionized formamide, 5 × Denhardt's solution, 0.1% SDS, 5% dextran sulfate, and 100 μg denatured salmon sperm DNA/ml; was probed for 18 h under the same conditions, with a reverse transcription–PCR (RT-PCR) product (fragment 2; table 1); and was labeled with $\alpha[^{32}P]$-dATP and a random priming labeling kit (Boehringer Mannheim). The blot was washed at 65°C in 0.3 × SSC (1 × SSPE = 150 mM NaCl; 15 mM Na citrate, pH 7.0) and 0.1% SDS before exposure to x-ray film.

PCR and DNA Sequencing

Overlapping RT-PCR products were obtained by use of the Titan One Tube RT-PCR kit (Boehringer Mannheim) and six different pairs of primers (RT-PCR products 1–6; table 1). Both strands of purified products were sequenced by use of a Thermo-Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Life Science) and human or bovine pNPI sequence–specific primers (Eurogentec). PCR products were synthesized from genomic DNA by use of Dynazyme *Taq* DNA polymerase (Biometra) and three different pairs of primers, to amplify portions of DNA that contained either the W795X mutation or the Q225X mutation (PCR products A and B, respectively [table 1]) or the deletion found in dermatosparactic calves (PCR product C).

Mutation Identification

To determine if codon Q225 was altered in patient 6, cDNA was amplified with the sense primer of primer pair B (table 1) and an antisense primer 174 bp downstream (5′-TGCAGCATGCCTGCGTGCCCT-3′) in a PCR program consisting of 94.5°C for 60 s, 52°C for 60 s, and 72°C for 30 s. The PCR product was purified on a 1% low-melting-temperature agarose gel (NuSieve GTG agarose; FMC), and was subsequently extracted from the gel by use of QIAquick™ spin columns (QIAGEN). The antisense amplification primer was used in an ABI PRISM®BigDye™ Terminator cycle sequencing reaction, and the products were analyzed by use of the ABI PRISM™ 310 Genetic Analyzer.

Sequence Determination at Three Polymorphic Sites in the pNPI Coding Region

To identify the nucleotide at the third position of the codons H286, D676, and D844, cDNA from fibroblasts of patient 6 was amplified by use of the following primer pairs: H286, the sense primer of primer pair B and the antisense primer of primer pair 2 (table 1); D676, primer pair 3 (table 1); and D844, the sense primer of pair 4 (table 1) and the antisense primer that was used for preliminary RT-PCR amplification to assay for the W795X mutation (35 cycles of 94°C for 1.5 min, 54°C for 1 min, and 72°C for 1.5 min).

Assembly of the Synthetic Competitor RNA

The sense and antisense strands of a synthetic DNA template were constructed by ligation of overlapping phosphorylated oligonucleotides, including oligonucleotide primers used for RT-PCR amplification of pNPI and spNPI mRNA (see RT-PCR Assay, below). Full length products were then purified and cloned into pSPT18 transcription vector (SP6/T7 transcription kit; Boehringer Mannheim). Synthetic RNA was generated from the linearized template plasmid by use of SP6 RNA polymerase and was purified (High Pure RNA Isolation kit; Boehringer Mannheim) and quantitated. RT-PCR amplification of this synthetic RNA was performed with pNPI or spNPI primer-generated products of 265 bp and 262 bp that were larger than those obtained from the wild-type mRNA (191 bp and 217 bp, respectively).

RT-PCR Assay

The RT-PCR amplifications were performed in an automated instrument (GeneAmp PCR system 2400 using a GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit; Perkin-Elmer) with two different pairs of $\alpha[^{32}P]$-labeled primers (T4 polynucleotide kinase; Boehringer Mannheim). The primer pairs specifically amplified either the 3′ end of human and bovine pNPI and lpNPI mRNA (table 1; RT-PCR product 7; bp 2043–2233 of the coding sequence) or the human spNPI mRNA (table 1; RT-PCR product 8; bp 43–259 of exon B) (fig. 4). For quantitative RT-PCR measurements, 10 ng of total RNA and a known copy number of standard synthetic RNA were used per 25 μl reaction mixture (final volume). The RT step (70°C for 15 min) was followed by a 2-min incubation at 95°C, for denaturation of RNA-DNA heteroduplexes, and then by amplification for 30 cycles. The PCR conditions were 94°C for 15 s, 66°C for 20 s and 72°C for 10 s. Samples were analyzed by autoradiography after electrophoresis on a 10% polyacrylamide gel. The films were assayed by densitometry (Imaging Densitometer model GS-700; Bio-Rad, Life Science).

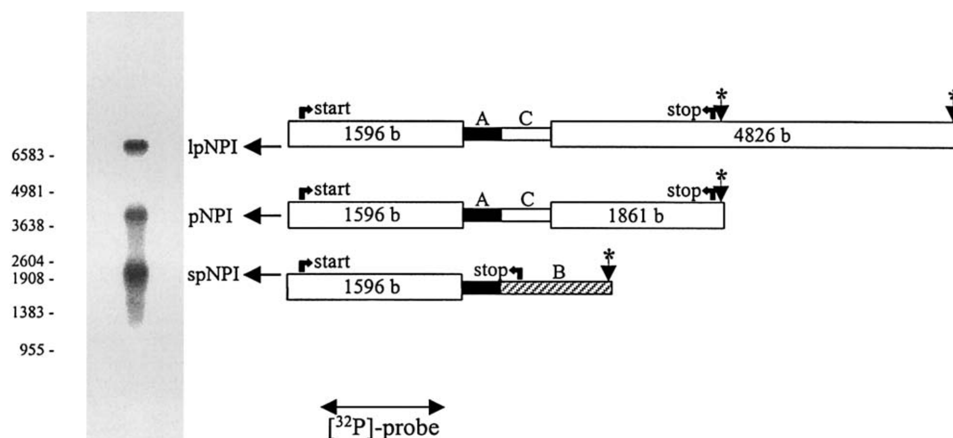

Figure 1 Northern blot analysis of human pNPI gene transcripts. Seven micrograms of mRNA extracted from human fibroblasts in culture were separated onto formaldehyde/agarose gel, transferred on nylon membrane, and hybridized with a labeled cDNA probe obtained by PCR. The double arrow indicates the region of the mRNAs recognized by the probe. Electrophoretic mobility of RNA molecular-weight markers (in bases) is indicated on the left. After autoradiography, three main bands are visible (lpNPI, pNPI, and spNPI). The structure of these three transcripts is represented on the right. Boxes A–C are exons reported in figure 3, and unblackened boxes represent multiexonic cassettes (not to scale). AATAAA polyadenylation signals (*) and starts and stops of translation are also indicated.

Allele-Specific RT-PCR Assay for the W795X Mutation

A preliminary RT-PCR amplification (Titan One Tube RT-PCR kit; Boehringer Mannheim) was performed with oligonucleotide primers (sense, 5′-CCGGGGA-GGTGGTGTCCATGAA-3′, from bp 1991–2012 of the coding sequence; anti-sense, 5′-CGGCGGCAGCCA-TACTTGGTGAA-3′ from bp 2648–2626) which do not discriminate between the two alleles. RT-PCR products were then purified on agarose gels and were diluted in order to achieve the same cDNA concentration in each sample. For allele-specific PCR amplification, the anti-sense oligonucleotide (5′-GGGATGACCAGAACGGT-GATGGT-3′; +81 to +59 if the A→G mutation is defined as +1) was ^{32}P-labeled to allow the quantitation of PCR products. The allele specificity was obtained by use of sense primers that had either a G (for the normal allele, 5′- GCCATGGGCGTGGA<u>T</u>TG-3′) or an A (for the mutated allele; 5′-TGCCATGGGCGTGGA<u>T</u>TA-3′) at the 3′ end. To improve specificity, a similar mismatch was introduced in both primers, changing G at position −3 to T (underlined in the sequences). Although this change moderately decreased PCR amplification of the specific allele, amplification of the other allele was completely prevented. Seventeen cycles of amplification (94°C for 15 s; 58°C for 15 s; and 72°C for 10 s) were performed with 1 ng preamplified cDNA, 4 pmol each oligonucleotide, and 0.4 U *Taq* DNA polymerase (Dynazyme; Biometra), for each 20 μl sample. PCR products were analyzed by electrophoresis through a 10% polyacrylamide gel and by autoradiography.

Results

Isolation of Multiple Human cDNAs

We used bovine pNPI cDNA clones (Colige et al. 1997) to screen a human cDNA library. Overlapping clones revealed two types of cDNA that differed both by size (lpNPI, 6,796 bp; and spNPI, 2,137 bp) and by the sequence of the 3′ ends. To measure their relative abundance and to search for additional transcripts, a northern analysis was performed with a probe that hybridized to the 5′ end of both products (fig. 1). In addition to the two expected mRNAs, a third message, of ~3,700 nucleotides (pNPI), was detected. Sequencing revealed that this transcript resulted from the use of an AATAAA signal situated at the beginning of the 3′ noncoding sequence of lpNPI mRNA (fig. 1). The two longer transcripts encoded an identical 1,211-amino-acid protein that displayed 93% identity with the bovine enzyme (fig. 2). The highest similarity was detected around the Zn^{++}-binding catalytic site, with no substitutions over a 111-amino-acid sequence and only four substitutions over a 305-amino-acid sequence. Other specific features of the bovine enzyme (four properdin repeats, two potential cleavage sites by mammalian subtilisins, one RGD sequence, and potential glycosylation sites mainly in the C-terminal domain [Colige et al. 1997]) were conserved, suggesting that these domains are important for the biological properties of pNPI.

The shorter transcript (spNPI) was identical to the long cDNAs, over the first 1,710 nucleotides, but the large 3′ end of the long forms was replaced by a 313-

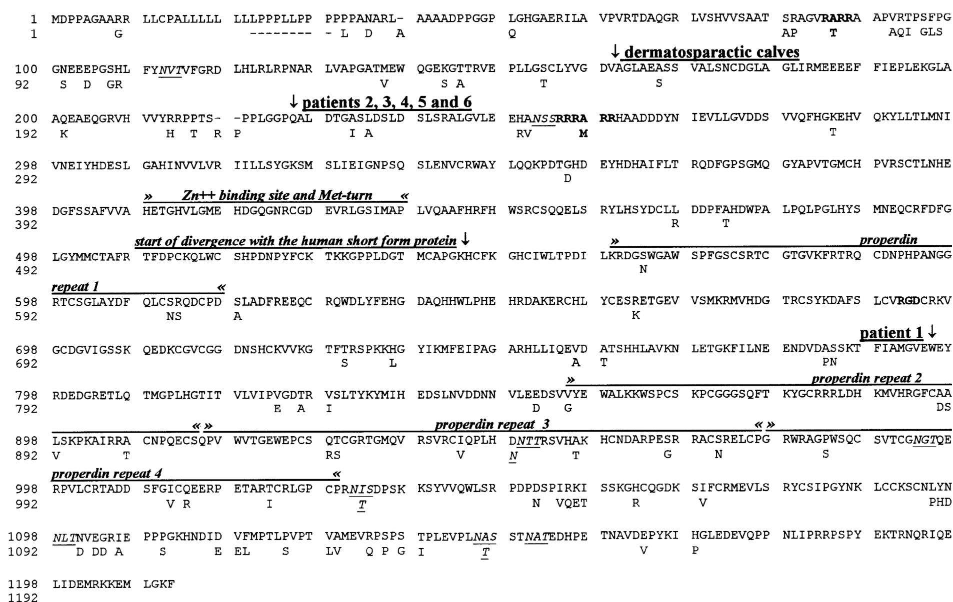

Figure 2 Deduced amino acid sequences of human and bovine pNPI. The human pNPI is the upper line, and the bovine sequence is the lower line (only positions where amino acids differ from those in the human sequence are shown). The two enzymes contain a signal peptide, composed mainly of Leu and Ala residues. Two potential cleavage sites, by mammalian subtilisins—RXRR and RRRXRR—and an RGD sequence are indicated in boldface at, respectively, amino acid residues 85–88, 254–259, and 691–693 of the human sequence. Potential glycosylation sites are in italic and underlined. The Zn^{++}-binding site and Met turn, the properdin repeats, and the positions where mutations were detected are indicated above the related sequences. The start of divergence, in human, between pNPI and spNPI is also indicated. For spNPI, the entire sequence downstream of Lys 543 is replaced by an unrelated 23-amino-acid sequence (FRPGAVAHACYPSTLGGQGRWIA encoded by 5′-TTCAGGCCGGGCGCGGTGGCTCATGCCTGTTATCCCAGCACTTTGGGAGGCCAAGGTAGGTGGATCGCCTGA-3′).

base sequence that terminated with a polyadenylation signal (AATAAA). Partial sequencing of this region of the gene indicated that this signal was located within an intronic sequence of pNPI and lpNPI pre-mRNAs. The spNPI message resulted from retention of a portion of this intron and the use of this polyadenylation signal (fig. 3). The open reading frame of the short message encoded a protein in which the entire C-terminal domain (His 544–Phe 1211) found in pNPI was replaced by a different 23-amino-acid sequence (fig. 2). The spNPI contains the Zn^{++}-binding catalytic site but lacks most of the potential glycosylation sites and the four properdin repeats that are thought to be important for intermolecular interactions (Tuszynski et al. 1992; Schultz-Cherry et al. 1995).

Mutations in pNPI in EDS Type VIIC

Once the human sequences were known, we searched for the molecular defects responsible for EDS VIIC in six unrelated individuals (Nusgens et al. 1992; Smith et al. 1992; Wertelecki et al. 1992; Petty et al. 1993; Reardon et al. 1995; Fujimoto et al. 1997; present study). RT-PCR assays demonstrated that mRNAs for pNPI were present in fibroblasts of four of the patients (fig. 4). The corresponding cDNAs were sequenced to identify mutations. In patient 1 (Nusgens et al. 1992), a G→A transition (2384G→A of the coding sequence) was found that changed the TGG tryptophan codon at position 795 of pNPI to a TAG stop codon (W795X) (figs. 1 and 5A). If translated, the resulting pNPI would contain the Zn^{++}-binding catalytic site and the first properdin repeat but would lack most of the C-terminus. Interestingly, this mutation does not alter the sequence of spNPI.

Sequence determination of the pNPI cDNA from fibroblasts of the parents of patient 1 revealed only the normal TGG codon (fig. 5*A*). At the genomic level, both parents were heterozygous for the wild-type and mutant alleles, whereas the patient was homozygous for the mutant allele (fig. 5*B*). To determine if the transcript with the premature-termination codon was present at low level in parental fibroblasts, an allele-specific RT-PCR assay was used (fig. 5*C*). With an oligonucleotide pair that allowed the amplification of the mutated allele, a strong signal was observed for the patient and a faint one was observed for both parental samples, consistent with nonsense-mediated decay of the altered message (Baserga and Benz 1992). Two different pairs of primers were used to measure the relative amounts of mRNA for spNPI and pNPI (see Assembly of the Synthetic Com-

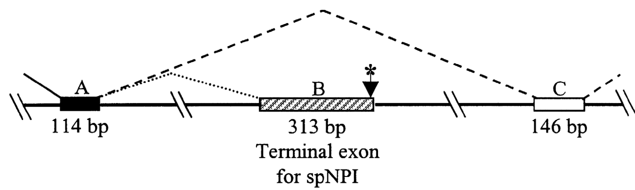

Figure 3 Alternative splicing of pNPI primary transcript. Alternative splicing and alternative use of an AATAAA polyadenylation signal (*) present in the middle of the pNPI gene are represented. Intronic sequences are represented by thick lines and exons are represented by boxes (A–C). Alternative splicing events producing spNPI or the two longer transcripts are indicated, respectively, by dotted or dashed lines.

petitor RNA, above) (fig. 4). For spNPI mRNA, which is not altered by the mutation, similar levels were detected in the cells of the normal individual, patient 1, and her parents (fig. 4*B*, lanes 1 and 4–7). In contrast, the pNPI mRNA steady-state level was reduced in cells from patient 1 and her parents (fig. 4*A*, lanes 5-7) compared with both that in the control individual (fig. 4*A*, lane 1) and that in spNPI mRNA.

The cDNA synthesized from RNA isolated from the cultured fibroblasts of patients 2 (Smith et al. 1992; Wertelecki et al. 1992), 3 (Petty et al. 1993), 4 (Fujimoto et al. 1997), and 6 (present study) were sequenced. The same C→T transition (673C→T of the coding sequence) was found that changed a Gln codon (CAG) to a stop codon (TAG) (Q225X) (fig. 6). In contrast to the mutation in patient 1, if this mutation allowed translation to occur, it would lead to truncation of spNPI and pNPI, such that both would lack the Zn^{++}-binding catalytic site (fig. 1).

The sequences of the genomic DNA from these four patients and from another unrelated patient (patient 5), from whom only genomic DNA was available (Reardon et al. 1995), indicated that all were homozygous at the mutation site (not shown). Samples from parents were available only for patients 2 and 6; analysis of the sequence indicated that all four patients were heterozygous for the mutation. Analysis of cDNA and genomic DNA from the parental cells of patient 2 was consistent with low levels of the mutation-bearing mRNA. Nonsense-mediated decay probably accounted for the reduction of both pNPI and spNPI messages in cells from patients 2–4, compared with the level measured in the normal individual (fig. 4).

When the cDNA or the genomic DNA from patients 2–5 was sequenced, all patients were found to be homozygous for the third-position nucleotides in the codons for histidine at position 286 (CAT) and for the aspartic residues at positions 676 (GAT) and 844 (GAT). In contrast, patient 6 was homozygous at position 286 but was heterozygous at the two other positions (GAC/T).

Mutation in pNPI in Bovine Dermatosparaxis

In the dermatosparactic calf, electrophoresis of short RT-PCR products demonstrated that there was a deletion in the 5′ end of the message (Fig. 7*A*). Sequence determination showed a 3-bp change, followed by a 17-bp deletion, in the beginning of the coding sequence, that changed the reading frame of the message (fig. 7*B*). If the mRNA is stable, this mutation would result in the synthesis of a truncated protein in which the entire sequence downstream of Val 153, including the catalytic site, would be replaced by a 24-amino-acid sequence (V-V-R-G-A-Q-Q-L-R-W-A-G-W-P-D-P-Y-G-R-G-G-I-L-Y-

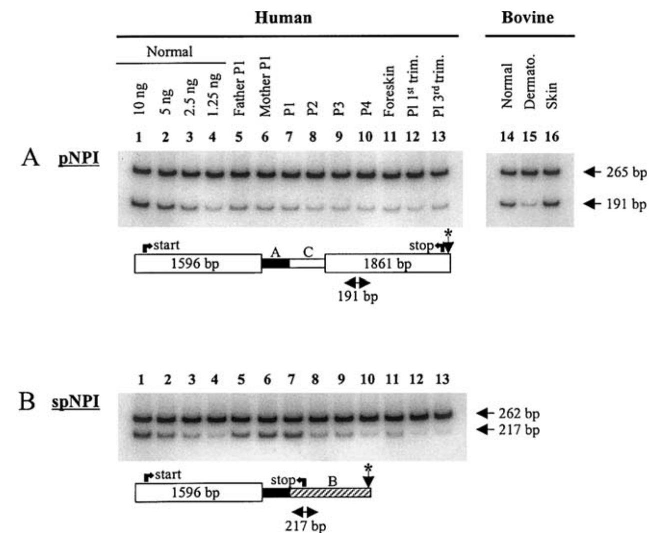

Figure 4 Analysis of pNPI and spNPI expression. The mRNA steady-state levels were assayed by RT-PCR using two different pairs of $\alpha[^{32}P]$-labeled primers and a synthetic RNA as an internal standard to monitor the efficiency of RT-PCR amplification. PCR-amplified products were separated on 10% polyacrylamide gels, were visualized after autoradiography, and were quantified. *A*, First pair of primers, which allowed the amplification of a 191-bp DNA product from pNPI mRNA and of a 265-bp product from the synthetic control RNA. The region of pNPI mRNA amplified with these primers is indicated by the double arrow. *B*, Second pair of primers, which allowed the amplification of a 217-bp product from the exon present only in spNPI mRNA (exon B; fig. 1) and of a 262-bp fragment from the control synthetic RNA. The transcript structure is as shown in figs. 1 and 3. In addition to two-fold serial dilutions of total RNA from a normal individual used as quantification control, total RNA (10 ng) from various sources was assayed. Lanes 1–10; Skin fibroblasts in culture from a normal individual (lanes 1–4), father of patient 1 (lane 5), mother of patient 1 (lane 6), patient 1 (lane 7), patient 2 (lane 8), patient 3 (lane 9), patient 4 (lane 10). Lane 11, Human foreskin. Lanes 12–13, Placental villi at the first (lane 12) and the third trimester (lane 13). Lanes 14 and 15, skin fibroblasts from a normal (lane 14) or a dermatosparactic calf (lane 15). Lane 16, bovine skin.

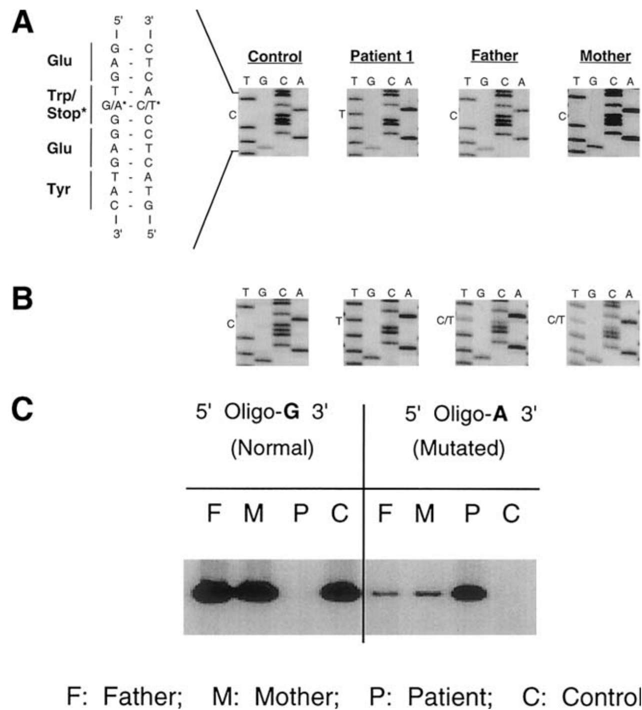

Figure 5 Mutation analyses of pNPI in the family of patient 1. DNA sequences of PCR-amplified cDNA (*A*) and genomic DNA (*B*) derived from a nonaffected control individual, from patient 1, and from her parents are illustrated. At the cDNA level (*A*), one mutation that changed a Trp codon to a stop codon (W795X) was found only in patient 1. At the genomic level, patient 1 is homozygous, and her parents are heterozygous (*B*). Allele-specific PCR amplification was performed on RNA from the same four individuals (*C*).

stop) (fig. 2). PCR analysis of the genomic DNA (PCR product C; table 1) revealed that dermatosparactic calves are homozygous for the deletion (not shown).

Discussion

The data presented here provide insights into the biology of the procollagen I N-proteinase and give direct evidence that EDS type VIIC and bovine dermatosparaxis result from alterations in the pNPI gene. A previous study had shown that procollagen I N-proteinase activity was not detectable in skin from patient 1 (Nusgens et al. 1992). Since the catalytic site of pNPI in this patient is not affected by the mutation, this finding means either that pNPI cannot be translated from the mutated mRNA or that the C-terminal region of pNPI contains domain(s) crucial for enzyme function. Quantitative RT-PCR assays (not shown) indicate that spNPI mRNA is present in the cytoplasm of fibroblasts, suggesting that it is probably translated. The spNPI transcript is not altered by the mutation which completely suppresses the procollagen I N-proteinase activity in patient 1, so it is unlikely that spNPI can efficiently process the N-propeptide. Recently, it was shown that the C-terminal domain of collagenase 3 (MMP13) is required for this enzyme to bind to triple-helical type I collagen and to perform its catalytic activity (Knaüper et al. 1997). We think that the properdin repeats, which are present in pNPI but are absent in spNPI and in pNPI of patient 1, could have a similar function—that is, to promote the binding of the active enzyme to type I procollagen. Thus, if the protein were translated from the abnormal message synthesized by the cells from patient 1, it would not be active. Analysis of cDNA of the other patients with EDS VIIC indicates that a mutation that prevents the synthesis of both spNPI and pNPI (patients 2–6) does not result in a phenotype more severe than a mutation that suppresses the synthesis of only pNPI (patient 1). These results further support the concept that spNPI has no significant aminoprocollagen peptidase activity. A physiological role for spNPI is, nonetheless, suggested by the relatively high level of its mRNA in skin and cultured fibroblasts and by variation of the pNPI/spNPI mRNA ratio in vivo (fig. 4*A* and *B*, lanes 11–13). Similar observations have been made for the procollagen carboxy-peptidase, also known as "BMP1" (Kessler et al. 1996; Li et al. 1996; Lee et al. 1997). This enzyme is responsible for the excision of the C-propeptide of type I, type II, and type III procollagens and can be detected, at the mRNA level, in the form of alternatively spliced transcripts that are differently expressed in various tissues (Takahara et al. 1994).

We were surprised that five unrelated individuals with EDS type VIIC who lived in the United States (Smith et al. 1992; Wertelecki et al. 1992; Petty et al. 1993; present study), in England (Reardon et al. 1995), and in Mexico (Fujimoto et al. 1997) had the same mutation. The mutation in patients 2–5 could be on the same allelic background, as judged on the basis of three other variants

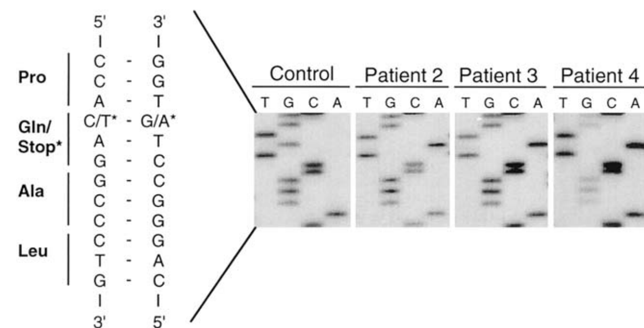

Figure 6 Mutation analysis for pNPI in patients 2–4. Sequences of the PCR-amplified cDNA derived from a normal individual and from patients 2–4 are illustrated. The same mutation that changed the Gln 225 codon to a stop codon Q225X) was observed in all three patients, as well as in patients 5 and 6 (not illustrated in this figure).

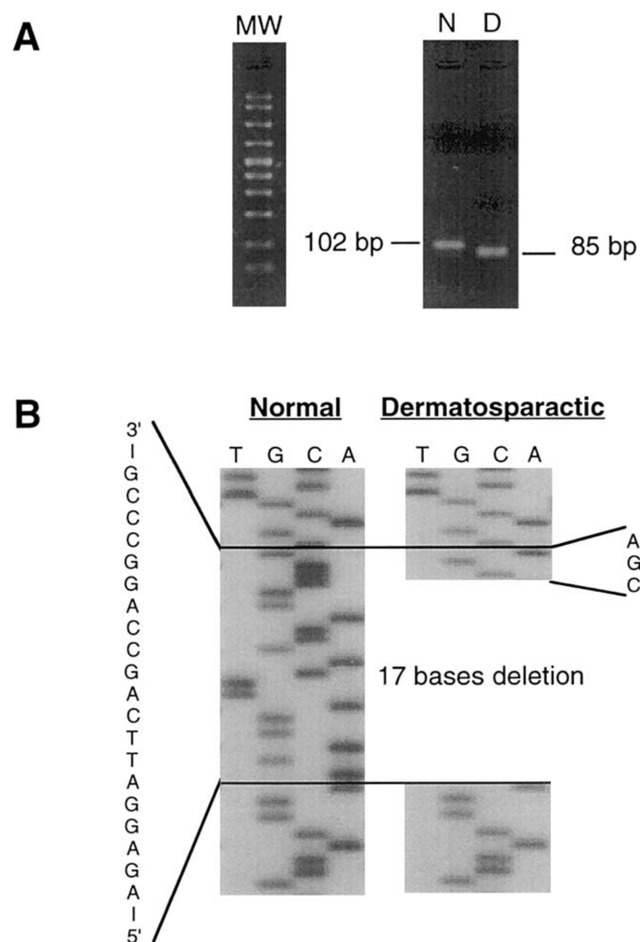

Figure 7 Mutation analysis of pNPI in dermatosparactic calf. *A*, Electrophoresis on agarose gel of RT-PCR products derived from normal (N) or dermatosparactic (D) calf mRNA. *B*, Sequence of the PCR-amplified cDNA derived from normal or dermatosparactic calf, around the deletion detected in panel *A*.

in the coding sequence of the gene. All have a T as the third base of the codons for His 286 (CAT), Asp 676 (GAT), and Asp 844 (GAT), whereas C or C+T was detected in seven other individuals. In patient 6, who is homozygous at position 286 but is heterozygous at the two other positions (GAC/T), it is not clear whether there is one additional allele or two since the phase relationships of the sequence variants have not been determined. An extended haplotype for the region surrounding the mutation has not been determined for any of the patients. The ethnic backgrounds of these five affected individuals differ: three are Ashkenazi Jewish , one is Hispanic/Mexican, and one is from an American family of undetermined background. The mutation appears to be on a different background in one of the Ashkenazi alleles. Thus the mutation appears to have occurred at least twice but is probably uncommon, since only five affected children have been identified. The mutation is not within a type of sequence in which recurrent mutation is frequent.

In cattle, fibroblasts from only one dermatosparactic calf were examined. However, there is little doubt that other affected calves have the same genomic alteration, since all known dermatosparactic calves in Belgium originated from the Belgian Blue cattle breed, which was selected by intensive inbreeding (Grobet et al. 1997) and had, as a common ancestor, the same bull. In dermatosparactic calves, the deletion in pNPI gene is situated 30 bp upstream of an intron/exon boundary as determined on the basis of human genomic DNA analysis. If it is assumed that the gene structure is the same in cattle, this would suggest that the alteration observed in dermatosparaxis is a true deletion, not a deletion resulting from a splice-site mutation and the use of a new donor site.

In EDS type VIIC and dermatosparaxis, the skin is more severely affected than other collagen-rich tissues (tendons, blood vessels, and bones) and this variation is correlated with the level of morphological alteration of the type I collagen fibrils. In cattle, the N-terminus of procollagen is barely processed in skin but is more extensively cleaved in bone and tendon. Since the mutation reported here would prevent the synthesis of active pNPI, this suggests that another enzyme, which has tissue-specific expression, can remove the amino-terminus of type I procollagen, although at a lower rate than that at which pNPI is removed. Recently, two proteins that have a high sequence homology with pNPI and that contain similar domains, including properdin repeats, have been described. One is a mouse protein associated with inflammation (Kuno et al. 1997), and the other was detected in human brain and placenta (Nagase et al. 1997). Their physiological roles have not been determined yet. However, the regulation of their expression and their tissue specificity suggest various functions for members of this new subfamily of metalloproteinases. These two metalloproteinases, or others of the same subfamily that have yet to be identified, are candidates for an alternative processing pathway of type I procollagen.

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Electronic-Database Information

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

Genbank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for human procollagen I N-proteinase cDNA [AJ003125])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for EDS type VIIC [MIM 225410])

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