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Identification of Two Splicing Mutations in the Collagen Type VII Gene (COL7A1) of a Patient Affected by the *Localisata* Variant of Recessive Dystrophic Epidermolysis Bullosa

Rita Gardella,[1] Laura Belletti,[1] Nicoletta Zoppi,[1] Dario Marini,[2] Sergio Barlati,[1] and Marina Colombi[1]

[1]Division of Biology and Genetics, Department of Biomedical Sciences and Biotechnology, and [2]Institute of Clinical Dermatology, University of Brescia, Brescia, Italy

Summary

Collagen type VII gene (COL7A1) has been demonstrated to be altered in several variants of dystrophic epidermolysis bullosa (DEB), with either recessive or dominant mode of inheritance. We have identified two mutations in a patient affected by a *localisata* variant of recessive DEB (L-RDEB), which is characterized by the less severe phenotype of the syndrome. These mutations are the first splicing mutations so far described for COL7A1 in DEB. One mutation is a paternally inherited A→G transition at position −2 of the donor splicing site of intron 3, which results in three aberrant mRNAs, depending on the skipping of exon 3, the usage of a cryptic donor site inside exon 3, or the maintenance of intron 3. The second mutation is a maternally inherited G→A transition at position −1 of the donor splicing site of intron 95, which induces the activation of a cryptic donor site 7 nt upstream the normal site and gives rise to a deleted mRNA, in addition to the normal one. All aberrant mRNAs show a shift of the reading frame, thus generating premature termination codons of translation. Allele-specific analysis of the transcripts has shown that the maternal mutation does not completely abolish the correct splicing of COLVII pre-mRNA, thus allowing, in the patient, the synthesis of a certain level of a functional protein. This result is compatible with the mild clinical L-RDEB phenotype observed in our patient.

Introduction

Dystrophic epidermolysis bullosa (DEB) is a hereditary skin disorder characterized by blisters formation below the lamina densa of the basement membrane, within the papillary dermis. DEB shows both dominant (DDEB) and recessive (RDEB) inheritance modes; moreover, on the basis of clinical manifestations, different subtypes of DEB have been described (Cooper et al. 1987; Bruckner-Tuderman 1993). The *localisata* variant of RDEB (L-RDEB) is a mild form in which blistering and scarring are predominantly localized to the extremities.

Microscopic analysis of the DEB patients' skin has shown morphological alterations and reduction or total absence of anchoring fibrils, the structures which mediate the attachment of the epidermis to the dermis and are mainly composed of type VII collagen (COLVII) (Keene et al. 1987; McGrath et al. 1993). Moreover, immunofluorescence microscopy with anti-COLVII antibodies in skin biopsies from RDEB patients evidenced the reduction, to variable extents, of COLVII at the dermal-epidermal junction (Bruckner-Tuderman et al. 1988). Type VII collagen gene (COL7A1), localized on chromosome 3p21, has therefore been suggested as the candidate gene in the etiopathogenesis of this disease. Linkage studies with intragenic RFLPs have confirmed the close association between both dominant and recessive DEB and COL7A1 in several families (Ryynänen et al. 1991, 1992; Hovnanian et al. 1992).

Type VII collagen molecule is a homotrimer, composed of three identical α1 chains. Each chain is synthesized as a proα1(VII) chain, containing a 145-kD noncollagenous N-terminal domain (NC-1), a 145-kD central collagenous domain, and a 30-kD noncollagenous C-terminal domain (NC-2) (Christiano et al. 1992, 1994*b;* Greenspan 1993). The proα1(VII) chains are encoded by the 32-kb COL7A1, containing 118 exons (Christiano et al. 1994*c*). Recently, different mutations in this gene have been identified in RDEB patients (Christiano et al. 1993, 1994*a,* 1995; Hilal et al. 1993; Christiano and Uitto 1994; Dunnill et al. 1994; Hovnanian et al. 1994). Since these are private mutations, the disease is extremely heterogenous, and, in general, the patients are compound heterozygous for two different mutations, the majority of which are nonsense or frameshift mutations leading to premature termination codons (PTCs). So far, mutations falling at the consensus splic-

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Address for correspondence and reprints: Dr. Marina Colombi, Division of Biology and Genetics, Department of Biomedical Sciences and Biotechnology, Via Valsabbina, 19, 25123 Brescia, Italy.
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ing sequences and altering COLVII mRNA maturation have not been described. Here we report two different COL7A1 mutations at the donors' splicing sites of introns 3 and 95, which affect the splicing of COLVII premRNA in an L-RDEB family.

Material and Methods

Skin Fibroblasts Cultures

Fibroblasts, obtained from skin biopsies, were grown in vitro at 37°C in modified Eagle's medium supplemented with 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Immunofluorescence Analysis

Skin cryosections from the L-RDEB family members and from a control subject were immunostained with the monoclonal antibody LH7.2 (Sigma). A rhodamine-conjugated anti-mouse IgG (Calbiochem-Novabiochem) was used as secondary antibody.

Oligonucleotides

In order to amplify the full-length COLVII cDNA and portions of COL7A1, we synthesized 25 pairs of oligonucleotides, designed according to the COLVII cDNA and the COL7A1 sequences with GenBank accession numbers L02870 and L23982, respectively.

The sequence and the location of the primer sets used for amplification of the mutant sequences were the following: C1: 5′-TGACCTGCACGCGCCTTTACGC-3′ (exon 2); C2: 5′-CCACAGCAAATAGCTTGACCCC-3′ (exon 4); C3: 5′-GACCCCTCAAGAGAGCCTGATACC-3′ (intron 2); C4: 5′-CCCGTCTGTGATCAGGATGCAG-3′ (exon 4); C5: 5′-TTGGGTTCCCGGGTCAGACAGG-3′ (exon 94); C6: 5′-CATGTCCCCCTTGGCACCCCGT-3′ (exon 101); C5–C7: 5′-CCCCAGGTGGTCCCACTGACCC-3′ (junction exons 95–96 in the wild-type cDNA); C5–C8: 5′-CCCCAGGTGGTCCCCCGGTGGT-3′ (junction exons 95–96 in the mutant cDNA); C9: 5′-GGTCTGGCAGGCCCCCCAGGGA-3′ (exon 95); C10: 5′-CTTGTCTCCTTTGAGTCCAGAGG-3′ (exon 96); and C9–C11: 5′-GCCCAGGCAGCCCTACTCCAGG-3′ (exon 97).

RT-PCR Analysis

Total RNA was purified from cultured skin fibroblasts by the guanidinium-thiocyanate method (Chomczynsky and Sacchi 1987); three micrograms of RNA were reverse transcribed in 50 μl of reaction solution, containing 75 mM KCl, 50 mM Tris HCl pH 8.3, 3 mM $MgCl_2$, 0.5 μg of random examers primers, 0.4 mM of each dNTP, 0.1 mM DTT, 10 units of RNasin, and 400 units of Moloney murine leukemia virus reverse transcriptase (RT). After 2 h of incubation at 37°C, the reaction was heated at 95°C for 10 min and quickly chilled in ice. The cDNA was amplified by PCR using 5 μl of the RT mixture in 50 μl of buffer 1 × (50 mM KCl, 25 mM Tris HCl, pH 8.5), containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of *Taq* DNA polymerase, and 30 pmol of each primer. Amplifications were carried out on a 9600 Perkin Elmer thermocycler, performing the hot-start method. For the majority of the oligo sets, the amplification protocol was 32 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 20 s, extension at 72°C for 30 s, and a final extension step of 5 min. For the C1–C2 set, the annealing temperature was 66°C, whereas for the C5–C7 and C5–C8 sets it was 72°C; for the C5–C6 set the extension step was 1 min.

DNA PCR and Restriction Analysis

Genomic DNA was purified from peripheral leukocytes or cultured skin fibroblasts by a standard method. PCR was performed using 200 ng of DNA and the same conditions described for cDNA amplification. For restriction analysis, 1/10 of the amplified products were digested with the appropriate enzyme, according to the manufacturer's instructions.

DNA Sequencing

After resolution on 1% low melting agarose (FMC), the amplified DNA fragments were excised and recovered by agarose digestion with the enzyme Gelase (Epicentre). Two-hundred nanograms of the purified fragments were sequenced on an Applied Biosystem 373A automatic sequencer by using the *Taq* DyeDeoxy terminators cycle sequencing kit, according to the ABI protocol.

Results

Description of the Clinical Case

The proband is a 14-year-old Italian male who is the only son of nonconsanguineous parents with no signs of EB in their family history. Clinical findings in the patient were blisters and erosions occurring since the 1st mo of life and mainly localized on knees, elbows, and ankles; and milia on the troncum and dystrophic nails. The teeth were normal, and extracutaneous involvements were absent. These phenotypical manifestations are consistent with the L-RDEB (Cooper et al. 1987; Bruckner-Tuderman 1993).

Study of COLVII Expression in Skin Biopsies

Immunofluorescence microscopy on the skin of the L-RDEB family members, using a monoclonal antibody anti-COLVII (LH7.2), showed a reduced staining of the dermal-epidermal junction in the proband, as compared to his parents and to a control donor (fig. 1). A decreased staining was also observed in the patient's parents' skin, if compared to control skin biopsy.

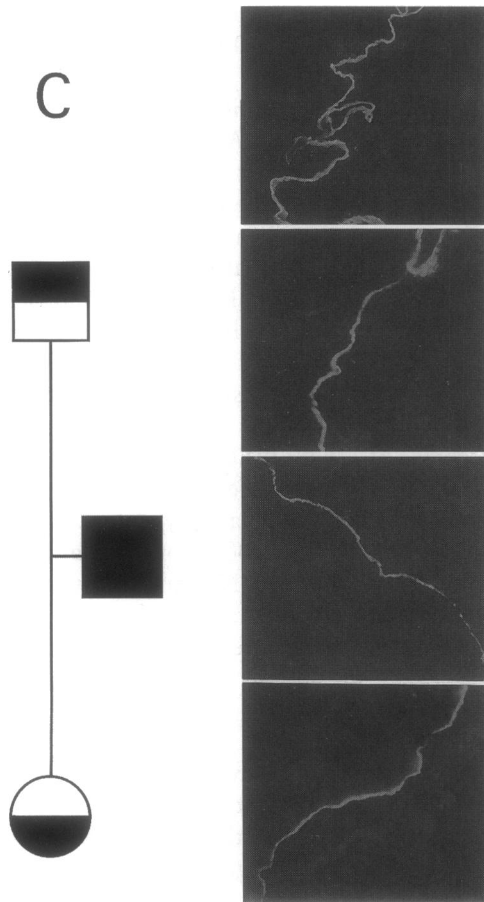


Figure 1 Immunofluorescence microscopy, with the anti-COL-VII monoclonal antibody LH7.2, on the cryosections of the dermal-epidermal junctions from the L-RDEB family and from a control donor (C).

Identification and Characterization of the Paternal Mutation

The search of mutations was performed on COLVII mRNA amplified by RT-PCR. For this purpose, total RNA purified from in vitro cultured skin fibroblasts was retrotranscribed and amplified using several oligonucleotides pairs that allow the amplification of the 9.2-kb full-length COLVII cDNA sequence (Christiano et al. 1994*b*).

The analysis by gel electrophoresis of the PCR reactions evidenced an anomalous pattern in the amplification product spanning exons 2–4, obtained using the oligonucleotides C1 and C2. As shown in figure 2*A*, the amplification with these primers evidenced three fragments, in addition to the 435-bp normal one. These anomalous fragments were also observed in the patient's father, whereas they were absent in his mother as well as in three unrelated RDEB subjects and in eight control individuals.

Sequencing analysis, performed on the electroeluted bands from the amplified cDNA of the patient, showed that all the aberrant fragments derived from altered splicing involving exon 3 (fig. 3*B*). The shortest and most abundant fragment (275 bp) is due to the skipping of the entire 160-bp exon 3; this transcript contains a PTC after the first 10 bases of exon 4. The second fragment (331 bp) lacks the last 104 bp of exon 3 and should originate from the activation of a cryptic exonic GT donor splicing site; this mRNA contains a PTC, located 61 nt downstream the cryptic site, in exon 4. The largest fragment (522 bp) contains exon 3 and the 87-bp-long intron 3; in this mRNA, a PTC at the 66th nucleotide of intron 3 is present.

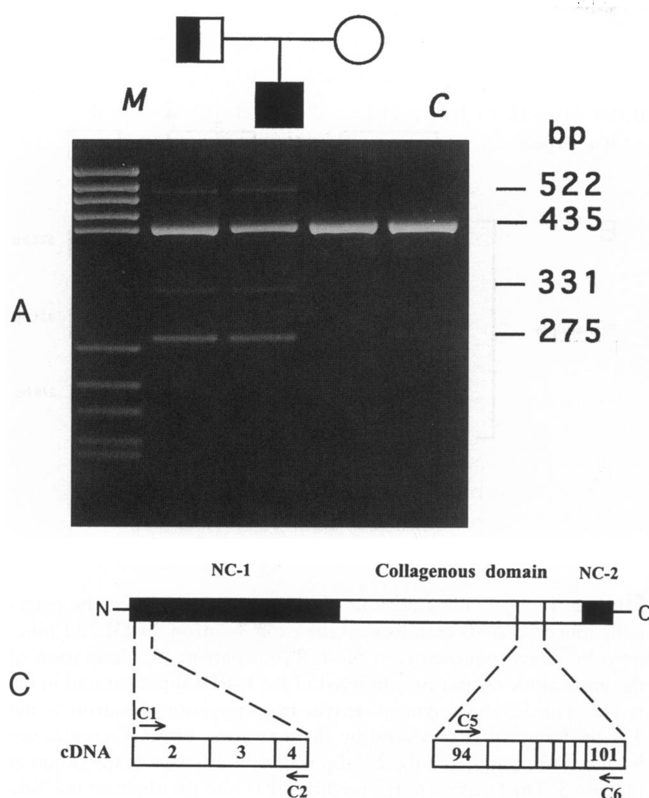
To identify the DNA mutation responsible for the anomalous splicing of exon 3, we sequenced the genomic region encompassing exon 3 and its flanking intronic boundaries. We found that the patient (fig. 3*A*) and his father are heterozygous for an A→G transition at the second-to-last nucleotide of exon 3, whereas the mother is homozygous for the normal allele. This point mutation converts a codon for lysine (AAG) in a codon for arginine (AGG). Moreover, the A→G transition should impair the correct splicing of exon 3, since this mutation falls at position −2 of a 5′ splicing site that, in 58% of the primate genes, is occupied by an A. Computation of the splicing consensus value, performed for −3 +6 donor consensus sequence, as described by Shapiro and Senapathy (1987), gives a value of 0.77 for the normal site and of 0.69 for the mutated site. These data support the involvement of the A→G substitution in the altered processing of exon 3.

This mutation has been reported by Christiano et al. (1994*b*) as a neutral DNA polymorphism, which can be recognized since it abolishes an *Sty*I restriction site. By DNA amplification with the oligo set C3–C4 and subsequent restriction digestion with *Sty*I, we screened 100 unrelated Italian control subjects for the presence of the A→G transition. The mutation was not observed in this sample and was only detected in the L-RDEB family, in the patient and his father (fig. 3C).

Identification and Characterization of the Maternal Mutation

A second alteration in COLVII cDNA was detected as a sovrannumerary slow-migrating fragment in the product obtained with the C5–C6 oligo set, which primes the amplification of a 407-bp fragment spanning exons 94–101, at the C-terminus of the collagenous domain (fig. 2*B*). This anomalous fragment was also observed in the mother of the patient, but it was absent in his father, as well as in three unrelated RDEB subjects and in eight control individuals.

Direct sequencing, performed on the electroeluted slow-migrating band, showed that it was an heteroduplex, composed of the normal COLVII cDNA se-



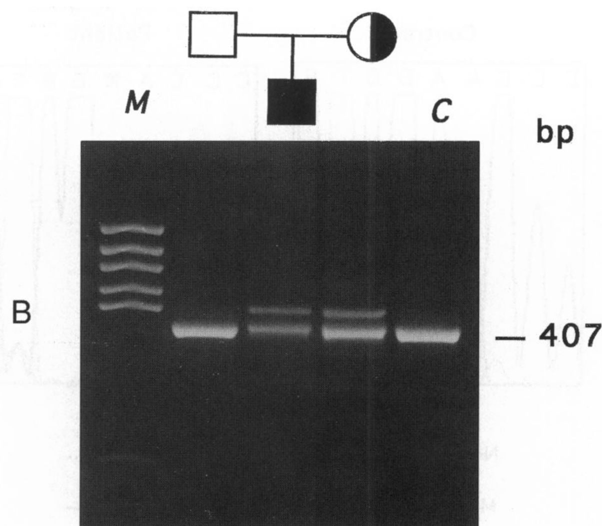


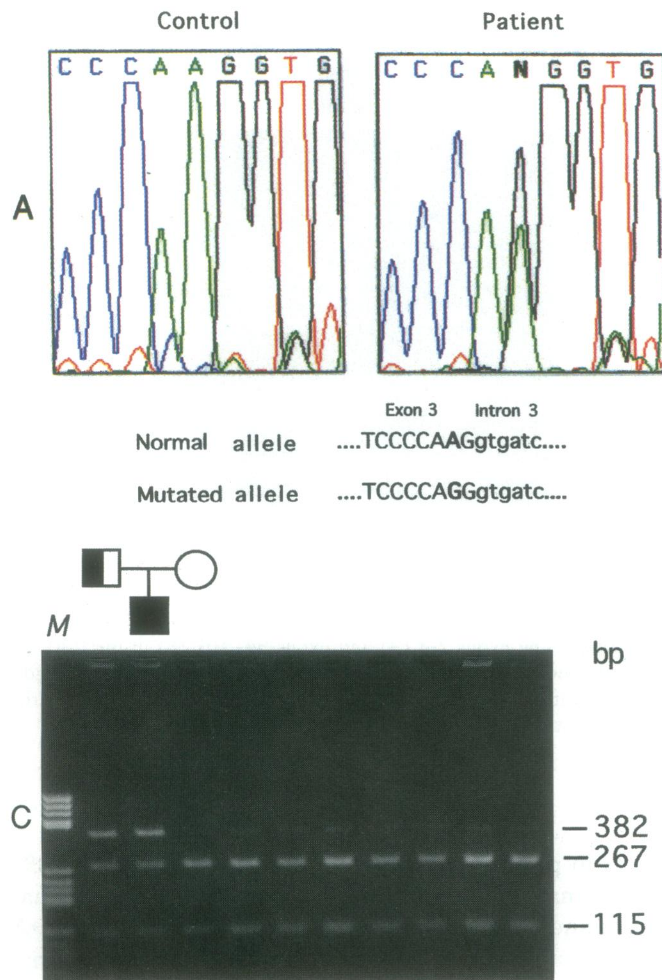
Figure 2 RT-PCR amplification of the COLVII cDNA regions encompassing exons 2-4 (*A*) and exons 94–101 (*B*) in the L-RDEB family and in a control (C) subject. M represents pBR322 DNA digested with *Hae*III. *C*, Localization of the set of primers used for the amplifications of these regions.

quence and of a deleted sequence missing the last 7 nt of exon 95. This deletion is predicted to cause a shift of the reading frame, leading to a PTC after the first 49 nts of exon 97. Thus, the aberrant mRNA should give rise to a truncated proα1(VII) chain, lacking the terminal portion of the collagenous domain and the entire NC-2 domain.

The presence of the deletion was also confirmed by PCR, specific either for the wild-type or for the mutated transcripts. These amplifications were performed using the primer C5 in combination with the primer C7, complementary to the wild-type cDNA sequence, or with the primer C8, which lacks the 7-bp deleted sequence and is complementary to the mutated cDNA molecule. As expected, the C5–C7 oligo set amplified the cDNA sequences derived either from the patient or from a control subject, while the C5–C8 oligo set amplified the deleted cDNA present only in the patient (not shown). In order to elucidate the molecular mechanism leading to the production of the aberrant COLVII mRNA, we amplified and sequenced the region encompassing the exon 95–intron 95 junction of COL7A1. This DNA region was obtained by PCR with the oligos C9 and C10, which amplify the entire exons 95 and 96 and the interrupting intron. A G→A transition at the last nucleotide of the exon 95 was found in one allele of the patient (fig. 4*B*) and of his mother. This point mutation does not lead to an amino-acid change, and it substitutes a G in position −1 of a 5′ splicing site. Since, in 78% of primate genes, this position is occupied by a G (Shapiro and Senapathy 1987), we concluded that the G→A substitution should reduce the utilization of the normal GT donor site of intron 95 and should activate a cryptic site localized 7 bp upstream from the normal one (fig. 4B), giving rise to the 7-bp deleted COLVII mRNA detected by RT-PCR analysis in the patient and his mother. Computation of the splicing consensus value (Shapiro and Senapathy 1987) gives the values of 0.87 and 0.75 for the wild-type and the mutated site at normal position, respectively, and a value of 0.80 for the cryptic site. These data are consistent with the hypothesis that the point mutation reduces the likelihood of utilization of the normal site, favoring the choice of the cryptic one.

In addition to the exonic mutation, the complete sequence of the intron 95 revealed five intronic variations, as compared to the published sequence (Christiano et al. 1994*c*), which were present in homozygosity in all the family members and in an unrelated control subject. These variations were insertion of a G at positions 13, 21, 83, and 88 and a C→T transition at position 84 (fig. 4C). Therefore, the size of the intron 95 resulted of 138 bp instead of the published 134 bp.

Since the G→A transition in COL7A1 exon 95 destroys an *Hph*I site, it was possible to screen a panel of control subjects for the presence of this mutation. The



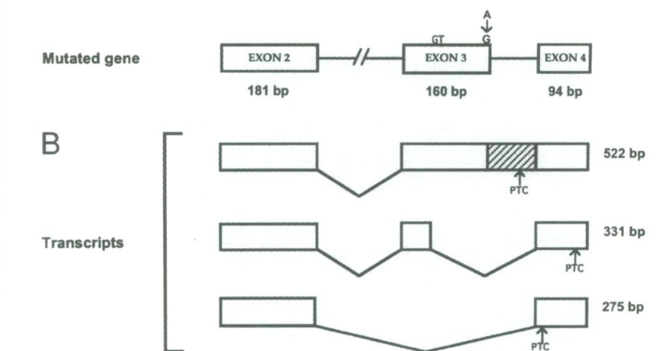


Figure 3 *A,* Identification of heterozygosity (N) for the paternally inherited A→G transition at the exon 3–intron 3 COL7A1 junction, by direct sequencing, in the L-RDEB patient. *B,* Generation of the anomalous transcripts observed in the L-RDEB patient and in his father. The 522-bp fragment derives from retention of intron 3, the 331-bp fragment is produced by the activation of a GT cryptic site localized in exon 3, and the 275-bp fragment arises from the skipping of exon 3. The position of the predicted PTCs in the aberrant mRNAs are also indicated. *C,* Detection of the A→G transition by *Sty*I restriction analysis on amplified genomic DNA. The patient and his father are heterozygous for the normal (267- and 115-bp fragments) and the mutated (382-bp fragment) allele, while the patient's mother and seven control subjects (lanes 5–11) are homozygous for the normal allele. M represents pBR322 DNA digested with *Hae*III.

amplification of the DNA of 100 unrelated subjects, using the C9–C10 oligo-set, followed by *Hph*I digestion, did not evidence the mutation (fig. 4*D*). These data strongly indicate that this point mutation is not a DNA polymorphism but a splicing mutation, which should be related to the disease.

Allele-Specific Analysis of the Transcripts

At the cDNA level, the paternal A→G substitution destroys an *Sty*I site, whereas the maternal G→A transition eliminates a *Bsm*FI site. Therefore, we analyzed these restriction site variations in order to identify the allelic derivation of the COLVII-amplified transcripts and to study the effect of these mutations on the maturation of the transcripts from the mutant alleles.

The analysis of the transcript region carrying the paternal A→G transition was performed on COLVII cDNA amplified with primers C1 and C2. In the control, after restriction digestion with *Sty*I, the 435-bp fragment was cleaved into the expected 337- and 98-bp fragments (fig. 5*A*). In the patient, in addition to these fragments, also those lacking the sequence containing the site and deriving from the aberrant splicing of exon 3 were detected (275 bp visible, 522 and 371 bp barely detectable). Since we did not observe any uncleaved 435-bp fragment, we concluded that all transcripts from the paternal mutated allele undergo altered processing of exon 3.

The analysis of the transcripts from the allele containing the maternal G→A transition was performed on COLVII cDNA amplified with the primers C9 and C11, which generate a 136-bp fragment internal to the C5–C6 amplimer. The small dimension of this amplified fragment allowed the detection of the 7-bp deleted cDNA (129-bp fragment), in addition to the heteroduplex band (fig. 5*B*). Restriction digestion with *Bsm*FI of the C9–C11 amplimer gave rise to the expected 86- and 50-bp fragments in the control. In the patient, in addition to these fragments, since the deletion of 7-bp regenerates a *Bsm*FI site, we observed a 79-bp band deriving from the cleavage of the mutated cDNA. Moreover, we observed an uncleaved 136-bp band, which corresponds to the correct spliced transcript derived

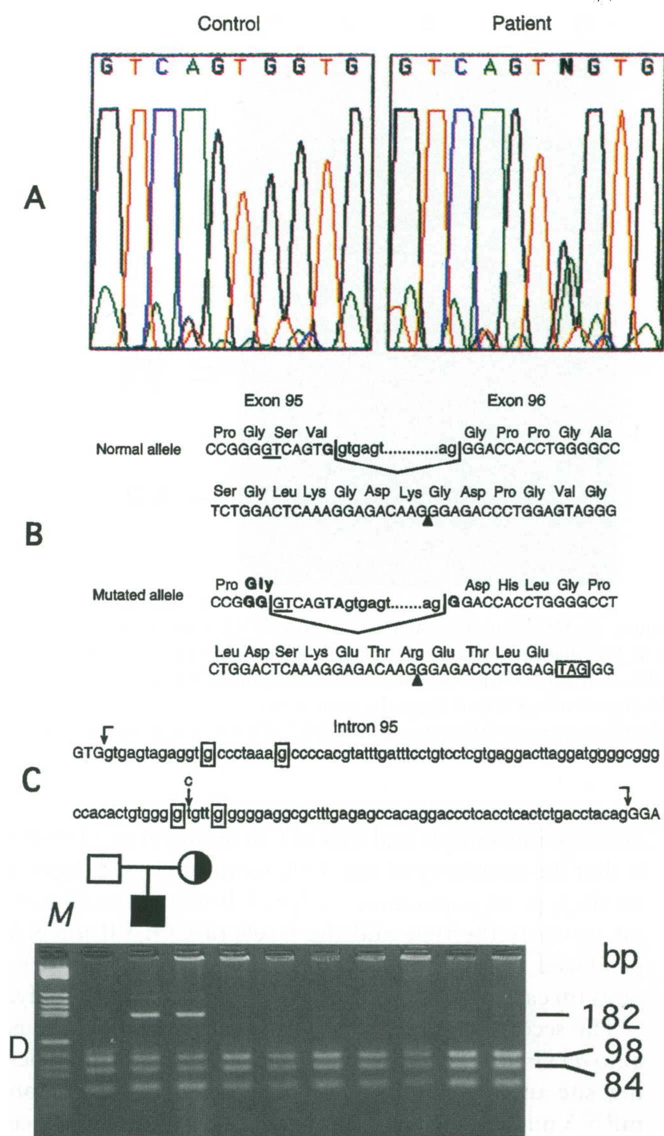


Figure 4 *A*, Identification of heterozygosity (N) for the maternally inherited G→A transition at the exon 95-intron 95 COL7A1 junction by direct sequencing in the L-RDEB patient. *B*, Mechanism giving rise to the aberrant 7-bp deleted mRNA. The cryptic GT splicing site activated by the G→A transition is underlined; the triangle indicates an intron; the PTC in exon 97 is boxed. *C*, Nucleotides variations detected in intron 95, as compared to the published sequence. The boxed letters indicate the insertion of a nucleotide, and the arrow indicates a base substitution. *D*, Detection of the G→A transition by *Hph*I restriction analysis of amplified genomic DNA. The patient and his mother are heterozygous for the normal (98- and 84-bp fragments) and the mutated (182-bp fragment) allele, whereas the patient's father and seven control subjects (lanes 5–11) are homozygous for the normal allele. M represents pBR322 DNA digested with *Hae*III.

from the mutated maternal allele. This observation suggests that the G→A substitution decreases, but does not suppress, the utilization of the normal 5′ splicing site. It should be noted that in the patient's parents the mutated alleles showed the same pattern of mRNA expression detected in the patient (not shown). In conclusion, the allele-specific analysis of the amplified COLVII mRNA indicates that the patient synthesizes a limited amount of normal COLVII mRNA deriving from the maternal mutated allele.

Discussion

We have investigated the COLVII mRNA of a patient with L-RDEB by RT-PCR analysis, and we have identified anomalous mRNAs deriving from aberrant processing of the exons 3 and 95 of COL7A1. Genomic sequencing revealed that the patient is a compound heterozygous for a paternal A→G transition at position −2 of the donor splicing site of intron 3 and for a maternal G→A transition at position −1 of the donor splicing site of intron 95.

The mutation at −2 position of the 5′ splicing site of intron 3 results in a complex aberrant pattern of splicing, giving rise to the three following mRNA forms: in one form the exon 3 is skipped; in the second form 104 nt at the 3′ end of exon 3 are missed, as a consequence of the activation of a cryptic GT site inside this exon; in the third form, the entire intron 3 is retained (fig. 3*B*). As reported in figure 2A, the most represented mRNA isoform is the one derived from exon 3 skipping, while the other two species are less abundant. These results are consistent with the data reported in other genes, for other splicing mutations, which mainly generate mRNAs skipping the mutated exon (Nakai and Sakamoto 1994). Since at the cDNA level the A→G substitution eliminates an *Sty*I restriction site, we could distinguish the allelic derivation of the transcripts and verify the effect of the mutation on the processing of the pre-mRNA from the paternal allele. We found that all transcripts carrying the A→G mutation undergo aberrant processing. The altered transcripts all contain a PTC before the end of exon 4, which should result in shorter proα1(VII) chains missing ~90% of the protein sequence. It is likely that these truncated chains undergo premature degradation and are not incorporated into the COLVII anchoring fibrils. The A→G transition is predicted to result in a Lys142Arg substitution. In light of the neutral nature of this change, and on the assumption that a correct splicing of the mutated transcript takes place, functional proα1(VII) chains should be produced. Therefore, the A→G mutation should have only a quantitative effect, leading to a decrease of the level of COLVII. However, the amount of COLVII synthesized by the normal allele is sufficient to give a normal phenotype, since the patient's father, who is carrier of the mutation, is clinically unaffected.

This A→G transition was previously reported by Christiano et al. (1994*b*) as a DNA polymorphism for

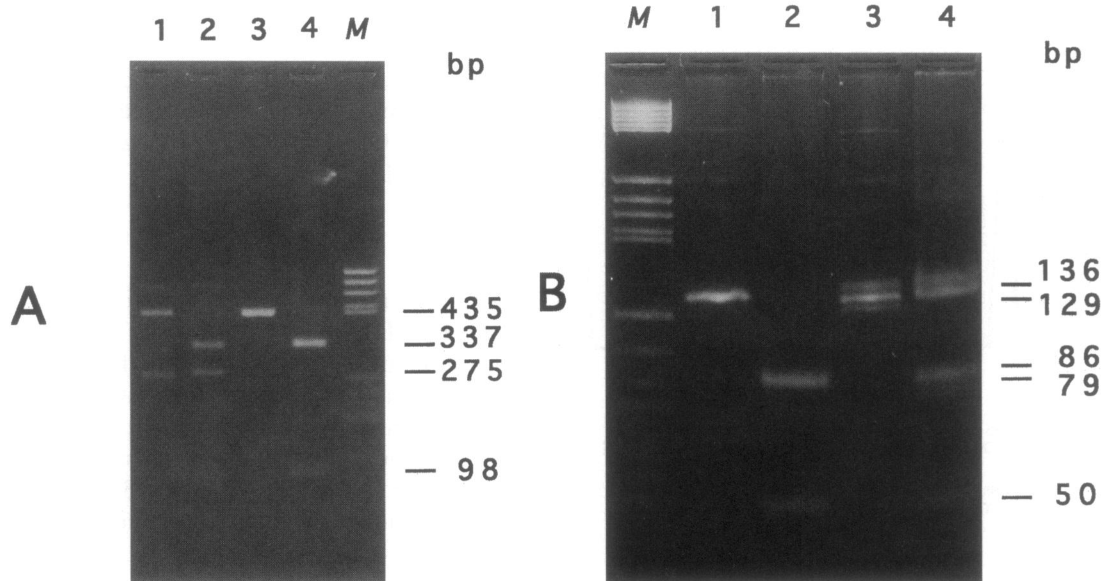


Figure 5 Allele-specific analysis of the amplified COLVII transcripts. *A, Sty*I restriction analysis of the cDNA region containing the paternal A→G transition. Control cDNA before (lane 3) and after (lane 4) *Sty*I digestion and patient cDNA before (lane 1) and after (lane 2) *Sty*I digestion are shown. M represents pBR322 DNA digested with *Hae*III. *B, Bsm*FI restriction analysis of the region containing the maternal G→A transition. Control cDNA before (lane 1) and after (lane 2) *Bsm*FI digestion and cDNA from the patient before (lane 3) and after (lane 4) *Bsm*FI digestion are shown. Electrophoresis was performed in 3% MetaPhor agarose. M represents pBR322 DNA digested with *Hae*III.

the *Sty*I restriction enzyme. Nevertheless, evidence suggests that this mutation is not a neutral polymorphism. The altered processing of exon 3 was observed only in the COLVII mRNA from the L-RDEB family members carrying the A→G mutation, whereas it was not detected in the mRNA from eight control subjects and in three unrelated RDEB patients. The A→G substitution was the only mutation found in exon 3 and in the exon-intron junctions potentially involved in the aberrant splicing of this exon. Moreover, following sequencing of the entire COLVII cDNA, we did not find evidence of other disease mutations, apart from the maternally inherited one. These data strongly indicate that the A→G mutation is responsible for the anomalous processing of exon 3. The restriction analysis of the transcripts expressed by skin fibroblasts of the family members showed that all pre-mRNAs transcribed from the mutated allele undergo anomalous processing, since the species corresponding to the correctly spliced transcript were not detected. Since the A→G transition completely inactivates the expression of COLVII, it cannot be a neutral mutation. In their report, Christiano et al. (1994*b*) analyzed a sample of 18 individuals and reported a frequency of .86 for the wild-type allele and a frequency of .14 for the allele carrying the A→G transition. We did not observe this mutation in a sample of 100 healthy unrelated Italian subjects, as well as in three RDEB families, not related to the family under study. The possible explanation for the differences observed between our sample and that of Christiano et al. (1994*b*) is that the frequency of the A→G mutation could depend on the type of population analyzed. It would be of interest to verify the type and the levels of COLVII mRNA produced by the heterozygous and/or by the homozygous (in case ever found) identified in the reported study.

The second mutation found in the L-RDEB patient is a novel G→A substitution at position −1 of the 5′ splicing site of intron 95. This mutation gives rise to an mRNA missing the last 7 nt of exon 95, as a consequence of the usage of a GT cryptic site located 7 bp upstream from the canonic site.

The mRNA analysis showed that a portion of the transcript with the mutation undergoes correct splicing; therefore, this transition reduces, but does not abolish, the utilization of the normal splice site. Since this G→A transition does not lead to an amino-acid change, the usage of the correct splicing site results in a normal protein.

On the contrary, the deleted mRNA contains a PTC in exon 97 and should result in truncated proα1(VII) chains lacking the last 335 amino acids of the C-terminus of the collagenous domain and the NC-2 domain. A COL7A1 deletion mutation causing a PTC formation in the collagenous domain has been reported in another RDEB patient, and it has been shown to interfere with the assembly of normal COLVII polypeptides through the formation of wild-type/mutant hybrids proteins, which were incorporated into the anchoring fibrils

(Christiano et al. 1994*a*). Therefore, it is likely that in both the L-RDEB patient and his mother the truncated α1(VII) chains, if produced, might perturb the assembly of the anchoring fibrils in a similar manner. It should be noted, however, that this mutation does not seem to have a dominant-negative phenotype, since the mother of the patient is unaffected.

The mutations identified in the L-RDEB family generate PTCs, which often cause the reduction of the levels of the affected mRNAs (McIntosh et al. 1993). In the patient's parents, we observed that the presence of one mutation resulting in PTC only slightly reduces the amount of COLVII mRNA (fig. 2); however, in the patient, the presence of both mutations giving rise to PTCs leads to a stronger reduction of the level of COLVII mRNA. Immunofluorescence analysis at the dermal-epidermal junction showed that also at the protein level both mutations lead to the reduction of COLVII, which is more evident in the patient (fig. 1).

Splicing mutations represent a common cause of genetic diseases (Krawczak et al. 1992). Although they most frequently affect the intronic canonic sites, mutations in the exonic consensus sequence have also been reported. In particular, a G→A nucleotide change at position −1 of the 5′ splicing consensus sequence has been characterized in the proα-2(I) collagen gene, in a patient affected by Ehlers-Danlos syndrome type VII (Weil et al. 1989), in the porphobilinogen deaminase gene, in a patient with intermittent porphiria (Grandchamp et al. 1989), in the β-globin gene, in a patient affected by thalassemia (Vidaud et al. 1989), and in an α-5(IV) collagen gene, in Alport patients (Lemmink et al. 1994). Alterations at position −2 seem to be a rare event, since, to our knowledge, the only mutation reported at this position is an A→G transition identified in the Menkes disease gene (MNK) of a patient affected by the occipital horn syndrome (Kaler et al. 1994). The low number of mutations reported at −2 position could reflect the lower stringency required at this position for recognition and binding of the U1 small ribonucleoprotein (SNRp) involved in the first step of splicing (Horowitz and Krainer 1994). However, in the case reported, the mutation falls in a sequence with suboptimal splicing capability, since the donor sequence of the COL7A1 intron 3 (AAGgtgatc) differs at four positions from the consensus donor sequence (CAGgtaagt). The most relevant variation involves the highly conserved +5 position, which in 84% of the primate genes is occupied by a G. Therefore, on the basis of these observations, it is conceivable that the mutation at −2 should not be tolerated by the splicing apparatus, with the consequent anomalous processing of exon 3.

More than 20 different mutations have been described in RDEB. Apart from a missense mutation, found in a patient affected by a mild (*mitis*) variant of RDEB (Christiano et al. 1993), all the other mutations are frame-shift or nonsense mutations leading to PTCs (Hilal et al. 1993; Hovnanian et al. 1993; Christiano et al. 1994*a*, 1995; Christiano and Uitto 1994). Homozygosity or compound heterozygosity for two PTC mutations has been observed in the Hallopeau-Siemens variant, the most severe, mutilating variant of RDEB (Christiano et al. 1994*a*, 1995; Christiano and Uitto 1994; Hovnanian et al. 1994), indicating that the presence of PTC mutations on both alleles is highly deleterious, since it leads to the lack of the full-length α1(VII) chains. Also the splicing mutations here reported result in aberrant mRNAs containing PTCs. However, as previously discussed, while the paternally inherited A→G transition in exon 3 should completely suppress the production of α1(VII) chains, the maternally inherited G→A transition should allow the partial synthesis of full-length α1(VII) chains. This evidence could explain the mild symptomatology observed in the L-RDEB investigated here.

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