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Compound Heterozygosity for a Recessive Glycine Substitution and a Splice Site Mutation in the COL7A1 Gene Causes an Unusually Mild Form of Localized Recessive Dystrophic Epidermolysis Bullosa

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Type VII collagen is the major component of anchoring fibrils, adhesion structures of stratified epithelia that span the basement membrane region and papillary dermis. Mutations in the gene *COL7A1* encoding type VII collagen cause dystrophic epidermolysis bullosa, a clinically heterogeneous autosomal dominant or recessive blistering disorder of the skin and mucous membranes. In this report, we investigate three siblings affected by an unusually mild form of localized recessive dystrophic epidermolysis bullosa who were shown to be compound heterozygotes for novel mutations affecting *COL7A1*. The maternally inherited mutation is a G→C transversion that converts a codon for glycine to a codon for arginine (G1347R). The paternal mutation is a neutral G→A transition at the last base of exon 70 (5820G→A) that alters the correct splicing of *COL7A1* pre-mRNA, giving rise to an aberrant mRNA carrying the in-frame skipping of exon 70 in addition to the full-length RNA transcript carrying the G→A substitution. Consistent with the normal levels of *COL7A1* mRNA transcripts detected by northern analysis, immunoblotting and immunofluorescence studies evidenced that the patient keratinocytes synthesize and secrete normal amounts of stable type VII collagen, which is correctly deposited at the dermal–epidermal junction. In addition, mutated type VII collagen molecules assemble to form numerous, normally shaped anchoring fibrils, as shown by electron microscopic examination. The combination of a recessive glycine substitution with a splice site mutation that permits partially correct splicing therefore leads to a normal expression of mutated type VII collagen molecules with marginally altered biologic activity, and to the extremely mild phenotype observed in our patients. ***Key words: anchoring fibrils/inherited blistering skin diseases/molecular genetics/type VII collagen. J Invest Dermatol 111:744–750, 1998***

Dystrophic epidermolysis bullosa (DEB) is a clinically heterogeneous group of autosomal dominant or recessive bullous disorders of the skin and mucous membranes characterized by blister formation consequent to varying degrees of trauma, healing with scarring, and nail dystrophy (Fine *et al*, 1991; Zambruno *et al*, 1998). The hallmark of DEB is the localization of the blister cleavage plane below the basement membrane zone (BMZ) at the level of the anchoring fibrils (Fine *et al*, 1991; Zambruno *et al*, 1998). The latter are attachment structures of stratified epithelia that connect the BMZ lamina densa to the upper dermis and are mainly composed of type VII collagen, a nonfibrillar collagen derived from the assembly of three pro-α1(VII) chains each containing a central collagenous domain flanked by two noncollagenous regions (Burgeson, 1993). The clinical heterogeneity of DEB is underscored by various biologic alterations in the anchoring fibrils, such as abnormalities in shape and number and a decreased/absent immunoreactivity for type VII collagen (Bruckner-Tuderman *et al*, 1989, 1995; McGrath *et al*, 1993; König *et al*, 1994), which, in turn, result from a range of mutations in type VII collagen gene (*COL7A1*) (Hovnanian *et al*, 1997; references herein).

In dominant DEB all mutations identified to date cause glycine

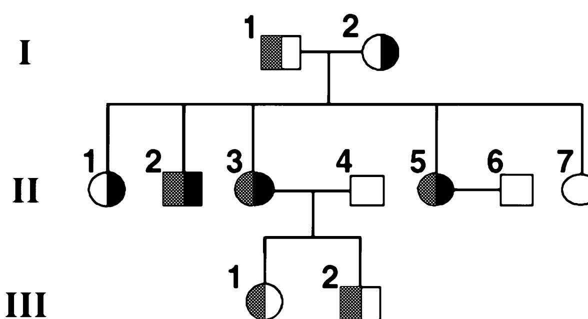

Figure 1. Pedigree of the localized RDEB family. The segregation of the glycine substitution G1347R (half black symbols) and the splice site mutation 5820G→A (half gray symbols) is shown.

Manuscript received June 2, 1998; revised July 20, 1998; accepted for publication July 27, 1998.

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Abbreviations: ASO, allele-specific oligonucleotide; DEB, dystrophic epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa.

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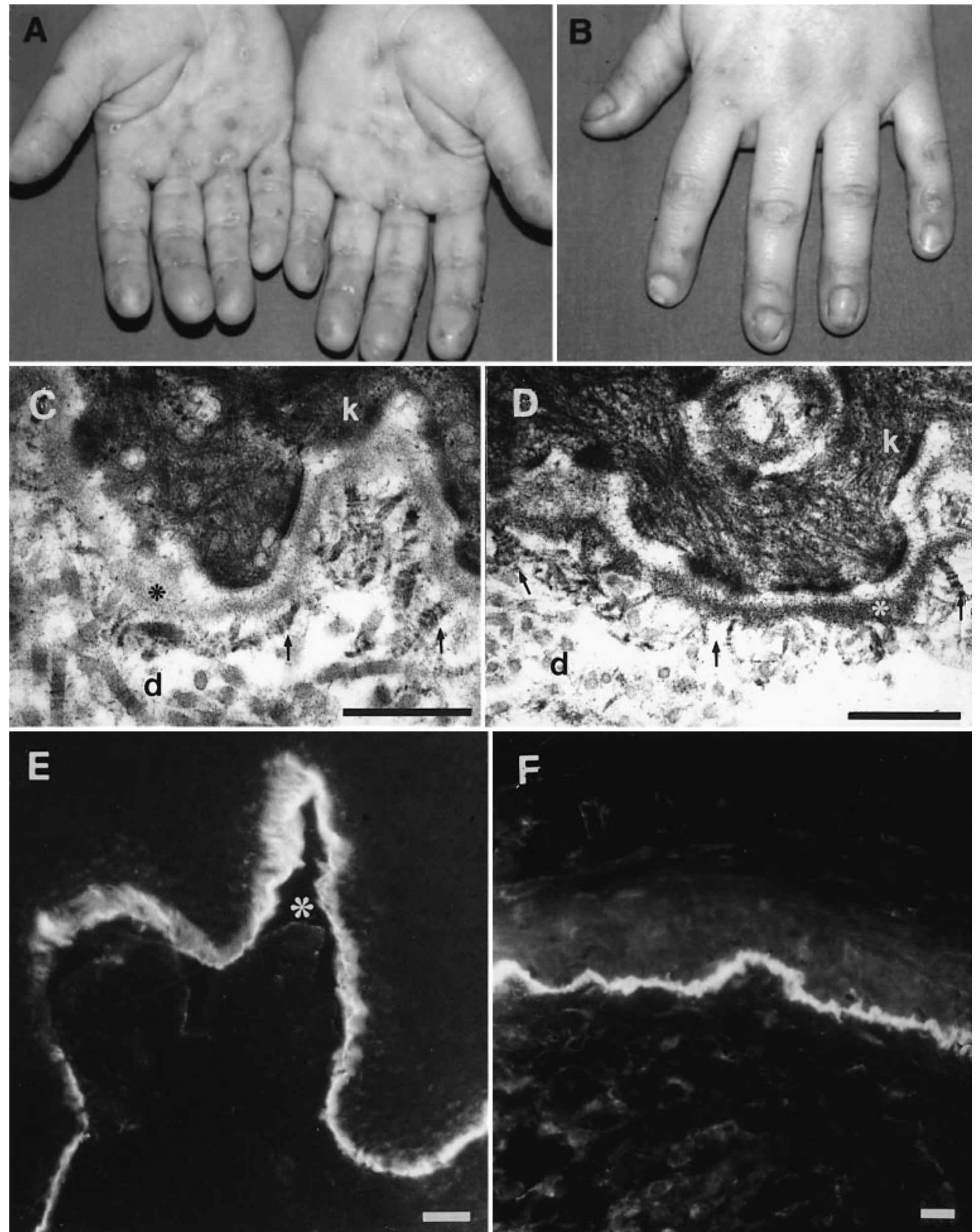

Figure 2. Clinical, ultrastructural, and immunofluorescence features of the localized RDEB family. Patient II.3 exhibits several small blisters, erosions, crusts, and scars on the hand palmar (*A*) and, to a lesser extent, dorsal (*B*) surfaces; mild dystrophy of fingernails is also present (*B*). Electron microscopy of the dermal–epidermal junction (DEJ) of uninvolved RDEB skin (*C*) reveals the presence of numerous, cross-banded anchoring fibrils (*arrows*), which appear similar to those detected in normal control skin (*D*) (k, basal keratinocyte, d, dermis; asterisk, lamina densa). By immunofluorescence microscopy, the linear labeling of the DEJ with monoclonal antibody LH7:2 against collagen VII is similar in control (*F*) and RDEB skin (*E*), where it is localized along the roof of an initial blister (asterisk). *Scale bars*: (*C*, *D*) 0.5 μm; (*E*) 16 μm; (*F*) 20 μm.

substitutions within the triple helix of type VII collagen, which are considered to exert a dominant negative effect by altering the folding of the collagen chains (Christiano *et al*, 1994a, 1996a, b; Lee *et al*, 1997; Winberg *et al*, 1997). In contrast, mutational events in the recessive DEB (RDEB) phenotypes vary considerably (Hovnanian *et al*, 1997 and references therein; Christiano *et al*, 1996b; Tamai *et al*, 1997; Winberg *et al*, 1997). In Hallopeau-Siemens disease, the genetic mutations often cause premature termination codons that inactivate the expression of the two *COL7A1* alleles through premature termination codon-mediated mRNA decay and result in the absence of type VII collagen and anchoring fibrils (Hovnanian *et al*, 1997 and references therein). In the generalized forms of RDEB, at least one other molecular mechanism has been pointed out that involves recessive missense mutations resulting in the synthesis of type VII collagen with decreased stability and/or altered function (Hovnanian *et al*, 1997 and references therein). Finally, two splicing mutations, one of which permits some correct splicing of *COL7A1* pre-mRNA, have been identified in the only patient with the localized form of RDEB so far characterized (Gardella *et al*, 1996).

We report three siblings affected by an unusually mild form of localized RDEB who are compound heterozygotes for a recessive glycine substitution and a splice site mutation. Analysis of the mutation consequences demonstrated that normal steady state levels of *COL7A1* mRNA are present in the patient keratinocytes leading to the secretion of stable type VII collagen molecules that form morphologically typical anchoring fibrils. The combined data provide evidence that normal expression of mutated type VII collagen molecules with marginally altered biologic activity are responsible for the extremely mild phenotype found.

MATERIALS AND METHODS

Patient data Complete clinical examination of all family members was carried out by two of the authors (G.S., G.Z.). Skin biopsies were obtained from patient II.3 (**Fig 1**), the patient's mother, and healthy controls and processed for immunofluorescence and electron microscopy studies, and keratinocyte cultures.

Electron microscopy studies Skin biopsy specimens were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, embedded in Epon resin, and then sectioned on a Reichert Ultracut E ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Philips CM100 transmission electron microscope (Eindhoven, The Netherlands).

Immunofluorescence analysis Frozen 5 μm thick skin sections were processed for immunofluorescence using a three-step biotin-streptavidin-

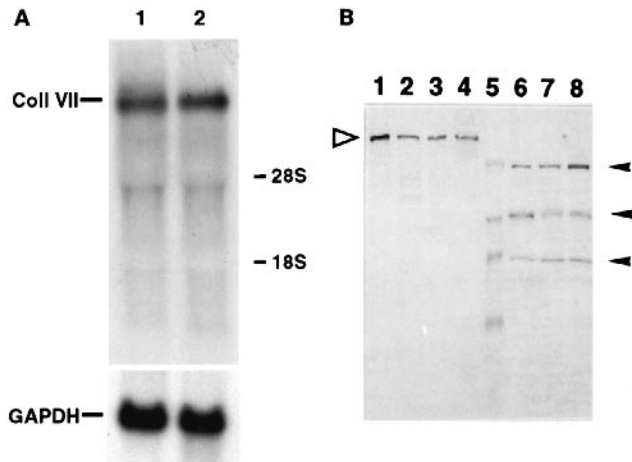

Figure 3. Northern blot and immunoblot analysis of collagen VII mRNA and protein levels in localized RDEB keratinocyte cultures. (*A*) Northern blot analysis of total RNA shows a signal of similar intensity for the collagen VII probe in patient II.3 (*lane 1*) and control keratinocytes (*lane 2*); GAPDH, glyceraldehyde-3-phosphate dehydrogenase probe used for loading control. (*B*) Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under denaturing conditions followed by immunoblotting with antibodies to collagen VII. Control keratinocyte extracts revealed a strong band of procollagen VII (*white arrowhead*; *lane 1*), similarly to extracts of proband II.3 (*lane 2*), the mother I.2 (*lane 3*), and a healthy unrelated control (*lane 4*). The keratinocyte extracts were subjected to limited pepsin treatment, and the digestion products were reacted with antibodies recognizing the triple-helical and the carboxy-terminal NC-2 domains of procollagen VII. The digestion removed the globular domains of procollagen VII and resulted in the appearance of three bands on the immunoblots: the intact triple helical domain and its amino-terminal half, the P2 fragment, as well as its carboxy-terminal half, the P1 fragment (black *arrowheads*, from top to bottom, respectively). In the extracts of probands II.3 (*lane 6*), the mother I.2 (*lane 7*), and a healthy unrelated control (*lane 8*), bands of similar intensity were observed, indicating that the mutations did not result in significant destabilization of collagen VII triple-helix. The 17 amino acid deletion caused by the splice site mutation in exon 70 is located amino-terminally adjacent to the *COL7A1* central hinge region, the main pepsin cleavage site that generates the P1 and P2 fragments. The glycine substitution G1347R resides well within the long triple helical fragment P2, more than 90 amino acid residues from the putative amino-terminal pepsin cleavage site of this fragment. *Lane 5* shows molecular weight standards, from top to bottom: 200, 120, 80, and 50 kDa.

fluorescein procedure, as described (Kanitakis *et al*, 1989). Expression of collagen VII was evaluated using monoclonal antibody (MoAb) LH7:2 (Cymbus Bioscience, Southampton, U.K.). Immunomapping of the epidermal basement membrane components was performed using the anti-laminin 5 MoAb GB3 (gift from G. Meneguzzi, INSERM U385, Nice, France) (Verrando *et al*, 1991); MoAb G0H3, directed against the α6 integrin subunit (gift from A. Sonnenberg, the Netherland Cancer Institute, Amsterdam, The Netherlands) (Sonnenberg *et al*, 1987); the anti-β4 integrin subunit MoAb 3E1 (Telios, San Diego, CA); MoAb HD121 and 1A8C, recognizing the hemidesmosomal proteins HD1 and BP180 (180 kDa bullous pemphigoid antigen, BPAG2), respectively (gift from K. Owaribe, Nagoya University, Nagoya, Japan) (Owaribe *et al*, 1991). The rabbit anti-serum to type IV collagen was purchased from Institut Pasteur (Lyon, France). The following sera and detection systems were employed: biotinylated horse anti-mouse IgG serum (Vector Laboratories, Burlingame, CA), biotinylated rabbit anti-rat IgG serum (Vector Laboratories), biotinylated goat anti-rabbit IgG serum (Vector Laboratories), and fluorescein-streptavidin (Amersham International, Little Chalfont, U.K.).

Keratinocyte cultures Human epidermal keratinocytes were cultivated on a feeder-layer of lethally irradiated 3T3-J2 murine fibroblasts (a gift from H. Green, Harvard Medical School, Boston, MA), as described (Zambruno *et al*, 1995).

Northern analysis For each sample, 20 μg of total RNA was separated by electrophoresis through a 1% agarose/formaldehyde gel, and transferred to Hybond-N+ nylon membrane in 20×sodium citrate/chloride buffer, as devised by the supplier (Amersham International). To detect the collagen VII transcripts, membranes were hybridized with a ^{32}P-labeled 523 bp collagen VII cDNA probe generated by reverse transcriptase-polymerase chain reaction (RT-PCR) and spanning the NC-1 domain of *COL7A1* from nt 734 to nt 1256. For loading control, membranes were hybridized with a probe corresponding to the ubiquitously expressed glyceraldehyde-3-phosphate dehydrogenase gene. Quantitation of autoradiograms was performed by densitometric scanning using a GS-750 densitometer (Biorad, Hercules, CA).

Protein extraction and immunoblot analyses The proteins of keratinocyte cultures were extracted with a neutral buffer containing 0.1 M NaCl, 0.020 M Tris-HCl, pH 7.4, 1% NP-40, and a mixture of proteinase inhibitors (Sonnenberg *et al*, 1991). The stability of procollagen VII in the extracts was analyzed by limited pepsin digestion. For that purpose, cell extracts were acidified with glacial acetic acid to a final concentration of 0.1 M, and the samples were incubated with 10 μg pepsin (Fluka, Deisenhofen, Germany) per ml for 2 h at 5°C. After neutralization with 0.5 M HEPES, pH 8.0, the samples were precipitated with ethanol and subjected to immunoblotting with antibodies to the triple helical and the carboxy-terminal (NC-2) domain of human collagen VII as described (Bruckner-Tuderman *et al*, 1995).

Mutation detection Genomic DNA, extracted from peripheral blood of the probands, the parents, and the controls was used as a template for amplification of individual exons of *COL7A1* gene, as described (Christiano *et al*, 1997). Specifically, to amplify a 445 bp region of *COL7A1* comprising exons 34–35, primers were (L) 5′ TGCTCTCTAAGTGTCTTCCC 3′ and (R) 5′ CCCACTACACATCACTTGCC 3′. Primers used for amplification of a 433 bp portion of *COL7A1* gene containing exons 69–70, were (L) 5′ TGAGTGCGGATGTTGGGTAG 3′ and (R) 5′ GCCCAAGTTCCCTTGA-GTGT 3′ (Christiano *et al*, 1997). For polymerase chain reaction (PCR) amplification, 100 ng of genomic DNA was used as template in 50 μl of reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 50 pmol of each primer, 20 μmol of each deoxynucleotide, and 2.0 U of AmpliTaq Gold polymerase (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ). PCR conditions were 94°C for 10 min; followed by 94°C for 30 s, T_m of primers for 30 s, and 72°C for 30 s (35 cycles). PCR amplification products were subjected to heteroduplex analysis by conformation-sensitive gel electrophoresis (Ganguly *et al*, 1993). If a heteroduplex band was detected, the PCR product was subjected to direct sequencing by Thermo Sequenase ^{33}P-radiolabeled terminator cycle method (Amersham International).

Mutation verification Allele-specific oligonucleotide (ASO) analysis was carried out to verify the exon 34 mutation and to assess its inheritance in the kindred, as described (Ruzzi *et al*, 1997). The ASO used were 5′ TACCGGCTCCC*C*CTTTGG 3′ for the wild-type allele, and 5′ TACCGGCTCCC*G*CTTTGG 3′ for the mutated allele. To verify the exon 70 mutation at the genomic level, aliquots of the corresponding amplification product were subjected to digestion with the restriction enzyme *Hph*I (New England Biolabs, Beverly, MA).

RT-PCR Because the exon 70 mutation occured at position −1 of the 5′ splicing site of intron 70, we looked for the presence of altered transcripts by RT-PCR. Approximately 2.5 μg of total RNA isolated from cultured keratinocytes (Chomczynski and Sacchi, 1987) were reverse-transcribed in a volume of 20 μl using SuperScript RNase H free reverse transcriptase (Gibco BRL, Gaithersburg, MD). PCR amplifications were performed using 1 μl of cDNA in a reaction volume of 25 μl. The oligonucleotides used were (L) 5′ GTTTTCCTGGTGT-CCCAG 3′ (nt 5702–5719) and (R) 5′ AGGCAGGAAGCTACCAGA 3′ (nt 5699–5916) (GenBank accession # L02870). Aliquots of the amplified products were analyzed on 3% agarose gel electrophoresis, subcloned into the pCR 2.1 TA vector (Invitrogen, San Diego, CA), and sequenced by standard dideoxynucleotide techniques (Sanger *et al*, 1977) using the Sequenase version 2.0 DNA Sequencing Kit (Amersham International). To estimate the relative initial amount of the two fragments identified, a PCR kinetic analysis was carried out as described (Salomon *et al*, 1992; Pié *et al*, 1997), with minor modifications. PCR amplification of cDNA was performed for different numbers of cycles (25–40) and aliquots of the amplification products were analyzed on 3% agarose gel electrophoresis. The gel was then blotted on a Hybond-N^+ nylon membrane according to the alkaline blotting procedure (Amersham International) and hybridized with a reverse γ^{32}P-labeled oligonucleotide 5′ GCAGTTTCCAGCAACCGATC 3′ spanning exon 71 (nt 5827–5846). In each lane, the peak areas of both cDNA bands were measured by densitometric scanning of autoradiograms and plotted as log area values *versus* the cycle number. Semi-logarithmic plots resulted in two straight lines with identical slopes over the range of 30–40 cycles, indicating a similar amplification efficiency of both fragments. To determine the relative difference in the initial amount of the two mRNA transcripts, the log area value was extrapolated to the intercept for each line.

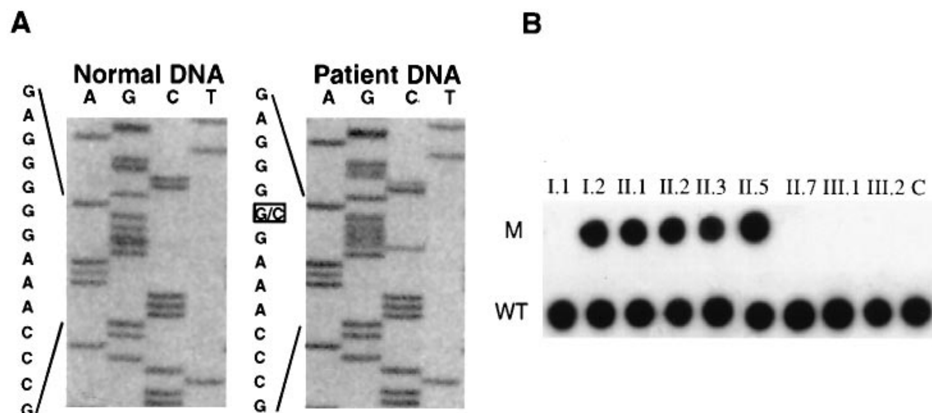

Figure 4. Identification of the heterozygous missense mutation G1347R in *COL7A1* gene of patient II.3 and inheritance in the kindred. (*A*) Total genomic DNA was subjected to PCR reaction using primers which amplify a 445 bp fragment spanning exons 34–35 of *COL7A1*. In comparison with the DNA of a normal control, sequencing of the patient's DNA reveals a G→C transversion at nucleotide position 4039 of exon 34 that changes a glycine codon to an arginine codon at amino acid residue 1347. The mutation is designated G1347R. (*B*) ASO analysis: hybridization of the 445 bp PCR product with a wild type (WT) and mutated (M) ASO shows the heterozygous state of the mutation in the three affected siblings (II.2, II.3, II.5), the patients' mother (I.2), and a healthy member (II.1) of the kindred. C, unaffected control individual.

RESULTS

Members of a family affected with an extremely mild form of localized RDEB display normal type VII collagen immunoreactivity and anchoring fibril morphology in the skin The patients were three Italian siblings (**Fig 1**, II.2, II.3, and II.5) aged 30, 28, and 27, respectively, born of nonconsanguineous and clinically unaffected parents. Two other siblings (**Fig 1**, II.1 and II.7) were unaffected and there was no family history of skin or genetic diseases. All the probands had been suffering from skin blisters and erosions since their first month of life. During early childhood, lesions were strictly localized on trauma-exposed sites, i.e., elbows, knees, hands, and feet, whereas, from early adulthood onwards, they appeared exclusively on the palmar and dorsal surfaces of hands (**Fig 2*A*, *B***) and, to a lesser extent, feet. The lesions resolved with mild scarring and milia formation (**Fig 2*A*, *B***). On examination, dystrophy of fingernails (**Fig 2*B***) and toenails was also observed, whereas mucosae, hair, and teeth showed no lesions.

The diagnosis of DEB was confirmed by ultrastructural examination of a skin biopsy from patient II.3, which localized the cleavage plane below the lamina densa of the cutaneous BMZ (not shown). In noninvolved skin, the numerous anchoring fibrils present along the BMZ appeared of normal length and thickness, and revealed a well-defined cross-banding pattern (**Fig 2*C*, *D***). Other components of the cutaneous BMZ, including hemidesmosomes and anchoring filaments, exhibited no significant abnormalities.

Antigen mapping was performed by immunofluorescence staining of frozen skin sections using antibodies recognizing the major components of the cutaneous BMZ. In areas of microblistering, the staining for the hemidesmosomal components $\alpha6\beta4$ integrin, BP180 (180 kDa bullous pemphigoid antigen, BPAG2), and plectin, as well as that for laminin 5 and type IV and VII collagens, was localized to the blister roof, indicating that tissue separation occurs below the lamina densa at the level of the anchoring fibrils, and confirming the diagnosis of DEB (not shown). Type VII collagen exhibited a strong, linear labeling along the BMZ, with an intensity comparable with that observed in normal control skin (**Fig 2*E*, *F***).

Collagen VII mRNA and protein levels are similar in localized RDEB and control keratinocytes The level of expression of the gene for type VII collagen was assessed by northern analysis of total RNA obtained from cultured keratinocytes of patient II.3, the patient's mother, and a healthy control. Densitometric analysis of the hybridization bands did not reveal any significant reduction of the steady state level of collagen VII mRNA in the patient as compared with the control (**Fig 3*A***).

Consistent with these results, immunoblotting analysis of cultured keratinocytes from patient II.3, the mother, and normal controls showed that comparable amounts of collagen VII could be extracted from all the cell cultures (**Fig 3*B***). A pepsin digestion under mild conditions resulted in fragmentation of collagen VII into the triple helix and the P1 and P2 fragments in all three extracts (Burgeson, 1993). The relative intensity and stability of the fragments were similar (**Fig 3*B***), implying that the genetic defects do not lead to significant destabilization or intracellular accumulation of collagen VII in the RDEB keratinocytes.

Mutation analysis reveals compound heterozygosity for a recessive glycine substitution and a splice site mutation in *COL7A1* gene The entire *COL7A1* gene was scanned by PCR amplification of individual exons and flanking intronic boundaries, followed by heteroduplex analysis of the PCR products using conformation-sensitive gel electrophoresis. Conformation-sensitive gel electrophoresis analysis of genomic DNA from patient II.3 showed two distinct shifts, corresponding to exons 34–35 and 69–70. Direct sequence analysis of the PCR product corresponding to exons 34–35 (445 bp) detected a G→C transversion at nucleotide position 4039 (4039G→C) of exon 34 that changes a glycine codon to an arginine codon at amino acid residue 1347 (GenBank, accession #L02870) (**Fig 4*A***). The mutation was therefore designated G1347R. ASO analysis confirmed the heterozygous state of mutation G1347R in the three affected siblings and in a healthy member of the kindred, and demonstrated the maternal inheritance (**Fig 4*B***). Screening 150 alleles from unrelated, healthy individuals by ASO analysis failed to disclose the presence of mutation G1347R.

Direct sequencing of the 433 PCR product corresponding to exons 69–70 and the flanking intronic regions detected a neutral G→A substitution at nucleotide position 5820 of the 5′ donor splice site of intron 70 (**Fig 5*A***). Mutation 5820G→A abolishes a restriction site for endonuclease *Hph*I. Digestion of the PCR product with this enzyme revealed that the three affected siblings, their father, and two unaffected family members were heterozygous for the mutation, whereas the mother did not demonstrate this genetic lesion (**Fig 5*B***). In addition, mutation 5820G→A was not present in 150 alleles from unrelated healthy individuals, confirming that this substitution does not represent a common polymorphism.

The splice site mutation results in an aberrant mRNA carrying the in-frame skipping of exon 70 and a full-length transcript carrying the G→A substitution The mutation 5820G→A occurs at position −1 of the 5′ donor splice site of intron 70. Because in primates 78% of genes harbor a G in this position, an aberrant splicing could be expected (Shapiro and Senapathy, 1987). To verify this possibility, mRNA obtained from cultured keratinocytes of patient II.3 was analyzed by RT-PCR using primers that amplify nucleotides 5702–

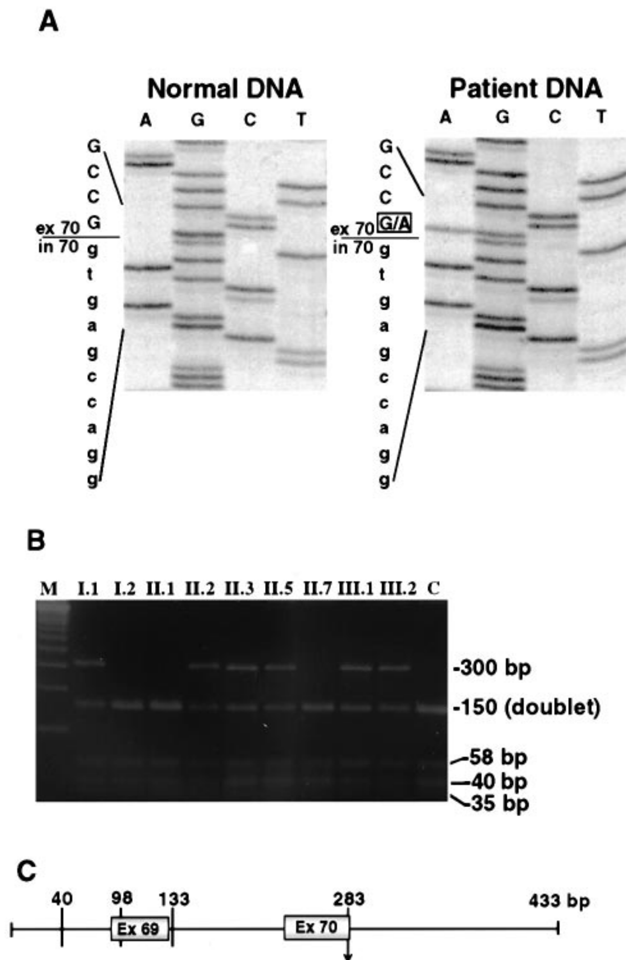

Figure 5. Identification of the heterozygous splice site mutation 5820G→A in *COL7A1* gene of patient II.3 and inheritance in the kindred. (*A*) Total genomic DNA was subjected to PCR reaction using primers that amplify a 433 bp fragment encompassing *COL7A1* exons 69–70. As compared with the DNA of a normal control, sequencing of the patient's DNA reveals a G→A substitution at the last nucleotide of exon 70 (nt position 5820). The mutation is designated 5820G→A. (*B*) Verification of mutation 5820G→A with *Hph*I restriction endonuclease digestion of the 433 bp PCR product. Five fragments (a 150 bp doublet, and 58, 40, 35 bp bands) are visible in the mother and an healthy unrelated control (*C*), and an additional 300 bp fragment (from the 150 bp doublet) is detected in the three affected siblings (II.2, II.3, II.5), their father (I.1), and two healthy family members (III.1, III.2), which is indicative of a heterozygous state for mutation 5820G→A. The markers for molecular size (100 bp DNA ladder, from 2000 to 100 bp) are in *lane M.* (*C*) Schematic representation of the localization of *Hph*I sites within the 433 bp amplified fragment, an *arrow* denotes the site abolished by mutation 5820G→A.

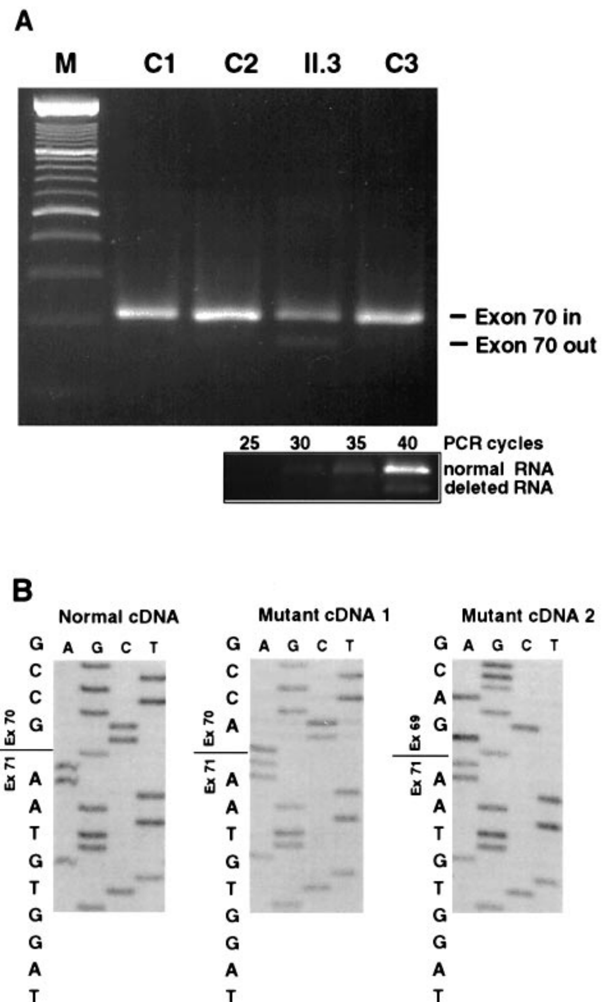

Figure 6. RT-PCR and cloning analysis of the effects of splice site mutation 5820G→A at the mRNA level. (*A*) The mRNA from cultured keratinocytes was analyzed by RT-PCR using oligonucleotide primers that amplify nt 5702–5916 of *COL7A1* cDNA. In addition to the expected major 215 bp band present in the controls (C1, C2, C3), analysis of the amplification product reveals a smaller band in patient II.3. The inset shows the electrophoretic pattern of the two amplified bands in relation to the number of PCR cycles. The markers for molecular size (100 bp DNA ladder, from 2000 to 100 bp) are in *lane M.* (*B*) As compared with a normal control, sequence analysis of a clone obtained from the 215 bp band (mutant cDNA 1) reveals a G→A substitution at the last nucleotide of exon 70. Isolation and cloning of the smaller band reveals an aberrant mRNA bearing an in-frame skipping of the entire exon 70 (48 bp) (mutant cDNA 2).

5916 of *COL7A1* cDNA. Analysis of the amplification product on 3% agarose gel revealed a clearly detectable, fast-migrating DNA fragment, in addition to the expected 215 bp band (**Fig 6*A***). Isolation, subcloning, and sequencing of the 215 bp cDNA band identified transcripts corresponding to a correctly spliced mRNA. Four of 32 independent clones carried mutation 5820G→A (**Fig 6*B***). Sequencing of cDNA clones derived from the fast-migrating band identified an abnormal mRNA transcript originating from the in-frame skipping of the entire exon 70 (48 bp) (**Fig 6*B***). In addition, the ratio between the mRNA species carrying the in-frame exon skipping and the full-length mRNA transcripts obtained by PCR kinetic analysis was 1:1.45 (see *Materials and Methods*, **Fig 6*A***), indicating that the internally deleted mRNA represents 40.8% of the total collagen VII mRNA.

DISCUSSION

In this report, we describe the molecular defect in three siblings suffering from an extremely mild form of localized RDEB. A search for mutations in the *COL7A1* gene demonstrated that the probands are compound heterozygotes for two novel mutations, the glycine substitution G1347R and the splice site mutation 5820G→A, which further extend the molecular heterogeneity of DEB.

The maternally inherited G→C transversion in exon 34 results in substitution G1347R within the second of the 20 collagenous subdomains that form the collagen VII triple helix (Christiano *et al*, 1994b). Substitution G1347R results in a recessively inherited condition, because two healthy family members were heterozygous carriers of the mutation. To date, several recessively inherited missense mutations, mainly glycine substitutions, within the triple helix of the *COL7A1* gene have been described in the Hallopeau-Siemens and mitis variants of generalized RDEB (Hovnanian *et al*, 1997 and references therein; Christiano *et al*, 1996a). Because heterozygous carriers are unaffected, these mutations are expected to act through mechanisms distinct from the dominant negative effect of other glycine substitutions reported in dominant DEB (Christiano *et al*, 1994a, 1996a, b; Lee *et al*, 1997; Winberg *et al*, 1997).

This study reports that a recessively inherited glycine substitution combined with a splice site mutation *in trans* results in a localized variant of RDEB. In our patient, the steady state level of type VII collagen mRNA comparable with normal controls is in agreement with similar data reported in other recessively inherited *COL7A1* missense mutations and with the observation that missense mutations do not usually alter RNA stability (Hovnanian *et al*, 1997). Immunoblotting and immunofluorescence analysis show type VII collagen levels similar to healthy controls, and ultrastructural observation reveals numerous, well-formed anchoring fibrils. These findings suggest that mutation G1347R not only allows synthesis, secretion, and deposition of stable collagen VII molecules, but also does not significantly interfere with the supramolecular assembly of collagen VII into anchoring fibrils.

The paternal mutation is a neutral G→A transition at position –1 of the donor splice site (5820G→A) of intron 70. In this study, we show that mutation 5820G→A alters the correct splicing of *COL7A1* pre-mRNA and generates a mRNA bearing a deletion of 48 nucleotides (transcript 2), and a full-length transcript carrying the G→A substitution (transcript 1). Transcript 2 carries an in-frame skipping of exon 70 and codes for a type VII collagen polypeptide with an internal deletion of 16 amino acids spanning the last 5 Gly V-X-Y repeat sequences of the tenth collagenous subdomain of the triple helix and the first of the 39 amino acids that form the nonhelical stretch located in the middle of the helix (Christiano *et al*, 1994b). Although transcript 2 accounts for about 40% of the total *COL7A1* mRNA and therefore represents the majority of the mRNA species produced from the paternal allele, minute amounts of correctly spliced RNA transcript 1 were also detected. In our patient, the normal steady state levels of *COL7A1* mRNA detected by northern analysis clearly indicate that, in addition to transcript 1, the far more abundant paternal transcript 2 carrying the in-frame skipping of exon 70 is also not degraded and contributes to the normal expression of collagen VII.

It has been shown that, in several genes, splice site mutations that permit some correct splicing lead to mild phenotypic disease variants (Weil *et al*, 1989; Akli *et al*, 1990; Aslanidis *et al*, 1996; Gardella *et al*, 1996; Posteraro *et al*, 1998). Interestingly, the only other case of localized RDEB characterized to date results from the combination of two splice site mutations, one of which affects position –1 of the donor splice site of intron 95 and causes partial correct splicing of the mutant *COL7A1* pre-mRNA (Gardella *et al*, 1996). Mutational analysis of additional patients is required to determine whether splice site mutations allowing some correct splicing represent a common mechanism in localized forms of RDEB.

In conclusion, our results indicate that both the maternal transcript carrying the recessive glycine substitution and the paternal aberrant transcripts 1 and 2 encode the stable collagen VII polypeptides visualized by immunofluorescence and immunoblotting. The subsequent supramolecular assembly of mutated collagen VII molecules into apparently normal anchoring fibrils with a marginal functional defect explains the extremely mild and localized phenotype in the probands who develop blistering lesions exclusively at sites of major, repeated traumatisms.

The authors thank L. Ruzzi for helpful discussion, N. De Luca and A. Bucci for technical assistance, and C. Secci for artwork. This work was supported by grants from Ministero della Sanità (Italy), EEC BIOMED 2 (n. BMHG4–97–2062), Telethon (Italy) (n. A.106), the Deutsche Forschungsgemeinschaft (DFG) (Br 1475/1–2 and Br 1475/2–2), and DEBRA U.K.

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