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# Characterization of 18 New Mutations in COL7A1 in Recessive Dystrophic Epidermolysis Bullosa Provides Evidence for Distinct Molecular Mechanisms Underlying Defective Anchoring Fibril Formation

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## Summary

**We have characterized 21 mutations in the type VII collagen gene (COL7A1) encoding the anchoring fibrils, 18 of which were not previously reported, in patients from 15 unrelated families with recessive dystrophic epidermolysis bullosa (RDEB). COL7A1 mutations in both alleles were identified by screening the 118 exons of COL7A1 and flanking intron regions. Fourteen mutations created premature termination codons (PTCs) and consisted of nonsense mutations, small insertions, deletions, and splice-site mutations. A further seven mutations predicted glycine or arginine substitutions in the collagenous domain of the molecule. Two mutations were found in more than one family reported in this study, and six of the seven missense mutations showed clustering within exons 72–74 next to the hinge region of the protein. Patients who were homozygous or compound heterozygotes for mutations leading to PTCs displayed both absence or drastic reduction of COL7A1 transcripts and undetectable type VII collagen protein in skin. In contrast, missense mutations were associated with clearly detectable COL7A1 transcripts and with normal or reduced expression of type VII collagen protein at the dermo/epidermal junction. Our results provide evidence for at least two distinct molecular mechanisms underlying defective anchoring fibril formation in RDEB: one involving PTCs leading to mRNA instability and absence of protein synthesis, the other implicating missense mutations resulting in the synthesis of type VII collagen polypeptide with decreased stability and/or altered function. Genotype-phenotype correlations suggested that the nature and location of these mutations are important determinants of the disease phenotype and showed evidence for interfamilial phenotypic variability.**

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## Introduction

Dystrophic epidermolysis bullosa (DEB) is a group of inherited skin diseases characterized by blistering and scarring of the skin after mild trauma. DEB is transmitted in an autosomal dominant or autosomal recessive pattern, both forms showing tissue separation beneath the basement membrane at the level of the anchoring fibrils (AF) (Fine et al. 1991; Bruckner-Tuderman 1993). Abnormalities in the number and morphology of AF are the characteristic hallmark in DEB patients' skin, and the expression of type VII collagen, the major component of AF, is frequently altered (Heagerty et al. 1986; Leigh et al. 1988; Bruckner-Tuderman et al. 1989). We and others have established linkage between the type VII collagen gene (COL7A1) and both dominant (DDEB) and recessive (RDEB) forms of DEB with no evidence for locus heterogeneity (Ryynänen et al. 1991; Al-Imara et al. 1992; Hovnanian et al. 1992; Dunnill et al. 1994*a;* Naeyaert et al. 1995). A number of COL7A1 mutations have now been reported in DDEB patients (Christiano et al. 1994*d*, 1995*a*, 1995*b*, 1996*b*) and RDEB patients (Christiano et al. 1993, 1994*a*, 1995*c*, 1996*a*, 1996*b*, 1996*c;* Hilal et al. 1993; Dunnill et al. 1994*b;* Hovnanian et al. 1994; Bruckner-Tuderman et al. 1995; Dunnill et al. 1996; Gardella et al. 1996; Shimizu et al. 1996), confirming the direct implication of this gene in both forms of the disease.

RDEB comprises several forms, the most severe of which is the Hallopeau-Siemens type (HS-RDEB; MIM number 226600). HS-RDEB is characterized by generalized blistering of the skin, leading to mutilating scarring of fingers and toes, flexural contractures, mucosal involvement, and poor prognosis due to malnutrition, anaemia, infectious complications, and the development of aggressive squamous-cell carcinoma. In this form of RDEB, AF are frequently absent, and immunofluorescence staining of skin is negative for type VII collagen antibodies (Bruckner-Tuderman et al. 1989). However, in a few cases with HS-RDEB, rare AF and immunoreactive type VII are seen at the dermo/epidermal junction (DEJ) (Rusenko et al. 1989; McGrath et al. 1993; Dun-

nill et al. 1996). Other forms of RDEB comprise the mitis, the inverse, and the localized types. These do not share the extreme severity of the HS-RDEB type and often show essentially normal or somewhat decreased but positive staining at the DEJ, with various abnormalities of AF (Bruckner-Tuderman et al. 1990).

COL7A1 is a complex and unusually compact gene consisting of 31,132 bp and 118 exons transcribed into a 9.2-kb mRNA (Christiano et al. 1994*b,* 1994*c*). Type VII collagen molecules are homotrimers, each proα1 (VII) polypeptide chain containing a central triple-helical collagenous domain (145 kD in size) flanked by both a large (145 kD) amino-terminal noncollagenous (NC-1) domain and a small (30 kD) carboxy-terminal noncollagenous (NC-2) domain (Burgeson et al. 1990; Christiano et al. 1994*b*). The collagenous domain consists of a repeating Gly-X-Y sequence that is disrupted 19 times by noncollagenous regions. The NC-1 domain consists of submodules with homology to adhesive proteins, including a cartilage matrix–protein motif, nine fibronectin type III–like domains, and a segment with homology to von Willebrand factor A domain (Christiano et al. 1992; Gammon et al. 1992). The NC-2 domain contains conserved cysteines involved in the formation of disulfide linkage between type VII collagen homotrimers (Greenspan 1993). During fibrillogenesis, type VII collagen molecules form antiparallel dimers that overlap through their NC-2 domains, which are subsequently proteolytically cleaved (Bruckner-Tuderman et al. 1995). AF are formed by the lateral aggregation of the collagenous domains of the dimers, and the NC-1 domains are thought to interact both with components of the basement-membrane zone and with anchoring plaques in the papillary dermis, thus securing the epidermis to the underlying dermis (Burgeson 1993).

It has been suggested that the nature and/or the location of mutations in COL7A1 determine the variations in clinical phenotype, type VII collagen expression, and AF formation (Hovnanian et al. 1993; Uitto and Christiano 1994). In support of this interpretation, a majority of mutations reported in HS-RDEB result in premature termination codons (PTCs) (Uitto et al. 1995), which are thought to lead to null alleles through PTC-mediated mRNA decay (Maquat 1995). In contrast, DDEB has been shown to be due to glycine substitutions in the collagenous domain of the molecule. However, glycine substitutions in the collagenous domain of type VII collagen, either present at the homozygous state or combined with a PTC, have also been reported in HS-RDEB, indicating that some glycine substitutions act in a recessive manner (Christiano et al. 1996*b;* Dunnill et al. 1996; Shimizu et al. 1996). Mutations in COL7A1 have also been described in RDEB mitis, the first of which was a methionine-to-lysine substitution in the NC-2 domain of COL7A1 (Christiano et al. 1993). PTCs associated with either a glycine substitution, a splice-site mutation, or a delayed termination codon have also been characterized in this clinicaly moderate form of RDEB (Christiano et al. 1996*c;* Shimizu H et al. 1996). Recently, two splicing mutations in COL7A1 have been described in a patient with the localized form of RDEB (Gardella et al. 1996). Overall, these results indicate that different combinations of COL7A1 mutations are associated with various clinical phenotypes.

In this study, we systematically screened the COL7A1 gene for mutations in 18 patients from 15 unrelated RDEB families. Sixteen patients were diagnosed as HS-RDEB, one patient had RDEB mitis, and another patient had inverse RDEB. COL7A1 mutations were identified in both alleles in all these patients. We have characterized 21 mutations, 18 of have not previously described. Fourteen mutations created PTCs, and seven mutations predicted glycine or arginine substitutions in the collagenous domain. We investigated the consequences of these mutations on COL7A1 transcript and on type VII collagen expression in a majority of these patients. Genotype-phenotype correlations were also analyzed in several families.

## Patients and Methods

### *Patients*

Eighteen patients from 15 unrelated families with RDEB were studied, 2 families (11 and 14) having more than one affected offspring (two and three, respectively). Families were of French, Pakistani, Portuguese, Spanish, or Tunisian origin. Consanguinity was known in five families (patients 3, 5, 6, 13, and 14). Sixteen patients presented with severe generalized RDEB (i.e., HS-RDEB), whereas one patient (patient 9) was affected with inverse RDEB and one patient (patient 12) with mitis RDEB. The diagnosis was based on family history, clinical examination, and electron-microscopic evaluation according to criteria as defined by Fine et al. (1991). Ethical-committee approval was obtained for the study.

### *Immunofluorescence Staining*

Indirect immunofluorescence staining was performed on cryosections of punch biopsies of skin from upper inner thigh, as described elsewhere (Bruckner-Tuderman et al. 1989). The primary mouse monoclonal antibody LH7:2 to the NC-1 domain of human collagen VII (Sigma Immuno Chemicals) was diluted 1:1,000, and a fluorescein isothiocyanate–conjugated secondary goat anti-mouse antibody (Sigma Immuno Chemicals) was diluted 1:100. Monoclonal antibody GB3 to laminin 5 (Valbiotech), diluted 1:100, was also used.

### *Standard Electron Microscopy and Immunoelectron Microscopy (IEM)*

Four-millimeter punch biopsies obtained from the inner thigh were processed for standard electron micros-

copy as described by Eady (1985). IEM was performed in patients 10, 12, and 14, by use of a preembedding technique adapted from Prost et al. (1984) and McGrath et al. (1993). Immunolabeling was performed with two primary antibodies: LH7:2 (diluted 1:2) and GB3 (diluted 1:10). A goat anti-mouse secondary antibody was used, prepared in one of two ways: either 1 nmol gold conjugated (Biocell) and diluted 1:3 or peroxidase labeled (Biosys) and diluted 1:10. Fixation in Karnovsky reagent was followed by silver enhancement, postfixation by osmium tetroxide, and embedding. Uncontrasted ultrathin sections were observed under a Jeol 1200 EX transmission-electron microscope. A biopsy of a surgical skin sample obtained from breast plastic surgery from a normal subject was used as a control.

### *PCR Amplification*

Genomic DNA was extracted from peripheral blood leukocytes by use of standard methods. PCR amplification of COL7A1 was performed by use of 68 pairs of oligonucleotide primers, spanning all 118 exons and flanking splice sites of the gene. The NC-1 domain (exons 1–28) was screened by denaturing gradient gel electrophoresis (DGGE) using 21 sets of primers (sequences are available, on request, from the authors). The collagenous domain (exons 29–112) and the NC-2 domain (exons 113–118) were studied by means of conformation-sensitive gel electrophoresis (CSGE) using 47 sets of primers described by Christiano et al. (in press). The conditions for PCR amplification consisted of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, melting temperature of the primers for 40 s, and 72°C for 30 s in a MJ Research PTC-100 thermal cycler. Splice-site mutations were further investigated by amplification of reverse-transcribed RNA from keratinocytes by use of COL7A1 cDNA primers encompassing the exons flanking the mutation (sequences are available, on request, from the authors).

### *DGGE Analysis*

One primer of each set used for DGGE analysis contained a GC-rich tail of 55–66 bases, to increase mutation detection efficiency (Sheffield et al. 1989). Denaturing conditions resulting in optimal resolution were selected by use of Lerman's MELT87 and SQHTX programs (Lerman and Silverstein 1987). The amplification products were subjected to electrophoresis on a 6.4% polyacrylamide gel containing a linearly increasing denaturant concentration of 50%–90% parallel to the direction of migration as described by Myers et al. (1987). The gels were run at 160 V and 60°C for 3–9 h.

### *CSGE Analysis*

The primers sets used for CSGE analysis are described by Christiano et al. (in press). After PCR amplification, the products from the patients and a normal subject were mixed and heated to 98°C for 5 min, followed by 68°C for 1 h, to induce the formation of heteroduplexes. The PCR products were loaded on a gel containing 10% acrylamide, 15% formamide, 10% ethylene glycol, and 0.5% glycerol-tolerant buffer (USB) and were subjected to electrophoresis at 500 V for 16 h, as described by Ganguly et al. (1993).

### *Sequence Analysis*

PCR products showing abnormal electrophoretic mobility were gel purified and subjected to direct automated sequencing using dideoxy-terminator cycle sequencing and an Applied Biosystems model 373A automated sequencer. Alternatively, PCR products were amplified by use of a biotinylated primer, and manual sequencing was performed by use of $\alpha[^{35}S]$-dATP and the Sequenase version 2.0 DNA sequencing kit (USB). All products were sequenced in the forward and reverse orientations. Some of the PCR products were subcloned into the pGEMT vector (TA cloning kit; Invitrogen), and the plasmid insert was sequenced.

### *Keratinocyte Culture, RNA Extraction, and Reverse Transcriptase–PCR (RT-PCR)*

Keratinocytes were cultured from 5-mm punch biopsies obtained from the patients, as described by Rheinwald and Green (1975) and Rochat et al. (1994). Total RNA was extracted from confluent cell layers, by use of the guanidium thiocyanate method described by Chomczynski and Sacchi (1987). cDNA was generated from total RNA by reverse transcription using random hexanucleotides (Pharmacia Biotech) and Moloney murine leukemia virus reverse transcriptase (Stratagene). Exonic primers flanking the mutation were used to amplify and sequence cDNA.

### *Northern Blot Analysis*

Keratinocyte total RNA (30 μg) was subjected to electrophoresis on 1% agarose in the presence of formaldehyde and was transferred to a Pall biodyne B membrane (Pall BioSupport). The cDNA probes for type VII collagen K131 (Parente et al. 1991) and for the ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene were radiolabeled with $\alpha[^{32}P]$-dCTP and were used as hybridization probes.

### *Restriction-Endonuclease Analysis of Amplified Genomic DNA*

Amplified genomic DNA was cleaved with the appropriate restriction enzymes (*Ava*II, *Bgl*I, *Bsp*MI, *Bsp*WI, *Bsp*HI, *Bsi*EI, *Bsi*YI, *Dde*I, *Fnu*4HI, *Msp*I, *Nla*III, *Pst*I, *ScrF*I, *Sty*I, *Taq*I, and *Xho*I) according to the manufacturer's recommendations (New England Biolabs and Boehringer Mannheim) and was analyzed on 2% agarose or 6% PAGE.

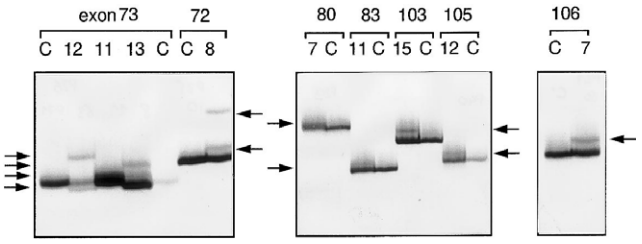


**Figure 1** CSGE analysis of PCR-amplified genomic DNA from seven exons of COL7A1 in RDEB patients. Abnormally migrating fragments are indicated by arrows. Numbers above the horizontal brackets denote exons; and numbers below the horizontal brackets denote patients (C = control).

### *Genotype Analysis*

Individuals from families 3, 4, 10, and 11 were genotyped by use of the microsatellite markers D3S1573 and D3S1076 flanking the COL7A1 locus at 3p21 (Christiano et al. 1996*a*). The following primers were used: D3S1573-L, 5′-TTCATTTTTGCTTATTAAGATATGC-3′; D3S1573-R, 5′-CCAGTAAATCACAGGGCTAT-3′; D3S1076-L, 5′-ATTCCCTGCATATGATCCCAA-CTG-3′; and D3S1076-R, 5′- ACCTAAGAGCAATCA-CTTAACTTAG-3′ (Christiano et al. 1996*a*). The PCR-reaction conditions were as described above, with an annealing temperature of 60°C. PCR products were separated on denaturing polyacrylamide gels (Sequagel; National Diagnostics) and were transferred to a Pall biodyne B membrane (Pall BioSupport). One PCR primer of each set was end-labeled with $\alpha[^{32}P]$-dCTP by use of terminal transferase (Boehringer Mannheim) and hybridized to membranes for 2 h at 42°C in Church buffer (Church and Gilbert 1984). Membranes were washed in 2 × SSC, 0.1% SDS at room temperature and were exposed to x-ray film.

## Results

The entire coding sequence of the COL7A1 gene and flanking intron boundaries were amplified and screened for mutation, by DGGE or CSGE. Representative examples of band shifts detected by CSGE analysis are shown in figure 1. Sequencing of variants showing abnormal electrophoretic mobility led to the identification of mutations on both COL7A1 alleles, for all the patients. Characteristics of these mutations are shown in table 1. Silent nucleotide substitutions located within introns and distant from splice sites were also identified, and they are listed in table 2.

### *Identification of COL7A1 Mutations*

*Nonsense mutations.*—Seven base substitutions that introduced PTCs into the coding sequence were identified, four of which were novel mutations (table 1). Patients 8 and 12 had a C→T transition at CGA arginine codons 185 and 2610, respectively, resulting in TGA stop codons; patient 1 had a C→T transition at CAG glutamine 281, leading to a TAG stop codon; and patient 11 had a G→T transversion at GGA glycine 2216, resulting in a TGA stop codon. Patients 2, 9, and 10 presented a C→T transition at CGA arginine codons 578, 236, and 1343, respectively, which had been reported elsewhere (Hovnanian et al. 1994; Dunnill et al. 1996). All these patients were compound heterozygotes, with the second COL7A1 mutation being either a small deletion (patient 1), a splice-site mutation (patient 2), or a missense mutation (patients 8–12).

*Frameshift mutations resulting in PTCs.*—Two small insertions and one small deletion in the coding region, creating shifts in the reading frame, were characterized in four unrelated patients. Patients 3 and 4 were homozygous for an insertion of $C_{325}$ and $G_{326}$ (325insCG) in exon 3, which predicted a TGA stop codon 113 bp downstream of codon 108. Patient 7 was a compound heterozygote for the insertion of $C_{6527}$ in exon 80 (6527insC), resulting in a TAA stop codon 337 bp downstream of codon 2176. Patient 1 demonstrated a 7-bp deletion in exon 5, which led to a TGA stop codon 22 nucleotides downstream of codon 173.

*Splice-site mutations.*—Four patients (patients 2 and 5–7) exhibited intronic splice-site mutations, three of which altered the invariant splice-donor or -acceptor sites' consensus sequences GT and AG. Patient 2 displayed a G→A change at the +1 position of the splice-donor site of intron 5 (682+1G→A). The analysis of RT-PCR products from this patient showed a larger band in addition to the band of normal size, the intensities of both bands being reduced in comparison with those in the control (fig. 2, lane 2). Direct sequencing of the band of normal size showed a normal sequence, whereas sequencing of the larger band revealed retention of the entire intron 5. This change creates a frameshift introducing a PTC at a TAG codon 33 bp after codon 227 (fig. 3*a*).

Patient 5 had a C→G substitution changing the variant C residue at position −3 of the splice-acceptor site of intron 2. After RT-PCR amplification, a band of larger size, in addition to a band of normal size, could be detected (fig. 2, lane 5). Both bands were reduced in intensity when compared with those in the control, although the intensity of the larger band was stronger than that of the band of normal size. Sequencing of the larger band showed the retention of the last 70 bases of intron 2, resulting from the activation of a cryptic splice-acceptor site TAG within intron 2. The retained 70 bp of intron 2 introduced a PTC (TAG) 41 bp after the cryptic splice site (fig. 3*b*). Direct sequence analysis of the band of normal size showed a normal sequence.

Patient 6 was homozygous for a 847-6 del5 mutation that removed nucleotides −2 to −6 from the splice-acceptor sequence of intron 6, introducing a T at the invari-

## Table 1

**Characteristics of COL7A1 Mutations in 15 Unrelated RDEB Patients**

| Patient[a] | Name of Mutation[b,c] | Nucleotide Change[c] | Consequence | Location | Protein Domain[d] | COL7A1 mRNA[e] | Type VII Collagen Protein[f] | Verification Method |
|---|---|---|---|---|---|---|---|---|
| 1 | 520del7 | GGGATCAAG→GG | Frameshift | Exon 5 | CMP | − | − | *Sau*3AI, *Mbo*I |
| | Q281X | 841C→T | Nonsense | Exon 6 | FNIII-1A | | | *Fnu*4HI |
| 2 | 682+1G→A | CTCgt→CTCat | Altered splicing[g] | Intron 5 | CMP/FNIII-1A | − | − | *Bsp*HI |
| | R578X[h] | 1732C→T | Nonsense | Exon 13 | FNIII-4B | | | *Xho*I |
| 3[i,j] | 325insCG (homozygous) | GAG→GCGAG | Frameshift | Exon 3 | CMP | + | − | *Bsp* WI |
| 4[j] | id. | GAG→GCGAG | Frameshift | Exon 3 | CMP | − | − | *Bsp* WI |
| 5[i] | 267-3C→G (homozygous) | cagGA→gagGA | Altered splicing[g] | Intron 2 | CMP | + | − | Sequencing |
| 6[i] | 847-6del5 (homozygous) | tgggcagGT→tgGT | Altered splicing[g] | Intron 6 | FNIII-1B | − | − | *Bsp* MI |
| 7 | 6527insC | CCTGG→CCCTGG | Frameshift | Exon 80 | Collagenous | − | − | *Bgl*I |
| | 7930-1G→C | agGGA→acGGA | Altered splicing[g] | Intron 106 | Collagenous | | | *Pst*I |
| 8 | R185X | 553 C→T | Nonsense | Exon 5 | CMP | ND | +/++ | Sequencing |
| | G1982W | 5944 G→T | Missense | Exon 72 | Collagenous | | | *Sty*I |
| 9 | R236X[k] | 706 C→T | Nonsense | Exon 6 | FNIII-1A | ND | +/++ | *Nla*III |
| | R2063G | 6187 C→G | Missense | Exon 74 | Collagenous | | | Sequencing |
| 10 | R1343X[k] | 4027 C→T | Nonsense | Exon 34 | Collagenous | ++ | +/++ | *Taq*I |
| | G2049E | 6146 G→A | Missense | Exon 73 | Collagenous | | | *Dde*I |
| 11[l] | G2049E | 6146 G→A | Missense | Exon 73 | Collagenous | ++ | +/++ | *Dde*I |
| | G2216X | 6646 G→T | Nonsense | Exon 83 | Collagenous | | | *Nla*III |
| 12 | G2025A | 6074 G→C | Missense | Exon 73 | Collagenous | ++ | +++ | *Bsi*YI |
| | R2610X | 7828 C→T | Nonsense | Exon 105 | Collagenous | | | *Dde*I |
| 13[i] | R2008G (homozygous) | 6072 C→G | Missense | Exon 73 | Collagenous | +++ | +/++ | *Bsi*EI |
| 14[i,l] | R2063W (homozygous) | 6187 C→T | Missense | Exon 74 | Collagenous | +++ | +++ | Sequencing |
| 15 | G2575R (homozygous) | 7723 G→A | Missense | Exon 103 | Collagenous | +++ | ND | *Msp*I |

[a] All are affected with HS-RDEB, except for patients 9 and 12, who are affected with inverse RDEB and RDEB mitis, respectively.
[b] Codons are numbered according to the COL7A1 cDNA sequence published by Christiano et al. (1994*b*) (GenBank L02870).
[c] Nucleotides are numbered according to the COL7A1 cDNA sequence published by Christiano et al. (1994*b*) (GenBank L02870). Bases within exons are denoted by uppercase letters; bases within introns are denoted by lowercase letters; and altered bases are underlined.
[d] CMP = cartilage matrix–protein motif; and FNIII = fibronectin type III–like domain.
[e] Investigated by northern blot analysis (see fig. 4).
[f] Analyzed by immunofluorescence of skin biopsy, with LH7:2 antibody to type VII collagen (see fig. 5). − = Undetectable; +/++ = reduced; +++ = normal; and ND = not determined.
[g] Effects on splicing are described in figure 3.
[h] Reported in different patients by Dunnill et al. (1994*b*, 1996).
[i] Known consanguinity.
[j] Two patients share same mutation and haplotype.
[k] Reported in the same patient by Hovnanian et al. (1994).
[l] Patient's siblings have the same mutation.

ant position −2. The analysis of RT-PCR products showed no band of normal size but did show a band of slightly smaller size, whose intensity was reduced when compared with that in the control (fig. 2, lane 6). Sequence analysis of this band revealed the removal of the first 13 bases of exon 7, which should originate from the activation of a cryptic splice-acceptor site CAG within exon 7. The resulting frameshift created a PTC at a TGA codon 40 bp downstream of codon 282 (fig. 3*c*).

Patient 7 had a G→C change at the −1 position of the acceptor site of intron 106 (7930-1G→C). RT-PCR products from this patient showed four bands: a smaller band (band d) and two larger bands (bands a and b), in addition to the band of normal size (band c) (fig. 2, lane 7). Direct sequencing of band c showed a normal sequence, whereas sequencing of band d revealed the skipping of exon 107, which predicts the in-frame deletion of 18 codons (G2644–M2661) (fig. 3*d*). Sequence analysis of bands a and b showed heteroduplexes composed of the normal COL7A1 cDNA sequence and of a sequence with retention of the entire intron 106. Retention of intron 106 predicted a TGA stop codon 251 bp after codon 2643 (fig. 3*d*).

*Mutations resulting in amino acid substitutions.*—Seven single-nucleotide changes leading to different amino acid substitutions were found in eight patients, mutation G2049E being present in two unrelated patients (patients 10 and 11). These missense mutations

**Table 2**

**Novel COL7A1 Polymorphisms Identified in Present Study**

| Name[a] | Location | Sequence | Distance to Nearest Exon (bp) | Screening Method | Frequency of Rarer Allele | |
|---|---|---|---|---|---|---|
| | | | | | Patients ($n$ = 18) | ($n$ = 50) |
| 9712G→A | intron 17 | accctg(g/a)ccagct | +14/exon 17 | *Scr*FI | .05 | .18 |
| 17761T→C | intron 51 | gagtca(t/c)ggcggg | +26/exon 51 | *Nla*III | .11 | .02 |
| 23946C→T | intron 75 | cttagt(c/t)ctgtga | +25/exon 75 | CSGE | .27 | .12 |
| 30344C→A | intron 103 | cctggg(c/a)cctggc | −96/exon 104 | *Ava*II | .05 | .30 |
| 31430C→T | intron 108 | acatga(c/t)agggag | +30/exon 108 | CSGE | .05 | .06 |

[a] Nucleotides are numbered according to genomic sequence in Genbank/EMBL, L23982.

changed glycine or arginine residues located within the collagenous domain of the type VII collagen molecule. Three patients (13–15) were homozygous for mutations R2008G, R2063W, and G2575R, respectively, in exons 73, 74, and 103, respectively (table 1). Five other patients were compound heterozygotes for a missense mutation and a nonsense mutation: patient 8 displayed a G1982W mutation in exon 72; patients 10 and 11 shared the same G2049E mutation in exon 73; patient 12 affected with RDEB mitis showed a G2025A mutation in exon 73; and patient 9, presenting with inverse RDEB, had a R2063G mutation in exon 74 (table 1). Interestingly, the replacement of the same arginine residue 2063 by a tryptophan or a glycine residue was associated with distinct forms of the disease—that is, with severe HS-RDEB in patient 14 and with inverse RDEB in patient 9, respectively. All mutations occurred at residues conserved among human, hamster, and mouse COL7A1 (Greenspan 1993; Kivirikko et al. 1996), suggesting a functional importance of these residues.

*Screening for the mutations.*—The inheritance of the mutations within the families studied was confirmed by either restriction-endonuclease digestion of PCR-amplified DNA or automated sequencing. The possibility that the missense mutations that we report stemmed from polymorphic variations was tested by screening for their presence in 50 unrelated controls. The presence of these mutations was not detected in any controls tested, suggesting that these changes do not correspond to rare polymorphisms. To investigate the possibility that these defects could be associated with RDEB in other families, we looked for their presence in 65 other RDEB patients who are currently being screened for COL7A1 mutations. Mutation 682+1G→A was found in an unrelated patient, as was mutation R236X. Mutations R2008G and 6527insC were identified in two and five other unrelated RDEB patients, respectively. These final nine patients are not included in this study, because we had no access to their RNAs. Finally, complete screening of the 118 exons of COL7A1 in the patients whom we report

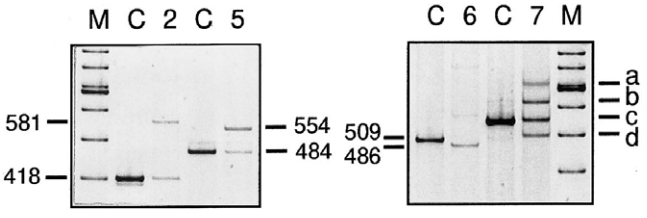


**Figure 2** RT-PCR amplification of the COL7A1 cDNA regions encompassing exons 3–6 (patient 2), exons 2–5 (patient 5), exons 5–8 (patient 6), and exons 102–110 (patient 7). The size (in bp) of the bands is indicated. Bands a–d correspond to the different transcripts observed in patient 7. C = control; and M = 100-bp ladder.

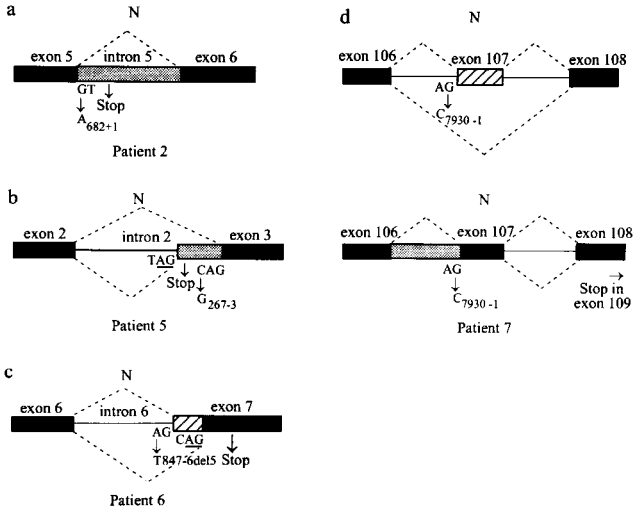


**Figure 3** Schematic representation of the effects of the splicing mutations identified in RDEB patients 2 and 5–7. Exons present in the mRNA are denoted by black boxes; exons and parts of exons absent are denoted by hatched boxes; and novel sequences are denoted by gray boxes. The broken V-shaped lines designate regions of pre-mRNA that are removed by normal splicing (N) or by abnormal splicing in the patient. Cryptic splice sites used for aberrant splicing are underlined. "Stop" denotes a premature-termination signal.

did not reveal additional nucleotide variation predicting an amino acid change or a splicing abnormality.

### *Genotype Analysis*

To investigate whether the recurrence of the 325insCG and G2049E mutations, observed in families 3 and 4 and families 10 and 11, respectively, resulted from independent events or from a common ancient mutation, haplotype analysis was performed by use of the microsatellite markers D3S1573 and D3S1076 flanking COL7A1 (Christiano et al. 1996*a*). These families were fully informative for at least one of these markers. Genotype analysis of families 3 and 4 showed that the 325insCG mutation segregated with the same alleles for D31076 and D3S1573, suggesting that patients from these families had inherited the same disease allele from a common ancestor. This was consistent with the common geographic origin of these patients, from villages closely located in the northern part of Portugal. In contrast, genotype analysis of families 10 and 11 showed that the G2049E mutation segregated with different alleles for both markers, suggesting the occurrence of independent events. This result was consistent with the absence of a common geographic origin for these families.

### *Northern Blot Analysis*

Northern blot analysis of RNA from keratinocytes in culture was performed on 13 patients and 2 age-matched controls (fig. 4). Patients 1, 2, 4, 6, and 7 showed absence of detectable COL7A1 mRNA. Patients 3 and 5 demonstrated markedly decreased COL7A1 mRNA levels, in comparison with those in their respective controls, C1 and C2. In contrast, patients 10–15 showed clearly detectable COL7A1 transcripts of normal size (9.2 kb). The quantity of RNA present in each lane was standardized by GAPDH hybridization. Densitometric-tracing analysis of the COL7A1 signal:GAPDH signal ratio showed a 40%–50% reduction of the COL7A1 mRNA steady-state levels in patients 10–12 (who are compound heterozygotes for a nonsense and a missense mutation), compared with those in their control, C2. No significant reduction was found in patients 13–15 (who are homozygous for a missense mutation), in comparison with their control, C1.

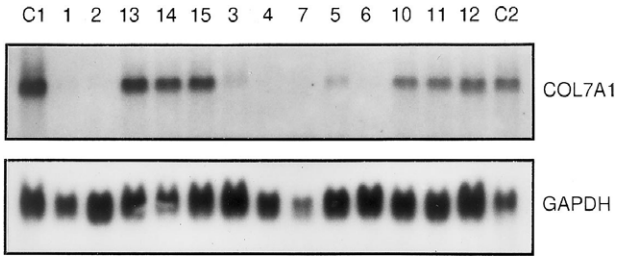


**Figure 4** Northern blot analysis of COL7A1 mRNA in primary keratinocytes from patients with RDEB and from controls. Total RNA (30 μg) was hybridized with α[$^{32}$P]-dCTP-labeled K131 COL7A1 cDNA probe (Parente et al. 1991). COL7A1 mRNAs are not detectable in patients 1, 2, 4, 6, and 7 and are present at extremely low levels in patients 3 and 5 (9.2 kb). In contrast, patients 10–15 display clearly detectable COL7A1 transcripts. The quantity of RNA present in each lane was standardized by GAPDH hybridization (*lower panel*). Patients are denoted by the same numerical designations used in table 1. "C1" and "C2" represent age-matched unrelated normal controls for patients 1–3 and 13–15 and for patients 4–7 and 10–12, respectively.

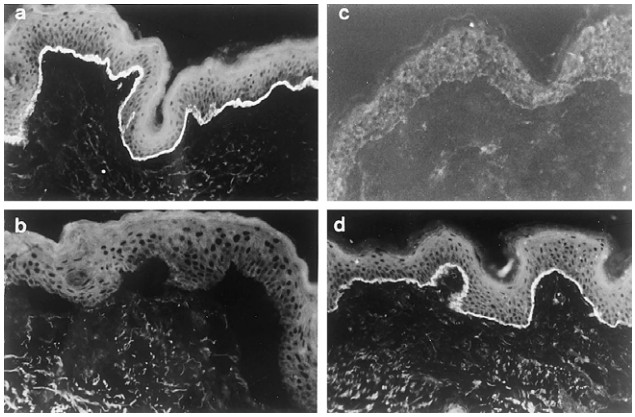


**Figure 5** Type VII collagen immunostaining of skin sections by use of LH7:2 antibody. *a,* Normal control showing strong and linear staining of the DEJ. *b,* Sections from patient 5 (267-3C→G/267-3C→G), revealing both absence of staining and presence of a dermo/epidermal cleavage. *c,* Faint and discontinuous staining at the roof of a blister in patient 13 (R2008G/R2008G). *d,* Strong and continuous staining along the DEJ similar to the control, observed in patient 14 (R2063W/R2063W). Magnification × 50.

### *Immunolocalization of Type VII Collagen*

*Immunofluorescence study.*—Immunofluorescence study of skin biopsies by use of LH7:2 antibody showed bright and linear staining along the DEJ in the control individual (fig. 5*a*). Staining was absent in patients 1–7, who were homozygous or compound heterozygous for mutations predicting PTCs (fig. 5*b*). In contrast, positive staining at the DEJ was seen in all patients tested who either were compound heterozygotes for a missense and a nonsense mutation or were homozygous for a missense mutation. Specifically, patients 8–11 and 13 showed reduced staining of the DEJ (fig. 5*c*), and patients 12 and 14 demonstrated strong staining of intensity comparable with that in the control (fig. 5*d*) (table 1).

*Standard electron microscopy and IEM.*—Standard electron microscopy of skin biopsy showed cleavage beneath the lamina densa and absence of normal AF, as defined by Tidman and Eady (1984), in all patients studied. Patients 10, 12, and 14 were then further studied by IEM. IEM using LH7:2 antibody showed a different pattern of staining in each patient. Patient 10 (R1343X/G2049E) showed absence of labeling of both the lamina

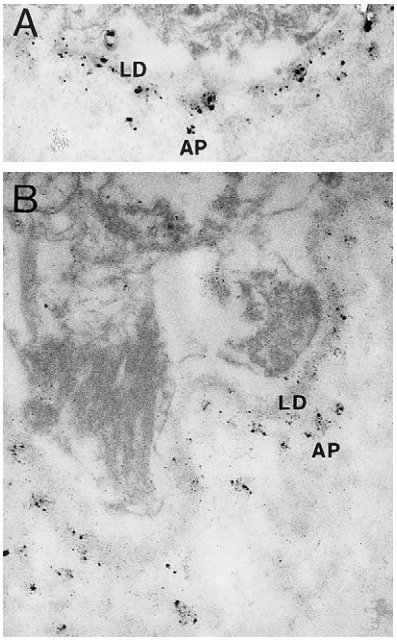


**Figure 6** IEM of skin, using the LH7:2 antibody. *A*, Control, showing labeling of both the lower part of the lamina densa and the anchoring plaques. AF are not seen because sections were not contrasted with lead citrate and uranyl acetate (Magnification × 4,500). *B*, Skin from patient 12 reveals subnormal staining of the lamina densa and of the anchoring plaques comparable to the control (Magnification × 9,750). LD, lamina densa; AP, anchoring plaque.

densa and the anchoring plaques. Patient 14 (R2063W/R2063W) showed labeling of the lamina densa, with no staining of the anchoring plaques. Last, patient 12 (G2025A/R2610X) showed labeling of both the lamina densa and the anchoring plaques, similar to what was seen in the control (fig. 6). Normal staining of both the lamina lucida and the adjacent lamina densa was obtained with GB3 antibody, in patients 10, 12, and 14.

## Discussion

In this study, we report the characterization of COL7A1 mutations in both alleles of 18 patients from 15 unrelated RDEB families. A total of 21 mutations were identified, of which 18 had not been reported previously. Of the nine mutations detected by DGGE, seven could also be disclosed by CSGE, whereas mutations Q281X (C→T at 841) and 267-3C→G did not produce detectable shift on CSGE. This is consistent with the efficiency of mutation detection by CSGE, which is estimated to be on the order of 80%, whereas DGGE using GC clamp is thought to permit detection of 100% of nucleotide variations (Prosser 1993). Eleven (61%) of the 18 novel mutations predicted premature termination of translation, and 7 were missense mutations, as summarized in table 1. We investigated the consequences of these mutations for type VII collagen transcript and protein detection. We conclude that the PTC-creating mutations that we have described result in mRNA instability and absence of type VII collagen polypeptide synthesis, whereas missense mutations have different effects on protein stability and function.

### *Consequences of COL7A1 Mutations for Mutant Transcripts and Protein Detection*

Of the 16 patients with HS-RDEB whom we studied, 4 were homozygous for a frameshift or a splice-site mutation (patients 3–6), and 3 presented different combinations of nonsense, frameshift, and splice-site mutations (patients 1, 2, and 7) (table 1). These mutations gave rise to PTCs and were associated with undetectable or drastically reduced COL7A1 transcript levels and absence of immunoreactive type VII collagen in skin. These results support a disease mechanism involving absence of protein synthesis, because of a PTC-mediated decrease in mRNA stability, as elsewhere reported for COL7A1 and other genes (Baserga and Benz 1988; Hilal et al. 1993; Maquat 1995). Because HS-RDEB patients often demonstrate undetectable type VII collagen polypeptide in the skin, it is likely that nonsense, frameshift, or splice-site mutations leading to PTCs are common causes of the most severe form of the disease. Interestingly, the splice-acceptor-site mutation identified in patient 7 gave rise to two different splice abnormalities—one resulting in the in-frame skipping of exon 107 and the other resulting in retention of intron 106, which led to a PTC. A complex aberrant pattern of splicing resulting from a mutation at the second-to-last nucleotide of exon 3 in COL7A1 has recently been reported in a patient with localized RDEB (Gardella et al. 1996). In the case of patient 7, the absence of detectable COL7A1 mRNA transcript on northern blot suggests that the principal effect of the splice-site mutation affecting intron 106 and of the 6527insC mutation is a profound reduction in the stability of the mRNA transcripts. In contrast, the mRNA analysis in patients 3 and 5, who are homozygous for a frameshift and a splice-site mutation leading to a PTC, respectively, revealed some detectable COL7A1 mRNA on northern blot (fig. 4, patients 3 and 5, lanes 7 and 10). This could be due either to unequal application of RNA, as indicated after normalization for GAPDH for patient 3, or to variable degradation of the unstable mRNA. In the case of patient 5, mutation 267-3C→G substitutes a purine residue for a pyrimidine, at position −3 of a splice-acceptor site, a position that, in vertebrates, is most often (frequency 97%) occupied by a pyrimidine (Shapiro and Senapathy 1987). This mutation allows for some normal splicing to occur, as shown by the presence of a reduced amount of normal COL7A1 transcripts after RT-PCR (fig. 2, lane 5), which may account for the faint COL7A1 signal seen on the northern blot.

The other RDEB patients who were studied (nine pa-

tients with HS-RDEB, one patient with RDEB mitis, and one patient with inverse RDEB) either were homozygous for a missense mutation (patients 13–15) or were compound heterozygotes for a nonsense and a missense mutation (patients 8–12) (table 1). Several glycine-to-arginine substitutions acting in a recessive manner have recently been reported in HS-RDEB and in RDEB mitis (Christiano et al. 1996*b,* 1996*c*). Four of the missense mutations that we report also change glycine residues in the collagenous domain of the molecule, whereas three other mutations change arginine residues. All of these mutations, with the exception of G2025A, are nonconservative and result in a change of evolutionarily conserved amino acids. Specifically, mutations G2049E, G2575R, and G1982W disrupt the repetitive Gly-X-Y sequence characteristic of collagens, either by replacing a glycine residue with a charged residue or by introducing a bulky chain in the center of the triple helix. Mutations R2008G, R2063G, and R2063W result either in the loss of an ionic charge or in the introduction of a bulky chain at external positions of the triple helix. These substitutions cosegregate with the disease phenotype in the families and were not observed in 100 normal chromosomes in unrelated healthy individuals. Therefore, it is unlikely that these changes represent polymorphisms.

Northern blot analysis in patients homozygous for a missense mutation (patients 13–15) showed no significant reduction of COL7A1 transcript levels, in agreement with the observation that missense mutations do not usually alter mRNA stability. Patients who were compound heterozygotes for a nonsense and a missense mutation (patients 10–12) showed a reduction, by approximately half, of the COL7A1 signal:GAPDH signal ratio, which is consistent with a decreased stability of the mutated allele bearing the nonsense mutation. Immunodetection of type VII collagen polypeptide in skin biopsies from these patients showed a normal pattern in patients 12 and 14 and showed reduced staining in patients 10, 11, and 13 (table 1). In all cases, electron microscopy showed absence of identifiable AF. Collectively, these results suggest that the missense mutations that we report lead to the synthesis of type VII collagen polypeptide with decreased stability and/or altered function, resulting in impaired AF formation.

### *Proposed Mechanism of Action of Recessive COL7A1 Missense Mutations*

In the absence of a model for recessive collagen disorders, the precise consequences of these mutations for type VII collagen polypeptide remain speculative. However, our results suggest that some of the COL7A1 missense mutations either decrease the stability or alter antigenic properties of type VII collagen, whereas others allow for the synthesis of stable but nonfunctional polypeptide. Because the LH7:2 antibody recognizes an epitope within the NC1 domain (Tanaka et al. 1994), it is unlikely that these mutations, which are located within the collagenous domain of the molecule, alter LH7:2 binding. Alternatively, missense mutations associated with deficient type VII collagen expression may disrupt structural domains of the molecule whose integrity is important for protein stability, as previously described for missense mutations in other genes, such as the dystrophin gene, the β-sarcoglycan gene, and the factor XIII gene (Prior et al. 1993; Bönnemann et al. 1996; Mikkola et al. 1996). In contrast, COL7A1 mutations associated with normal immunodetection of type VII collagen would lead to the synthesis, the secretion, and the deposition, at the DEJ, of mutated molecules unable to assemble into functional AF. This suggests that these amino acids are located in domains of the molecule that are critical for the proper function of the type VII collagen protein. Interestingly, the R2008G mutation that we describe in patient 13 occurs in an RGD (arg-gly-asp) sequence, which is a potential cell-attachment motif. Thus, in addition to its potential effect on AF formation, this mutation may impair the interaction between AF and extracellular components such as fibronectin.

We suggest that the missense mutations that we describe alter homotrimer formation and/or higher-ordered fibril assembly in homozygotes and in compound heterozygotes. Because heterozygous carriers are unaffected, the mechanisms of action of these mutations would be distinct from the dominant-negative effect of other glycine substitutions reported in dominant DEB. The reasons why some glycine substitutions result in dominant DEB, whereas others act in a recessive manner, are unclear. However, these mutations occur at different glycine residues within the 19 different collagenous subdomains of the molecule. It is thus possible that these amino acids are located within motifs of different importance for protein processing, folding, stability, and function of type VII collagen polypeptide.

### *Distribution of COL7A1 Mutations*

Analysis of the distribution of the COL7A1 mutations reported here shows that six of the seven missense mutations are located within exon 72, exon 73, or exon 74, within a region encompassing 82 amino acids next to the hinge region. The apparent "clustering" of these mutations just after the major interruption of the collagenous domain, a region thought to be involved in flexibility of the molecule, may be of biological relevance. However, mutational analysis of additional patients is required in order to determine whether it represents a region prone to mutations.

### *Genotype-Phenotype Correlations and Variability of the Disease for the Same Mutation*

Genotype-phenotype analysis showed that, of the five patients who were compound heterozygotes for a mis-

sense mutation and a mutation leading to a PTC (patients 8–12), three were affected with severe RDEB (patients 8, 10, and 11), one presented with RDEB mitis (patient 12), and another had inverse RDEB (patient 9). Because PTCs are likely to lead to the absence of protein synthesis, owing to mRNA instability, these patients are expected to express only the mutated allele bearing the missense mutation. The difference in the phenotype would thus depend on the nature and/or position of the amino acid substitution and, possibly, on differences in genetic background and external factors. In that context, it is of interest that the R2063G and R2063W mutations, which occurred at the same arginine 2063 residue in patients 9 and 14, respectively, were associated with inverse RDEB and HS-RDEB, respectively, suggesting that the nature of the amino acid change for a given position within the molecule may influence the phenotype.

Finally, the severity of the disease differed between patients showing the same mutation. Specifically, patients 3 and 4 (20 and 8 years of age, respectively), who were from unrelated families, were homozygous for the same 325insCG mutation, but severe dysphagia was noted in patient 4 only. The disease phenotype also showed considerable variability among affected family members. The oldest affected brother in family 14 (16 years of age) presented with severe mittenlike deformities of the hands at the age of 8 years, whereas his two younger affected brothers (11 and 4 years of age) did not develop syndactyly. Similarly, the young affected sib (12 years of age) from family 11 showed severe fusion of the fingers, whereas her older sister (25 years of age) did not. These observations illustrate inter- and intrafamilial phenotypic variability occurring for the same mutation and underscore the role of genetic backgrounds and/or environmental factors in expressivity of the disease.

Our results further illustrate the extensive diversity of mutational events that lead to the RDEB phenotype. They provide evidence for distinct consequences of these mutations for both type VII collagen mRNA and protein stability and function, and thus they contribute to a better knowledge of the molecular mechanisms involved in defective AF formation in RDEB.

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