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Recurrent Nonsense Mutations within the Type VII Collagen Gene in Patients with Severe Recessive Dystrophic Epidermolysis Bullosa

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Summary

The generalized mutilating form of recessive dystrophic epidermolysis bullosa (i.e., the Hallopeau-Siemens type; HS-RDEB) is a life-threatening disease characterized by extreme mucocutaneous fragility associated with absent or markedly altered anchoring fibrils (AF). Recently, we reported linkage between HS-RDEB and the type VII collagen gene (COL7A1), which encodes the major component of AF. In this study, we investigated 52 unrelated HS-RDEB patients and 2 patients with RDEB inversa for the presence, at CpG dinucleotides, of mutations changing CGA arginine codons to premature stop codons TGA within the COL7A1 gene. Eight exons containing 10 CGA codons located in the amino-terminal domain of the COL7A1 gene were studied. Mutation analysis was performed using denaturing gradient gel electrophoresis of PCR-amplified genomic fragments. Direct sequencing of PCR-amplified products with altered electrophoretic mobility led to the characterization of three premature stop codons, each in a single COL7A1 allele, in four patients. Two patients (one affected with HS-RDEB and the other with RDEB inversa) have the same C-to-T transition at arginine codon 109. Two other HS-RDEB patients have a C-to-T transition at arginine 1213 and 1216, respectively. These nonsense mutations predict the truncation of ~56%–92% of the polypeptide, including the collagenous and the noncollagenous NC-2 domains. On the basis of linkage analysis, which showed no evidence for locus heterogeneity in RDEB, it is expected that these patients are compound heterozygotes and have additional mutations on the other COL7A1 allele, leading to impaired AF formation. These results indicate that stop mutations within the COL7A1 gene can underlie both HS-RDEB and RDEB inversa, thus providing further evidence for the implication of this gene in RDEB.

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Introduction

Dystrophic epidermolysis bullosa (DEB) is a group of inherited diseases characterized by trauma-induced blistering of the skin and mucous membranes, resulting in atrophic scars and milia (Fine et al. 1991; Briggaman 1992; Epstein 1992; Lin and Carter 1992). DEB occurs in dominant (DDEB) or recessive (RDEB) forms, each form comprising subtypes of different clinical presentation and severity. All forms of DEB display cleavage beneath the lamina densa and variable anchoring fibril (AF) defects on electron microscopy (EM) (Hashimoto et al. 1976; Tidman and Eady 1985). RDEB comprises the Hallopeau-Siemens type (HS-RDEB), the generalized mitis type, and the localized and inversa forms. HS-RDEB (McKusick 226600) is the most severe form of DEB and is responsible for widespread mucocutaneous blistering leading to fusion of the digits, nail loss, flexural contractures, esophageal strictures, as well as oral and ocular erosions. Malnutrition, anemia, and growth retardation commonly occur, and squamous-cell carcinomas may develop (Briggaman 1992; McGrath et al. 1992). Patients with HS-RDEB demonstrate absent or markedly altered AF on EM and display absence or marked diminution of staining of basement-membrane zone with antibodies against type VII collagen (Heagerty et al. 1986; Bruckner-Tuderman et al. 1988, 1989; Leigh et al. 1988).

Type VII collagen is the major component of AF (Sakai et al. 1986; Burgeson et al. 1990). The COL7A1 cDNA and gene have recently been cloned (Parente et al. 1991; Christiano et al. 1992*c*; Gammon et al. 1992; Greenspan 1993), and the human COL7A1 gene has been mapped to chromosomal region 3p21 (Parente et al. 1991). COL7A1 is a complex and unusually compact gene (32 kb) that contains 118 exons separated by small introns and is transcribed into a 9.2-kb mRNA encoding a 340-Kd protein (Christiano et al. 1992*a*).

We previously reported linkage between HS-RDEB and the COL7A1 gene by using an intragenic *Pvu*II polymorphic marker, with a lod score of 3.97 at $\theta = 0$ in 19 affected families (Hovnanian et al. 1992). In support of the direct implication of the COL7A1 gene in RDEB, a missense mutation and an insertion-deletion generating a premature

stop codon have been recently characterized in the COL7A1 gene in the homozygous state in two offspring affected with the mitis form of RDEB (Christiano et al. 1993) and in a patient with HS-RDEB (Hilal et al. 1993).

To screen for additional mutations, we based our strategy on the observation that CpG dinucleotides in a number of human disease genes have been shown to be prone to C-to-T mutations, as the result of hypermutability of 5-methyl-cytosine to thymine, leading to premature stop codons when this occurs at the first base of CGA (arginine) codons (Youssoufian et al. 1986; Cooper and Youssoufian 1988; Maddalena et al. 1988; Amselem et al. 1991; Reiner and Thompson 1992). We therefore selectively screened COL7A1 exons containing CGA arginine codons where a C-to-T transition would give rise to a premature stop codon. We used denaturing gradient gel electrophoresis (DGGE) of GC-clamped PCR-amplified genomic fragments to screen eight COL7A1 exons containing 10 CGA arginine codons in the amino-terminal region of the COL7A1 gene. Direct sequencing of PCR-amplified products with altered electrophoretic mobility led to the identification of three nonsense mutations occurring at different codons for arginine in three unrelated HS-RDEB patients and in one patient with RDEB inversa. These mutations predict the synthesis of dramatically truncated polypeptides likely to impair AF formation in these patients.

Patients and Methods

Patients

HS-RDEB families.—Fifty-two HS-RDEB families having at least one affected living offspring were investigated. In five families, patients were known to be born from consanguineous unions. Six families had more than one affected living sibling. HS-RDEB was diagnosed according to clinical and EM criteria as defined by Fine et al. (1991). Families II–IV each had one affected sibling (II-3, 4 years old; III-5, 7 years old; and IV-6, 19 years old, respectively) (fig. 2). The parents were unaffected and not consanguineous and were of French (families II and III) and Turkish (family IV) origin. Patients II-3, III-5, and IV-6 have been affected, since birth, with widespread blisters resulting in scarring and milia with pseudosyndactyly of the fingers and/or the toes. Oral blisters, microstomia, and ankyloglossia were present, as well as growth retardation and anemia. Esophageal stenosis was diagnosed in patients III-5 and IV-6. EM examination of skin of these patients revealed tissue cleavage beneath the lamina densa, either with absence of AF or with rudimentary and thin filaments reduced in number and without distinct crossbanding.

RDEB inversa families.—Two families, each with one sibling affected with RDEB inversa, were studied. Parents were not consanguineous and were not affected. Patient I-3, from family I, is an 11-year-old girl who has neck, axilla, groin, and oral blistering involvement, sparing the hands and feet as well as the rest of the body. Severe and recurrent esophageal stenosis has required several dilations. The patient from the other family had a similar clinical appearance. Both patients displayed cleavage beneath the lamina densa, lacked normal AF, but had rudimentary AF diminished in number on EM examination of skin biopsies.

Amplification of Genomic DNA by PCR

Genomic DNA was extracted from peripheral blood cells as described elsewhere (John et al. 1991). The primers designed for DNA amplification of COL7A1 exons 6, 13–15, 19, and 34, as well as the 3′ end of exon 5 and 12 containing CGA arginine codons, are complementary to the published sequence of the COL7A1 cDNA (Christiano et al. 1992*c*) or to intronic regions and are indicated in table 1. One of the PCR primers of each set bore a 5′ GC-rich segment (GC-clamp) in order to create a high-melting-temperature domain, thus allowing detection, by DGGE analysis, of all nucleotide variations, even those located in the highest-melting-temperature regions (Sheffield et al. 1991). The primers used to investigate the intragenic *Pvu*II and *Alu*I polymorphic sites have been described elsewhere (Christiano et al. 1992*b*). PCR amplification was performed as described by Saiki et al. (1988), using 1 μg of genomic DNA in 100 μl of 1.5 mM $MgCl_2$, 10 mM Tris pH 8.3, 25 mM KCl, 200 mM of each dNTP, 10 pM of each primer, and 2.5 U of *Taq* polymerase (Cetus) at the following conditions: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 s, for 35 cycles, with a final extension for 7 min at 72°C.

DGGE Analysis

This technique is based on the principle that single-base-pair variations in a double-stranded DNA molecule change its melting temperature and that these differences in melting temperature can be detected by electrophoresis through an increasing gradient of denaturants (Myers et al. 1987). Twenty microliters of each of the PCR-amplified genomic products were subjected to electrophoresis at 160 V at 60°C in a 6.4% polyacrylamide gel containing a linearly increasing denaturant concentration (100% denaturant = 7 M urea and 40% formamide), parallel to the direction of migration as described elsewhere (Sheffield et al. 1991). The denaturant concentrations and the running time are indicated in table 1. Gels were stained with ethidium bromide and were photographed under UV light.

Computer Analysis

The melting behavior of exons 6, 13–15, 19, and 34 of the COL7A1 gene, as well as the flanking intronic sequences, were analyzed with Lerman's MELT87 and SQHTX programs (Lerman and Silverstein 1987). These computer algorithms predict the melting behavior of a DNA fragment, on the basis of its nucleotide composition and sequence. This information was used to design PCR primers for DGGE analysis, as well as to determine the

Table 1

Primer Sets Used for Amplification, and DGGE Conditions

Primer[a]	Sequence	Location	CGA Codon Tested (aa position)[b]	Amplified Segment (bp)	Denaturant Range (%)	Running Time[c] (h)
P1	5′ CTTCTTCTTCTTCGTCAATG 3′	Exon 5	99	206	...[d]	...[d]
P2	5′ GGGTCGGGGTCAGGAG 3′	Intron 5				
P3	5′ GGCAGTGCTGATTCCATCCT 3′	Intron 5	109	384	50–90	4
P4	5′ (60GC) ACCCCGCAGCCGCACACT 3′	Exon 7				
P5	5′ GTAACAGACCTGCAAGCCAC 3′	Exon 12	398	187	50–90	3
P6	5′ (60GC) GAGAGGGCTGGAGGTACAC 3′	Intron 12				
P7	5′ (55GC) CCCCATGGCCCTTCTCAC 3′	Intron 12	451	552	40–80	4
P8	5′ CTCATTGGTCCCTTTGGCAG 3′	Intron 14	486			
P9	5′ (60GC) CCAGGGCTGCGGGTTGTG 3′	Exon 14	542	484	40–80	8
P10	5′ TCTGAGGGAGGAGGGAGTG 3′	Intron 15	555			
P11	5′ (60GC) TGCACTGCCTCTCTGTTCTC 3′	Intron 17	716	567	40–80	6
P12	5′ ACGCCCCGCCCAGCCTC 3′	Intron 19				
P13	5′ (65GC) TTCTTTGCTCTCTAATGTCTTC 3′	Intron 33	1213	255	50–80	4
P14	5′ CATGTCCAGCTATTCCTATTC 3′	Intron 34	1216			

[a] Primer sets P7–P8, P9–P10, and P13–P14 were designed to study arginine codons 451 and 486, 542 and 555, 1213 and 1216, respectively, during the same DGGE run.

[b] Corresponds to the numbering of amino acid residues by Christiano et al. (1992*c*).

[c] For migration at 160 V.

[d] PCR product obtained with the set of primers P1–P2 was directly sequenced without prior DGGE analysis.

electrophoretic conditions resulting in optimal resolution and heteroduplex formation.

Direct Sequencing of PCR-amplified Genomic DNA

The sequence of DNA samples showing altered electrophoretic mobility was determined after asymmetric amplification using an unequal primer ratio (exon 6:primer 3/primer 4 = 1/50 pmol; and exon 34:primer 13/primer 14 = 50/1 pmol) as described by Gyllensten and Erlich (1988). The single-stranded amplified genomic DNA was ethanol precipitated and sequenced with either primer 3 (CGA-109 in exon 6) or primer 14 (CGA-1213 and 1216 in exon 34) by the dideoxynucleotide termination method (Sanger et al. 1977). Products from the sequencing reaction were electrophoresed on a 6% polyacrylamide denaturing gel and were autoradiographed.

Southern Analysis

Genomic DNA was digested separately with *Bam*HI, *Bst*XI, and *Pst*I restriction enzymes (New England Biolabs), according to the manufacturer's specifications. The DNA fragments were separated in a 1% agarose gel and were transferred to a Hybond C-extra membrane (Amersham). Prehybridization and hybridization of the filters with the 1.9-kb cDNA probe for human COL7A1 (K131) were performed as described by Ryynänen et al. (1992).

Restriction-Endonuclease Analysis of Amplified Genomic DNA

Twenty microliters of the 100 μl of amplified genomic DNA were cleaved with the appropriate restriction enzyme (*Nla*III, *Taq*I, *Ava*I, *Pvu*II, or *Alu*I), according to the manufacturer's recommendations (New England Biolabs), were electrophoresed on a 6% polyacrylamide gel, and were stained with ethidium bromide.

Results

DGGE Analysis of Exonic Regions Containing Arginine Codons

Twelve CGA arginine codons were identified within the published sequence of the COL7A1 cDNA (fig. 1). Seven sets of PCR primers for amplification of genomic DNA segments containing 10 of these CGA codons were designed on the basis of the cDNA and flanking intron sequences. Eight of the CGA codons studied were located within the amino-terminal NC-1 domain and two CGA codons were located within the 5′ end of the collagenous domain of the COL7A1 gene (fig. 1). The following exons containing these CGA codons and corresponding splice junctions were studied: exons 6 (FN-1A), 13 (FN-4B), 14 (FN-5A), 15 (FN-5B), 19 (FN-7B), and 34 (collagenous domain). The 3′-end region of exons 5 (CMP) and 12 (FN-4A), encompassing CGA-99 and CGA-398, respectively,

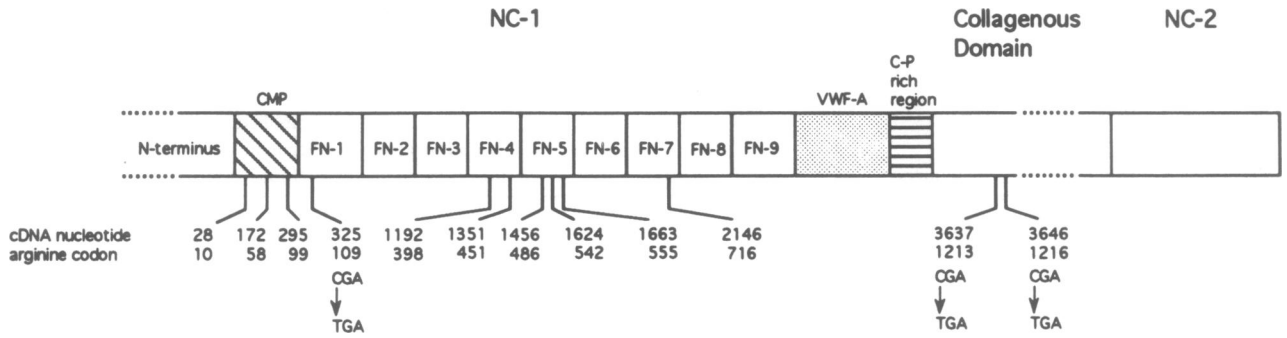

Figure 1 Schematic representation of the partial human type VII collagen cDNA. The subdomains of the amino-terminal noncollagenous domain (NC-1) are indicated: cartilage matrix protein (CMP), fibronectin type III repeats (FN-1–FN9), the A domain of von Willebrand factor (VWF-A), and the cysteine-and-proline–rich region (C-P rich region). The positions of the CGA codons for arginine and the nonsense mutations characterized in this study are indicated.

were also analyzed. The cumulative length (1,095 bp) of these exons represents ~12% of the COL7A1 cDNA. The adjacent intronic regions analyzed, including the corresponding splice junctions, represent an additional 1,316 bp.

Characterization of Nonsense Mutations

The study of 52 unrelated HS-RDEB patients and 2 patients with RDEB inversa by PCR-DGGE analysis led to the detection of three DGGE variants displaying altered electrophoretic patterns (fig. 2). Analysis of CGA codon 109 within exon 6 revealed the same altered electrophoretic pattern in two patients, one presenting HS-RDEB, the other affected with RDEB inversa: a homoduplex with decreased mobility and heteroduplexes was seen, in addition to the homoduplex of normal electrophoretic mobility (fig. 2*a*, patient I-3 in family I and II-3 in family II). Direct sequencing of single-stranded amplification products from both patients revealed a heterozygous C-to-T transition at nucleotide position 325 (numbering is as in Christiano et al. 1992*a*) (fig. 3*a*). This substitution converts arginine 109 to a premature stop codon (R109X). Analysis of genomic DNA of the parents of both patients showed that, in each case, one of the parents (I-2 and II-1, respectively) carried this mutation.

In two other HS-RDEB patients, the amplified DNA fragment containing CGA codons 1213 and 1216 within exon 34 revealed two different DGGE patterns distinct from that of the control (fig. 2*b* and *c*). In each case, both a homoduplex with reduced mobility and heteroduplexes were noted, in addition to the homoduplex comigrating with the control (fig. 2*b* and *c*). Direct sequencing of the amplification product showed a C-to-T transition in one of the COL7A1 alleles, converting arginine codon 1213 into a stop codon (R1213X) in patient III-5 from family

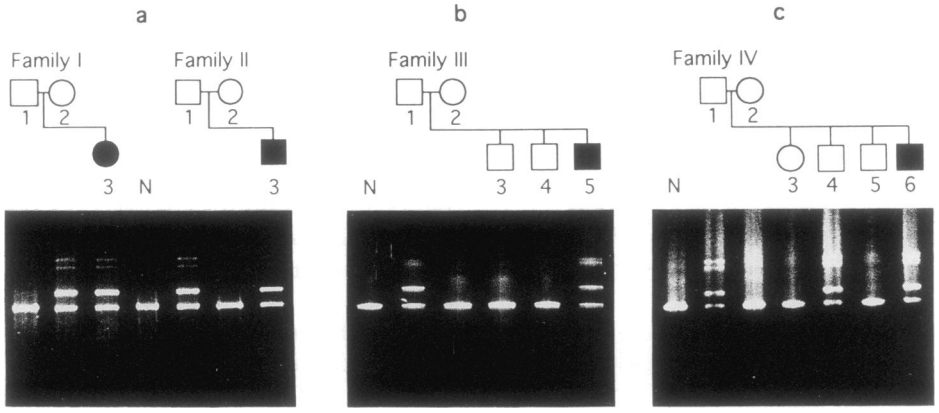

Figure 2 Pedigree of families I–IV, and DGGE analysis of PCR-amplified fragments of exon 13 that contain the CGA codon for arginine 109 (in families I and II *a*]) and of exon 34 containing the CGA codon for arginine 1213 (family III *b*]) and 1216 (family IV *c*]). *a*, Patients I-3 (affected with RDEB inversa) and II-3 (affected with HS-RDEB) showing a heterozygous pattern with homoduplexes and heteroduplexes. The same pattern was also observed in patient I-3's mother (I-2) and in patient II-3's father (II-1). *b* and *c*, Patients III-5 (*b*) and IV-6 (*c*) (both affected with HS-RDEB) displaying two homoduplexes with distinct heteroduplexes. This pattern was also seen in patient III-5's father (III-1 *b*]) and in patient IV-6's father (IV-1) and in one of his unaffected siblings (IV-4 *c*]). Lanes N, Normal control.

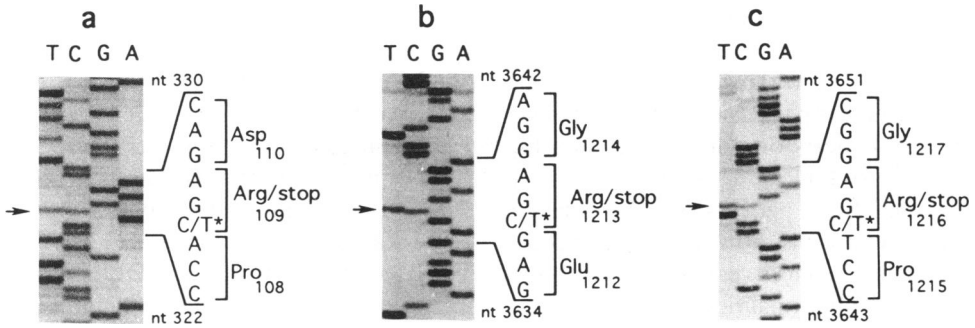

Figure 3 Sequence showing the C-to-T mutation at CGA codons for arginine 109, 1213, and 1216 in the COL7A1 gene. The sequence is shown for nucleotides indicated between the brackets. The asterisk and arrow mark the C-to-T transition in the sense strand of one COL7A1 allele converting an arginine codon (Arg) into a stop codon. *a*, Arg-109/stop mutation in patients I-3 (RDEB inversa) and II-3 (HS-RDEB). *b*, Arg-1213/stop mutation in patient III-5 (HS-RDEB). *c*, Arg-1216/stop mutation in patient IV-6 (HS-RDEB).

III (fig. 3*b*). The same C-to-T transition converted arginine codon 1216 into a stop codon (R1216X) in one allele in patient IV-6 (family IV) (fig. 3*c*). DNA analysis of members of families III and IV revealed that the R1213X mutation was carried by the father in family III (III-1), whereas the father (IV-1) and one of the unaffected offspring (IV-4) in family IV were heterozygotes for the R1216X mutation (fig. 2*b* and *c*).

The R109X mutation creates a *Nla*III restriction site, whereas the R1216X mutation abolishes a *Taq*I and an *Ava*I site, as confirmed by endonuclease digestion of amplified genomic DNA corresponding to these regions in patients I-3/II-3 and IV-6, respectively. The R1213X mutation does not affect any known restriction-enzyme–recognition sites. These three mutations were not found by using DGGE of PCR-amplified genomic DNA from either the other 48 unrelated patients with HS-RDEB or the other patient, with RDEB inversa. The absence of the R109X and the R1216X mutations was also verified in 50 unrelated individuals with no feature of epidermolysis bullosa, by endonuclease digestion of PCR-amplified DNA, with *Nla*III and *Taq*I restriction enzymes, respectively. Screening for additional mutations occurring at the seven other CGA codons for arginine that are located in the CMP domain (exon 5, CGA-99), in the fibronectin 4A domain (FN-4A) (exon 12, CGA-398), in FN-4B (exon 13, CGA-451), in FN-5A (exon 14, CGA-486), in FN-5B (exon 15, CGA-542 and CGA-555), and in FN-7B (exon 19, CGA-716) was negative in all the 54 RDEB patients studied.

Absence of Large Deletion at the COL7A1 Locus in Families I–IV

We investigated the possibility of a gross rearrangement or deletion in the other COL7A1 allele in patients I-3, II-3, III-5, and IV-6. Southern analysis of genomic DNA after digestion with *Bam*HI, *Bst*XI, and *Pst*I restriction enzymes, using the COL7A1 cDNA clone K131 as a probe, did not reveal abnormal bands or reduction in the intensity of autoradiographic signals, in comparison with the control. The presence of two COL7A1 alleles was further documented by restriction-endonuclease analysis of amplified COL7A1 regions containing the previously described *Pvu*II and *Alu*I intragenic polymorphic sites: patients I-3, III-5, and IV-6 showed heterozygosity at the *Pvu*II polymorphic restriction site, whereas patient II-3 was heterozygous at the *Alu*I polymorphic site.

Discussion

We recently reported linkage between HS-RDEB and the COL7A1 gene (Hovnanian et al. 1992), the candidate gene for RDEB, which encodes the major component of AF. In the present study, we investigated 52 HS-RDEB patients and 2 patients with RDEB inversa, for mutations within the COL7A1 gene. The observation that CpG dinucleotides are mutation hot spots in a number of human disease genes led us to selectively screen CpG dinucleotides in COL7A1 exons, for mutations changing CGA arginine codons to TGA premature stop codons. Such a strategy has previously allowed the characterization of nonsense mutations resulting from C-to-T transitions at CGA arginine codons in several human genetic diseases (Maddalena et al. 1988; Reiner and Thomson 1992).

We characterized three premature termination codons at CGA (arginine)—codons 109, 1213, and 1216—which were present in a single COL7A1 allele in four unrelated RDEB patients, in the total of 54 patients studied. The mutation at codon 109 occurs in a patient affected with HS-RDEB and in a patient presenting RDEB inversa, while the mutations at position 1213 and 1216 are found in two HS-RDEB patients. The possibility that these patients may be affected with DDEB, whose locus has also been shown to be linked to the COL7A1 gene (Ryynänen et al. 1991, 1992; Al-Imara et al. 1992; Gruis et al. 1992), is highly unlikely for several reasons. First, patients II-3, III-5, and IV-6 have mutilating scarring that has led to the fusion of the fingers and/or toes, a clinical feature that has not been

reported in DDEB but that is considered specific for HS-RDEB (Fine et al. 1991; Briggaman 1992). Second, subjects I-2, II-1, III-1, IV-1, and IV-4 are carriers for these mutations and unaffected, whereas patients I-3, II-3, III-5, and IV-6, each of whom also bears one of these mutations, display severe epidermolysis bullosa. This is not consistent with the possibility that these patients may be affected with DDEB, whose penetrance is thought to be complete (Sybert and Holbrook 1992). Third, patients I-3, II-3, III-5, and IV-6 had major AF abnormalities on EM examination of skin biopsy: they lacked recognizable AF or had rudimentary AF markedly reduced in number, both of which are suggestive of severe RDEB and which have not been reported in DDEB (Tidman and Eady 1985; McGrath et al. 1993).

At present, only a few mutations in the COL7A1 gene in patients with RDEB have been published. Specifically, a homozygous methionine-to-lysine mutation in the NC-2 domain of COL7A1 has been characterized in two offspring with the mitis form of RDEB (Christiano et al. 1993). We reported a homozygous insertion-deletion leading to a premature stop codon within the FN-5A domain of the COL7A1 gene in a patient with HS-RDEB (Hilal et al. 1993). Recently, four other mutations have been identified in three patients with HS-RDEB (Christiano et al. 1994), each of these mutations resulting also in a premature termination codon in the NC-1 domain of the COL7A1 gene. Although these mutations do not occur in CGA codons for arginine, these results raise the possibility that premature stop codons in the COL7A1 gene may underlie HS-RDEB in a number of cases. The R109X mutation that we describe is the first identification of a gene defect in RDEB inversa. The very low incidence of this form of RDEB has until now hindered linkage analysis. The present study's results provide genetic evidence that COL7A1 mutations underlie both HS-RDEB and RDEB inversa, which is consistent with AF abnormalities reported in both forms of the disease (Bruckner-Tuderman et al. 1992).

The mutations that we report are located within the amino-terminal region of the COL7A1 gene and predict early translation termination. The stop codon R109X occurs within exon 6 (FN-1A domain) located at the beginning of the NC-1 domain, whereas the R1213X and R1216X mutations are located within exon 34 at the amino-terminal end of the collagenous domain (fig. 1). A possible consequence of these mutations would be the synthesis of truncated polypeptides lacking the collagenous and the NC-2 domains of the molecule, which have been proposed to be critical for homotrimer assembly and triple helix formation (Burgeson et al. 1990). Alternatively, premature termination codons have often been shown to lead either to mRNA instability in human disease genes (Baserga and Benz 1988; Kadowaki et al. 1990; Hamosh et al. 1991) or, occasionally, to aberrant splicing (Dietz et al. 1993), and both possibilities have not been excluded in the cases that we describe.

On the basis of linkage analysis, there is at present no evidence for locus heterogeneity in HS-RDEB. Indeed, linkage analysis using the intragenic *Pvu*II polymorphic marker in 19 informative HS-RDEB families previously showed no recombinants between the COL7A1 gene and the disease locus (Hovnanian et al. 1992), suggesting that both defects leading to HS-RDEB lie within the COL7A1 alleles in the families studied. Therefore, it is expected that patients I-3, II-3, III-5, and IV-6 are compound heterozygotes at the COL7A1 locus and must bear, on the other COL7A1 allele, additional mutations leading to impaired AF formation.

Thus far, the search for the molecular defect within the second COL7A1 allele remains negative in our patients. In particular, we did not find evidence for loss of a COL7A1 allele through gross deletion or gross rearrangement leading to a hemizygous state: no abnormal bands were seen on Southern blots, and heterozygosity was found at the intragenic polymorphic *Pvu*II or *Alu*I sites within COL7A1 in families I–IV.

We also searched for mutations within the seven other CGA codons tested within the NC-1 domain, as well as for other mutations and small rearrangements within the exonic regions and splice junctions surrounding these arginine codons. Except for a previously described *Alu*I polymorphic site within exon 14 (Christiano et al. 1992*b*), we did not detect additional nucleotide variation in either these patients or the other 50 RDEB patients studied. Since the GC-clamped DGGE method that we used has been shown to be extremely sensitive for the identification of single base changes (Sheffield et al. 1991), our data indicate that these CGA codons and corresponding exons 6, 13–15, 19, and 34 of the COL7A1 gene are not prone to mutations in the patients studied, and we are currently studying other regions of the COL7A1 gene.

Further characterization of COL7A1 mutations will determine whether premature stop codons are common causes for RDEB, with some regions more prone to mutations, and may prove useful in defining regions of the COL7A1 molecule that are critical for AF formation. Delineation of the molecular defects within the COL7A1 gene in HS-RDEB will also improve genetic counseling for this life-threatening form of RDEB.

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