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Modulation of disease severity of dystrophic epidermolysis bullosa by a splice site mutation in combination with a missense mutation in the COL7A1 gene

Jan-Olof Winberg[1,*], Nadja Hammami-Hauasli[2], Øivind Nilssen[3], Ingrun Anton-Lamprecht[4], Susan L. Naylor[5], Karen Kerbacher[5], Mirjam Zimmermann[6], Peter Krajci[7], Tobias Gedde-Dahl Jr[8] and Leena Bruckner-Tuderman[2]

[1]Biochemistry Department, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway, [2]Department of Dermatology, University of Münster, Münster, Germany, [3]Department of Medical Genetics, University Hospital of Tromsø, Tromsø, Norway, [4]Institute für Ultrastrukturforschung der Haut, University of Heidelberg, Heidelberg, Germany, [5]Department of Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, TX, USA, [6]Department of Dermatology, University Hospital Zürich, Zürich, Switzerland, [7]Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, National Hospital and University of Oslo, Oslo, Norway and [8]Institute of Forensic Medicine and Department of Dermatology, National Hospital and University of Oslo, Oslo, Norway

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Dystrophic epidermolysis bullosa (EBD) is a clinically heterogeneous skin disorder, characterized by abnormal anchoring fibrils (AF) and loss of dermal–epidermal adherence. EBD has been linked to the COL7A1 gene at chromosome 3p21 which encodes collagen VII, the major component of the AF. Here we investigated two unrelated EBD families with different clinical phenotypes and novel combinations of recessive and dominant COL7A1 mutations. Both families shared the same recessive heterozygous 14 bp deletion at the exon–intron 115 boundary of the COL7A1 gene. The deletion caused in-frame skipping of exon 115 and the elimination of 29 amino acid residues from the pro-α1(VII) polypeptide chain. As a result, procollagen VII was not converted to collagen VII and the C-terminal NC-2 propeptide which is normally removed from the procollagen VII prior to formation of the anchoring fibrils was retained in the skin. All affected individuals also carried missense mutations in exon 73 of COL7A1 which lead to different glycine-to-arginine substitutions in the triple-helical domain of collagen VII. Combination of the deletion mutation with a G2009R substitution resulted in a mild phenotype. In contrast, combination of the deletion with a G2043R substitution led to a severe phenotype. The G2043R substitution was a *de novo* mutation which alone caused a mild phenotype. Thus, different combinations of dominant and recessive COL7A1 mutations can modulate disease activity of EBD and alter the clinical presentation of the patients.

INTRODUCTION

Epidermolysis bullosa (EB) is a group of genodermatoses in which mild mechanical trauma results in skin blistering (1,2). The affected structure is the dermal–epidermal basement membrane (BM) zone that attaches the epidermis to the dermis in the skin (3,4). There is a large variation in the clinical severity of EB, from perinatally lethal cases to mild seasonal blistering. Based on the precise ultrastructural localization of the blisters, EB has been divided into three major subgroups. In EB simplex, the blistering level is within the basal keratinocytes just above the BM. In junctional EB, the split occurs within the BM and in EB dystrophica (EBD), below the BM (5).

EBD can be inherited either dominantly or recessively. Four clinically distinct recessive EBD groups are recognized according to the distribution of the lesions: localized, inverse, generalized non-mutilating and generalized mutilating EBD (1,2). Ultrastructural analysis of the EBD subtypes has revealed various degrees of morphological abnormalities of the anchoring fibrils (AF), ranging from normal appearance to total absence. In most patients with severe mutilating EBD, clinically unaffected skin lacks AF and collagen VII, although exceptions have been reported (6–8). In less severe EBD subtypes, collagen VII can be

*To whom correspondence should be addressed. Tel: +47 77 64 54 88; Fax: +47 77 64 53 50; Email: janow@fagmed.uit.no

immunologically detected in the skin, but the ultrastructure of the anchoring fibrils often appears abnormal (9–11).

Collagen VII is the major, if not sole structural protein of the anchoring fibrils (4). It is synthesized as a procollagen that contains a large N-terminal globular domain (NC-1), a triple-helical domain and a C-terminal propeptide (NC-2). Procollagen VII monomers form antiparallel dimers with overlapping NC-2 domains. These dimers are presumably stabilized by interchain disulfide bridges, and the NC-2 propeptide is removed during biosynthesis and polymerization of collagen VII to anchoring fibrils (12,13). The triple-helical domains contribute to the ultrastructurally recognizable central cross-banded part of the anchoring fibrils. The NC-1 domains mediate the attachment of the fibrils into the BM (14).

The gene for collagen VII, COL7A1 is located at chromosome 3p21. The length of the gene is 31 132 bp, and it contains 118 exons giving rise to a 8832 bp mRNA transcript which codes for a pro-α1(VII) chain of 2944 amino acids (15). Genetic linkage analysis of informative REBD mutilans families indicated that the collagen VII gene was the candidate gene for REBD (16,17). A number of mutations have been reported in patients with recessive, severe mutilating EBD. Interestingly, most of them have been either homozygous or heterozygous mutations leading to premature termination codons (PTC) in the COL7A1 gene (18–22). Most of the affected individuals were compound heterozygotes carrying two different PTCs, a fact that explains the lack of collagen VII and anchoring fibrils in the skin. In contrast, far fewer mutations have been found in patients with less severe forms of EBD. Glycine substitutions in the triple-helical domain of collagen VII were reported in dominant EBD (23–26). Two other reports dealt with mutations in localized or inversa EBD. A patient with localized recessive EBD carried a homozygous missense mutation in exon 112 (22) and another two splicing mutations (27) and a patient with EBD inversa carried one nonsense mutation, but the other mutation of the same patient remained unknown (20).

Recently we identified a sporadic case of EBD with a heterozygous deletion mutation in the COL7A1 gene that led to exon skipping and interference with the processing of procollagen VII to collagen VII (13). However, lack of family history and unavailability of any family members for analysis impeded further investigation. In the present study we have further characterized this patient of Polish origin, as well as some members of her family. In addition, an unrelated Norwegian EBD family [Family X of Gedde-Dahl (28)] which now consisted of two affected individuals was investigated. One of the mutations of this family was the same 14 bp deletion in the exon–intron 115 border as the Polish patient. The mutation was also found in several unaffected family members of both families, but absent in >200 other unrelated individuals. The second mutation in the Polish and Norwegian families were different, but both led to glycine substitutions in the triple-helical domain of collagen VII. These families illustrate how different combinations of dominant and recessive mutations can determine the clinical severity in EBD.

RESULTS

Clinical and genealogical description

Family EB10. This north-Norwegian non-consanguineous EBD family was first published by Gedde-Dahl (28). The family shows a widespread divergent Norwegian and Swedish ancestry and is not associated with other EDB families in Norway. The maternal ancestors of EB10-1 are widespread over Northern Norway, while her husband EB10-95 and the father of her two children is from Southern Norway.

The proband EB10-1 is a 58 year-old female with generalized, non-mutilating EBD who was followed by one of the authors (TG-D) for over 30 years. Blistering and scarring started with finger blisters at the age of 3 days and became generalized, with equally severe blistering on the trunk as on the extremities. The lesions were more severe than those seen in dominant EBD Pasini and without development of albopapuloid papules. Repeated subungual blistering resulted in loss of all nails by the age of 7 years, but synecchias or mutilation of the fingers and toes never developed. Moderate oral blistering was accompanied by rapid decay of the teeth. Up to early adulthood, she was severely handicapped by EB, but since then the blistering activity has diminished, leaving the skin erythematous, thin, atrophic and vulnerable. Trauma induced acral blisters still occur almost daily.

Her now 26 year-old second son EB10-98 also developed finger blisters at age of 3 days, but the trauma induced blistering remained localized to the extremities, with rare blisters on the trunk, ears or in oral mucosa. No albopapuloid Pasini lesions developed. The course of the disease was strikingly milder than that of the mother, and the patient is only moderately handicapped. Hence, he presented with a characteristic clinical phenotype of localized EBD. Careful clinical examination (TG-D) revealed no signs of EBD by any of the five siblings or by the other son of EB 10-1.

Family LB4. The now 15 year-old female patient LB4-1 was born as the first child of healthy non-consanguineous Polish parents. No other members of the family had had a skin disease or a genetic disorder. LB4-1 presented at birth with localized absence of the skin on one leg. At the age of 2 weeks the first blisters were noted. The tendency to generalized blistering slowly diminished over the first years of life, and only trauma-exposed sites, e.g. hands, feet, elbows and knees remained affected. This tendency continued, resulting in localized acral blistering. The blisters healed with erythematous scarring, development of milia, Pasini papules and nail dystrophy. Most toe nails were dystrophic and finger nails remained unaffected or dystrophic. Oral blistering was rare, and esophagus involvement was not observed. Thus, this patient showed the characteristic clinical phenotype of localized EBD. Careful clinical examination (LB-T) revealed no signs of EBD by the mother or the half-brother of LB 4-1. The father was not available for examination.

Electron microscopy

EB10-1. Both by light and electron microscopy, non-lesional intact skin from the upper buttock/perilumbal area (i.e. a predilection site) of the patient EB10-1 showed an unsplit intact dermo–epidermal junction. Hemidesmosomes and other basement membrane components appeared normal in the investigated biopsy samples. Anchoring fibrils were present in nearly normal amounts and most were of normal length (Fig. 1). Some of the anchoring fibrils were especially long and a fraction of these were connected to the basal lamina with both ends (Fig. 1). All anchoring fibrils, however, were thinner than normal, slightly hypoplastic and without a clear and specific banding pattern.

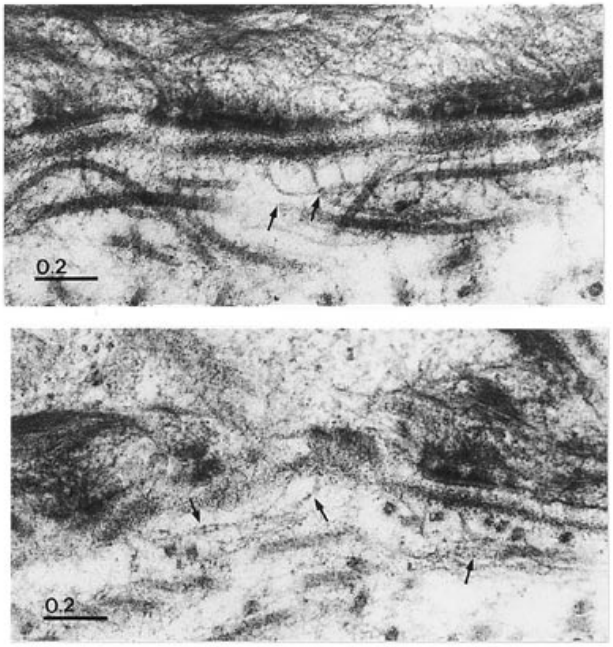

Figure 1. Intact dermo-epidermal junction of EB10-1 with nearly normal amounts of anchoring fibrils (arrows) that are long and slender but significantly thinner than normal, occasionally with a faint cross banding; some fibrils insert in the basal lamina with both ends. Hemidesmosomes are present in normal frequency and with the normal ultrastructure that is, however, only revealed in perpendicular sections. Magnification ×43 000; the bar indicates 0.2 µm.

LB4-1. Intact skin exhibited a reduced number of anchoring fibrils which appeared diffuse and lacked a well defined cross-banding pattern. The electron microscopic findings of the skin of LB4-1 have been published previously (13).

Indirect immunofluorescence (IF) analysis

Antigen mapping confirmed the clinical diagnosis of EBD in patients EB10-1, EB10-98 and LB4-1. Skin biopsies from unaffected areas were stained with IF technique using antibodies to well defined components of the BM zone. In all biopsies, microscopic blisters were observed, and all antibodies stained the blister roof, indicating that the tissue separation occurred below the BM, at the level of anchoring fibrils.

Since in normal skin procollagen VII is processed to collagen VII, we postulated that defective processing could underlie EBD in some patients. After more than 50 normal unrelated control skin biopsies showed lack of immunoreaction with antibodies to the NC-2 domain of procollagen VII, skin of 81 EBD and 26 other EB patients was examined using IF staining. Only EB10-1 and LB4-1 exhibited a strongly positive linear fluorescence at the BM (Fig. 2). This finding indicated that the NC-2 propeptide had not been removed from procollagen VII. In contrast, the skin of the affected proband EB10-98 did not show positive staining, implying that he did not carry the abnormality leading to persistence of the NC-2 domain.

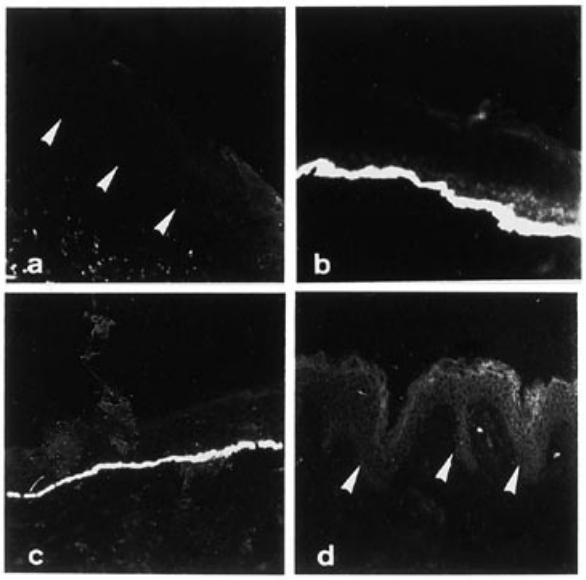

Figure 2. Indirect immunofluorescence staining of control and EBD skin with antibodies to the C-propeptide of procollagen VII. Healthy control skin (**a**), LB4-1 (**b**), EB10-1 (**c**), EB10-98 (**d**). In (a) and (d), arrowheads point to the BMZ.

Detection of abnormal COL7A1 alleles and identification of mutations

Messenger RNA isolated from the cells of patients EB10-1 and EB10-98 was subjected to RT-PCR analysis with primers that cover the region corresponding to the NC-2 domain of procollagen VII. Similarly to controls, the analysis of EB10-98 showed only one cDNA fragment of the expected size of 439 bp. In contrast to this, two fragments were found in the samples of EB 10-1, one with the expected normal size, and the other one ~90 bp shorter (Fig. 3A). Direct sequencing demonstrated skipping of 87 bp in the shorter PCR product (Fig. 3B). The deleted region corresponded exactly to exon 115 in the COL7A1 gene, suggesting a splice site mutation resulting in in-frame exon skipping. This showed that similar to LB4-1 (13), EB10-1 lacked exon 115 in one of the allelic mRNA transcripts.

Genomic DNA from EB10-1 and EB10-98 was amplified by PCR using primers covering exons 114–116 and introns 114, 115 and part of intron 116. DNA sequencing of the amplimers demonstrated a heterozygous 14 bp deletion in EB10-1. This deletion was localized at the exon–intron 115 border, corresponding to nucleotides 33 563–33 576 of the COL7A1 gene (Fig. 4), which is identical to that previously found in LB4-1 (13). Thus the skipping of exon 115 in the mRNA transcript is in accordance with the removal of the 5′ donor splice site of intron 115. Sequencing of corresponding genomic DNA from EB10-98 revealed only normal sequence of this region of the COL7A1 gene (Fig. 4).

To identify the other mutation in EB10-1 and hence the mutation transmitted to EB10-98, genomic DNA from these two patients was amplified by PCR, using primers that covered all exons of the COL7A1 gene, and the amplimers were subjected to heteroduplex analysis. Examination of a PCR product spanning exon 73 of COL7A1 demonstrated a band of altered mobility in

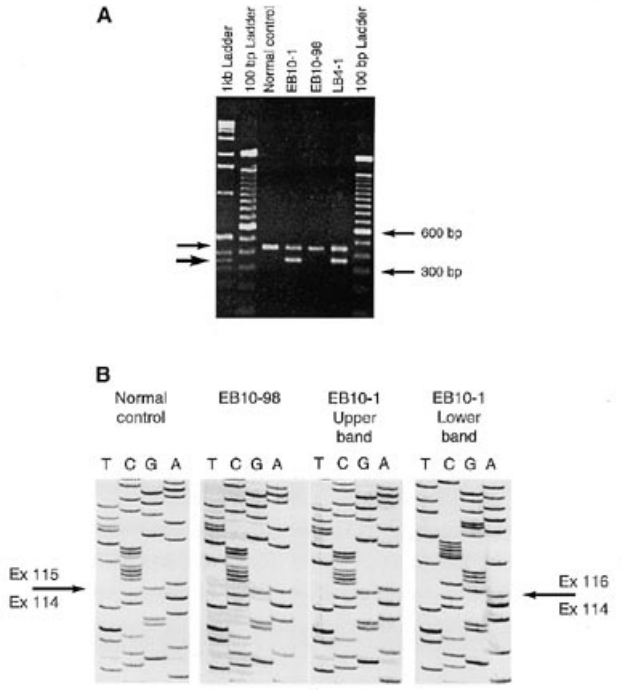

Figure 3. Analysis of procollagen VII cDNA from the two EBD patients in family EB10. (**A**) Agarose gel electophoresis of products of RT-PCR with primers Col11F and Col10R from normal control and patients EB10-1, EB10-98 and LB4-1. The samples from patients EB10-1 (generalized EBD) and LB4-1 (localized EBD) showed two PCR products, one of the expected size of 439 kb (thin arrow), and one that was ~80–90 bp shorter (thick arrow). Both the son of EB10-1 with localized EBD, EB10-98, and the control showed only one band (thick arrow). On the outmost lanes as size markers a 1 kb DNA ladder, and on the next lanes a 100 bp ladder from 1500 to 100 bp (Gibco Life Technologies). (**B**) Nucleotide sequence of cDNA from normal control, EB10-98, EB10-1 upper band and EB10-1 lower band as shown in (A). Note, in the cDNA sequence of the EB10-1 lower band, exon 116 follows exon 114, which shows skipping of exon 115.

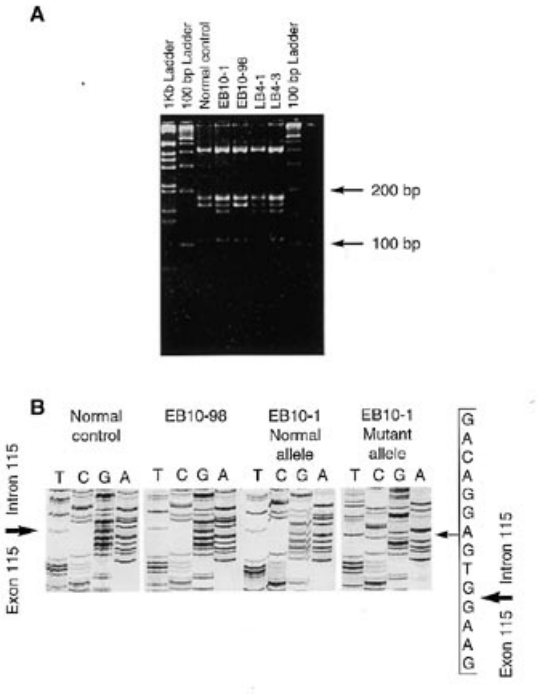

Figure 4. Deletion mutation in the collagen VII gene. (**A**) Genomic PCR products were generated from normal control, EB10-1, EB10-98, LB4-1 and LB4-3 with primers ColE114 and Col10R. The PCR products were digested with *Pst*I and *Sph*I, and separated on a high resolution agarose gel. (**B**) Characterization of the mutant allele. DNA sequence of genomic DNA from normal control, EB10-98, normal allele of EB10-1 and mutant allele of EB10-1. The 14 bp deleted from the mutant allele are shown between the brackets (thin arrow). The exon 115–intron 115 borders are indicated with thick arrows.

the two affected individuals, compared to unrelated unaffected individuals (Fig. 5A). Sequencing of the PCR products of EB10-1 and EB10-98 (Fig. 5B) revealed a heterozygous G-to-A transition at nucleotide position 6127 in the cDNA sequence (nucleotide 23 592 in the genomic DNA sequence). This mutation resulted in substitution of a glycine (GGG) by an arginine (AGG) at amino acid position 2043 in the collagen VII chain. This mutation was designated G2043R.

To identify the second mutation in the Polish patient LB4-1, genomic DNA from this patient and her mother LB4-3 was amplified by PCR, using primers that covered all exons of the COL7A1 gene, and the amplimers were subjected to heteroduplex analysis. Once again, the PCR product which spanned exon 73 of COL7A1 demonstrated a band of altered mobility in the affected individual, in contrast to the mother who showed only one band (Fig. 6A). The PCR products were subcloned and sequenced. LB4-1 (Fig. 6B) revealed a heterozygous G-to-A transition at nucleotide position 6025 in the cDNA sequence (nucleotide 23 490 in the genomic DNA sequence). This nucleotide change resulted in substitution of a glycine (GGA) by an arginine (AGA) at amino acid position 2009 and was designated G2009R.

Verification and inheritance of the mutations

Members of families EB10 and LB4 were screened for the presence of the 14 bp deletion mutation. IF staining with the NC-2 antibodies showed presence of procollagen VII in the skin of several members of the EB10 family. The unaffected brothers EB10-2 and EB10-4, the unaffected sister EB10-5, and the unaffected son EB10-96 were all positive. The unaffected sister, EB10-6 and the affected son, EB10-98, were negative. Other family members were not examined with this technique. The unaffected mother and half-brother of patient LB4-1 were now available for examination. The mother, LB4-3, showed positive staining while the half-brother, LB4-4, did not.

To confirm the 14 bp deletion mutation at the genomic level and to examine the inheritance of the mutation within the families, DNA from family members was analysed with RFLP. A *Pvu*II restriction site resides within the deleted region of the COL7A1 gene (nucleotides 33 563–33 576). In addition there are two *Pst*I sites, one in exon 115 and one in intron 115, and a *Sph*I site in intron 115. For RFLP analyses, PCR was performed on genomic DNA with primers ColE114 and Col10R giving rise to a 872 bp fragment. Figure 7A shows the *Pvu*II digest of the PCR products. In normal controls, two bands appeared and in heterozygous individuals with the 14 bp deletion, there was an additional band corresponding to the undigested fragment. In addition to the patients EB10-1 and LB4-1, the unaffected family members EB10-2, EB10-4, EB10-5, EB10-96 and LB4-3 carry the

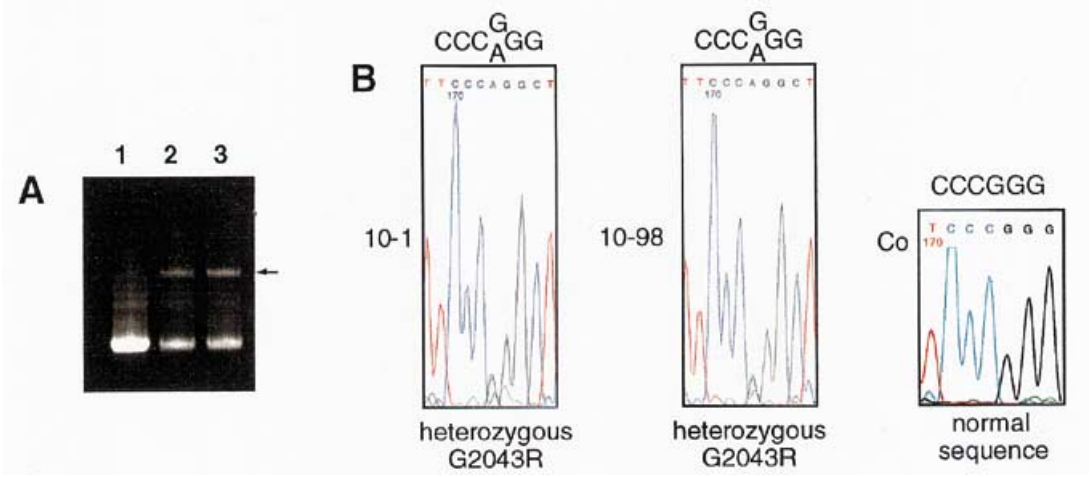

Figure 5. Glycine substitution mutation in family EB10. (**A**) Heteroduplex analysis of the PCR product containing exon 73 revealed a heteroduplex band (arrow) in EB10-1 (lane 2) and EB10-98 (lane 3), but not in a control (lane 1). (**B**) Automated sequence analysis of the patients' DNA revealed a heterozygous G-to-A transition at nucleotide position 6127 of the cDNA, designated G2043R. The mutation was not present in a normal control.

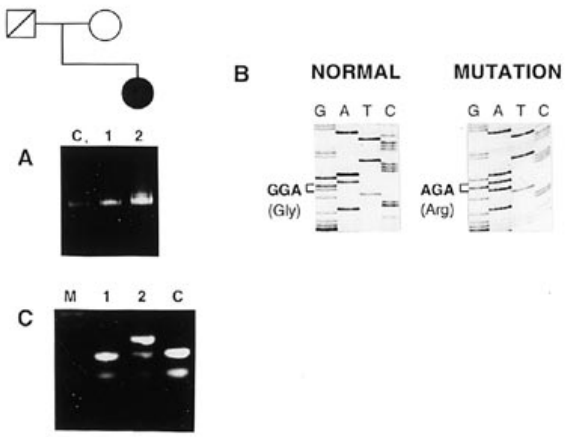

Figure 6. Glycine substitution mutation in family LB4. (**A**) Heteroduplex analysis of the PCR product containing exon 73 revealed a heteroduplex band in the patient LB4-1 (lane 2), but not in the mother (lane 1) or in a control (lane C). (**B**) Sequence analysis of the patients' DNA revealed a heterozygous G-to-A transition at nucleotide position 6025 of the cDNA, designated G2009R, but not in a normal control. (**C**) The mutation was verified by the loss of a *Dsa*I restriction enzyme site. The PCR products of a control (lane C) and the mother (lane 1) were digested to two fragments of 199 and 88 bp. The heterozygous patient showed an additional uncleaved band of 287 bp (lane 2).

deletion. Neither the mildly affected EB10-98, nor the other healthy members of family EB10 had this mutation. These results were verified by *Pst*I and *Sph*I digest of the PCR amplified genomic DNA as shown in Figure 4A for some of the family members. Here normal controls gave rise to four bands and those carrying the deletion to five bands, since the 14 bp deletion creates a new, slightly faster migrating band. There was complete agreement of the carrier analysis with IF and RFLP methods.

Members of the EB10 family was also screened for the presence of the G-to-A mutation at nucleotide 23 592 in exon 73. This mutation resulted in the loss of a *Sma*I restriction site, CCCGGG. In a normal allele, *Sma*I cleaved the 287 bp PCR product into three fragments of 149, 93 and 45 bp. However, in the mutant allele it gave rise to only two fragments of 149 and 138 bp. As shown in Figure 7B, only EB10-1 and EB10-98 carried the mutation in the EB10 family. This shows that the mutation in EB10-1 is a *de novo* mutation in the paternal allele, i.e. the one inherited from EB10-8 and which does not carry the 14 bp deletion.

The G-to-A mutation in exon 73 of the LB4-1 patient resulted in the loss of a *Dsa*I restriction site, CCGTGG. In the normal allele, the enzyme cleaved the 287 bp fragment into two fragments of 199 and 88 bp, while the mutant fragment remained uncleaved. Three fragments of 287, 199 and 88 bp occurred after *Dsa*I treatment of the PCR product from LB4-1, while the mother and a normal control only showed the two fragments of 199 and 88 bp (Fig. 6C). This confirmed the mutation in the patient, but not in the mother or the control.

Screening for the mutant COL7A1 alleles

To investigate the possibility that the 14 bp deletion mutation was associated with EBD in other patients, or a normal polymorphism in the population, two approaches were taken: IF staining of skin biopsies and RFLP. As mentioned above, skin of 142 unrelated individuals with or without EB from different European countries showed no positive reaction in IF staining with antibodies to the NC-2 domain. The 14 bp deletion was not present in 200 chromosomes from additional unrelated control individuals as shown by *Pvu*II digestion of ColE114–Col10R PCR fragments amplified from genomic DNA. These findings demonstrate that the 14 bp deletion, and hence the retention of the NC-2 domain of procollagen VII in the skin, do not represent a normal polymorphism in the population.

The G-to-A mutation at position 6027 in exon 73 was not present in 150 chromosomes from unrelated control individuals as shown by *Dsa*I digestion of the PCR fragment amplified from genomic DNA. This shows that this mutation is not a common polymorphism.

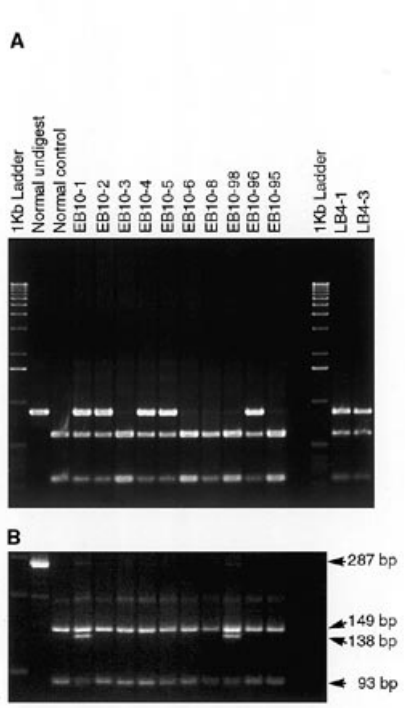

Figure 7. Mutation analysis using RFLP of PCR products from genomic DNA. (**A**) Genomic DNA of families EB10 and LB4 was amplified with primes ColE114 and Col10R. The resulting 872 bp fragment was digested with *Pvu*II and separated on an agarose gel. (**B**) In the EB10 family, the PCR product of 287 bp which contained exon 73 was digested with *Sma*I and separated on an agarose gel.

Haplotype analyses

The haplotype of the EB10 family at chromosome 3p21 was examined with polymorphic markers flanking the COL7A1 gene as well as using markers within the gene. Figure 8 shows that the 14 bp deletion followed the allele carrying the haplotype marked in red, 3;7;3;B1;A1;+;del14bp;163;19;1;144 (from *D3S1260* to *D3S1578*) in family EB10. This allele was inherited from the mother of EB10-1, EB10-7, as verified by the *Pvu*II polymorphism in exon 21 of the COL7A1 gene. This allele had also been inherited by the three healthy siblings of EB10-1, EB10-2, EB10-4 and EB10-5, and also by her healthy son EB10-96, but not the EBD affected son EB10-98. These results are consistent with the carrier analysis of the deletion allele using either IF or PCR/RFLP analysis. The allele carrying the G2043R substitution in EB10-1 was derived from her father, EB10-8, as this mutation had been transferred to her diseased son, EB10-98. However, as this mutation occurred only in the two patients, this must be a *de novo* germline mutation in EB10-8. In the EB10 and LB4 families, the chromosome carrying the 14 bp deletion had an identical haplotype (3;B1;A1;+;del14bp;163) of all six intragenic COL7A1 markers (only two is shown) and of the two flanking markers (S1235 and S1029), while the outer flanking markers differed (Fig. 8).

DISCUSSION

Ultrastructural and immunochemical observations have attributed skin fragility in EBD to changes in AF and their main component, collagen VII (2,29). Linkage studies and mutation analyses supported this assumption (22). In dominant EBD that usually shows a milder phenotype, all mutations reported so far resulted in glycine substitutions in the triple-helical region (23–26,30). These substitutions destabilize the collagen triple-helix, and if incorporation of such helices into supramolecular aggregates occurs, they are likely to influence the stability of AF and integrity of the skin. Most patients with recessive EBD are compound heterozygotes carrying two different COL7A1 gene defects, as shown by mutation analyses (22) and haplotyping studies (Gedde-Dahl *et al*., unpublished). In severe EBD mutilans, both mutations often result in PTC, low mRNA levels and lack of collagen VII protein in the skin (21). An interesting compound heterozygous constellation in this disease group was reported recently: one mutation led to a PTC, and the other caused a glycine substitution in the triple-helix, a combination that also resulted in a very severe clinical phenotype (26). This is probably due to intracellular accumulation and degradation of the allelic product containing the glycine substitution as shown in other dominant EBD patients (31). In contrast, an EBD patient with a milder phenotype carried a mutation that resulted in a PTC and a point deletion in the penultimate exon of the COL7A1 gene that altered the reading frame of the last 25 amino acids, overriding the normal termination codon and added seven amino acids to the pro-α1(VII) chain before termination (32). Although only a limited number of mutations is known, it becomes clear how extensive the molecular heterogeneity of EBD is. The combination of two different mutations leading to different consequences for the multimeric collagen VII aggregates can account for a vast variety of biological and clinical phenotypes. In this context, certain simpler constellations can be predicted: mutations leading to two PTC cause a severe mutilating phenotype, whereas a PTC in combination with another mutation can cause a severe or a mild EBD phenotype depending on the second mutation, but is harmless when combined with a normal allele, as seen in healthy carriers.

In the present study, two patients were compound heterozygous for novel combinations of an exon skipping mutation and two different glycine substitutions, and the third patient had only a dominant glycine substitution as summarized in Table 1 and Figure 9. The combinations resulted in distinctly different clinical phenotypes. In the EB10 family, both affected individuals were carriers of a G2043R substitution in the triple-helical region of the collagen VII molecule. Mutation analysis and haplotyping studies showed that the G-to-A transition at position 6127 (cDNA) is a *de novo* germline mutation in EB10-8. It is interesting to note that an identical mutation has previously been found in a large Italian EBD family, with a dominant inheritance of the disease in three generations (24). As this is a *de novo* mutation in the Norwegian family, two identical events have occurred independently. Because this mutation is neither a common polymorphism nor a prevalent mutation in other EBD families (24), it is not likely that this site in the COL7A1 gene is particularly susceptible for mutations.

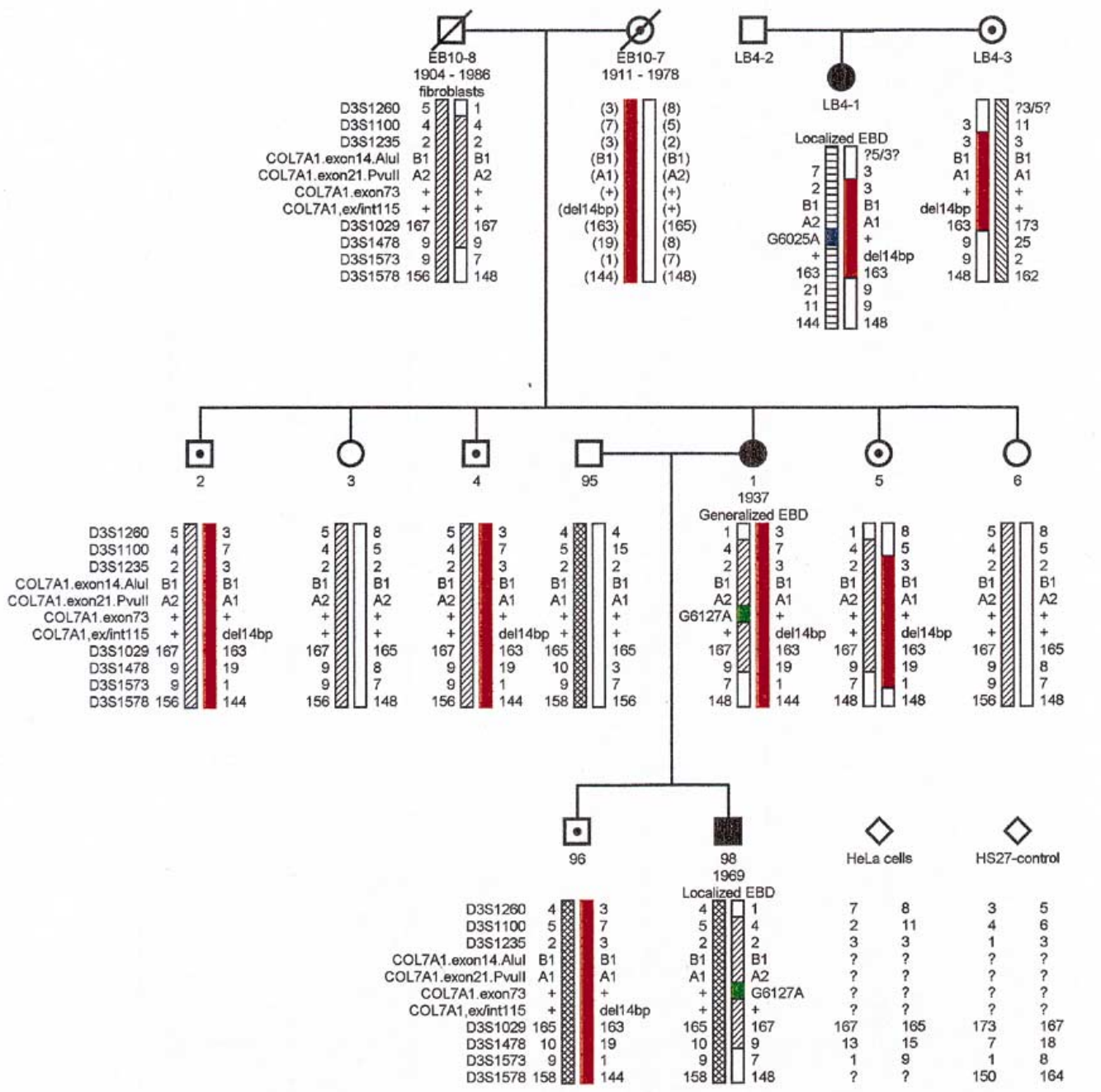

Figure 8. Genotypes at known polymorphic loci within the COL7A1 gene and microsatellite loci surrounding this gene at chromosome 3p21–22 in the EB10 and LB4 families. The three telomeric (top) and the three centromeric (bottom) microsatellite markers differ in their order from that established in the CEPH families (44, Genome Data Bank Maps C3M77 and C3M113) in that we place *D3S1578* centromeric to *D3S1573*. The reason is that in addition to the present family (...S1478;S1573×S1578 crossover in the maternal chromosome of EB10-5 or a mutation in S1578) we have observed another family with S1235;S1573×S1578 crossover or S1578 mutation (Gedde-Dahl *et al*., unpublished). This makes the CEPH position of S1573 as flanking unlikely. The gene order and orientation of *D3S1029* and COL7A1 are as yet unknown. They can be either side of *D3S1235*. The position given here is tentative and based on our current family studies (Gedde-Dahl *et al*., unpublished). It also differs from the pter–S1029-(0.005)-S1235-(0.003)-S1573-(0.003)-COL7A1(*Pvu*II) order used by Christiano *et al*. (30).The genetic distance is 10 cM for S1573-S1235 (45) and still larger from S1260 (on 3p24.2–p22) to S1573 (on 3p21.2–p14.2). Notice that EB10-5 carries a maternal double crossover chromosome. The father EB10-8 appeared homozygous for the commonest allele in all six intra-COL7A1 RFLPs (in exons 3, 14, 21, 30, 84 and 118; only two are shown here), as well as for the closest flanking markers. Therefore, the paternally derived chromosome from S1100 to S1478 are non-informative in the children. Hence, EB10-1 and EB10-5 may have inherited the same COL7A1 haplotypes from both parents except for the G2043R mutation in EB10-1, which is a *de novo* mutation inherited from the father. Therefore, the two haplotypes may be identical at all positions except at position 23 592 in the genomic DNA of the COL7A1 gene. The haplotype for EB10-7 was reconstructed on the basis of information on the genotypes of her children. The chromosome containing the 14 bp deletion of COL7A1 in the Polish LB4 family is of the same intragenic (but common) COL7A1 haplotype, and with the same flanking S1235 and S1029 alleles as in the Norwegian EB10 family. Based on Christiano *et al*. (42), the frequency of the intragenic alleles at exons 3, 14, 21, 30, 84 and 118 of the 14 bp deleted COL7A1 gene are as follows: 0.86, 0.77, 0.40, 0.82, 0.90 and 0.94, respectively. The flanking S1235*3 allele has a frequency of 0.13, while the S1029*163 allele is outside the previously recorded 175–165 bp range, alleles 1–6 (Genome Data Bank). No recombination has been observed in our families between the markers S1235 and S1029. Therefore, we assume that also the S1029*163 allele has a low frequency.

Table 1. Summary of the present study

Patient/ EBD type	Genomic DNA		cDNA	Protein		Skin	
	Exon/intron affected	mutation	mutation	procollagen VII	collagen VII	IF staining with NC2 antibodies antibodies	anchoring fibrils from EM studies
LB4-1 localized	Ex 73 Ex–In 115 border	G23490A Δ33563–33576 (14 bp del)	G6025A Ex 115 in-frame skipping	G2009R 29 amino acids deleted	G2009R NC-2 not cleaved off	positive	reduced number, diffuse, lack of clear banding pattern
EB10-1 generalized	Ex 73 Ex–In 115 border	G23592A Δ33563–33576 (14 bp del)	G6127A Ex 115 in-frame skipping	G2043R 29 amino acids deleted	G2043R NC-2 not cleaved off	positive	normal amount, thinner than normal, slightly hypoplastic, lack of clear banding pattern
EB10-98 localized	Ex 73	G23592A	G6127A	G2043R	G2043R	negative	EM studies not done

Clinically, EB10-1 is more severely affected than her son, EB10-98. In contrast to her son, she also carried an aberration in her maternal COL7A1 allele, the 14 bp deletion that resulted in the skipping of exon 115 and elimination of 29 amino acids from the pro-α1(VII) polypeptide, but left the remaining C-terminus intact. The eliminated sequence includes the putative cleavage site for procollagen C-proteinase (13). The deletion is unlikely to be a dominant disease-causing mutation since five healthy family members also were carriers of this aberration. Neither can it be regarded as a normal polymorphism in the population, since it was not found in 92 unrelated EB patients or more than 150 unrelated healthy individuals, i.e. in more than 484 chromosomes. Therefore, our data strongly suggest that the deletion is a recessive disease-causing mutation that is pathogenic at least in combination with another aberration that can act in synergy, but harmless when combined with a normal allele. Thus, EB10-1 carries a dominant mutation in one allele and a recessive mutation in the other allele, while EB10-98 carries only the dominant mutation (Table 1). This is consistent with the difference in clinical severity between these two probands, since dominant EBD generally presents with milder symptoms.

Another interesting constellation appears in the LB4 family. The patient with localized EBD has inherited from the maternal side the same 14 bp deletion as in the EB10 family, but she carried another missense mutation in exon 73, resulting in G2009R substitution in the triple-helical domain. This mutation alters a conserved RGD motif (Fig. 9) which is also found in collagen VII from mouse (33). Unfortunately, her father who was reported to be unaffected was not available for examination. Therefore, a mild form of EBD cannot be excluded in the father. Several possibilities appear with respect to the inheritance of this missense mutation. It may reside in the paternal allele of the patient. Another possibility is a *de novo* mutation in the maternal allele, and hence two mutations exist in the same allele. Therefore, it is not possible to predict from the pedigree whether the G2009R substitution acts dominantly or recessively. All dominantly inherited EBD investigated so far are due to glycine substitutions in the triple-helical domain of the collagen VII molecule, however, silent glycine substitutions were found in several EBD families with recessive inheritance (25). This is in contrast to similar mutations in other collagen genes, where all glycine substitutions in the triple-helical domains result in dominant inheritance of disease (34–36).

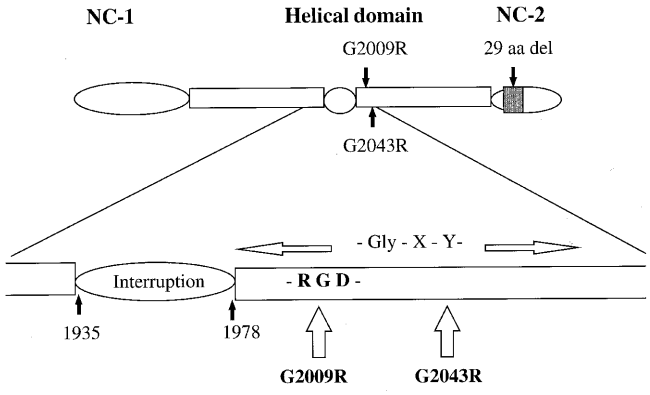

Figure 9. A schematic presentation of a procollagen VII monomere and the localization of the mutations found in the two EBD families. Table 1 summarizes the findings in the three patients. The G2009R substitution eliminates one of the four -RGD- sequences from collagen VII. The G2043R substitution is located in the middle of a long uninterrupted collagenous sequence. The numbers in the lower part of the figure refer to the position of amino acid residues.

The difference in clinical severity between LB4-1 (localized) and EB10-1 (generalized) might be attributed to the position of their glycine substitutions in the collagen VII triple-helix. In normal individuals, the triple-helix is interrupted at several positions, with the longest non-helical stretch of 39 amino acids in the middle of the helix. One could hypothesize that glycine substitutions close to this interruption have less deleterious effects on the stability of the molecule than substitutions within a strictly helical sequence. The G2009R mutation of LB4-1 is located 30 amino acid residues C-terminal from the non-helical sequence, within an RGD sequence, whereas the G2043R substitution of EB10-1 is located further towards the C-terminus, in the middle of a long uninterrupted -Gly-X-Y- repeat (Fig. 9).

The contribution of the 14 bp deletion mutation to the phenotype in both patients is presumably similar, although the precise molecular consequences of defective procollagen VII

processing remain elusive at present. Ultrastructural changes of AF in the two patients were observed, both having fibrils with diffuse and ill defined cross-banding pattern. However, these AF aberrations did not provide an obvious explanation for the difference in AF stability and clinical severity between these two patients. In this context it may be of interest that whereas the retention of the C-propeptide of collagen VII is harmful only in combination with another aberration, the retention of other collagen propeptides can have deleterious consequences. An example is the heritable connective tissue diseases, Ehlers–Danlos syndromes VII A–C, in which exon skipping mutations or reduced activity of the procollagen N-proteinase distort the N-terminal processing of collagen I (37–39). This may be explained by the different supramolecular aggregation of collagens I and VII. Collagen I forms heterotypic quarter staggered fibrils with other collagens, whereas collagen VII may just self-aggregate and condense laterally in a non-staggered manner to the AF. Whether the abnormally processed collagen VII molecules are incorporated into the fibrils remains to be shown.

To obtain more information on the inheritance of the EBD and the origin of the 14 bp deletion in the Norwegian and Polish families, genealogical and haplotyping studies were performed. In both families, the 14 bp deletion mutation was of maternal origin. The fact that two families have an identical deletion might be either due to the same mutational event in two unrelated individuals in a susceptible region of the gene, or due to common ancestry on the maternal side of the two families. The apparent rarity of this mutation in EBD families as well as in the general population argues against the former hypothesis. In this context, it is of interest that one of the ancestors of EB10-7 may originate from Schleswig-Holstein in Germany, an area that is geographically close to Poland. It is also noticeable that in these two families, the maternal 3p21 haplotype carrying the COL7A1 14 bp deletion had identical alleles in both the intragenic COL7A1 markers and in the two closest flanking markers S1235 and S1029 (Fig. 8). The COL7A1 intragenic marker alleles were of the common type, while the two flanking marker alleles were rare (Fig. 8). Therefore, we cannot exclude the possibility of a common ancestor of the 14 bp deletion in these two families.

MATERIALS AND METHODS

Electron microscopy

Electron microscopy of skin biopsy samples of intact non-lesional normal appearing skin from the generalized non-mutilating EBD patient EB10-1 was performed as described previously (10,40).

NC-2 domain-specific antibodies and indirect immunofluorescence

Bacterial fusion proteins corresponding to the NC-2 domain of procollagen VII were designed on the basis of the 3′ cDNA sequence of the COL7A1 gene and produced as described elsewhere (13). Antibodies were raised to these fusion proteins and affinity purified. The antibodies recognized procollagen VII, but not collagen VII, since the antigen sequence corresponded solely to the NC-2 propeptide of procollagen VII (13). The antibodies were employed for immunofluorescence staining of cryosections of normal and EBD skin. As controls, domain-specific collagen VII antibodies against the NC-1 domain and the triple helical region were used. The stainings were carried out using standard techniques, with biotin-avidin amplification of the signals (13). Diagnostic antigen mapping of EBD skin was carried out with antibodies to bullous pemphigoid antigens 1 and 2, integrins α6 and β4, laminins 1 and 5, and collagens IV and VII as described elsewhere (2).

RNA isolation, cDNA synthesis

Total RNA was isolated from normal and EBD fibroblasts with TRIzol reagent according to the manufacturer's specifications (BRL, Gaithersburg, MD, USA). The RNA was dissolved in RNase-free water and the concentration adjusted to ~1 µg/µl. Deoxyoligo-dT(18) primed cDNA synthesis was carried out with SuperScript^{TM}II reverse transcriptase essentially as described by the manufacturer (BRL, Gaithersburg, MD, USA) but with the following exceptions: RNA was denatured with 100 pmol deoxyoligo dT at 90°C for 10 min prior to primer extension which was performed at 50°C for 1 h.

PCR, cloning, DNA sequencing and RFLP analysis

Genomic DNA was isolated from either peripheral blood lymphocytes or skin fibroblasts from the three EBD patients, their relatives and unrelated control individulas, and used as template for amplification of genomic sequences within COL7A1.

Specific primers for the NC-2 domain of collagen VII were designed based on the published cDNA sequence (41,42; DDBJ/EMBL/GenBank accession no. L23982). The following flanking primers with internal restriction sites (underlined) which covered the 3′ end encoding the NC-2 domain of procollagen VII were used:

Col 11F: 5′-AGGAC<u>GAATTC</u>TTGTGCGCCAAGAGATCAGTC-3′;
Col 10R: 5′-CATGC<u>AAGCTT</u>GTCCCCTGGCTCTGGACCAC-3′

An internal primer ColE114 (5′-CCAGTTCATCGCATCTG-GATCAC-3′) was made for RFLP analysis and for DNA sequencing of genomic clones. RT-PCR with primers Col11F and Col10R was carried out under the following cycling conditions: denaturation at 95°C for 4 min and then 34 cycles at 95°C for 1 min, 59°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. PCR on genomic DNA was performed with primers Col11F and Col10R essentially as described above but with 32 cycles and with extension time of 2 min and 30 s. The PCR products were purified with Qiaex particles (QIAGEN Ltd, Dorking, UK) prior to *Eco*RI/*Hind*III digestion and ligation into *Eco*RI/*Hind*III treated M13mp19. Recombinant clones were propagated on JM101. cDNA clones were sequenced with the –40 M13 universal primer and genomic clones were sequenced with primer ColE114. DNA sequencing was performed using kit reagents from U.S.B. (Cleveland, OH, USA). For RFLP analyses, genomic DNA was amplified with primers ColE114 and Col10R with denaturation for 4 min at 94°C followed by 30 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were digested with *Pvu*II, or *Pst*I and *Sph*I, overnight, and then separated on a 3.5% MetaPhor agarose gel according to the manufacturer's specifications (FMC, BioProducts, Rockland, ME, USA).

Amplification of exon 73 of COL7A1 (nucleotide positions 5980–6180 in the cDNA; DDBJ/EMBL/Genbank accession no. L23982) was carried out using primers based on the flanking

intronic sequences. This resulted in a 287 bp fragment and the primers used were those described by Christiano *et al.* (24):

upstream primer: 5′-GGGTGTAGCTGTACAGCCAC-3′
downstream primer: 5′-CCCTCTTCCCTCACTCTCCT-3′

Genomic DNA (100 ng) was used as template for the PCR reaction, and the amplification conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s, in a Perkin Elmer 9600 thermal cycler (Perkin Elmer Cetus, Weiterstadt, Germany). Amplification reaction contained 10× PCR buffer (Gibco-BRL Life Technologies, Eggenstein, USA), 1 U *Taq* polymerase (Gibco-BRL), 0.2 mM dNTP and 1 mM primers each in a total volume of 50 µl. Aliquots of 5 µl of the PCR products were analysed by 1.5% agarose gel electrophoresis. For heteroduplex analysis, 5 µl of the PCR product was denatured for 5 min at 95°C, cooled slowly down to 38°C and analysed by Mutation Detection Gel Electrophoresis (MDE, FMC BioProducts, Rockland, M, USA). PCR products of detected heteroduplexes were analysed by either automated sequencing (Applied Biosystems, Genome-Express, Grenoble, France) or subcloned with a T/A Cloning Kit (Invitrogen, Leek, The Netherlands) and manual sequencing using the Sequenase Sequencing Kit (USB, Cleveland, OH, USA). PCR products were digested with either *Sma*I or *Dsa*I overnight, and then separated on a 1.5% agarose gel or on a 3.5% MetaPhor agarose gel.

Haplotype analyses

PCR assays as described by Theune *et al.* (43) were performed on DNA extracted from the EB10 and LB4 families. For these assays, one of each PCR primer pair was end-labelled with [γ-^{32}P]dATP using polynucleotide kinase (1 µg of primer, 20–30 µCi [^{32}P]dATP, PNK buffer, and 1 U PNK, incubated at 37°C for 30 min). The PCR was completed using Techne 96-well plates for 35 cycles. The product diluted 1:1 in a formamide loading dye was denatured for 2–4 min at 95°C, then loaded onto a pre-warmed formamide gel (32% formamide, 7% acrylamide, 8 M urea and a Tris-borate buffer). The gel was run at 75 W for 1–5 h, then fixed in 10% acetic acid, 10% methanol solution and dried under vacuum at 85°C. An X-ray film was exposed at –70°C with an intensifying screen for 30 min to up to a week.

COL7A1 alleles at exons 3, 14, 21, 30, 84 and 118 were analysed with PCR of genomic DNA from the EB10 and LB4 family members, using primers, restriction enzymes and conditions as described by Christiano *et al.* (42).

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ABBREVIATIONS

AF, anchoring fibrils; EB, epidermolysis bullosa; EBD, dystrophic EB; BM, basement membrane; PTC, premature termination codons.

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