Hilal L, Rochat A, Duquesnoy P et al.A homozygous insertion-deletion in the type VII collagen gene (COL7A1) in Hallopeau-Siemens dystrophic epidermolysis bullosa. Nat Genet 5:287-2...

ARTICLE *in* NATURE GENETICS · DECEMBER 1993

Impact Factor: 29.35 · DOI: 10.1038/ng1193-287 · Source: PubMed

A homozygous insertion-deletion in the type VII collagen gene (*COL7A1*) in Hallopeau–Siemens dystrophic epidermolysis bullosa

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The Hallopeau–Siemens type of recessive dystrophic epidermolysis bullosa (HS-RDEB) is a life-threatening autosomal disease characterized by loss of dermal-epidermal adherence with abnormal anchoring fibrils (AF). We recently linked HS-RDEB to the type VII collagen gene (*COL7A1*) which encodes the major component of AF. We describe a patient who is homozygous for an insertion-deletion in the FN-4A domain of the COL7A1 gene. This defect causes a frameshift mutation which leads to a premature stop codon in the FN-5A domain, resulting in a marked diminution in mutated mRNA levels, with no detectable type VII collagen polypeptide in the patient. Our data suggest strongly that this null allele prevents normal anchoring fibril formation in homozygotes and is the underlying cause of HS-RDEB in this patient.

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Hereditary epidermolysis bullosa (EB) is a group of autosomal diseases characterized by blistering of the skin and mucous membranes after minor trauma. They are classified according to the level of tissue separation within the skin into simplex, junctional and dystrophic forms, each form being further subdivided into different clinical subtypes[1,2]. The simplex forms show intraepidermal cleavage within the basal keratinocytes and arise from mutations in the keratin 5 and 14 genes[3–7]. Junctional EB demonstrates cleavage within the lamina densa and the genes encoding nicein/kalinin/epiligrin are candidate genes in these forms of EB[8]. Dystrophic EB (DEB), which occurs in both dominant (DDEB) and recessive (RDEB) forms, displays cleavage beneath the lamina densa at the level of the anchoring fibrils (AF). AF are attachment structures composed of collagen VII and contribute to the adherence of the lamina densa to the underlying dermis[9,10]. Abnormalities in the number and/or the integrity of AF[11,12] as well as reduced or absent immunofluorescence (IF) staining using antibodies against type VII collagen in the skin of DEB patients[13–20], have implicated the COL7A1 gene in the pathogenesis of both dominant and recessive forms of DEB. In particular, in generalized mutilating RDEB — the Hallopeau-Siemens type (McKusick no. 226600) — AFs may be completely lacking, and labelling of the skin with monoclonal and polyclonal antibodies against type VII collagen is most often negative[14–16,21]. This life-threatening form of the disease is the most severe type of RDEB, responsible for extensive mucocutaneous blistering leading to pseudosyndactyly and mitten-like deformities of the hands and feet, loss of nails, joint contractures, esophageal strictures and complications such as growth retardation, anaemia and squamous cell carcinomas[2].

Cloning of the *COL7A1* cDNA[22] and identification of a highly informative intragenic *Pvu*II RFLP allowed us and others to demonstrate close linkage between *COL7A1* gene localized on chromosomal region 3p21 and DDEB[23–26] as well as with the Hallopeau-Siemens form of RDEB[27]. Specifically, linkage was established between *COL7A1* and the Cockayne-Touraine form of DDEB (McKusick no. 131800) in three Finnish families (combined lod score=8.77 at θ=0.00)[23,24], and two Dutch families (combined lod score=6.08 at θ=0.00)[25]. Linkage of DDEB to the marker *D3S2* close to the *COL7A1* locus was also reported in three British families with DDEB[26]. Finally, a lod score of 3.97 at θ=0 with *COL7A1* was obtained in 19 families with the Hallopeau-Siemens form of RDEB[27]. These data strongly indicate that *COL7A1* is the candidate gene for DEB harbouring the mutations in both dominant and recessive forms of DEB, with no evidence for non-allelic heterogeneity thus far.

The type VII collagen molecule is a homotrimer $[\alpha 1(VII)]_3$ with each pro-α chain consisting of a 145 kD N-terminal noncollagenous (NC-1) domain, a 145 kD central collagenous domain, and a 30 kD C-terminal noncollagenous domain (NC-2)[10]. Recent advances in the cloning of the *COL7A1* cDNA and gene have provided insight into the molecular and functional organization of the type VII collagen[28]. The NC-1 domain consists of multiple domains with homology to adhesive molecules: a cartilage matrix protein, nine consecutive fibronectin III domains, and the A domain of von Willebrand factor[28,29]. The central collagenous domain is made of Gly-X-Y repeats interrupted by non-helical segments. The NC-2 domain contains the cysteine residues involved in

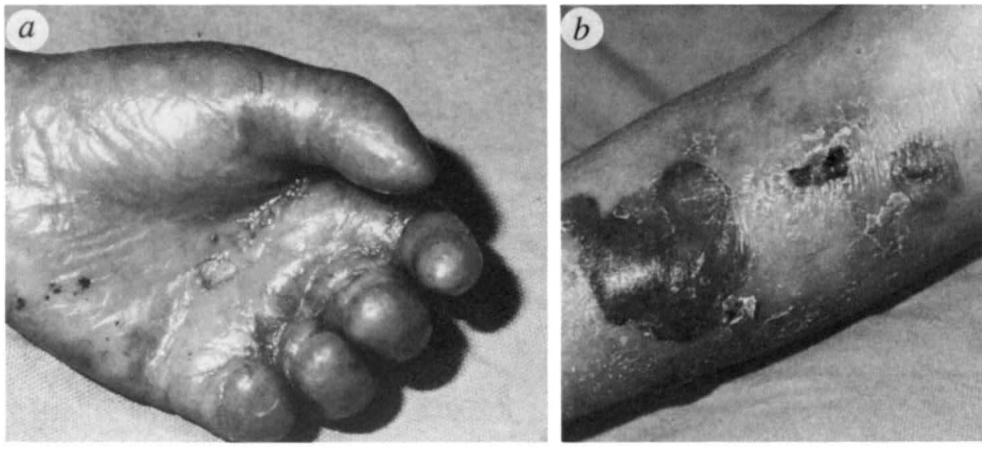


Fig. 1 Clinical features of the patient at 4 years of age, with generalized mutilating recessive dystrophic epidermolysis bullosa. *a*, Scarring of the fingers and the palmar surface of the hand, with post-bullous erosions of the palm, early pseudosyndactyly of fingers and absence of nails. *b*, Large haemorrhagic blister with dystrophic scars on the patient's leg.

interchain disulphide bonds.

In light of the different modes of inheritance and the severity of the phenotype, it is likely that different types of mutations within type VII collagen underlie DDEB and RDEB. The wide range of clinical variation between patients presenting with differing clinical forms of RDEB (Hallopeau–Siemens, generalized non-mutilating, inversa, localized, and mitis forms), as well as variations in AF abnormalities and in IF staining patterns in skin from RDEB patients, also suggest that distinct *COL7A1* mutations cause the different subtypes of RDEB. Christiano *et al.* recently reported a homozygous Met-to-Lys substitution in a highly conserved region of the NC-2 domain in the COL7A1 gene in two siblings presenting with the mitis form of RDEB[30]. We now report a homozygous insertion-deletion in *COL7A1* in a patient with the Hallopeau–Siemens form of RDEB. This rearrangement causes a frameshift and leads to a premature stop codon in the FN-5 domain of *COL7A1*. It results in markedly decreased levels of mutated *COL7A1* mRNAs, with absence of detectable truncated type VII collagen polypeptides. These data strongly suggest that homozygosity for this null allele causes the disease in this patient.

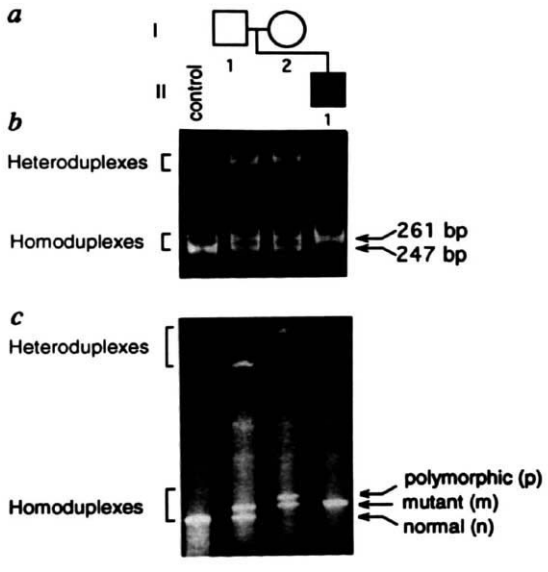


Fig. 2 Detection of a *COL7A1* PCR product of the FN-4A domain with abnormal mobility, co-segregating with the disease. *a*, Partial pedigree of the family. The affected offspring (II-1) is designated by the filled symbol. *b*, Migration pattern observed on non denaturing 6% acrylamide gel: in both parents (I-1, I-2), a larger extra-band (261 bp) and heteroduplexes are seen in addition to the wild-type band (247 bp). The patient (II-1) displays only the larger band, with no wild-type band. *c*, DGGE revealed the presence of a band (m) of abnormal electrophoretic mobility in the three members of the family, and an additional band (p) detected only in the mother.

Clinical description

The proband (II-1) (Fig. 1) is a 4-year-old boy, the only child of clinically healthy parents who came from the same village in Spain. There was no previous history of a blistering disease in the family. He was affected since birth with generalized and scarring skin blistering that has led to pseudosyndactyly of the fingers. The patient had to undergo surgical repair of pseudosyndactyly of his right hand at 4 years of age. On clinical examination milia are present and nails are absent. The patient had normal dentition with blistering of mucous membranes, and no evidence of oesophageal strictures. Electron microscopy (EM) examination of a skin biopsy showed tissue cleavage beneath the lamina densa with no recognizable anchoring fibrils.

Detection of abnormal *COL7A1* alleles

We first screened for gross deletions within *COL7A1* in our patient by Southern blot analysis. No band of abnormal size was found after digestion of genomic DNA with *Bam*HI, *Bstx*I, *Pst*I, *Pvu*II or *Taq*I restriction enzymes, using K131, 421 and 623 *COL7A1* cDNA clones as probes. Additional screening for mutations in the coding sequences and the intron-exon boundaries of *COL7A1* corresponding to the published sequence of the cDNA[28] was performed using denaturing gradient gel electrophoresis (DGGE) analysis[31]. The GC-clamped amplified products from genomic DNA encompassed exons and splice junctions, and were tested on 6% non-denaturing acrylamide gel prior to DGGE. Upon screening the NC-1 domain in our patient, amplification of part of the fourth fibronectin domain (FN-4A) with primers p1 and p2 (see Methodology), revealed the presence of a band of larger size (261 bp) and no band of normal size (247 bp) (Fig.

2*a,b*). Analysis of the family showed that both parents (I-1 and I-2) displayed this larger extra band, with heteroduplexes, in addition to the band of normal size seen in the control (Fig. 2*a,b*). DGGE analysis of the same PCR-amplified genomic fragment confirmed that the father was a heterozygote, with a wild-type band (n), a band with reduced mobility (m) and heteroduplexes (Fig. 2*c*). The mother showed no band with normal electrophoretic mobility in comparison to the control. We observed one band co-migrating with the paternal (m) band, a second band (p) with further decreased mobility, and heteroduplexes different from the father (Fig. 2*c*). Therefore, the mother was heterozygous for the (m) allele, with a (p) allele different from the normal allele (n). In contrast, the patient showed a unique band co-migrating with the (m) band, and the absence of heteroduplexes confirmed that he was homozygous for the (m) allele. These data indicated that the (m) allele was associated with the disease in this family. To investigate the nucleotide change underlying the different melting behavior of (m) and (p) alleles in this family, we sequenced these alleles in the parents and the patient.

Characterization of insertion-deletion in *COL7A1*

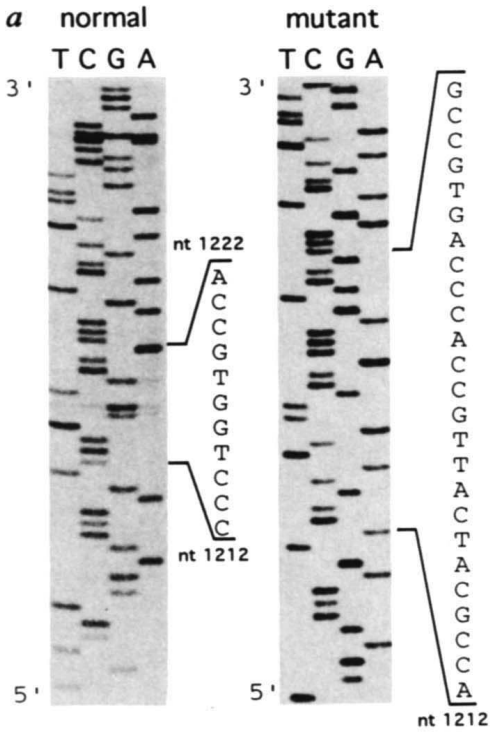
Direct sequencing of the patient's PCR-amplified genomic fragment of the FN-4A domain revealed the replacement of a 11 basepair (bp) fragment (nucleotide 1212–1222) by a 25 bp fragment (Fig. 3*a*). This insertion-deletion results in a frameshift leading to a premature stop codon in the FN-5A domain, 87 amino acids downstream from Pro405. Detailed comparison of the normal and mutated DNA sequences revealed the presence of two motifs which are repeated in the mutant (a 10 bp motif 1 and an 11 bp motif 2 boxed in Fig. 3*b*), located immediately at the 3' end of the deletion. This rearrangement was confirmed by direct sequencing of reverse transcriptase-PCR products from cultured keratinocytes as well as from skin samples from the patient, thus demonstrating that the mutated gene was transcribed in the keratinocytes of the patient. Sequencing of cloned alleles from both parents revealed that they were heterozygous for the same insertion-deletion.

Screening for the mutated *COL7A1* allele

To investigate the possibility that this molecular defect could be associated with RDEB in other affected patients, we screened for its presence in 53 unrelated patients with the Hallopeau-Siemens form of RDEB. DGGE of PCR-amplified genomic DNA generated with primers p1 and p2 detected no abnormal migration pattern in any of these patients, with respect to a normal control. The absence of this insertion-deletion was also verified in 100 unrelated control individuals without EB.

Characterization of maternal polymorphism

Direct sequencing of the maternal p band showed a G-to-A transition at nucleotide 1244, predicting an Arg415His conversion in exon 12 coding for the FN-4A domain (data not shown). This substitution abolishes a *Fsp*I restriction enzyme site, as confirmed by endonuclease digestion of PCR amplified genomic DNA from the mother using this restriction enzyme. Screening for this substitution by *Fsp*I endonuclease digestion of PCR amplified genomic DNA from 100 unrelated subjects without EB, detected this change in the heterozygous state in one normal subject and in the homozygous state in another unaffected control subject, suggesting that this transition is a rare polymorphic variation, unrelated to the EB phenotype.



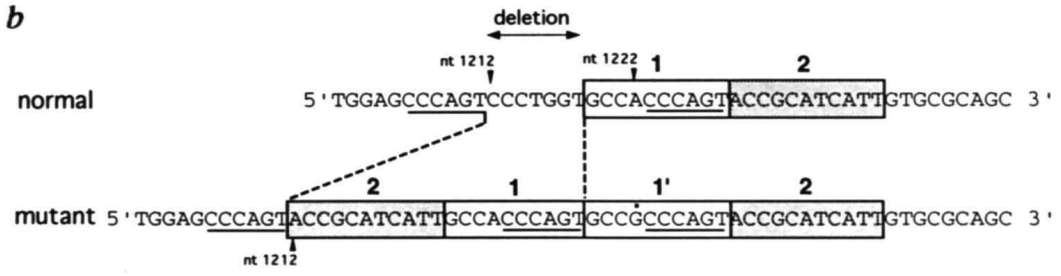


Fig. 3 Characterization of the mutant allele, with representation of duplicated motifs within the mutated sequence. *a*, Normal allele, with the sequence between nucleotide 1212 and 1222 indicated between brackets; mutant allele, with the new sequence replacing the normal sequence from nucleotide 1212 to 1222, indicated between brackets. *b*, The new sequence consists of the duplication in the same orientation of motif 1(boxed) (motif 1' has one base pair variation indicated by a dot) and motif 2 (grey box). A 6 bp direct repeat (CCCAGT), which may have favoured nucleotide pairing, is underlined.

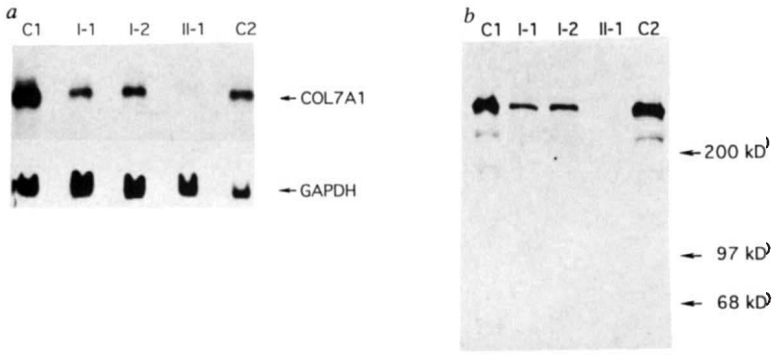


Fig. 4 Northern blot of total RNA and immunoblot analyses from keratinocyte cultures. *a*, Northern blot *COL7A1* transcripts (9 kb in size) were clearly detectable in RNAs from the age matched controls (C1, C2), at lower levels in both parents (I-1, I-2), and at extremely low levels in the patient (II-1). The quantity of RNA present in each lane was standardized by GAPDH hybridization. *b*, Immunoblot analyses with LH7:2 NC-1 specific monoclonal antibody to type VII collagen: decreased levels of type VII collagen protein of normal size (290kD) were seen in both parents (I-1, I-2) compared to the controls (C1, C2), whereas no type VII collagen polypeptide was detectable in the patient (II-1). 200 µg of protein extract were loaded in each lane. Ponceau Red staining prior to transfer indicated that the amount of total protein was comparable in each sample. The mobilities of molecular weight markers are indicated to the right.

Northern blot analysis

Northern analysis showed markedly decreased *COL7A1* transcript levels in the patient, with no significant difference in size as compared to the control (Fig. 4*a*). A smaller diminution of the *COL7A1* mRNAs steady-state levels was observed in both parents (Fig. 4*a*). Densitometric tracing analysis of the *COL7A1* signal to the GAPDH signal in the father and the mother showed a 40–50% diminution in comparison to the normal controls.

Immunoblot and immunofluorescence studies

The expected 61 kD peptide was not found on immunoblot analyses from cultured keratinocyte extracts from the patient using the LH7:2 monoclonal antibody against the NC-1 domain (Fig. 4*b*). Both parents showed decreased levels of type VII collagen protein of normal size (290 kD) compared to the control (Fig. 4*b*). Densitometric tracing of these bands revealed a 40–50% diminution of the type VII collagen protein signal in parents in comparison to the control. Immunofluorescence studies of skin sections with LH7:2 antibody showed an absence of immunostaining in the patient, whereas the staining was normal in both parents in comparison to the control (Fig. 5).

Discussion

We have screened for mutations within the COL7A1 gene in a patient presenting with the generalized, mutilating form of RDEB (the Hallopeau-Siemens type). We have characterized an insertion-deletion in the FN-4A domain in *COL7A1* which cosegregates with the disease, being present in the heterozygous state in both parents, and homozygous in genomic DNA from the patient. This insertion-deletion causes a shift in the translational reading frame resulting in a premature termination codon in the FN-5 domain. Although consanguinity was not known by the parents, both came from the same small village in Spain, and inheritance of the same defective *COL7A1* allele from a common ancestor is possible. Furthermore, the possibility of hemizygosity of the patient was unlikely, as no gross deletion was observed in this region of the COL7A1 gene. Therefore, homozygosity of the patient for the mutated *COL7A1* allele is highly probable.

The genetic mechanism(s) responsible for this rearrangement remain(s) unclear. The normal sequence has been rearranged as follows: motif 1 is flanked on its 5' end by motif 2, and on its 3' side by a repetition of motif 1 differing by an A to G substitution (motif 1') located immediately upstream to another motif 2 (Fig. 3*b*). The existence of a six nucleotide repeat CCCAGT (underlined in Fig. 3*b*) located immediately adjacent to the 5' end of the replaced sequence, as well as to the 5' end of the duplicated motif 2, may have favoured accidental nucleotide pairing and deletion of the intervening 7 bp sequence CCCTGGT (Fig. 3*b*). However, unequal crossing over could not account for the position of motif 2 at the 5' end of motif 1. Alternatively, this small rearrangement could have been generated during the repair process of the gap, which could have been filled with copies of adjacent sequences 1 and 2 during replication. Position of motif 2 at the 5' end of motif 1, as well as the A to G change observed in motif 1 (1') would have been generated during the same process. Finally, we found no evidence for other sequences predisposed to recombine, such as inverted repeats, intronic Alu repeats, or A and T rich domains, in the region encompassing this rearrangement.

As a result of the premature termination codon, both patients' alleles are predicted to encode one truncated type VII collagen polypeptide with an abnormal 3' end, lacking the FN-5 to 9 domains, the A domain of von Willebrand factor, as well as the complete collagenous and non-collagenous-2 domains of the protein. As has been described for a number of nonsense mutations in different genes[32], one of the biological consequences of such a mutant allele is a decrease in the steady state level of *COL7A1* mRNA as shown on northern blots (Fig. 4*a*). In contrast, splicing of the pre-mRNA in the region encompassing this insertion-deletion was not altered. Specifically, sequencing of exons 12 (FN-4A), 13 (FN-4B) and 14 (FN-5A) from the *COL7A1* cDNA from the patient showed no evidence for exon skipping, a mechanism by which a nonsense mutation, or intraexonic deletion, can result in the skipping of the exon bearing the mutation[33].

Immunofluorescence of skin sections and immunoblot analysis of cultured keratinocyte extracts from the patient failed to detect type VII collagen polypeptide. Possible explanations for this absence include marked instability of the mutant transcript, as suggested by decreased mRNA levels, and/or rapid degradation of the mutant polypeptide. Alternatively, the mutated polypeptide may be stable and secreted, but not detected by the LH7:2 monoclonal antibody we used. On the basis of the cDNA sequence

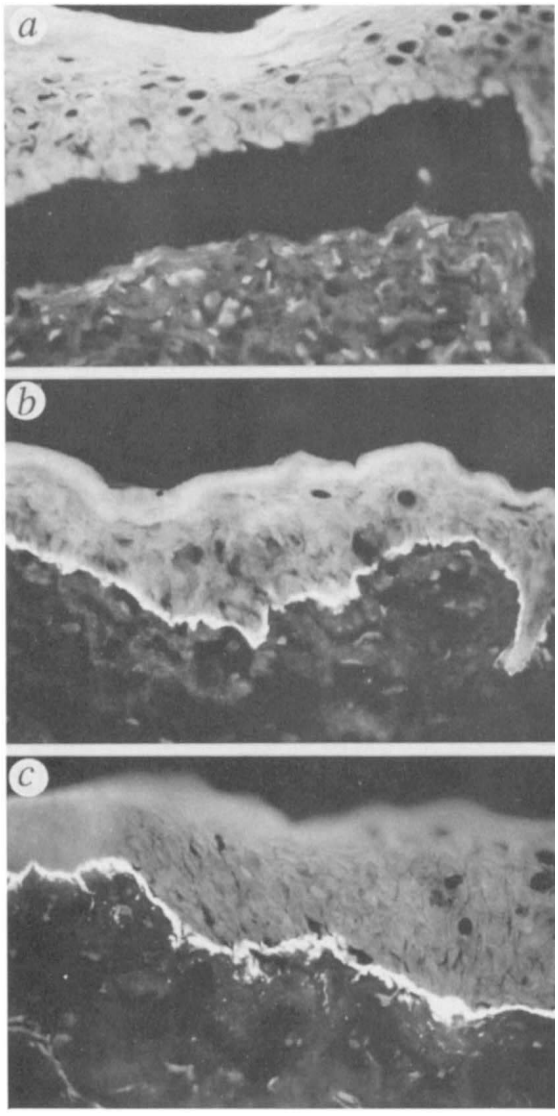


Fig. 5 Immunofluorescence of skin with LH7:2 antibody to type VII collagen. *a*, Absence of labelling of the basement membrane zone (BMZ) in skin of the patient with dermal-epidermal cleavage. *b*, Normal staining at the BMZ in the father and in the mother (not shown). c, Linear fluorescence at the BMZ in a normal control (magnification x400).

isolated from a human keratinocyte library using this antibody[34], LH7:2 recognizes an epitope between FN-2A and FN-5B in the NC-1 domain of COL7A1, and thus may recognize an epitope located downstream of the frameshift mutation in FN-4A. However, if stable, the shortened polypeptide encoded by the mutated allele would lack 79% of the normal molecule, including its collagenous domain by which collagen molecules associate into a triple helix, and its C-terminal domain by which the assembly of pro-α collagen chains is thought to initiate. According to the critical function of these domains, we infer that this markedly truncated polypeptide would be functionally inactive and prevent anchoring fibril formation in homozygotes. Alternatively, it would not interfere with normal triple-helical assembly in heterozygotes, thus permitting normal anchoring fibril formation, as suggested by normal appearance of anchoring fibrils in the parents of the proband.

Very few mutations in other collagen genes have been described to cause recessive inherited diseases, mainly osteogenesis imperfecta (OI). Among these defects, a homozygous 4 nucleotide frameshift deletion in the C-terminal region of COL1A2 resulting in a polypeptide which did not associate in heterotrimers in heterozygotes caused recessive type III OI in homozygotes[35]. In lethal type II OI, an A to G nucleotide substitution occurs at an obligatory AG splice site in *COL1A2* (ref. 36), and a G to A homozygous transition in the splice donor site of intron 14 in *COL1A1* results in skipping of exon 14 (ref. 37).

Interestingly, the mutated *COL7A1* allele described here results in about a 50% reduction in normal type VII collagen molecules in the unaffected parents of the proband. Thus, unlike other structural genes whose inactivation leads to a dominant disease phenotype such as *COL1A1* in type I OI[38], a twofold reduction in the amount of type VII collagen protein had no significant phenotypic consequence.

This insertion-deletion in the *COL7A1* allele is unique among mutations producing HS-RDEB in the 53 unrelated patients we have studied, suggesting that the molecular defects within the COL7A1 gene causing this form of RDEB is heterogeneous. Allelic heterogeneity is consistent with the variations in the severity of the disease, in AF alterations, as well as in immunofluorescence and immunoelectron microscopy staining with antibodies to collagen VII in the skin from HS-RDEB patients[39]. Similarly, the homozygous Met to Lys substitution in *COL7A1* recently described in *mitis* RDEB was not found in eight unrelated RDEB patients suggesting that different *COL7A1* mutations may also be responsible for the other subtypes of RDEB[30].

Characterization of a homozygous mutation in *COL7A1* leading to the synthesis of a functionally inactive polypeptide is of potential interest for gene therapy. It should permit targeted correction of the defective gene in keratinocytes of the patient for autologous grafts. Further delineation of mutations in the COL7A1 gene and elucidation of their consequences at the protein level, will provide insight into the different mechanisms responsible for abnormal AF formation in RDEB and DDEB, allowing for possible genotype-phenotype correlations and the development of gene therapy.

Methodology

Transmission electron microscopy. A skin sample for electron microscopy analysis was obtained from the hand of the patient during surgical correction of the fusion of the digits. A skin biopsy of the groin after rubbing was also obtained from both parents after informed consent.

Keratinocyte culture, RNA extraction and RT-PCR. Keratinocytes were cultured from skin samples obtained from the proband and his parents as described[40]. Total RNA was extracted from confluent cell layers according to Chomczynski[41], and cDNA was generated from total RNA by reverse transcription using random hexanucleotides (Pharmacia).

Immunoblotting and immunofluorescence staining (IF). For immunoblotting, sub-confluent cultured keratinocytes from the patient, his parents and two age-matched control individuals were extracted with 8 M urea, 2% SDS, 0.05 M Tris, pH 6.8, 0.1 M DTT for 1 min at 95 °C, and dialysed against electrophoresis sample buffer with protease inhibitors as described[21]. The samples were separated

with sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 4.5–15% gradient gels, transferred to nitrocellulose filters, and reacted with the monoclonal antibody LH7:2 to the NC-1 domain of human collagen VII (Sigma), followed by incubation with peroxydase-conjugated rabbit antimouse immunoglobulins (Dako, Denmark). The peroxidase reaction was detected using the enhanced chemiluminescence (ECL) immunodetection system (Amersham). Immunofluorescence staining was performed on 5 μm cryosections of normal or EB skin, using the monoclonal antibody LH7:2 to the NC-1 domain of human collagen VII and a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse (Sigma) The immunoreactions were carried out at room temperature for 30 min.

Southern and northern analyses. For Southern analyses, genomic DNA was extracted from peripheral blood cells as described[42]. 10 μg of DNA was digested to completion with 20 U of *Bam*HI, *Bstx*I, *Pst*I, *Pvu*II and *Taq*I restriction enzymes. The DNA fragments were separated in a 1% agarose gel and transferred to a Hybond C-Extra membrane (Amersham). For northern analyses, 30μg of total RNA extracted from keratinocyte cultures was electrophoresed through a formaldehyde/agarose gel and transferred to a Hybond C-Extra membrane (Amersham). Prehybridization and hybridization of the Southern and northern blot filters with the probes for type VII collagen K131, 421, 623 and K131, respectively, were performed as described[43].

Primers for PCR, DGGE and direct sequencing. The primers designed for DGGE analysis of genomic DNA fragments encompass exons and splice junctions, and are complementary to the published sequence of the *COL7A1* cDNA[28]and to adjacent intronic regions. The attachment of GC-rich segments to the ends of amplified genomic fragments made it possible to detect nucleotide changes located in high temperature melting domains. The optimal denaturing conditions of the GC-clamped amplified products were determined using Lerman's Melt 87 and SQHTX programs[44]. A segment of the FN-4A domain of *COL7A1* was amplified using an exonic primer1 (5'–GTAACAGACCTGCAAGCCAC–3') (nucleotide 1150–1169) and a GC-clamped intronic primer2 (5'–CG60-GAGAGGG-CTGGAGGTACAC–3') located between exon 12 (FN-4A) and 13 (FN-4B).

The sets of primers used to amplify cDNA for direct sequencing of the mutated sequence are primer3 (5'–CG55-CGCCTCAC-ACTCTACACTC–3') (nucleotide 1060–1078) and primer4 (5'–AGCCCAGCCTGAACGTCATC–3') (nucleotide 1325–1306), as well as primer3 and primer5 (5'–CGCAGTACCGACACAGCCA–3') (nucleotide 1625–1607). The numbers refer to the nucleotides position according to A. Christiano *et al.*[28].

Denaturing gradient gel electrophoresis (DGGE). Genomic DNA was amplified with AmpliTaq (Perkin Elmer Cetus) at the following conditions: 94 °C for 1 min, 55 °C 1 min, 72 °C 1 min 30 s for 40 cycles, with a final extension of 7 min at 72 °C. The amplified products were first tested on a non denaturing 6% polyacrylamide gel, and subsequently subjected to electrophoresis on a vertical gel containing a linearly increasing 50–90% denaturing gradient parallel to the direction of the electrophoresis as described[31]. The denaturing gel was run at 160V for 3 h at 60 °C temperature, stained with ethidium bromide and photographed.

Direct PCR sequencing. Genomic DNA template: Asymmetric PCR was performed as described[45] using primer1 and 2 in a ratio of 1:50 and Ampli*Taq* (Perkin Elmer Cetus). PCR conditions were 60 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min 30 s at 72 °C. The single-stranded amplified genomic product was sequenced with primer1 by the dideoxynucleotide termination method[46].

cDNA template: Two different fragments were generated by asymmetric amplification using the same PCR conditions. A fragment was first amplified to characterize the insertion-deletion using primers 3 and 4 in a ratio of 50:1, and sequenced as described above with primer 4. A second fragment encompassing FN-4A, FN-4B and FN-5A was generated by asymmetric PCR to investigate the possibility of exon skipping using primers 3 and 5 in a ratio of 50:1, and sequenced with primer 5.

Cloning and sequencing of PCR-amplified genomic DNA. PCR products were separated by agarose gel electrophoresis in 4% NuSieve GTG agarose, and the 247, 261 bp fragments were removed and eluted using ultrafree-MC filters (Millipore). The eluted DNA was phenol-chloroform extracted, ethanol precipitated. 10 ng of the isolated material was cloned using the TA cloning kit according to manufacturer's instructions (Invitrogen). Plasmid DNA was prepared using Qiagen-tip 100 columns. The purified DNA was alkali-denaturated and the sequence was determined by the chain-termination method[46].

***Fsp*I digestion of PCR products.** One-tenth of the PCR-amplified genomic products generated with primers 1 and primers 2 was digested for 3 h with *Fsp*I restriction enzyme (10 U) (New England Biolabs), and analysed on 6% polyacrylamide gel electrophoresis.

Received 21 June; accepted 9 August 1993.

Acknowledgements

We thank P. Morier, D. V. Egloff, P. Roggero, J-P Bossi, L. Bruckner-Tuderman, R. Grossman, the Epidermolyses Bulleuses Association Entraide, A. Bullot and the RDEB families who participated in this study for their help and contribution to this work. This work was supported by grants from the Association Française contre les Myopathies (AFM), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Ministère de la Recherche et de l'Espace (MRE 92N60/0696), the European Economic Community (Contrat Genome CT 91-0027) and National Institutes of Health grants P01-AR38923 and T32-AR07561. A.M.C. is a recipient of a Dermatology Foundation Career Development Award.

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