European Journal of Human Genetics (1999) 7, 293–300

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ARTICLE

Donor splice site mutation in keratin 5 causes in-frame removal of 22 amino acids of H1 and 1A rod domains in Dowling-Meara epidermolysis bullosa simplex

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Epidermolysis bullosa simplex (EBS) arises from mutations within the keratin 5 and 14 (K5 and K14) genes which alter the integrity of basal keratinocytes cytoskeleton. The majority of these defects are missense mutations in the rod domain, whose locations influence the disease severity. We investigated a large family dominantly affected with the Dowling-Meara form of EBS (EBS-DM). Sequencing of amplified and cloned K5 cDNA from cultured keratinocytes revealed a 66 nucleotide deletion in one allele corresponding to the last 22 amino acid residues encoded by exon 1 (Val164 to Lys185). Sequencing of amplified genomic DNA spanning the mutant region revealed a heterozygous G-to-A transition at +1 position of the consensus GT donor splice site of intron 1 of K5. This mutation leads to the use of an exonic GT cryptic donor splice site, located 66 nucleotides upstream from the normal donor splice site of intron 1. The corresponding peptide deletion includes the last five amino acids of the H1 head domain and the first 17 amino acids of the conserved amino terminal end of the 1A rod domain, including the first two heptad repeats and the helix initiation peptide. The shortened polypeptide is expressed in cultured keratinocytes at levels which are comparable to the normal K5 protein. This is the first splice site mutation to be reported as a cause of EBS-DM. Owing to the functional importance of the removed region, our data strongly suggest that shortened keratin polypeptide can impair keratin filament assembly in a dominant manner and causes EBS-DM.

Keywords: keratin 5; epidermolysis bullosa simplex; splice site mutation; cytoskeleton

Introduction

Epidermolysis bullosa simplex (EBS) comprises a group of hereditary skin diseases characterised by blistering of the skin following mild physical trauma as a result of cytolysis within the basal epidermal cells.[1,2] EBS is commonly inherited in an autosomal dominant fashion with an incidence of 1 in 50 000. Three major different subtypes are recognised. The Dowling-Meara form of EBS (EBS-DM) is the most severe type where blistering can be generalised, occurs in clusters, is often associated with hyperkeratosis of palms and/or soles

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Received 6 October 1998; accepted 10 November 1998

and is distinguished from other forms by clumping of keratin filaments in the basal epidermal cells. The Koebner type (EBS-KS) shows milder generalised blistering, whereas the Weber-Cockayne form (EBS-WC) is the mildest form of EBS, with blistering confined to the hands and feet.

Studies of transgenic mice expressing mutant keratins, together with linkage and mutation analyses in EBS patients and *in vitro* point mutagenesis studies have established that defects in either of the genes encoding the basal epidermal keratins K5 and K14 underlie most EBS cases.[3–8] EBS mutations are most often missense mutations located within the central helical rod domain of K5 or K14. The precise location of the amino acid changes within the keratin molecules correlates with the degree to which they impair keratin filament assembly and with disease severity.[9,10] Specifically, EBS-DM mutations occur within the highly conserved α-helical end segments of helix 1A and 2B and induce major defects in keratin filament elongation.[4,6,11–16] In particular, R125C and R125H mutations in K14 account for the majority of EBS-DM cases.[10,11] Mutations in EBS-K are more internal to the α-helical rod domain,[5,17–20] whereas mutations in EBS-WC have been found in the non-helical linker (L1-2) region of K5 and K14, and in the H1 head domain of K5.[12,21–26] Recently, a missense mutation in the non-helical V1 head domain of K5 was identified in patients with EBS with mottled pigmentation.[27,28]

We investigated a multigenerational family dominantly affected with EBS-DM for mutations in K5 cDNA. We describe abnormal splicing of the last 66 nucleotides in exon 1 caused by a genomic mutation at the consensus donor splice site of intron 1. We show that this mutation co-segregates with the disease in this family and results in the synthesis of a shortened polypeptide lacking important functional domains of K5.

Materials and Methods

Clinical Description

We studied a family of French ancestry, with a three-generation history of blistering disease affecting eleven members in an autosomal fashion with complete penetrance (Figure 1). All affected individuals suffered since birth from recurrent and general blistering after mild physic trauma. Nonscarring blistering occurs at any body site, often in clusters, predominantly on hands, elbows, feet, knees and the face. Oral blistering was sometimes noted. Disease exacerbation was observed during the summer. Moderate hyperkeratosis of palms and soles was present in several affected individuals. No milia were noted, and hair, nails and teeth were normal. Electron microscopic examination of a skin biopsy obtained after rubbing an intact area showed cleavage within the basal keratinocytes with clumping of keratin filaments. The basement membrane zone, hemidesmosomes and anchoring fibrils were normal. The overall presentation was very suggestive of the Dowling-Meara form of EBS.

Keratinocyte Culture, RNA Extraction and Reserve Transcriptase-PCR

Keratinocytes were cultured from 5-mm punch biopsies obtained from patient II-4 as described by Rheinwald and Green.[29] Total RNA was extracted from confluent cell layers using the guanidium thiocyanate method described by Chomczynski and Sacchi.[30] After reverse transcription using random hexanucleotides (Pharmacia, St Albans, UK) and Moloney murine leukaemia virus reverse transcriptase (Stratagene, Cambridge, UK), cDNA was used as a template in a 50 μl PCR mixture containing 10 picomoles of each specific K5 primer, 200 μM dNTPs, 1 × PCR buffer with 1.5 mM $MgCl_2$ and one unit of AmpliTaq DNA polymerase (Perkin Elmer, Warrington, UK). The coding sequence of keratin 5 cDNA was amplified using overlapping sets of primers: K5F318 5'-CTGAGCTCTGTTCTCTCCA-3'/K5R868 5'-GGGGTCGATTTGCAGGTTG-3' and K5F649 5'-GTTTGGTGCTGGTGCTGG-3'/K5R1080 5'-AGCTGCCTCCTGAGGTTG-3' for exon 1; K5F950 5'-CGGTTCCTGGAGCAGCAG-3'/K5R1381 5'-GTTGTTGTCCATGGAGAGG-3' for exons 2–4; K5F1284 5'-ATGAGATTAACTTCATGAAGATG-3'/K5R1812 5'-AGCAGCTTGCGGTAAGTGG-3' for exons 5–7 and K5F1711 5'-GAAGGCCAAGCAGGA-CATG-3'/K5R2123 5'-CAAATTTGACGCTGGAGCTG-3' for exon 8. Primers are numbered according to GenBank accession number M21389. Thirty-five cycles of PCR were performed at the following conditions: 94°C for 30 s, 55°C for 45 s, 72°C for 10 s. An aliquot of the PCR products was run on 6% acrylamide gels before sequencing and stained with ethidium bromide.

PCR-amplification of Genomic DNA Genomic DNA was extracted from peripheral leukocytes and was used as a template for PCR amplification. The genomic region of K5 spanning the donor splice site of exon 1 was amplified using primers K5F649 and K5R1080.

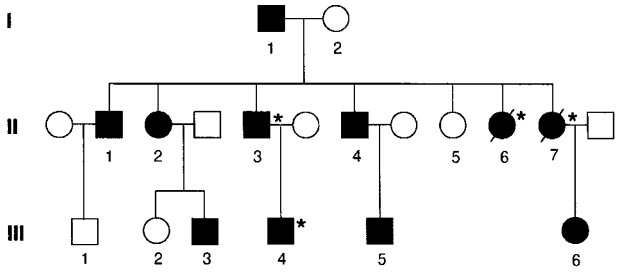

Figure 1 *Pedigree of the family studied with EBS-DM. Shaded symbols indicate affected individuals; open symbols, unaffected persons; slashed symbol, deceased individuals. Four family members whose DNA could not be assayed for mutation detection are indicated by asterisks.*

Sequencing of PCR Products Amplified material was sequenced directly or was cloned into the pGEM-T cloning vector (Promega) prior to sequencing, according to manufacturer's instructions. Sequencing was performed in both directions by the dideoxy-nucleotide method using Sequenase version 2.0 (United States Biochemical, Cleveland, USA) using vector primers T3 and T7 and insert-specific primers for sequencing reactions. The donor splice site mutation was identified by sequencing and confirmed by *Hph*I restriction enzyme digestion (New England Biolabs, Hitchin, UK).

Immunoblotting The cytokeratins were extracted and analysed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting as previously described.[31] Antibodies to keratin 5 tested were BL18, a rabbit polyclonal raised against the last nine residues of K5 (TSSSRKSFK),[32] and RCK102, a mouse monoclonal antibody specific for K5 and K8.[33] The secondary antibodies were goat anti-rabbit or rabbit anti-mouse IgG conjugated to alkaline phosphatase (Dako, Bucks, UK). The blots were scanned on a 'Bio Image Whole Band Analyzer' to determine the relative proportions of the normal and mutant keratin 5 bands.

Results

Mutation Analysis

Overlapping regions of keratin 5 cDNA were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) of keratinocyte RNA from patient II-4 and were analysed on 6% acrylamide gels prior to sequence analysis. Products obtained with primers K5F649 and K5R1080 revealed a shorter band and two heteroduplexes in addition to the band of normal size seen in control keratinocytes (Figure 2). Cloning and sequencing of these PCR products showed the in-frame removal in several clones of the last 66 nucleotides (881 to 946) encoding Val164 to Lys185 from exon 1, and normal sequence in other clones (Figure 3a). No other aberrant K5 cDNA sequence could be detected.

To identify the keratin 5 gene defect underlying this change, genomic DNA spanning exon 1 and its flanking donor-splice site, was PCR-amplified from patient peripheral lymphocytes using primers K5F649 and K5R1080. Analysis of the PCR-amplified products on a 6% acrylamide gel showed a band of normal size (1016 bp) and no evidence of a shorter band or heteroduplexes. Cloning and sequencing of these PCR products demonstrated a single base GT-to-AT change in the consensus donor splice site of intron 1 in several genomic clones (Figure 3b). Other clones from this patient contained the normal sequence. Direct sequencing of PCR-amplified genomic DNA confirmed that the patient was heterozygote for this mutation designated 946 + 1 G→A. This mutation involves the invariant GT donor-splice consensus sequence, suggesting that it would alter splicing of keratin 5 mRNA. In fact, it is likely that this mutation leads to the use of a GTGAG cryptic donor splice site of exon 1, located 66 nucleotides upstream from the normal splice site of intron 1 (Figure 4a). Abnormal splicing of exon 1 removes the last five amino acids of the H1 head domain and 17 amino acids of the 1A rod domain of K5, including the first two heptad repeats (Figure 4b).

Screening for the Mutation

The 946 + 1 G→A mutation abolishes a restriction enzyme site for *Hph*I (GGTGA) and yields digestion fragments of 231 and 785 bp from the mutant allele, and 231, 66 and 719 bp from the normal allele. *Hph*I digests of PCR amplified genomic DNA from the patient and six other affected individuals in this family confirmed that they were heterozygotes for this mutation. In contrast, this mutation was not observed in any of the unaffected members of the family tested and was not

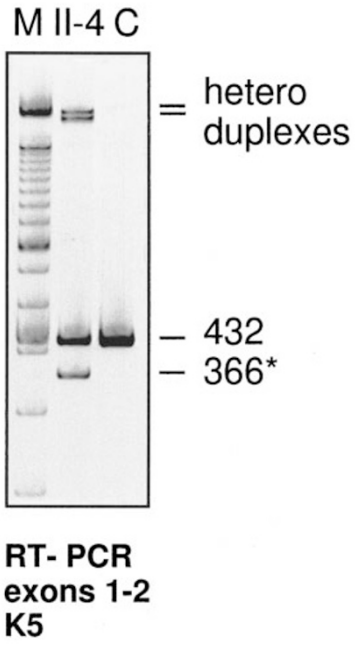

Figure 2 *RT-PCR amplification spanning the junction between exons 1 and 2 of K5 cDNA. In patient II-4, electrophoresis in a 6% acrylamide gel of RT-PCR products obtained with primers K5F649 and K5R1080 showed an extra band (366 bp)(asterisk) migrating faster than the band of normal size (432 bp) and two heteroduplexes of reduced mobility. C, normal control; M, 100 bp marker.*

present in 50 unrelated controls, suggesting that it is unlikely to be a polymorphic site.

Immunodetection of the Keratin 5 Mutant

Expression of the keratin 5 polypeptide was studied by immunoblotting of protein extracts isolated from culture keratinocytes from the patient and a control (Figure 5). The normal keratinocytes extracts contained a unique K5 peptide of normal size. In contrast, patient extracts showed a smaller band in addition to the peptide of normal electrophoretic migration. Scanning of these signals showed that the intensity of the smaller band was between 66 and 84% of the normal band in the patient extracts, indicating that substantial amounts of the mutant keratin 5 were expressed.

Screening of K5 cDNA for Additional Nucleotide Changes

Further sequencing of the K5 cDNA from patient II-4 showed two nucleotide variations located in the V1 and H1 domains of K5, none of which changed the corresponding encoded amino acids: a previously reported C-to-T transition at the third base of the leucine codon 117 was present on both K5 alleles and a T-to-C transition was identified at the third base of the threonine codon 210 on the normal K5 allele. The previously described G138E polymorphism in the H1 domain of the K5 gene which is known to result in an abnormal migration pattern of the polypeptide variant was not found in our patient.[34] Finally, sequencing of the entire rod domain of K5 did not reveal other

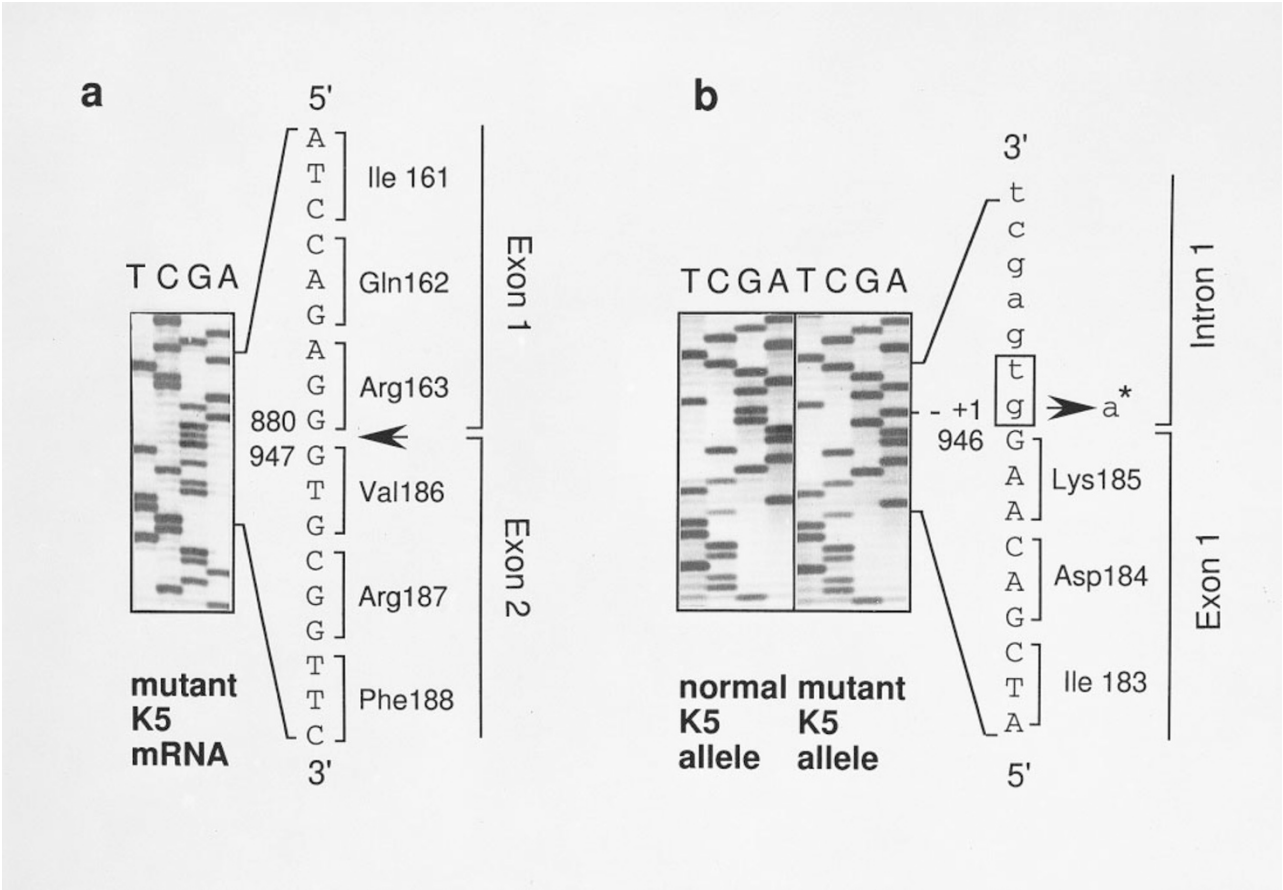

Figure 3 *Identification of abnormal splicing of exon 1 associated with a donor splice site mutation of intron 1 in the* K5 *gene. (***a***) In the patient, sequencing of clones containing the short RT-PCR product (366 bp) revealed the removal of the last 66 nucleotides of exon 1 (from 881 to 946)(arrowhead) of K5 transcripts. (***b***) Sequence analysis of cloned PCR products from the patient's amplified genomic DNA showed in several clones, a g-to-a mutation (arrowhead and asterisk) at the consensus gt donor splice site (boxed) of intron 1. Other genomic clones showed a normal sequence. The exon 1 and 2 sequence is indicated by upper case letters, the intron 1 sequence by lower case letters. Numbering of the amino acids and nucleotides refers to the cDNA sequence in GenBank no. M21389.*

nucleotide variation predicting an amino acid change or a splicing abnormality.

Discussion

We describe a family with EBS-DM where affected individuals are heterozygous for a donor splice site mutation of intron 1 of the keratin 5 gene. This mutation results in abnormal splicing of the last 66 nucleotides of exon 1 by activation of a cryptic exonic splice site. The corresponding peptide deletion spans the last 5 amino acids of the H1 head domain and the first 17 amino acids of the 1A rod domain of K5 including the two first heptad repeats. The shortened K5 polypeptide is expressed in amounts which are comparable to the normal K5 protein, suggesting that it retains sufficient integrity to interfere with keratin filament formation and causes EBS-DM in this family.

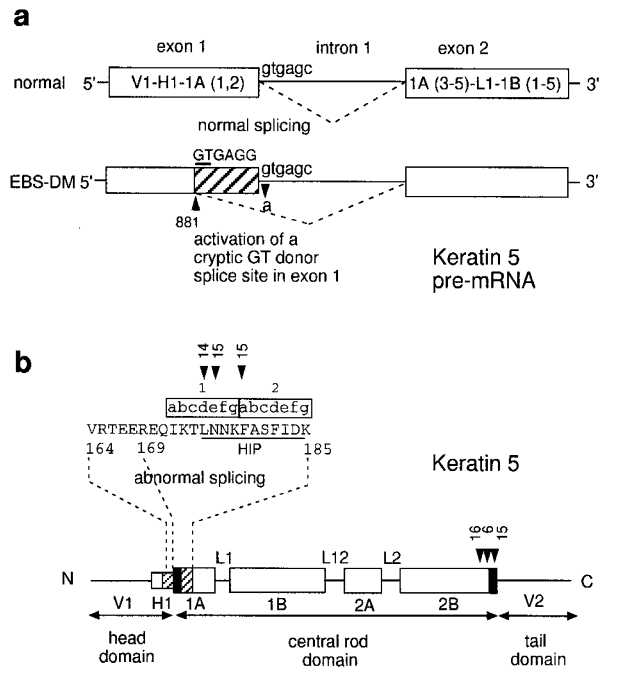

Figure 4 **a** *Schematic representation of abnormal splicing of exon 1 of K5. Exons and parts of exons present in the mRNA are shown as blank boxes. The abnormally spliced sequence is shown as a box with hatching. The donor splice site mutation of intron 1 (gt→at) leads to abnormal splicing of the last 66 bp of exon 1 after the activation of a cryptic GT donor splice site (underlined) within this exon. The numbers in brackets following domains 1A and 1B indicate the heptad repeats encoded by the corresponding exon.* (***b***) *Schematic diagram of the mutated keratin 5 polypeptide. Abnormal splicing (hatching) of the last 22 amino acids of exon 1 removes the last 5 amino acid residues of the H1 domain and the first 17 amino acids of the 1A domain, including the first two heptad repeats with the helix initiation peptide (HIP) (underlined, black boxed). Capitals indicate amino acid residues in the one letter code, small letters (a–g) represent their position within the first two heptad repeats of domain 1A of keratin 5. The position of other known K5 mutations in EBS-DM are indicated by arrowheads and reference numbers. The different segments of the head domain (V1 and H1), the rod domain (coiled-coil α helical segments 1A, 1B, 2A, 2B and corresponding non-helical linker regions L1, L1-2, L2) and the tail domain (V2) are indicated.*

Donor Splice Site Mutation of Intron 1 Activates a Cryptic Splice Site with High Potential for Splicing

The G-to-A transition that we describe occurs at the +1 donor splice position of an intron, a position which has been shown to be invariant in almost all examined eukaryotic genes.[35] Examples of mutations at this position causing activation of a cryptic splice site or resulting in exon skipping have been documented in several other disease-causing genes.[36] In the case that we report, mutation of the normal donor splice site of intron 1 results in the use of a cryptic GT splice site of exon 1 which is part of a AGGTGAGG consensus sequence. This sequence is almost identical to the natural occurring donor splice site of intron 1 of keratin 5 (AGgtgagc) and to the consensus sequence (AGgtgagt) found at the 5' end of a majority of eukaryotic introns.[35] We calculated the scores of these splicing sequences as described by Shapiro and Senapathy.[35] We found scores of 90.87 for the normal splice

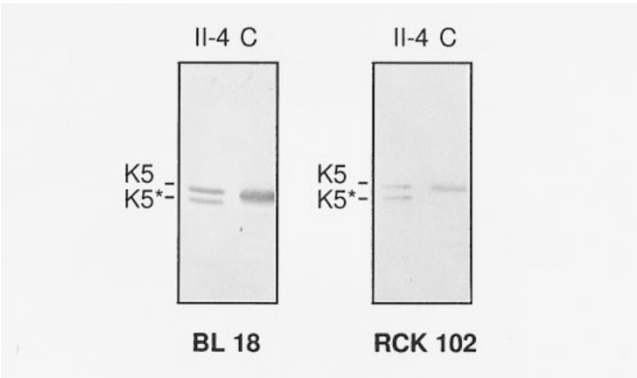

Figure 5 *Immunoblots of cytoskeletal extracts from EBS-DM and normal keratinocyte cultures. Keratins were extracted and resolved by electrophoresis on 10 and 2% (w/v) polyacrylamide–bisacrylmide gels, transferred to nitrocellulose and probed with specific keratin 5 antibodies: BL18, a rabbit polyclonal antibody to keratin 5, and RCK102, a monoclonal mouse antibody to keratins K5 and K8. II-4, cytokeratin extract from DM-EBS patient II-4 keratinocyte cultures; C, cytokeratin extract from normal keratinocyte cultures. The normally migrating K5 polypeptide is labelled as K5, while the faster migrating abnormal K5 is labelled as K5*.*

site (AGgtgagc), 72.62 for the mutated splice site (AGatgagc) and 92.15 for the cryptic splice site (AGGTGAGG) within exon 1. These values support the possibility that the mutation of the natural donor splice site of intron 1 has favoured the use of the cryptic splice site of exon 1 with higher statistical score.

Consequences of Abnormal Splicing of Exon 1

Abnormal splicing of exon 1 results in the inframe removal of the last 5 amino acids (VRTEE) of the H1 region, and the first 17 amino acids of the 1A domain including the first two heptad repeats (IKTLNNK-FASFIDK) of K5 (Figure 4b). These sequences are highly conserved among human type II keratins and intermediate filaments from distant species (Figure 6). Several lines of evidence indicate that they play an important role in keratin filament formation. Specifically, deletion experiments of the head domain of K5, including the H1 sequences immediately adjacent to the start of the rod domain, resulted in alteration of elongation and lateral alignment of keratin filaments in *in vitro* filament assembly studies.[37] Also, competition experiments with synthetic peptides to the H1 domain of keratin 1 have suggested that the H1 domain in type II keratins is important for stability and alignment of keratin filaments, possibly through interactions with L2 and 2B sequences.[38] These observations indicate that the head of type II keratins, but not of type I keratins (which do not possess a H1 region), is crucial for keratin filament formation.[7,39]

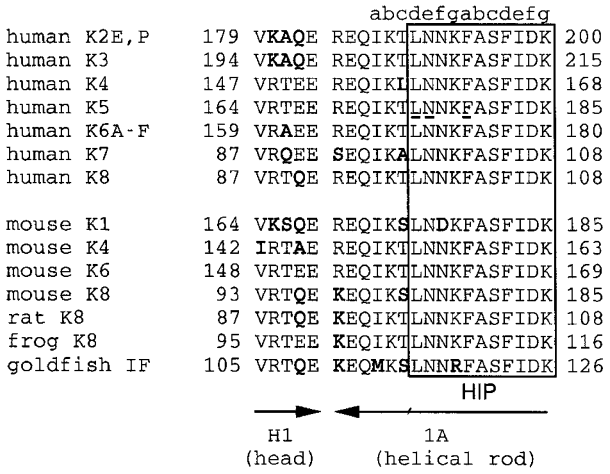

Figure 6 *Conservation of the last 22 amino acids of exon 1 of K5 among human type II keratins and intermediate filaments from different species. The numbers indicate the position of the first and last residues of the sequences. The position (a–g) of the residues relative to the first and second heptad repeats of the 1A domain are indicated. Letters in bold differ from the K5 sequence. The helix initiation peptide sequence (HIP) is boxed. The position of the three mutations previously reported in the HIP sequence of K5 in EBS-DM patients[14,15] is underlined.*

Furthermore, the two first heptad repeats of the 1A domain of K5 are located at the highly conserved amino terminal rod end of the molecule, spanning the LNNKFASFIDK sequence known as the helix initiation peptide (HIP). It is notable that the three other mutations described in the 1A domain of K5 in EBS-DM (L174F, N176S and F179S)[14–15] and all 16 K14 mutations reported in EBS-DM have been identified within the HIP sequence of these genes.[8] In support of the importance of this region in keratin filament assembly, several deletion and mutagenesis studies have previously provided compelling evidence that defects in this conserved HIP sequence are very disruptive to filament elongation, consistent with the head to tail alignment of keratin heterodimers.[40–42] For these reasons, we believe that the removal of this sequence from the K5 polypeptide, together with the elimination of the adjacent part of the H1 domain, impairs keratin filament formation and causes EBS-DM.

Proposed Mechanism of Disease

Protein analysis of keratinocyte extracts showed that significant amounts of shortened keratin 5 polypeptide is expressed *in vitro*, suggesting that the mutated protein retains sufficient structural integrity to interact with its K14 partner and impairs keratin filament formation in a dominant negative manner. During the formation of coiled-coil heterodimers, it is possible that the association of the shortened K5 polypeptide with its K14 partner leaves the first and second heptad repeats of K14 unpaired, whereas the other heptad repeats of both molecules could align normally to establish the largest number of hydrophobic interactions. However, according to the current model for keratin molecule assembly, the absence of H1 and 1A end sequences from K5 is expected to alter both head-to-tail alignment of dimers, whose rod ends have been suggested to overlap slightly during protofilament formation, and lateral association of staggered K5–K14 dimers at the protofilament and protofibril level.[39,43] Interestingly, despite the fact that keratin aggregates could be seen in basal keratinocytes of skin biopsies from patient II-4, immunofluorescence staining of cultured keratinocytes from the same patient with antibodies BL18 and

RCK102 showed no evidence for filament abnormalities. This result is consistent with the observation that cultured cells from EBS patients have to be stressed before any aggregates can be seen.[44]

Genotype–Phenotype Correlation

This is the first report of a keratin mutation in EBS-DM which is not a straightforward missense mutation. All the previously reported mutations have been within the helix initiation or the helix termination peptide of K5 or K14, mutations in K5 being less frequently reported than K14 mutations (Figure 4b). In other forms of EBS, two previous keratin non-missense mutations have been described: a three-nucleotide deletion (ΔE375K14) was reported in EBS-WC,[24] and a mutation of the acceptor splice site of intron 1 in keratin 14 leading to a premature termination codon and absence of protein expression in a kindred with severe recessive EBS.[45] Other splice-site mutations in keratin genes associated with diseases have not been reported to our knowledge.[8]

Despite the large size of the defect, the patients from this family exhibited features which are similar to classical EBS-DM. This is in contrast to a recently reported severe case of EBS-DM caused by a Y129D mutation in the conserved amino-terminal end of helix 1A of keratin 14.[13] This suggests that some missense mutations can be more disruptive on keratin filament formation than in-frame removal of complete sequences from the H1 and 1A domains. Finally, the case that we describe provides new evidence for heterogeneity in the nature of keratin defects underlying EBS and their phenotypic consequences.

Acknowledgements

We are grateful to Dr Ervin Epstein Jr and Jeannette Bonifas for providing primers for amplification of K5 cDNA. We thank the family for its participation in this study, Dr C Blanchet-Bardon for referring the family to our laboratory, Isabelle Guillemin for her assistance in this work and Sarah Compton for her critical reading of the manuscript. This work was supported by the Dystrophic Epidermolysis Bullosa Research Association, DEBRA UK (ELR & EBL, AH), the Cancer Research Campaign (Grant no. SP2060/01 to EBL) and the Epidermolyse Bulleuse Association d'Entraide (EBAE) (AH). AH holds a DEBRA UK fellowship. ELR and M-O R-P contributed equally to this work. Part of this work was previously published as an abstract for the 1994 annual meeting of the Society for Investigative Dermatology in *J Invest Dermatol* 1994, **102**: 610, abstract 518.

References

1 Fine JD, Bauer EA, Briggaman RA *et al*: Revised clinical and laboratory criteria for subtypes of inherited epidermolysis bullosa: a consensus report by the subcommittee on diagnosis and classification of the national epidermolysis bullosa registry. *J Am Acad Dermatol* 1991; **24**: 119–135.
2 Fuchs E: Genetic skin disorders of keratin. In: Scriver CR, Beaudet AL, Sly WS, Valle D (ed.). *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill: New York, 1995, **3**, pp 4421–4437.
3 Vassar R, Coulombe PA, Degenstein L, Albers K, Fuchs E: Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. *Cell* 1991; **64**: 365–380.
4 Coulombe PA, Hutton ME, Letai A, Hebert A, Paller AS, Fuchs E: Point mutations in human keratin 14 genes of epidermolysis bullosa simplex patients: genetic and functional analyses. *Cell* 1991; **66**: 1301–1311.
5 Bonifas JM, Rothman AL, Epstein EH Jr: Epidermolysis bullosa simplex: evidence in two families for keratin gene abnormalities. *Science* 1991; **254**: 1202–1205.
6 Lane EB, Rugg EL, Navsaria H *et al*: A mutation in the conserved helix termination peptide of keratin 5 in hereditary skin blistering. *Nature* 1992; **356**: 244–246.
7 Fuchs E, Weber K: Intermediate filaments: structure, dynamics, function and disease. *Annu Rev Biochem* 1994; **63**: 345–382.
8 Corden LD, McLean WHI: Human keratin diseases: hereditary fragility of specific epithelial tissues. *Exp Dermatol* 1996; **5**: 297–307.
9 Letai A, Coulombe PA, McCormick MB, Yu Q-C, Hutton E, Fuchs E: Disease severity correlates with position of keratin point mutations in patients with epidermolysis bullosa simplex. *Proc Natl Acad Sci USA* 1993; **90**: 3197–3201.
10 Fuchs E: Intermediate filaments and disease: mutations that cripple cell strength. *J Cell Biol* 1994; **125**: 511–516.
11 Stephens K, Sybert VP, Wijsman EM, Ehrlich P, Spencer A: A keratin 14 mutational hot spot for epidermolysis bullosa simplex, Dowling-Meara: implications for diagnosis. *J Invest Dermatol* 1993; **101**: 240–243.
12 Chen H, Bonifas JM, Matsumura K, Ikeda S, Leyden WA, Epstein EHJ: Keratin 14 gene mutations in patients with epidermolysis bullosa simplex. *J Invest Dermatol* 1995; **105**: 629–632.
13 Chan Y-M, Cheng J, Gedde-Dahl TJ, Niemi K-M, Fuchs E: Genetic analysis of a severe case of Dowling-Meara epidermolysis bullosa simplex. *J Invest Dermatol* 1996; **106**: 327–334.
14 Nomura K, Shimizu H, Meng X: A novel *K5* gene mutation in Dowling-Meara epidermolysis bullosa simplex. *J Invest Dermatol* 1996; **107**: 253–254.
15 Stephens K, Ehrlich P, Weaver M, Le R, Spencer A, Sybert VP: Primers for exon-specific amplification of the *KRT5* gene: identification of novel and recurrent mutations in epidermolysis bullosa simplex patients. *J Invest Dermatol* 1997; **108**: 349–353.
16 Irvine AD, McKenna KE, Bingham A, Nevin NC, Hughes AE: A novel mutation in the helix termination peptide of keratin 5 causing epidermolysis bullosa simplex Dowling-Meara. *J Invest Dermatol* 1997; **109**: 815–816.

17 Dong W, Ryynänen M, Uitto J: Identification of a leucine-to-proline mutation in the keratin 5 gene in a family with the generalized Köbner type of epidermolysis bullosa simplex. *Hum Mutat* 1993; **2**: 94–102.
18 Humphries MM, Sheils DM, Farrar GJ *et al*: A mutation (Met-to-Arg) in the type I keratin (K14) gene responsible for autosomal dominant epidermolysis bullosa simplex. *Hum Mutat* 1993; **2**: 37–42.
19 Yamanishi K, Matsuki M, Konishi K, Yasuno H: A novel mutation of Leu 122 to Phe at a highly conserved hydrophobic residue in the helix initiation motif of keratin 14 in epidermolysis bullosa simplex. *Hum Mol Genet* 1994; **3**: 1171–1172.
20 Stephens K, Zlotogorski A, Smith L *et al*: Epidermolysis bullosa simplex: a keratin 5 mutation is a fully dominant allele in epidermal cytoskeleton function. *Am J Hum Genet* 1995; **56**: 577–585.
21 Rugg EL, Morley SM, Smith FJD *et al*: Missing links: Weber-Cockayne keratin mutations implicate the L12 linker domain in effective cytoskeleton function. *Nat Genet* 1993; **5**: 294–300.
22 Chan Y-M, Yu Q-C, Fine J-D, Fuchs E: The genetic basis of Weber-Cockayne epidermolysis bullosa simplex. *Proc Natl Acad Sci USA* 1993; **90**: 7414–7418.
23 Chan Y-M, Yu Q-C, Christiano AM *et al*: Mutations in the non-helical linker segment L1-2 of keratin 5 in patients with Weber-Cockayne epidermolysis bullosa simplex. *J Cell Sci* 1994; **107**: 767–774.
24 Chen MA, Bonifas JM, Matsumura K, Blumenfeld A, Epstein EHJ: A novel three-nucleotide deletion in the helix 2B region of keratin 14 in epidermolysis bullosa simplex: ΔE375. *Hum Mol Genet* 1993; **2**: 1971–1972.
25 Matsuki M, Hashimoto K, Yoshikawa K, Yasuno H, Yamanishi K: Epidermolysis bullosa simplex (Weber-Cockayne) associated with a novel missense mutation of asp (328) to val in linker L12 domain of keratin 5. *Hum Mol Genet* 1995; **4**: 1999–2000.
26 Humphries MM, Mansergh FC, Kiang AS *et al*: 3 keratin gene mutations account for the majority of dominant simplex epidermolysis bullosa cases in Ireland. *Hum Mutat* 1996; **8**: 57–63.
27 Uttam J, Hutton E, Coulombe PA *et al*: The genetic basis of epidermolysis bullosa simplex with mottled pigmentation. *Proc Natl Acad Sci USA* 1996; **93**: 9079–9084.
28 Irvine AD, McKenna KE, Jenkinson H, Hughes AE: A mutation in the V1 domain of keratin 5 causes epidermolysis bullosa simplex with mottled pigmentation. *J Invest Dermatol* 1997; **108**: 809–810.
29 Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975; **6**: 331–334.
30 Chomczynski P, Sacchi N: Single step method of RNA isolation of acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
31 Rugg EL: Detection and characterisation of keratins by immunocytochemistry and immunoblotting. In: IL Watt F (ed). *Keratinocyte Methods*. Cambridge University Press: Cambridge, 1994, pp 127–148.
32 Purkis PE, Steel JB, Mackenzie IC, Nathrath WBJ, Leigh IM, Lane EB: Antibody markers of basal cells in complex epithelia. *J Cell Sci* 1990; **97**: 39–50.
33 Broers JLV, Carney DN, Rot MK *et al*: Intermediate filament proteins in classic and variant types of small cell lung carcinoma cell lines: A biochemical and immunochemical analysis using a panel of monoclonal and polyclonal antibodies. *J Cell Sci* 1986; **83**: 37–60.
34 Wanner R, Förster H-H, Tilmans I, Mischke D: Allelic variations of human keratins K4 and K5 provide polymorphic markers within the type II keratin gene cluster on chromosome 12. *J Invest Dermatol* 1993; **100**: 735–741.
35 Shapiro MB, Senapathy P: RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res* 1987; **15**: 7155–7174.
36 Krawczak M, Reiss J, Cooper DN: The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum Genet* 1992; **90**: 41–54.
37 Wilson AK, Coulombe PA, Fuchs E: The roles of K5 and K14 head, tail, and R/KLLEGE domains in keratin filament assembly *in vitro*. *J Cell Biol* 1992; **119**: 401–414.
38 Steinert PM, Parry DAD: The conserved H1 domain of the type II keratin 1 chain plays an essential role in the alignment of nearest neighbor molecules in mouse and human keratin 1/keratin 10 intermediate filaments at the two- to four-molecule level of structure. *J Biol Chem* 1993; **268**: 2878–2887.
39 Steinert PM: Structure, function, and dynamics of keratin intermediate filaments. *J Invest Dermatol* 1993; **100**: 729–734.
40 Albers K, Fuchs E: Expression of mutant keratin cDNAs in epithelial cells reveals possible mechanisms for initiation and assembly of intermediate filaments. *J Cel Biol* 1989; **108**: 1477–1493.
41 Coulombe PA, Chan Y-m, Albers K, Fuchs E: Deletions in epidermal keratins leading to alterations in filament organization *in vivo* and in intermediate filament assembly *in vitro*. *J Cell Biol* 1990; **111**: 3049–3064.
42 Fuchs E: Genetic skin disorders of keratin. *J Invest Dermatol* 1992; **99**: 671–674.
43 Fuchs E, Chan Y-M, Paller A, Yu Q-C: Cracks in the foundation: keratin filaments and genetic disease. *Trends Cell Biol* 1994; **4**: 321–326.
44 Morley SM, Dundas SR, James JL *et al*: Temperature sensitivity of the keratin cytoskeleton and delayed spreading of keratinocyte lines derived from EBS patients. *J Cell Sci* 1995; **108**: 3463–3471.
45 Jonkman MF, Heeres K, Pas HH *et al*: Effects of keratin 14 ablation on the clinical and cellular phenotype in a kindred with recessive epidermolysis bullosa simplex. *J Invest Dermatol* 1996; **107**: 764–769.