Mutations in the Rod 1A Domain of Keratins 1 and 10 in Bullous Congenital Ichthyosiform Erythroderma (BCIE)

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Bullous congenital ichthyosiform erythroderma is a human hereditary skin disorder in which suprabasal keratinocytes rupture. Recent reports have implicated keratins K1 and K10 in this disease. Here we describe four diverse keratin mutations that are all significantly associated with this disease. Two of these are in the helix 1A subdomain of the type II keratin 1, giving a serine-to-proline substitution in codon 185 and an asparagine-to-serine substitution in codon 187. In the analogous region of type I keratin 10, an arginine-to-proline and an arginine-to-serine transition in codon 156 have been identified. All four mutations create restriction fragment length polymorphisms that were used exclude the mutations from 120 normal chromosomes. Insertional polymorphism (in the V2 subdomains of the non-helical tails of K1 and K10) was excluded as the cause of the phenotypic heterogeneity observed within one family. *Key words: EHK (epidermolytic hyperkeratosis)/intermediate filaments. J Invest Dermatol 102:24–30, 1994*

Keratins are the major intermediate filament proteins found in the cytoskeleton of epithelial cells. These proteins assemble as coiled-coil heterodimers consisting of a specific type I (acidic) and type II (basic) keratin [1–3]. Dimers undergo further polymerisation to form keratin intermediate filaments by an assembly process that has yet to be fully defined. Keratins are expressed as pairs in a tissue-specific manner. In the basal cells of epidermis in the skin, synthesis of keratins K5 (type II) and K14 (type I) predominate, whereas in the outer suprabasal layers of the epidermis, keratins K1 (type II) and K10 (type I) constitute the majority of the cellular protein.

A direct role for keratins in maintaining the structural integrity of skin has recently been demonstrated by findings that certain point mutations in either of the K5 and K14 keratin genes are strongly associated with the autosomal dominant blistering disorder epidermolysis bullosa simplex (EBS), where blistering occurs through lysis of epidermal keratinocytes in the basal cell layer [4–6]. Similarly, suprabasal splitting by cytolysis has been found to occur in BCIE [7,8], a dominant disorder characterised by pronounced hyperkeratosis, ichthyosis, and erythroderma [9,10]. This condition is also referred to as epidermolytic hyperkeratosis (EH, EHK). Recently, mutations associated with this disease have been reported in keratins 1 and 10 [11–13]. It has been shown by *in vitro* experiments that artificial mutations in these regions of keratins can affect the appearance of the filaments they form [14]. It is likely that *in vivo* the pathogenic keratin mutations compromise the resilience of the intracellular supportive cytoskeleton of the keratinocytes, rendering them less resistant to physical trauma.

In this study, we report mutations creating a serine-to-proline substitution and an asparagine-to-serine substitution in the helix 1A subdomain of the keratin 1 polypeptide, seen in affected members of two families carrying BCIE. Previously, all mutations found in the conserved 1A domain have been within type I keratins, and mutations in type II keratins have all been in other domains (head H1, helix 2B). These are the first examples of mutations in the helix 1 domain of a type II intermediate filament protein. We also report arginine-to-proline and arginine-to-serine mutations in helix 1A of keratin 10 producing the disease. These four diverse mutations in helix 1A of suprabasal epidermal keratins all produce severe BCIE phenotypes. Knowledge of where the mutation-sensitive points are in the proteins will help us to understand how keratin filaments are assembled and how they function, and will clarify the nature of these mechano-bullous diseases and lead to development of better treatment.

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Abbreviations: BCIE, bullous congenital ichthyosiform erythroderma; EBS, epidermolysis bullosa simplex; EH, EHK, epidermolytic hyperkeratosis; TBE: Tris borate EDTA buffer.

MATERIALS AND METHODS

Transmission Electron Microscopy Perilesional skin biopsy specimens were fixed in half-strength Karnovsky fixative, post-fixed in osmium tetroxide, dehydrated in ethanol, and embedded in Epon, largely as described [8] but with additional *en bloc* staining with uranyl acetate. Ultrathin sections

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were further stained with uranyl acetate and lead citrate and examined in a Jeol 100CX electron microscope operating at 60–80 kV.

Immunoelectron Microscopy The specimens, derived from the same skin biopsy as the tissue used for transmission electron microscopy (above), were divided into subsamples no larger than 0.5 mm^3, soaked for 1 h in 20% glycerol in phosphate-buffered saline (PBS) as a cryoprotectant, plunge-frozen in liquid propane at −190°C using a Leica KF80 apparatus, subjected to freeze substitution in methanol using Leica CS-Auto apparatus, and embedded in Lowicryl K11M resin [8]. Ultrathin sections were collected on nickel grids and incubated with rabbit polyclonal antiserum to keratin 1 (RbaK1 [15]). After extensive buffer washes, further incubations were performed using appropriate 1-nm gold-conjugated secondary antibodies (Auroprobe One, Amersham International plc., Amersham, Bucks, UK). The gold particles were silver enhanced (IntenSE M, Amersham, UK) for easy recognition. Negative control studies included the use of normal rabbit serum instead of the specific primary antibody.

Polymerase Chain Reaction (PCR)

Keratin 1: A 578-bp genomic DNA fragment was amplified by primers 5′ TTC CGG GTC TGG GTA CCG AAG 3′ (designated K1p1, sense strand) and 5′ CAC CTT TGT CAA TGA AGG AGG CAA 3′ (K1p7, antisense strand). Primer K1p1 is located at the start of the head domain coding sequence; K1p7 spans the exon1/intron1 junction in the 1A domain.

Keratin 10: A 590-bp genomic DNA fragment was amplified by primers 5′ GAT ACA GCT CAA GCA AGC ACT ACT 3′ (K10p14, sense strand, head domain) and 5′ GTA GTA TTT GCT GTA GTC ACG AGG C3′ (K10p11, antisense strand, linker L1 domain).

PCR Conditions: Approximately 500 ng of genomic DNA was used per 100μl reaction containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 10% dimethylsulfoxide, 0.5 μg/ml each primer, and 2.5 U Taq polymerase (Amplitaq). After an initial incubation at 94°C for 5 min, PCR was performed for 30 cycles consisting of 94°C for 30 seconds, 60°C for 1 min, 72°C for 2 min on a DNA Thermal Cycler (Perkin Elmer Cetus, CA).

Cycle Sequencing PCR fragments were resolved on 1.5% low—melting-point agarose gels and purified using Gelase (Epicentre, USA). Purified templates (50 fmol approximately) were sequenced using the DS-Cycle sequencing system (BRL, Bethesda, MD) with [^{32}P] end-labeled primer. The following PCR program was used for sequencing: 20 cycles consisting of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, followed by 10 cycles of 94°C for 30 seconds, 72°C for 60 seconds. Samples were resolved on standard 6% denaturing sequencing gels. K1 PCR fragments were sequenced initially with K1p7, and the reverse strand in the region of the mutation was also sequenced using K1p8 (5′ TCT GCC CTC CTG GTG GCA TAC AAG 3′) for confirmation. Similarly, K10 PCR fragments were sequenced using K10p11 in the first instance and the opposite strand sequenced with K10p7 (5′ GGA GGA GAT GGT GGC CTT CTC TCT 3′) for confirmation of mutations.

Mutation Screening by Restriction Fragment Length Polymorphism (RFLP)

Family 1: PCR was performed with primers K1p7 and K1p8 as described above with the following modifications. The PCR volume was reduced to 50μl. The PCR program consisted of an initial incubation at 94°C for 5 min, followed by 30 cycles consisting of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds. Following PCR, 10 μl of 5 × MaeIII buffer and 1 U MaeIII restriction enzyme (BCL) was added per 40 μl PCR reaction mixture. After overnight digestion at 55°C, samples were analyzed on 4% Nusieve agarose TBE minigels. The 159-bp PCR product amplified from normal alleles contains two MaeIII sites producing fragments of 27, 40, and 92 bp. Digestion of the mutant allele yields fragments of 27 and 132 bp so that an additional 132 bp band was observed in affected heterozygotes.

Family 2: PCR was performed using primers K1p20 (5′ AGG TGA AGT CTC GAG AAA GGG 3′) and K1p21 (5′ CGT TCG CAA AAC ATC CTT TT 3′) using the reaction conditions above with the following program: 94°C for 30 seconds, 55°C for 1 min, 72°C for 1 min, 30 cycles. Following PCR, 10 μl 5 × *DdeI* buffer and 1 U *DdeI* was added per 40 μl PCR reaction. After overnight incubation at 37°C, digests were analyzed as above. The 193-bp fragment produced does not normally contain a *DdeI* site. However, the mutation in this family produces a *DdeI* site 36 bp from one end; digestion of the PCR product from the affected individual results in the appearance of additional bands of 157 and 36 bp due to the mutant allele as well as the 193-bp band.

Families 3 and 4: PCR was performed using primers K10p11 and K10p24 (5′ GGC CTT CTC TCT GGA AAT GA 3′) using the reaction conditions above with the following program: 94°C for 30 seconds, 50°C for 1 min, 72°C for 1 min, 35 cycles. Following PCR, 5 μl of 10 × *AciI* buffer was added to 45 μl PCR product. Spermidine was added to a final concentration of 1 mM and the reaction heated to 65°C for 15 min before digestion with 2 U *AciI* at 37°C for more than 2 h. Digests were analysed as above. Digestion of normal alleles (195 bp) yields fragments of 59 and 136 bp. The mutations found in both families 3 and 4 disrupt the *AciI* site so that an undigested 195 bp band is indicative of either mutation.

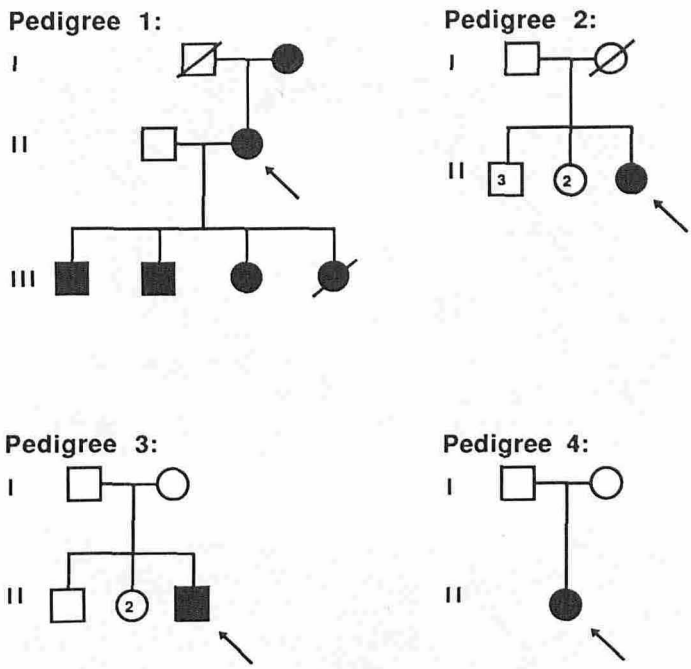


Figure 1. Pedigrees of the four families under study, carrying bullous congenital ichthyosiform erythroderma (BCIE) of Brocq. *Closed symbols* show affected individuals, *open symbols* unaffected; *squares* represent *male* and *circles, female*. *Crossed symbols* represent deceased subjects. *Arrow* indicates the proband.

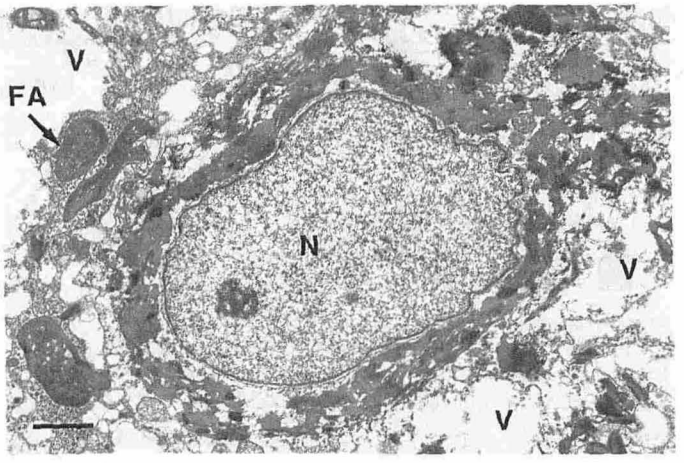
Protein Structure Predictions The plots shown in Fig 6 show the results of analysis carried out on the complete amino acid sequence of helix 1A of human keratin 1 plus the α-helical portion of the H1 subdomain of the non-helical head region (residues 172–214 inclusive), using the Garnier algorithm of GeneWorks 2.1 (Intelligenetics Inc.). The analogous region of K10 was also analyzed. Predictions were based on calculations carried out using eight residues on either side of the target residue as recommended [16].

V2 Domain Polymorphism in Keratins 1 and 10 The V2 subdomain of K10 was amplified by PCR using the primers described elsewhere [17] with the following modifications. The thermal cycling program used was as described for K1p1 and K1p7 (above) and 10% dimethylsulfoxide was added to the reaction mix. Formamide and glycerol were omitted from the reaction. The resulting reaction product was analyzed on 3% Nusieve TBE gels.

The V2 subdomain of K1 was amplified as above with the following primers: K1p12 (sense strand) = 5′ GTG AGC ACA AGC CAC ACC AGC 3′ and K1p13 (antisense strand) = 5′ TTG TTA GTG ATG CTG GGG GAG 3′. The PCR fragments were visualized by end labeling of one primer and separation on a 6% sequencing gel. This reaction produced a 475-bp product from all members of the family analyzed.

RESULTS

Electron Microscopy A skin biopsy from an affected individual (the proband in pedigree 1 [Fig 1]) in a family carrying BCIE was examined by transmission electron microscopy (Fig 2). Blistering was found to occur within the suprabasal cell layers of the epidermis. Figure 2 illustrates the filament abnormalities observed in this patient as pronounced aggregates in the granular (Fig 2*a*) and spinous (Fig 2*b*) layers of the epidermis. Basal cells appeared normal. The diagnosis was therefore confirmed as BCIE, and distinguished from other ichthyoses or disorders causing hyperkeratosis. Figure 2*a* shows that much of the aggregated keratin forms a ring or shell



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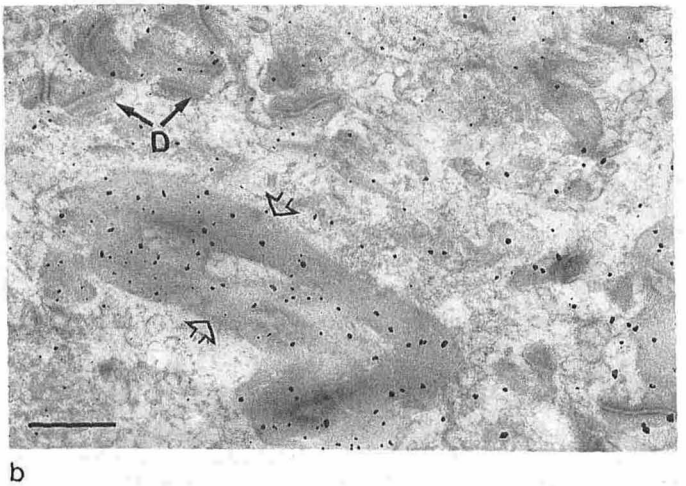

b

Figure 2. Transmission electron micrographs showing abnormal keratin tonofilament aggregates in the granular *(a)* and spinous *(b)* layers of skin affected by epidermolytic (BCIE) hyperkeratosis. *a)* Instead of forming a normal network, the keratin filaments are tightly aggregated into wreath-like distribution around the nucleus (N), or in discrete peripheral filament aggregates (FA). The cytoplasm has become vacuolated (V) where normal filaments are lacking. *Scale bar,* 1 μm. *b)* Immunogold-silver enhanced labeling of Lowicryl K11M ultrathin section using a rabbit antiserum to keratin 1. Clear specific labeling is shown in a large keratin filament aggregate *(open arrows)* plus more peripheral aggregates, some of which are associated with intact desmosomes (D). *Scale bar,* 1 μm.

around the nucleus, which is still separated from the nuclear envelope by a thin rim of cytoplasm. Other material of different electron density is associated with this, probably other components of differentiating keratinocytes. Filament clumps were shown to be selectively stained with specific antisera to K1 and K10 by immunoelectron microscopy (Fig 2*b*), implying the involvement of at least one of these two copolymerizing keratins in the disease. Very similar results were obtained for individuals from families 2–4 in this study. Figure 2*b* also demonstrates that desmosomes are still present and appear to be intact, and that some filamentous material is still attached to them.

PCR Sequencing

Keratin 1 Sequence Corrections: Genomic DNA was extracted from skin fibroblast cultures and/or blood samples from four affected and one unaffected member of family 1 (Fig 1*a*). Similarly, genomic DNA was obtained from the affected member and the unaffected individuals in family 2.

A 578-bp fragment of genomic DNA comprising the coding sequence for the V1 and H1 subdomains of the non-helical head region and most of the helix 1A domain of the rod region of K1 was amplified by PCR. Amplified DNA from affected and unaffected members of the family and unrelated normal individuals was sequenced directly by the cycle sequencing method. A number of sequence differences were observed, between the sequence obtained from the normal controls and the previously published sequence [18], i.e., C_{1081} (not T), C_{1172} (not T), A_{1283} (not C), and A_{1288} (not C). Two of these changes alter the predicted amino acid sequence (Ser now becoming Pro_{147} and Gln becoming Lys_{184}). Protein sequence alignment comparisons show that these amino acid alterations increase the homology of K1 with the other type II keratins (not shown) and therefore have a probability of being correct. These changes were found to be consistent in six unrelated control samples and probably represent errors in the original sequencing of this gene as predicted [13]. They have been submitted to the genetic database of the European Molecular Biology Laboratory (EMBL), Heidelberg (accession number X69725)

Keratin 1 Mutations: By direct sequencing, all four affected members of family 1 were found to be heterozygous for a $T_{1286}C$ transition in codon 185 of K1, which thus encoded a proline residue instead of the normal serine (Fig 3, EMBL accession number X69758). The unaffected member did not have this mutation, nor was it present in six unrelated control sequences. The mutation was confirmed by sequencing of the reverse strand using an internal primer. The mutation occurs in the recognition site for the restriction enzyme *MaeIII* thus allowing rapid screening for this mutation in the general population (Fig 4). No change in this site was observed in 60 unrelated normal individuals, i.e., 120 separate alleles, indicating that this change is unlikely to be a polymorphism in the general population.

The affected individual in family 2 was found to be heterozygous for a $A_{1293}G$ transition in codon 187 of K1, producing a asparagine to serine transition (Fig 3). The mutation was absent from the six unaffected members of the family. The base change in this family creates a new *DdeI* site allowing screening for this mutation in the normal population. No additional site was observed in 60 unrelated normal individuals (Fig 4).

The positions of these mutations, together with two other mutations in K1 previously reported to be present in patients with BCIE [11–13], are shown in Fig 5 relative to the consensus domain structure of keratin polypeptides. Like the mutations in K5 and K14 that cause the analogous disease EBS, and those reported in K10 causing BCIE, the mutations appear to cluster in two highly conserved regions around i) the start (in H1, 1A) and ii) the end (helix termination peptide) of the α-helical rod domain, with the exception of one mutation in helix 2B of K14 reported to cause a Koebner form of EBS [5]. This is consistent with transfection experiments [19,20] and *in vitro* filament assembly studies [21,14] with keratins carrying deletions and point mutations in the ends of the rod domain, which indicate that these regions are essential for normal polymerization. The mutations reported here occur close to the start of the rod domain, although the altered S_{185} residue is not so highly conserved as those previously observed to be mutated. In contrast, the mutated N_{187} residue in family 2 is very highly conserved between all type I, II, and III intermediate filament proteins, thus one would expect mutations here to have some effect.

Keratin 10 Mutations: A 590-bp fragment of the K10 gene was amplified by PCR. This fragment includes most of the head domain, helix 1A and linker L1. This was directly sequenced with the reverse primer initially used for amplification and the mutations were confirmed by sequencing of the sense strand with an internal primer.

The affected individual in family 3 was found to be heterozygous for a point mutation $G_{2140}C$ that produces an arginine-to-proline substitution in codon 156 at the start of the helix 1A domain of K10 (Fig 3). Similarly, the affected individual in family 4 was found to have a point mutation $C_{2139}A$ that changes arginine to serine in the same codon of K10 (Fig 3). The unaffected parents in both these

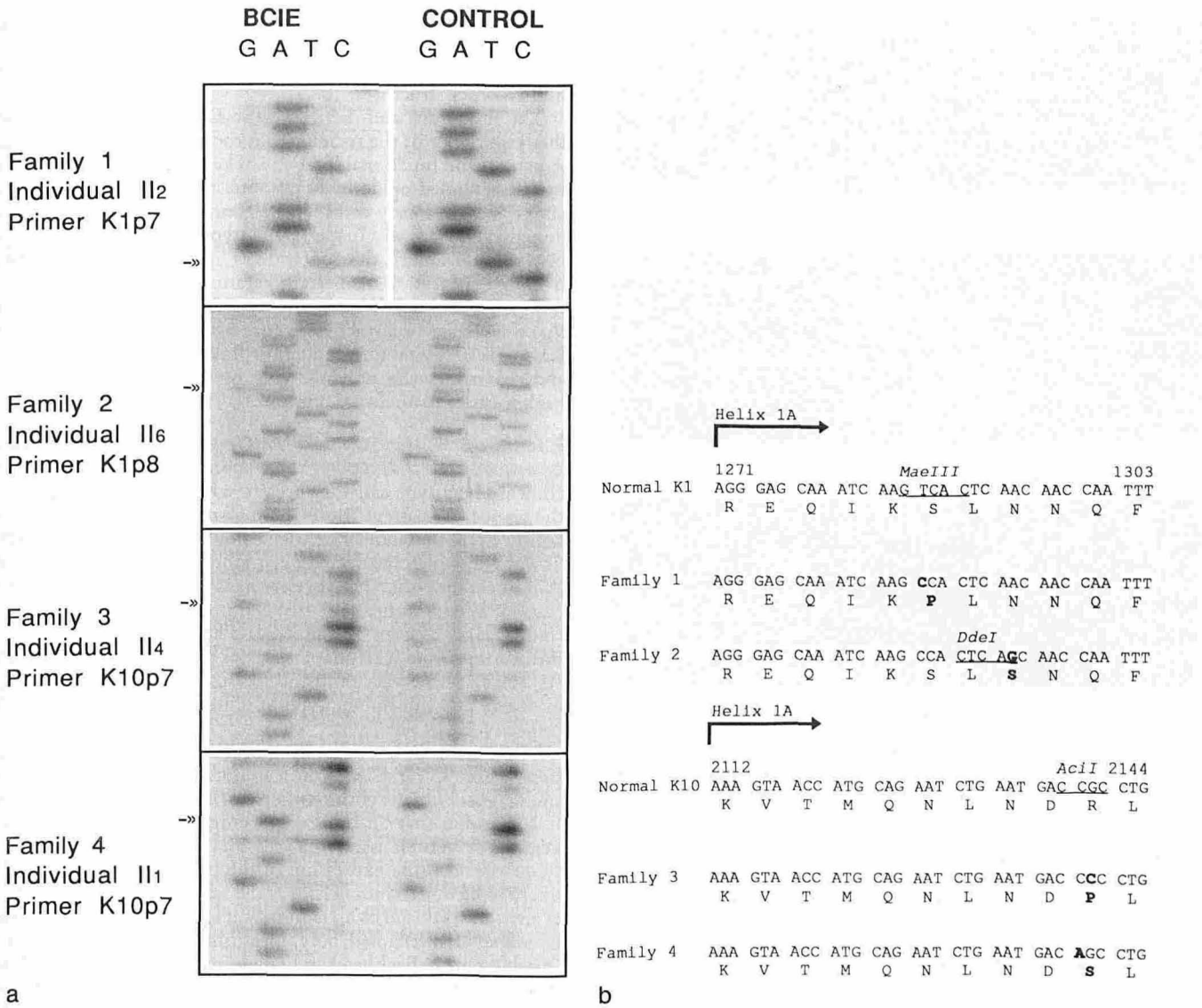

Figure 3. Identification of mutations in keratins 1 and 10. *a)* Excerpts from sequencing gels of DNA amplified from affected members (*left tracks,* BCIE) in the four families under study, compared with DNA samples derived from four separate unaffected unrelated controls (*right tracks,* control). Termination bases in each track are indicated above (G, A, T, C). Sequences shown from families 1 and 2 are from the gene encoding keratin 1, and those for families 3 and 4 are from the keratin 10 gene. Marks indicate positions of mutations. Primers K1p7, K1p8, and K10p7 used for sequencing reactions are as described in the *Materials and Methods* section. Note that the K1p7 sequences are from the non-coding strand and the gel has been reversed for ease of reading. *b)* Genomic DNA sequences in the helix 1A domains of K1 and K10 as read from a longer region of the above gels, and the predicted amino acid sequences (single letter nomenclature below each DNA codon). Bases numbers 1271–1303 of K1 and 2112–2144 of K10 are shown. *Bold type* indicates mutated bases and amino acids. The recognition sequences of restriction endonucleases affected by the various mutations as used for population screening are underlined.

families had no alterations in this region by direct sequencing or PCR screening.

Both mutations observed in families 3 and 4 disrupt a recognition site for the restriction enzyme *AciI*. A PCR-based *AciI* RFLP assay was used to screen unaffected members of both families and a population of 60 unrelated, unaffected individuals. An additional band was only seen in PCRs from the two affected individuals (Fig 4). The positions of these and previously reported mutations [11–13] within the protein are shown in Fig 5. The affected residue R_{156} is completely conserved within the type I and type III intermediate filament proteins and thus a point mutation at this position is likely to be detrimental to the assembly and/or structural integrity of keratin intermediate filaments.

Effects of Mutations on Predicted Protein Structure

Substitutions in Keratin 1: At position 185 of human K1 there is a serine residue. Most human type II keratins have threonine at this position, although this residue is leucine in human K4 and alanine in human K7. Analyses of this domain according to the Garnier method [16] predict a tendency to form α-helix. In comparison, a proline substitution at residue 185 would cause a major disruption of the helix according to Garnier predictions (Fig 6), consistent with the helix forming values of [22,23]. Interestingly, the threonine alternative observed at this position in most type II keratins increases the predicted helicity slightly, and the natural sequences observed in K4 and K7 both give a considerably stronger prediction of α-helicity (plots not shown). Subtle variations such as this between keratins may reflect their individual tissue-specific functional requirements.

By contrast, the K1 $N_{187}S$ mutation observed in family 2 is a relatively conservative transition because both serine and asparagine are uncharged under physiologic conditions. Asparagine, however, has a slightly larger side chain. Garnier prediction for this mutation in the 1A domain of K1 indicates that the tendency to form part of

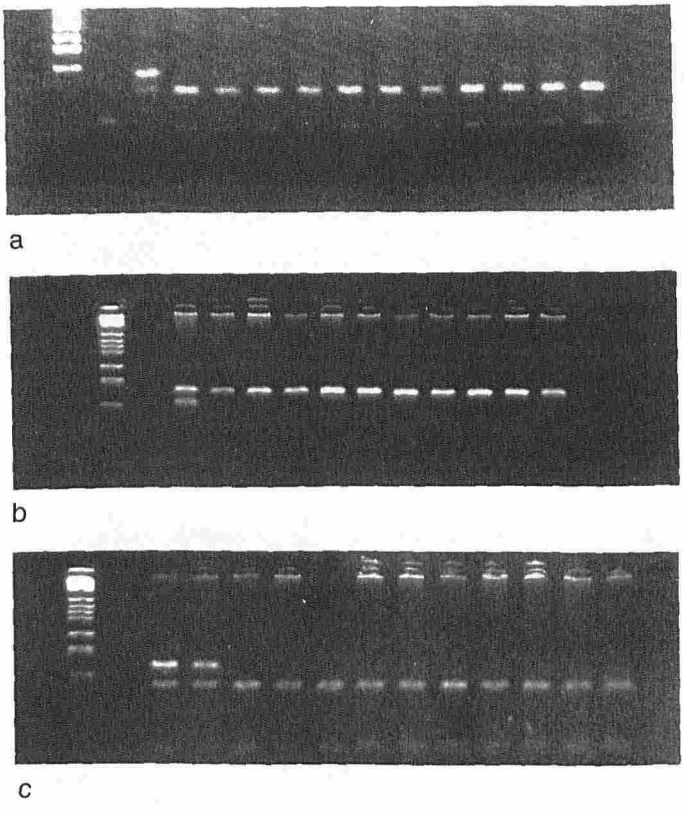

Figure 4. Screening for the BCIE mutation by restriction fragment length polymorphism analysis. Tracks number from left to right. *a)* PCR amplification a 132-bp fragment of human keratin 1 from genomic DNA. Track 1, DNA ladder (BRL, type VI). The smallest fragment is 154 bp. Track 2, PCR water control. Track 3, proband from BCIE family 1; track 4, unaffected family member; tracks 5–14, normal unrelated control samples. The mutation destroys a recognition site for the restriction endonuclease *MaeIII* and therefore creates a restriction fragment length polymorphism. The occurrence of the mutation in PCR-amplified DNA is revealed by the presence of an additional large undigested 132-bp band following digestion with *MaeIII*. An additional band was observed in all five affected members of the family under study, and was not seen in samples from 60 normal unrelated individuals. *b)* PCR amplification a 193-bp fragment of human keratin 1 from genomic DNA. Track 1, DNA ladder. Track 2, PCR water control. Track 3, proband from family 2. Tracks 4–13, normal unrelated control samples. The affected individual has an additional smaller 157 bp band following digestion with the restriction enzyme *DdeI*, as the mutation in this case creates a new *DdeI* site not present in the controls. *c)* PCR amplification a 195-bp fragment of human keratin 10 from genomic DNA and digestion with *AciI*. Track 1, DNA ladder. Track 2, PCR water control. Tracks 3 and 4, probands from families 3 and 4, respectively. Tracks 5–14, normal unrelated control samples. Normal control samples are digested to give 59- and 136-bp fragments. The fragments amplified from affected heterozygous individuals have an additional uncut band because either mutation disrupts this site.

an α-helix is unchanged. Interestingly, the phenotype of the patient carrying this mutation is severe and very similar to that observed to that of patients in family 1 carrying the $S_{185}P$ mutation. It appears therefore that it is not merely the α-helical conformation of this part of the 1A domain that is necessary for the correct function of K1/10 intermediate filaments, but that residues in this region are involved in other important interactions. This is consistent with current thinking on the higher-order filament assembly of keratin dimers, where there is evidence for an overlap of about 10 amino acids between the ends of rod domains in the assembled filaments [24].

Substitutions in Keratin 10 The K10 mutations observed in families 3 and 4 produce similar Garnier predictions to that obtained for the K1 mutations above (Fig 6). The $R_{156}P$ mutation is again predicted to be highly disruptive to the α-helical conformation of this domain, whereas the $R_{156}S$ mutation is much less damaging to the predicted α-helix formation. In this case, a positively charged side chain is replaced by a polar, non-charged side chain so one might expect this to adversely affect the formation of dimers or higher-order structures.

The mutations seen here to cause BCIE therefore demonstrate the importance of the α-helical 1A rod domain in maintaining the structural and functional integrity of keratin intermediate filaments in differentiated epidermal cells. Substitution of a helix-breaking amino acid seriously disrupts the conformation of this functional domain and leads to a disease phenotype in two cases. In two other instances, although the mutation is apparently less damaging to α-helix conformation, aberrant keratin filaments are observed histologically and the disease phenotype is similar. This demonstrates that the precise amino acid sequence of this part of the 1A domain of K1 and K10 is vital for production of functionally correct filaments and underlines the probable role of these conserved regions in higher-order filament assembly [24].

Phenotypic Variation and V2 Domain Polymorphism An aspect of BCIE that has yet to be addressed is the finding that the clinical phenotype can vary greatly within families, and also with the age of the patient. Both these forms of variation are found in family 1 reported here. The affected individual in generation II (Fig 7) was very severely affected in childhood, with the majority of the skin hyperkeratotic. The severity has markedly decreased with age (patient is now in her mid 40s) and now only the hands, feet, and some flexor surfaces are affected. The patient does not use any medication. Patient III_1 has an similar phenotype, whereas patients III_2 and III_3 have very mild and localized changes. The occurrence of severe and mild phenotype in family 1 is indicated as S or M, respectively, in Fig 7.

These findings are interesting because each of these patients are shown here to carry the identical mutation in K1. We have made similar observations within other BCIE kindreds (not shown here). It is possible that an otherwise benign polymorphism, either in keratins or in their associated accessory proteins, may have a modifying affect on the expression of the phenotype. The effect is unlikely to be due to mosaicism, as the lesions are not obviously focal even in the proband, who is already at least the second generation to be affected. Insertional polymorphism is known to occur in the variable non-helical head and tail domains of K1 and K10 [16,25]. Recent studies imply that although these end domains may not be essential for filament assembly, they are probably involved in maintaining the stability of 10 nm intermediate filament network [26,27]. Because the V1 and V2 domains are the regions that vary most between keratins, these domains are thought to influence the qualities of the filaments required in specific tissues, such as strength or flexibility. Insertional polymorphism of this type might therefore be involved in phenotypic variation within BCIE. To test this, the V2 domains of K1 and K10 were amplified by PCR from genomic DNA taken from all members of the family. No size variation was observed in the V2 domain of K1 in the family, with all members being homozygous.

Insertional polymorphism, however, was observed in the K10 V2 domain, where two alleles differing by approximately 75 bp were seen, representing an insertion of about 25 amino acids. However, variation in the severity of the phenotype clearly does not segregate with this polymorphism, as shown in Fig 7.

DISCUSSION

The patients in the families under study expressed the hallmarks of BCIE, the occurrence of blistering and keratin tonofilament aggregation within the suprabasal layers of epidermis. Immunoelectron microscopy showed that K1 and K10 were prominent components of the filament clumps. DNA sequencing revealed underlying genetic defects in the 1A domain of keratins 1 and 10. In K1 these were $S_{185}P$ and $N_{187}S$; in K10 mutations $R_{156}P$ and $R_{156}S$ were found. These mutations are not present in the normal population. DNA encoding the ends of the rod domains of K1 and K10 have now been sequenced from 10 families with BCIE in this laboratory; only these

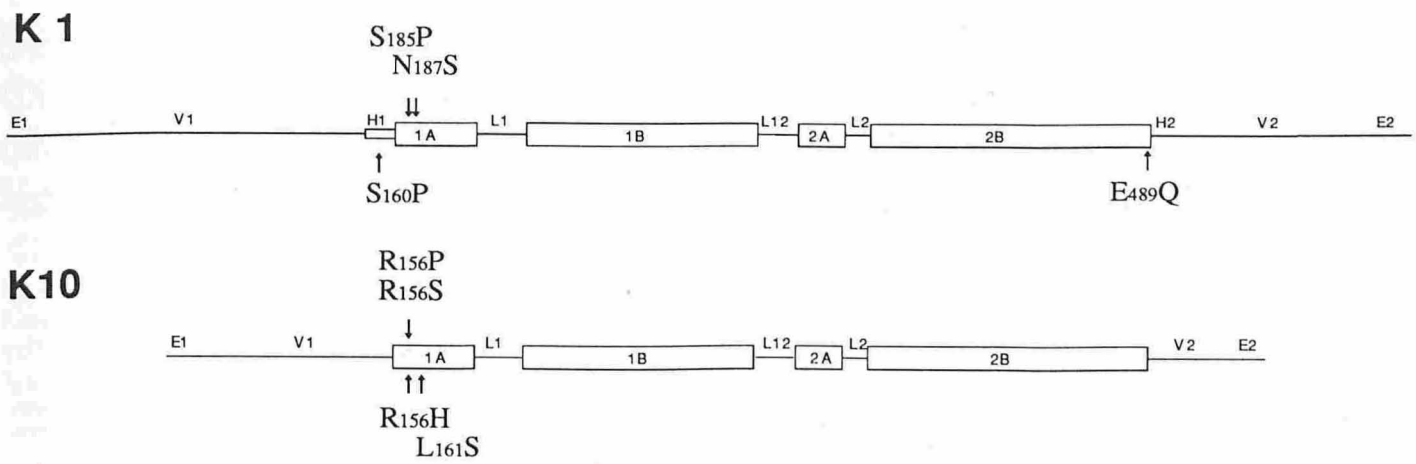

Figure 5. Schematic diagram of human keratins 1 and 10 showing consensus protein domain structure and the positions of mutations reported here in BCIE patients both here (shown above protein diagram) and by other groups (shown below protein diagram).

four families show mutations in these regions. It is supposed that the genetic defects in the other six kindreds lie elsewhere in these keratins, or in other epidermal differentiation keratins such as K2e, or in a non-keratin gene(s).

The importance of the helix 1A domain in assembly and/or integrity of the K1/K10 filament networks in suprabasal keratinocytes is demonstrated by these mutations, which are analogous to the K5/K14 dependence on the same domain for filament integrity in the basal cells in EBS. The early appearance of reciprocal clustering of mutations at the start of the type I rod domain and at the end of the type II rod domain was probably due to small sample numbers; it is now known that mutations at either end of the central rod domain of either a type I or type II keratin can cause blistering disorders. It seems probable that EBS-causing mutations will also be found at the start of helix 1A of K5. To date there have been no mutations found in the helix termination peptide of a type I keratin. Whether this is because such mutations are non-pathologic, or are lethal, or whether this apparent bias is still due to small sample size remains to be seen.

Although the mutation K1 $S_{185}P$ observed here is not in as highly a conserved residue as those reported previously, the transition from serine to proline in this α-helical domain is predicted to be highly disruptive. It appears that the 1A domain must exist in an α-helical conformation or the cytoskeletal properties of the filament network are significantly compromised. Whether the defects interfere with nucleation, elongation, or higher-order filament assembly is unclear.

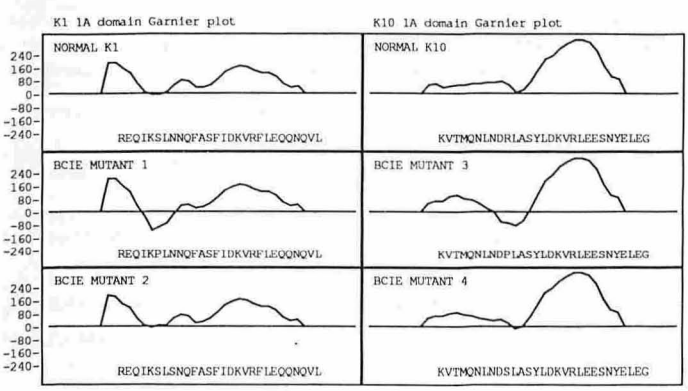

Figure 6. Garnier α-helicity plots of normal keratin 1 *(left)* and keratin 10 *(right)* helix 1A domains and the four mutant keratins described here. Proline is seen to cause severe disruption of the α-helix in either protein; however, the K1 $N_{187}S$ and the K10 $R_{156}S$ mutations have comparatively mild effects on the predicted helicity of this region.

In contrast, the $N_{187}S$ occurs in a residue that is conserved between all type I, II, and III intermediate filament proteins characterized thus far. The transition from asparagine to serine is semi-conservative, both amino acids having uncharged polar side chains. However, this mutation nevertheless produces a clinical phenotype that is as severe as those observed with proline substitutions demonstrating that even slight changes in this highly conserved region of the 1A domain cannot be tolerated for functional filament formation.

The K10 mutations reported here both affect the conserved residue R_{156} in the 1A domain. The mutation $R_{156}H$ in K10 has been previously reported to be linked to BCIE/EHK, and the analogous arginine (R_{125}) of K14 has also been found to be mutated to histidine or cysteine in patients with the Dowling-Meara form of EBS. Of all the keratin mutations identified to date in mechano-bullous diseases of which we have information, at least 14 of 32 affect exactly this arginine residue, so it is not surprising to find two more in the four mutations we document here. The DNA motif CpG, in which the C is often methylated in mammalian coding sequences, occurs in

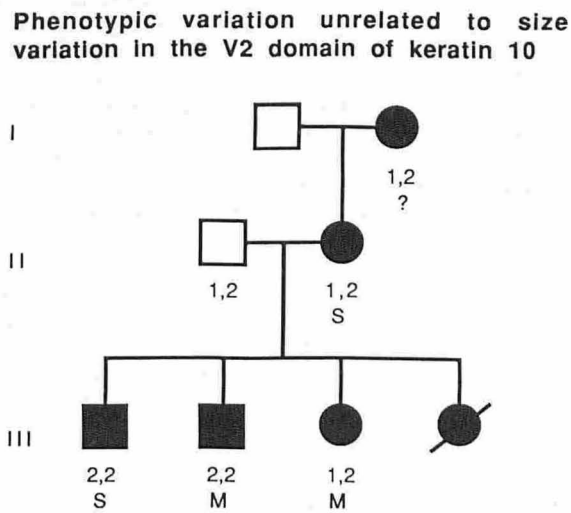

Figure 7. Pedigree showing the occurrence of severe (S) or mild (M) phenotype within BCIE family 1 under study. Results of PCR analysis of the polymorphic V2 domain of keratin 10 are shown. Allele 1 is approximately 525 bp; allele 2 is approximately 450 bp. A tandem repeat (essentially GGC repeating units) occurs in the coding sequence and thus the gene product of allele 1 has an additional 25 amino acids (approximately) in the tail domain. However, this extended tail domain is seen to be unrelated to the observed phenotypes.

this arginine codon in both K10 and K14. Methyl-C can be spontaneously deaminated to become T, resulting in C-T (or G-A mutations). The arginine-to-histidine and arginine-to-cysteine mutations reported represent the two possible products of deamination of the methyl-C on the antisense and sense strands, respectively. The two mutations described here in this codon involve C-A and G-C transitions and therefore CpG deamination events are not the only cause of BCIE mutations in this codon, although these are probably more common. This residue is very highly conserved in the type I keratins and the range of mutations seen so far in this codon in the 1A domain show that a variety of amino acid substitutions are deleterious to the *in vivo* function of the K1/10 cytoskeleton.

Taken together, the mutations reported here in K1 and K10 emphasise the importance of the 1A domain in the function of keratin intermediate filaments. It is interesting to note that all the helix 1A mutations found thus far that are associated with filament aggregation and severe clinical phenotype are clustered near the start of the rod domain. The only mutation seen at the internal end of this domain ($E_{144}A$ in K14) produces a very mild recessive EBS phenotype despite being a non-conservative amino acid substitution [28]. Heterozygous individuals in that particular family showed no clinical features of the disease. There is a marked difference in the degree of conservation observed between the amino-terminal start of 1A (which is highly conserved between the various keratins) and the carboxyl-terminal end of the domain (which is only moderately conserved). Although the sample number is still small, there would appear to be a broad correlation between disease severity and the position of the mutation within helix 1A. This in turn correlates well with data suggesting that the first 10 or so residues of 1A are involved in overlap interactions between dimers in the assembly of keratin filament structures [24]. From this, one would predict that mutation in these residues (or close by) would have much more serious effects on filament formation than mutations near the carboxy-terminal terminal end of helix 1A. This is supported by all the mutations reported to date.

The possible role of known polymorphisms in V2 domains of K1 and K10 in the marked phenotypic variation exhibited in this family was investigated, but results failed to indicate any relationship between size of these domains and phenotypic heterogeneity. Genetic linkage analysis of a large kindred with highly polymorphic DNA markers may reveal the involvement of non-keratin genes encoding accessory proteins in this phenomenon. Data have been reported that suggest linkage of the rare Ogna form of EBS to loci on chromosome 8 [29]. Because all keratin genes cloned to date have been found to map to gene clusters on chromosomes 12q and 17q, it seems possible that a non-keratin gene may be involved in this disorder. Genes for keratin-associated or regulatory proteins may play a hitherto unrecognized role in keratin filament assembly, structural integrity of the cytoskeleton or in phenotypic variation of these diseases. Identification and characterization of more accessory proteins may be essential to gain complete understanding of keratin filament biology.

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