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Mutations in the non-helical linker segment L1-2 of keratin 5 in patients with Weber-Cockayne epidermolysis bullosa simplex

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SUMMARY

Keratins are the major structural proteins of the epidermis. Analyzing keratin gene sequences, appreciating the switch in keratin gene expression that takes place as epidermal cells commit to terminally differentiate, and elucidating how keratins assemble into 10 nm filaments, have provided the foundation that has led to the discoveries of the genetic bases of two major classes of human skin diseases, epidermolysis bullosa simplex (EBS) and epidermolytic hyperkeratosis (EH). These diseases involve point mutations in either the basal epidermal keratin pair, K5 and K14 (EBS), or the suprabasal pair, K1 and K10 (EH). In severe cases of EBS and EH, mutations are found in the highly conserved ends of the α-helical rod domain, regions that, by random mutagenesis, had already been found to be important for 10 nm filament assembly. In order to identify regions of the keratin polypeptides that might be more subtly involved in 10 nm filament assembly and to explore the diversity in mutations within milder cases of these diseases, we have focused on Weber-Cockayne EBS, where mild blistering occurs primarily on the hands and feet in response to mechanical stress. In this report, we show that affected members of two different W-C EBS families have point mutations within 1 residue of each other in the non-helical linker segment of the K5 polypeptide. Genetic linkage analyses, the absence of this mutation in >150 wild-type alleles and filament assembly studies suggest that these mutations are responsible for the W-C EBS phenotype. These findings provide the best evidence to date that the non-helical linker region in the middle of the keratin polypeptides plays a subtle but significant role in intermediate filament structure and/or intermediate filament cytoskeletal architecture.

Key words: genetic skin disease, keratin, intermediate filament

INTRODUCTION

At the interface between the traumas of the environment and the body, the epidermis serves a protective function, which it manifests by an extensive cytoskeletal architecture of 10 nm intermediate filaments (IFs) of type I and type II keratins (reviewed by Albers and Fuchs, 1992). As basal epidermal cells cease to divide and move towards the skin surface, they switch from expressing K5 (type II) and K14 (type I) to a new keratin pair, K1 (type II) and K10 (type I) (Fuchs and Green, 1980; Roop et al., 1987). This switch is accompanied by an increase in filament bundling that may enhance the ability of keratins to survive the ensuing destructive phase of terminal differentiation.

Each IF is composed of ~20,000-30,000 polypeptides that, in vitro, assemble into complex 10 nm structures in the absence of any apparent auxiliary proteins or factors (reviewed by Conway and Parry, 1988; Albers and Fuchs, 1992). Approximately 32 polypeptides constitute the diameter of an IF, and approximately 500-1,000 polypeptides linked end-to-end are needed to give rise to a 20-40 μm length.

The typifying feature of a cytoskeletal IF polypeptide is a central 310 amino acid α-helix, referred to as the rod domain. An IF rod contains heptad repeats of hydrophobic residues that enable intertwining of a second IF protein along this hydrophobic seal, thereby forming a coiled-coil dimer, which is the basic IF subunit. Interspersed by three short non-helical linker regions (L1, L1-2 and L2), the rod is subdivided into four segments, referred to as helix 1A, 1B, 2A and 2B. Type I and type II keratin rod domains form parallel, unstaggered, coiled-coil heterodimers, which in IF assembly then align, in an anti-parallel manner, in a complex hierarchy of lateral and end-to-end associations. Recent chemical crosslinking studies and computer modeling suggest that IF assembly involves both staggered (amino end overlap) and unstaggered interactions between dimers (Geisler et al., 1992; Steinert et al., 1993). In addition, models of end-to-end interactions between dimers invoke a small overlap between the amino-terminal ends of the 1A helices of one dimer and the carboxy-terminal ends of the 2B helices of another (Steinert et al., 1993). This configuration provides an explanation for the importance of these segments, first recognized from their high degree of sequence conserva-

tion and from molecular mutagenesis studies (Albers and Fuchs, 1987, 1989; Raats et al., 1990, 1991; Wong and Cleveland, 1990; Gill et al., 1990; Lu and Lane, 1990; Hatzfeld and Weber, 1991a; Letai et al., 1992).

The IF rod is flanked by non-helical head (amino) and tail (carboxy) segments that are variable in size and in sequence. Given the diversity of head and tail sequences and their protrusion on the IF surface, these domains likely impart specialized functions to IFs, a postulate supported by molecular mutagenesis studies (Coulombe et al., 1990; Lu and Lane, 1990; Hatzfeld and Weber, 1990b; Bader et al., 1991; Wilson et al., 1992).

Recently, transgenic mice were engineered to express epidermal keratin mutants that perturb 10 nm filament assembly in a dominant negative fashion (Vassar et al., 1991; Coulombe et al., 1991a; Fuchs et al., 1992). These mice exhibit skin blistering upon mild physical trauma. In the most severe cases, tonofilament clumping and cell degeneration occur in either basal or suprabasal cells, depending upon whether a K14 or a K10 mutant gene is expressed. These features are the hallmarks of severe forms of the autosomal dominant diseases epidermolysis bullosa simplex (EBS; a basal cell disorder) and epidermolytic hyperkeratosis (EH; a suprabasal cell disorder). The degree of blistering and filament disorganization is a function of the level of transgene product and the degree to which a mutation perturbs filament assembly (Coulombe et al., 1991a).

Recent studies using improved markers have shown that the genetic defects of several families with moderate to mild forms of EBS map to human chromosomes 17 or 12 (Bonifas et al., 1991; Ryynanen et al., 1991; McKenna et al., 1992; Chan et al., 1993), at locations corresponding to the loci for type I and type II keratin gene clusters, respectively (Rosenberg et al., 1988, 1991; Lessin et al., 1988). Unequivocal evidence that human EBS arises from genetic defects in K5 and K14 came from sequencing these genes cloned from patients. Thus far, ten severe (Dowling-Meara, D-M) EBS cases have been shown to contain point substitutions in either their K5 or K14 genes. In our first study of D-M EBS, we found a K14 R125:C point mutation in one case and a K14 R125:H mutation in another (Coulombe et al., 1991b). Seven additional D-M EBS cases with the R1-25:C/H mutation have now been identified (Fuchs and Coulombe, 1992; Stephens et al., 1993). Affected members of another D-M EBS family have a K5 E475:G mutation (Lane et al., 1992). It is noteworthy that the D-M mutations are in the highly conserved rod ends of K5 and K14, i.e. regions especially critical for IF assembly (Letai et al., 1992; Steinert et al., 1993). Point mutations have also been found in these domains of the K1 and K10 genes in EH (Cheng et al., 1992; Rothnagel et al., 1992).

In contrast to D-M, two moderate (Koebner, K) EBS cases have proline mutations that map within the α-helical rods of K14 or K5, but outside the conserved ends (Bonifas et al., 1991; Dong et al., 1993). It is notable that a genetically engineered proline within a few residues of these naturally occurring ones is not as disruptive to IF formation as more subtle mutations within the rod ends (Letai et al., 1993).

So far, the mildest cases of EBS and EH have keratin gene mutations that map outside the α-helical rod. A family with mild EH has a 160L:P point mutation in the head domain of K1 (Compton et al., 1992; Chipev et al., 1992), and affected members of two unrelated mild (Weber-Cockayne, W-C) EBS families have the point mutation, I161:S, in the head domain of K5 (Chan et al., 1993). Intriguingly, these mutations reside in a portion of the head domain that is not only conserved among type II keratins, but is also important for filament assembly (Wilson et al., 1992; Chipev et al., 1992).

Exploring the mild forms of EBS and EH has begun to provide valuable insights into mutations that can have subtle effects on 10 nm filament assembly. It is remarkable that the mutations are clustered in the three mild cases of EBS and EH studied thus far. To determine whether there are mutations in other regions of the keratin polypeptides that might contribute to mild forms of these diseases, we explored the genetic basis of two additional incidences of W-C EBS. In this report, we attribute the genetic basis of EBS in these families to either of two K5 point mutations separated by a single amino acid and located within the non-helical linker segment (L1-2) separating helices 1 and 2. We provide functional studies to show that these mutations are likely to be responsible for the subtle perturbations on keratin filament networks that exist within affected members of these families. When combined with other recent reports, this study reveals a hitherto unappreciated, but marked importance of a segment of the L1-2 linker in IF structure.

MATERIALS AND METHODS

Biopsies and blood samples

One clinically affected member of each family donated skin biopsies for culturing keratinocytes and for ultrastructural studies. Blood samples (15-20 ml) were taken for isolation of genomic DNA.

Chromosome mapping and DNA sequence analysis

Nucleotide primers were selected for polymerase chain reaction (PCR) amplification of DNAs encompassing polymorphic sites at or near the keratin clusters of chromosomes 17 and 12. PCRs were performed on genomic DNA isolated from blood of family members. DNAs were then resolved by gel electrophoresis, as described (Weissenbach et al., 1992). Primers: D17S800 (AMF200zf4), GGTCTCATCCATCAGGTTTT and ATAGACTGTGTACTGGGCATTGA (Weissenbach et al., 1992); K1, ATAACTGAGCTTCCTCTTGC and GGATCCCCGGCCTCCTATGG; 1G12A/CA, ACTTCAGGAGGTTAAGAATGAATCC and CCTGGCTACTATGTTGGACAGC; 262B3/CA, CTAGCTTTCTACTGGATTTCCTTTC and TGGATCGACATGACCACA. DNA probe/enzyme systems used were: pWV214/*Hin*fI (locus COL2A1; Weaver et al., 1989), pCMM86/*Hin*fI (locus D17S74; Nakamura et al., 1988a); pcEFD33/*Msp*I (locus D12S14; Nakamura et al., 1988b).

RNAs from cultured keratinocytes were primed with random hexamers and reverse transcribed into cDNA (Coulombe et al., 1991b). In some cases, genomic DNAs from blood were used, in which case, exon-specific K5 primers were used. All reactions were amplified by PCR and sequenced, using biotinylated primers and solid-phase DNA sequencing, or CircumVent (New England Biolabs, Beverly, MA). K5 primers were chosen from the published sequence (Lersch et al., 1989). PCR/sequencing were always repeated in duplicate to verify that putative mutations did not arise from polymerase artifacts.

After confirming the presence of a mutation in the cDNA, the entire rod domain region of K5 was amplified as a 1276 bp fragment using primer set K5-5′1B (sense: TTCAGGAACCGGTTTGG) and K5 VIII-3′ (antisense: AGATGTTGACTGGTCCA). The fragment was subcloned into pCRII plasmid vector (TA Cloning System, Invitro-

gen, San Diego, CA) according to the manufacturer's specifications. Upon screening, clones were isolated and sequenced using standard dideoxy sequencing procedures.

PASA analysis of the N:K mutation

To detect the K5 N:K mutation, PCR amplification of specific alleles (PASA) was used with the following primers: wild-type, sense-strand primer (WT+), CAAGCGTACCACTGCTG; wild-type, antisense-strand primer (WT−), CAGGTCCAGGTTGCGGTTG (G is wild type); mutant antisense-strand primer (Mut−), CAGG TCCAGGTTGCGGTTT (T is in one allele of affected family 34 members).

FPLC purification of keratins, filament assembly and quantitation

Bacterially expressed keratins were isolated from inclusion bodies and purified by a Mono-Q anion exchange column (Coulombe et al., 1990). Keratins were concentrated by filtration through Centricon-10 units (Amicon; W. R. Grace & Co., Boston, MA). K5 and K14 or corresponding mutants were mixed in a 1:1.05 ratio (K5:K14) and purified as a heteromeric complex by FPLC. All buffers contained 10 mM β-mercaptoethanol and were at pH 7.5. Complexes were equilibrated by dialysis against the following buffers: (1) 9.5 M urea, 10 mM Tris-HCl (10 hours, RT); (2) 4 M urea, 10 mM Tris-HCl (12 hours, RT); (3) 1 M urea, 10 mM Tris-HCl (2 hours, 4°C); (4) 5 mM Tris-HCl buffer (2 hours, 4°C), twice. Filaments were negatively stained (1.25% uranyl acetate) and examined by EM (Coulombe et al., 1990).

For quantitation, random series of micrograph negatives from each experimental condition were examined under a Nippon Kogaku shadowgraph. Filament widths were measured from 20 randomly selected filaments. Quantitative analyses were made using the Macintosh StatView program. The variation in filament width among a given population was indicated by the coefficient of variance. Lengths were estimated by determining the total length of filaments in a given micrograph and dividing by 2× the number of free ends.

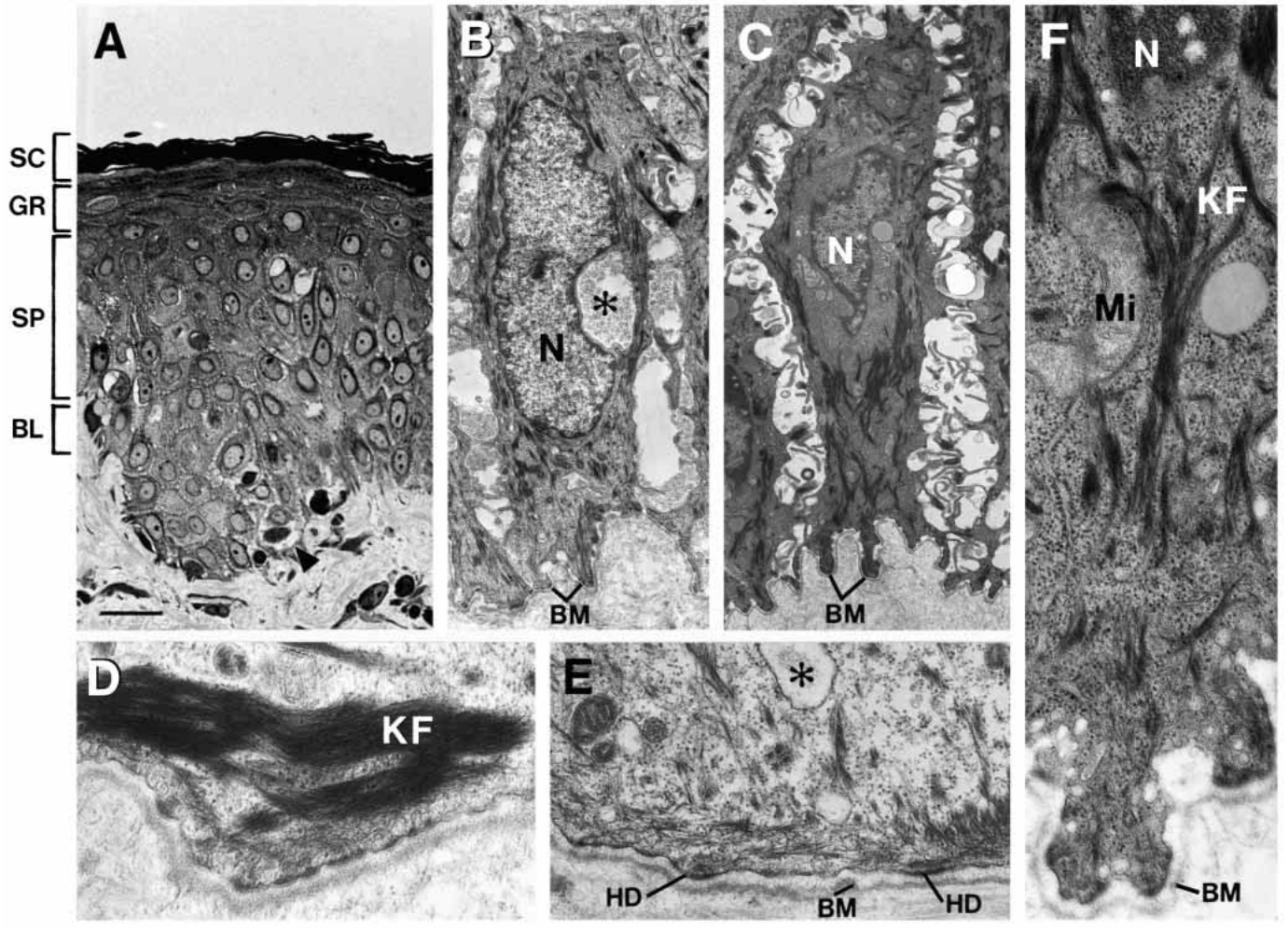


Fig. 1. Histology and ultrastructure of W-C EBS. (A) Semi-thin section of epidermis from W-C EBS 33.12. Note isolated cytolyzed cell in basal layer (arrowhead). (B) Ultrastructure of basal cell from 33.12 skin. Note cytolysis (asterisk), found in isolated basal cells along the basement membrane (BM). (C) Ultrastructure of a control basal layer. (D) Thick keratin filament bundle from 33.12 basal cell. (E) Disoriented and atypically short keratin filaments from 33.12 basal cell. (F) Classical keratin cytoskeleton in a portion of a control basal cell. Note: upon review of many micrographs, differences shown were representative, but not found in all micrographs. Bars: 18.2 μm (A), 1.5 μm (B), 1.8 μm (C), 0.3 μm (D), 0.5 μm (E) and 0.5 μm (F). N, nucleus; BL, basal layer; SP, spinous layer; SC, stratum corneum; GR, granular layer; KF, keratin filaments; HD, hemidesmosomes; Mi, mitochondria, BM, basement membrane.

Linkage analyses

Linkage analyses were performed using the MLINK program of the Linkage Analysis Package II, version 5.10 (Dr J. Ott, Columbia University, NY, NY).

RESULTS

Diagnosis of EBS families

W-C EBS patients of two families used for this study exhibited mild blistering of palmar and plantar skin. Light microscopy revealed typical signs of basal cell degeneration (arrowhead in Fig. 1A; reviewed by Anton-Lamprecht, 1983), while suprabasal layers displayed no major abnormalities. Ultrastructural analyses uncovered additional traits of EBS (Fig. 1B, compare with normal basal cell in 1C). Approximately 10-20% of basal epidermal cells displayed mild perturbations, including: (a) signs of vacuolization or cytolysis, often adjacent to the nucleus (asterisk in frame B); (b) an overall increase in lateral bundling of tonofilaments in some cell regions (frame D, W-C EBS 33·12); (c) disorientation of a disproportionate number of short filaments in other cell regions (frame E); and (d) a sometimes peculiar concentration of mitochondria around the nucleus (not shown). However, in contrast to more severe (D-M) EBS, basal keratin was filamentous and not clumped in amorphous aggregates. Moreover, although classical wild-type keratin networks (frame F) were relatively rare in W-C EBS skin, some W-C EBS keratin cytoskeletons were difficult to distinguish from wild-type.

Linkage analyses of W-C EBS families 33 and 34

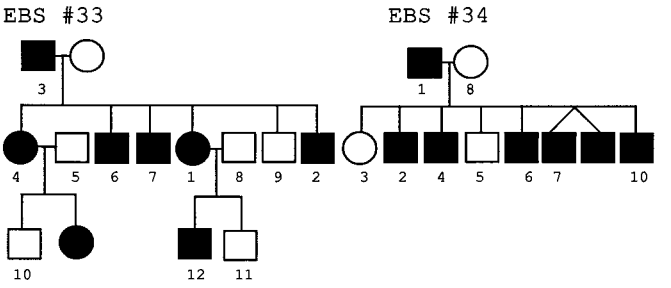
The two families used for our study (see Fig. 2) were sufficiently large to provide strong linkage data (Table 1). The AFM200zf4 marker at the D17S800 locus, near the K14 gene on chromosome 17 (17q12-q21; Rosenberg et al., 1988), has six allelic variations (Weissenbach et al., 1992). Electrophoretic separation of genomic PCR fragments encompassing this polymorphic site revealed that no single D17S800 allele cosegregated with the EBS disease in families 33 and 34 (Fig. 2). Similarly, pCMM86 at the D17S74 locus has four allelic variations (Nakamura et al., 1988a), none of which cosegregated with the disease. With the AFM200zf4 marker, the lod score at θ=0.01 was −4.21 for both families. With the pCMM86 marker, the lod score at θ=0.01 was −4.21 and −2.08 for EBS 34 and 33, respectively. These data indicate that the genetic defects for these two families do not reside on chromosome 17.

To assess whether EBS in families 33 and 34 is linked to 12q12-q14, where the K5 gene resides (Rosenberg et al., 1991), we used three polymorphic markers. pEFD-33.2 at locus D12S14, has four allelic variations (Nakamura et al., 1988). Allele 1 cosegregated with the EBS disease in family 34, with a lod score at θ=0.00 of 1.51 (Table 1). 1G12A/CA and 262B3/CA each have five allelic variations and were discovered in a cosmid that maps to 12q13 and that contains a keratin-like sequence. Both markers revealed a chromosome 12 allele cosegregating with the EBS disease in both families (Fig. 2). The combined lod score at θ=0.00 was 3.92 and 3.28 for markers 1G12A/CA and 262B3/CA, respectively (Table 1). Taken together, these data provide strong evidence of linkage to the K5 gene in both families.

A methionine to threonine point mutation within the L1-2 linker segment of the K5 rod domain

Since all of the mild cases of EBS and EH thus far examined have mutations within the head domain of the type II keratin polypeptide, we first searched this region of K5 for a mutation. However, sequences corresponding to the K5 head domain that had been amplified from keratinocyte mRNAs of EBS 33.12 and 34.1 showed no mutations.

We next amplified and sequenced remaining regions of 33.12 K5 mRNAs. A single point substitution at nucleotide 980 (A of the first ATG = 1; Lersch et al., 1989) distinguished one K5 allele from wild-type (Fig. 3A). The transition caused a methionine (ATG) to threonine (ACG) mutation at codon 327 in the L1-2 linker segment. Both A and G occurred in the sequencing ladder of the non-coding strand (T and C in the coding strand). In contrast, the control sequence from wild-type keratinocyte mRNA showed only an A at this position (Fig. 3B). When subcloned, some cDNAs contained only the mutant G (Fig. 3C), while others contained the wild-type A (Fig. 3D). This confirmed the existence of both mutant and



WC-EBS33

Ind	K1	1G12A/CA	262B3/CA	D17S800	K5/Nco1	COL2A1	D17S74
33.1	2/2	2/4	3/3	4/4	1/2	1/1	2/2
33.2	2/2	2/4	3/3	4/5	1/2	1/1	2/3
33.3	2/2	3/4	2/3	4/5	1/2	1/1	2/3
33.4	2/2	3/4	3/3	4/4	1/2	1/1	2/3
33.5	1/2	1/2	3/4	3/4	1/1	1/2	1/3
33.6	2/2	2/4	3/3	2/5	1/2	1/1	2/2
33.7	2/2	2/4	3/3	4/4	1/2	1/1	2/2
33.8	1/2	3/3	1/3	2/5	1/1	3/3	2/2
33.9	2/2	2/3	2/3	4/4	1/1	1/1	2/2
33.10	2/2	2/3	3/4	3/4	1/1	1/2	1/3
33.11	1/2	2/3	3/3	2/4	1/1	1/3	2/2
33.12	2/2	3/4	1/3	4/5	1/2	1/3	2/2

WC-EBS34

Ind	K1	1G12A/CA	262B3/CA	D17S800	K5/PASA	D12S14	COL2A1	D17S74
34.1	2/2	2/3	3/4	1/4	1/2	1/2	1/3	1/4
34.2	2/2	2/3	1/4	2/4	1/2	1/2	1/1	1/2
34.3	2/2	3/3	1/3	1/2	1/1	2/2	1/3	3/4
34.4	2/2	2/3	1/4	2/4	1/2	1/2	1/1	1/2
34.5	2/2	3/3	1/3	2/4	1/1	2/2	1/1	1/2
34.6	2/2	2/3	1/4	1/2	1/2	1/2	1/1	3/4
34.7	2/2	2/3	1/4	1/2	1/2	1/2	1/1	2/4
34.8	1/2	3/3	1/2	2/2	1/1	2/2	1/3	2/3
34.10	2/2	2/3	1/4	1/2	1/2	0/0	0/0	0/0

Fig. 2. Pedigrees of W-C EBS families 33 and 34 and chromosomal segregation of seven polymorphic loci. Patient numbers were assigned to members for which blood was obtained (skin biopsies and blood from 33.12 and 34.1). Closed symbols, members clinically affected with W-C EBS; open symbols, unaffected individuals; squares, males; circles, females. For each polymorphic marker, each PCR band corresponding to a unique allelic variation was arbitrarily assigned a number, and these numbers for each pair of alleles are listed in the tables as X/Y for each locus and patient.

Table 1. Linkage analyses of WC-EBS families 33 and 34

Pairwise lod scores between WC-EBS33 and various markers

	Recombination fraction						
Marker	0.00	0.01	0.05	0.10	0.20	0.30	0.40
K1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1G12A/CA	2.11	2.07	1.91	1.70	1.26	0.79	0.32
262B3/CA	1.47	1.45	1.34	1.20	0.90	0.56	0.20
K5/Ncol	2.41	2.37	2.21	2.00	1.54	1.02	0.45
COL2A1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D17S800	–99.99	–4.21	–2.16	–1.33	–0.58	–0.23	–0.05
D17S74	–99.99	–2.08	–1.37	–0.98	–0.49	–0.20	–0.05

Pairwise lod scores between WC-EBS34 and various markers

	Recombination fraction						
Marker	0.00	0.01	0.05	0.10	0.20	0.30	0.40
K1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1G12A/CA	1.81	1.78	1.65	1.49	1.13	0.72	0.28
262B3/CA	1.81	1.78	1.65	1.49	1.13	0.72	0.28
D12S14	1.51	1.48	1.37	1.23	0.92	0.58	0.21
K5/PASA	1.81	1.78	1.65	1.49	1.13	0.72	0.28
COL2A1	–99.99	–0.81	–0.19	0.02	0.12	0.09	0.03
D172A1	–99.99	–4.21	–2.16	–1.33	–0.58	–0.23	–0.05
D17S74	–99.99	–4.21	–2.16	–1.33	–0.58	–0.23	–0.05

wild-type sequences in the cDNA pool. Complete sequencing of 33.12 K5 cDNAs did not uncover additional mutations.

Cosegregation of the 327 M:T mutation with the disease in W-C family EBS 33

The 327 M:T mutation obliterated an *Nco*I site, enabling a rapid assessment of whether the mutation cosegregated with the EBS disease (Fig. 4, top gel). When digested with *Nco*I, a control 1330 bp genomic PCR DNA encompassing this site was cleaved to 830 and 500 bp (lane C). In contrast, PCR DNAs from 7 affected members of family 33 were ~50% (on a molar basis) undigested. Alleles from unaffected family members were homozygous for this site, as were genomic DNAs from 78 normal individuals. Thus, the loss of this site cosegregated with the disease, and it was not found within the general population.

An N:K point mutation within the L1-2 linker segment of the K5 rod domain

For family 34, we amplified and sequenced the K5 coding sequence from genomic DNAs. Surprisingly, a point substitution was detected at nucleotide 987 in one of the K5 alleles of affected member 34.1 (Fig. 5A). The transversion caused an asparagine (AAC) to lysine (AAA) mutation at codon 329 in the L12 linker segment of K5 and a mere codon away from the substitution discovered in family 33. Both G and T occurred in the sequencing ladder of the noncoding strand (C and A in the coding strand). This was true for only affected and not

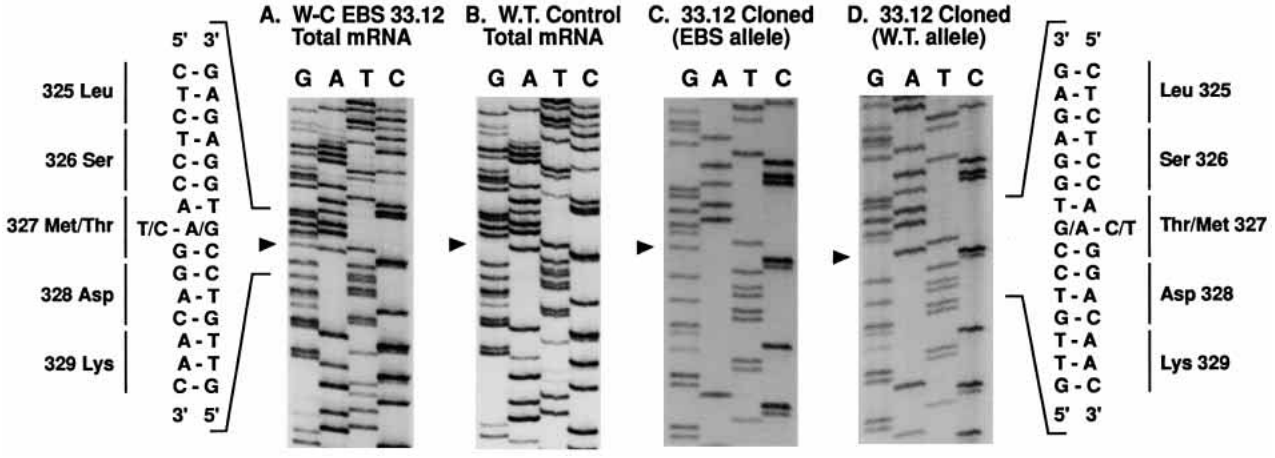


Fig. 3. T to C transition at codon 980 in one of two alleles of W-C EBS member 33.12. PCR was used to amplify K5 sequences from mRNA of 33.12 epidermal keratinocytes. Amplified fragments from two independent rounds of PCR were subjected to DNA sequencing, either directly or after subcloning. Note T to C transition (A to G in noncoding strand) at nucleotide 980 (codon 327) in one of two alleles (arrowhead). Sequences are from total K5 PCR mixture of 33.12 mRNA (A) or control mRNA (B); cloned cDNA from mRNAs of mutant 33.12 allele (C) or wild-type 33.12 allele (D).

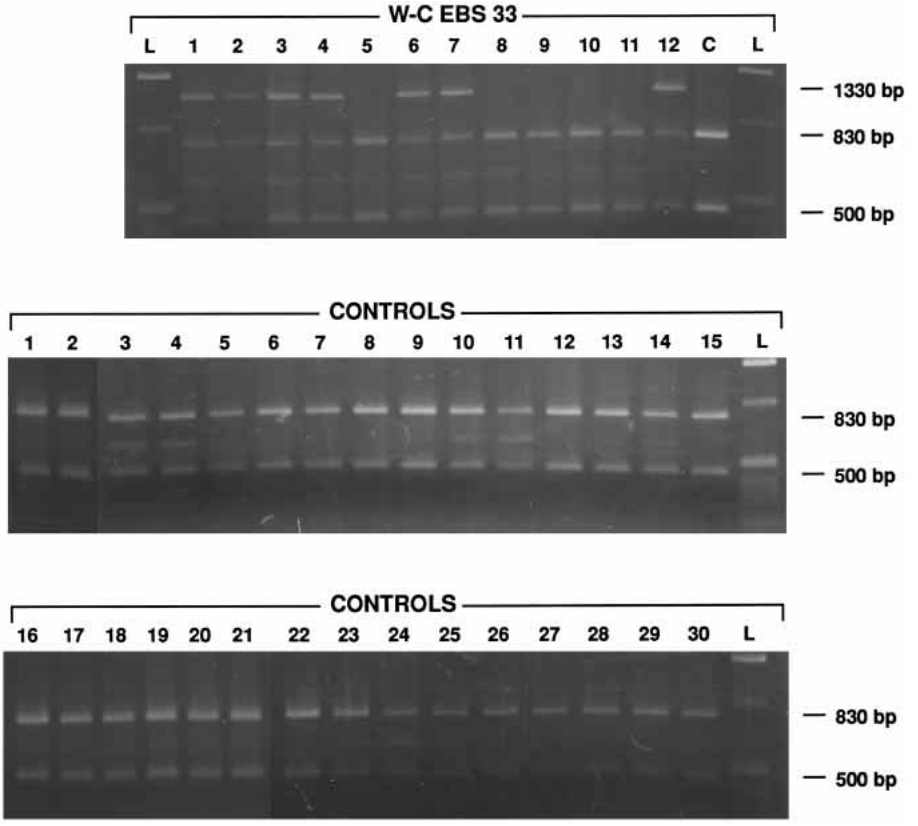


Fig. 4. Cosegregation of the 327 M:T mutation with the disease in W-C EBS family 33. Genomic DNAs were PCR amplified to produce a 1300 bp K5 DNA fragment encompassing the T to C transition at nucleotide 980. DNAs were digested with *Nco*I, which cleaved wild-type DNA, but not DNA containing the 980T:C mutation, to 830 bp and 500 bp fragments. Following digestion, DNAs were resolved by agarose gel electrophoresis, and stained with ethidium bromide. Top row: L, DNA ladder; C, control DNA; remaining lanes, DNAs from W-C EBS 33 members, 33.1-33.12, from left to right. Second and third rows, genomic DNAs from blood of first 30 of 78 normal individuals.

unaffected members of the family (examples in Fig. 5). Complete sequencing of 34.1 K5 cDNAs did not uncover additional mutations.

Cosegregation of the K5 329N:K mutation with the disease in W-C Family EBS 34

The 329 N:K mutation neither created nor obliterated a known restriction endonuclease site. Therefore, we resorted to allele-specific PCR assays (PASA) to rapidly assess whether the mutation cosegregated with the EBS disease in family 34 (Fig. 6). A wild-type primer set generated a 420 bp PCR fragment from DNAs of all family members, as well as from 65 control DNAs of normal individuals. In contrast, a mutant primer set only generated a 420 bp PCR fragment from genomic DNAs of affected family members. No PCR fragment was generated with the mutant primer set and DNAs from unaffected members or from 65 control DNAs. Thus, the 329N:K lesion occurred in a heterozygous fashion, correlated with the

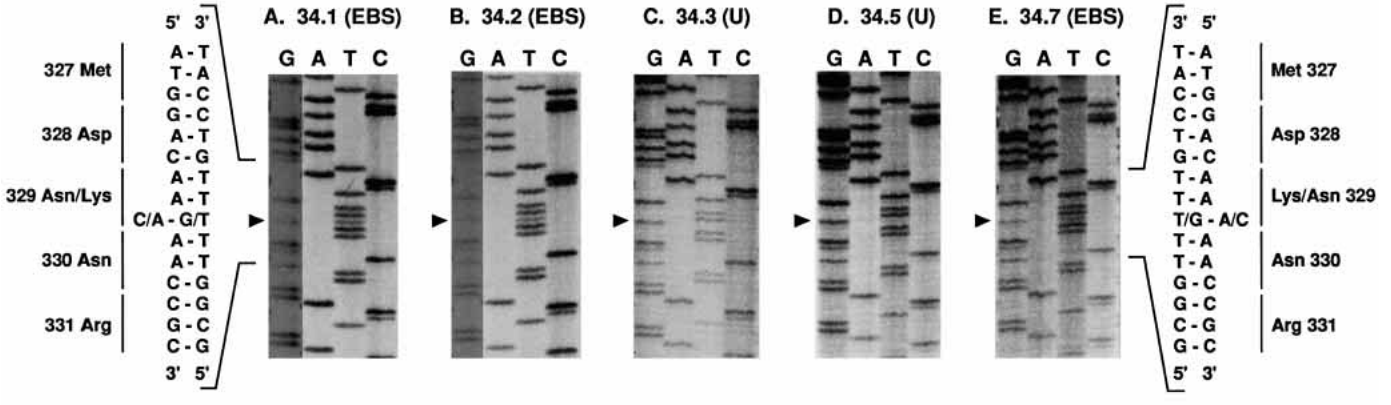


Fig. 5. C to A transversion at codon 987 in one of two alleles of affected individuals from W-C EBS family 34. PCR was used initially to amplify K5 sequences from 34.1 genomic DNA. Sequencing revealed a heterozygous C to A transversion (G to T in non-coding strand) at codon 329 of K5 (A). PCR was then used to amplify the region encompassing the putative 987 C to A transversion from genomic DNAs of each member assigned a number in Fig. 2. Shown are sequences from representative examples of non-coding strands from affected (EBS) and unaffected (U) individuals. The mutation cosegregated with affected members of W-C EBS family 34.

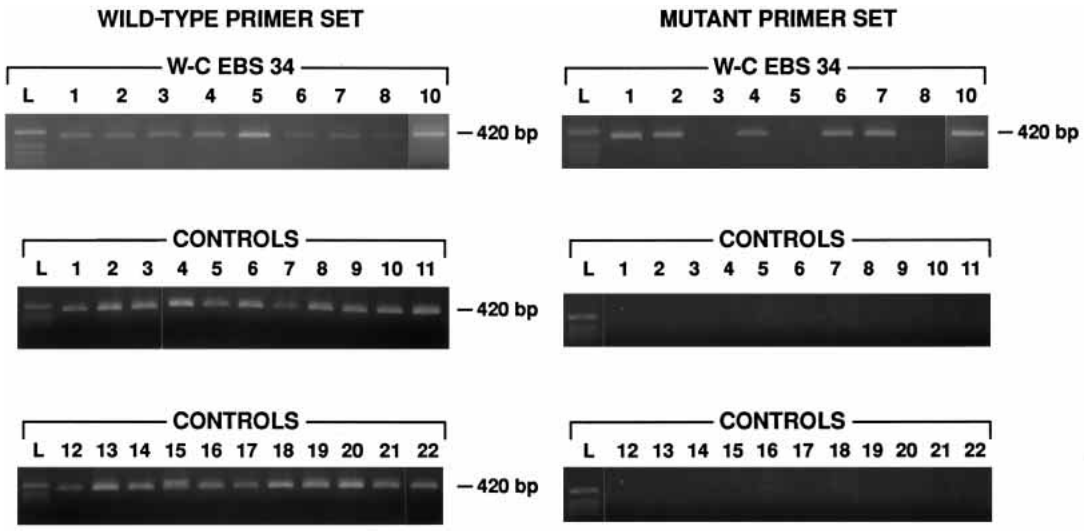


Fig. 6. Primer-specific amplification of the 329 N:K mutation in affected members of W-C EBS family 34. Genomic DNAs were PCR amplified using a common 3′ primer and one of two different 5′ primers. One 5′ primer was specific for the wild-type C residue at nucleotide 987, and the other was specific for the mutant A residue at nucleotide 987 (see Materials and Methods). DNAs were resolved by agarose gel electrophoresis, and stained with ethidium bromide. Left gels show bands generated by wild-type primer set; right gels show bands generated by mutant primer set. L, DNA ladder; W-C EBS 34 DNAs are listed in order from 34.1 to 34.10. Shown also are reactions from genomic blood DNAs of first 22 of 65 controls. Note that all samples generated an amplified fragment of 420 bp with the wild-type specific primer set, but only the affected members generated an amplified fragment with the mutant-specific primer set.

disease phenotype and was not likely to be a polymorphic variation.

The M327:T and N329:K mutations in linker 1-2 perturb the ability of keratin 5 to assemble with its partner into 10 nm filaments

Previously, we engineered 125R:H/C mutations in human K14 cDNA and demonstrated that these point mutations were sufficient to generate a type I keratin that was perturbed in its ability to form proper 10 nm filaments in vitro (Coulombe et al., 1991b; Cheng et al., 1992). To ascertain whether the M327:T and N329:K mutations can also elicit aberrations in 10 nm filament assembly, we engineered these mutations in human K5 cDNA, and used the pET expression vector to express the mutant proteins in bacteria. Following FPLC anion exchange chromatography, preparations of wild-type and mutant K5 proteins were then combined with wild-type K14, and the tetrameric complexes were isolated by a second round of FPLC (see Materials and Methods). As judged by SDS-polyacrylamide gel electrophoresis, these preparations were pure (Fig. 7A).

In two independent rounds of IF assembly using two separate preparations of purified keratins, filaments formed from wild-type K5 and K14 were long (>2,000 nm) and uniform (11.3 nm ± 0.7 nm diameter) (Fig. 7B). In contrast, filaments formed from either M327:T or N329:K mutant K5 and wild-type K14 were somewhat shorter (650-1190 nm and 520-1210 nm, respectively) and less uniform (Fig. 7C and D). Most notably, these mutant filaments were often unraveled, a feature that was also seen in the wild-type preparation, albeit to a far lesser extent (arrowheads in Fig. 7 denote unraveling). The greater degree of unraveling in mutant filaments was reflected in their increased diameter: the diameter of the unraveled filaments in the two mutant preparations was 24.7±0.7 and 22.1±0.7 nm, respectively, whereas the diameter of the unraveled filaments in the wild-type preparation was 19.7±0.7 nm. Overall, perturbations in 10 nm filament structure were more subtle than those seen previously for the R125:C/H K14 mutations (Coulombe et al., 1991b; Cheng et al., 1992). However, the differences were nevertheless readily discernable. These functional studies provide strong evidence that the L1-2 point mutations in K5 are functionally responsible for the W-C EBS phenotype in these two families.

DISCUSSION

A number of lines of evidence indicate that the T to C transition at nucleotide 980 of K5, and the C to A transversion at nucleotide 987 of K5, underlie the genetic bases for W-C EBS in families 33 and 34, respectively: (a) these mutations were not found in 130-150 wild-type alleles; (b) the mutations were carried only by affected family members; (c) the mutations were inherited in an autosomal dominant fashion, characteristic of the pattern of inheritance in these families; and (d) the mutations found were in the K5 gene on chromosome 12 (Rosenberg et al., 1991), and the peak combined lod score at $\theta=0$ was 3.92 for chromosome 12 (–8.42 at $\theta=0$ for chromosome 17).

Residues 327 and 329 of K5 are in the non-helical linker 1-2 segment of the IF polypeptide (Fig. 8). Residue 329 of K5 is asparagine in all type II keratins, but is otherwise not conserved. Residue 327 is methionine in all type II keratins and in some type I and III IF polypeptides. Interestingly, it has recently been reported that a family with K-EBS has a K14 M:R mutation in the equivalent L1-2 residue to our K5 M327:T

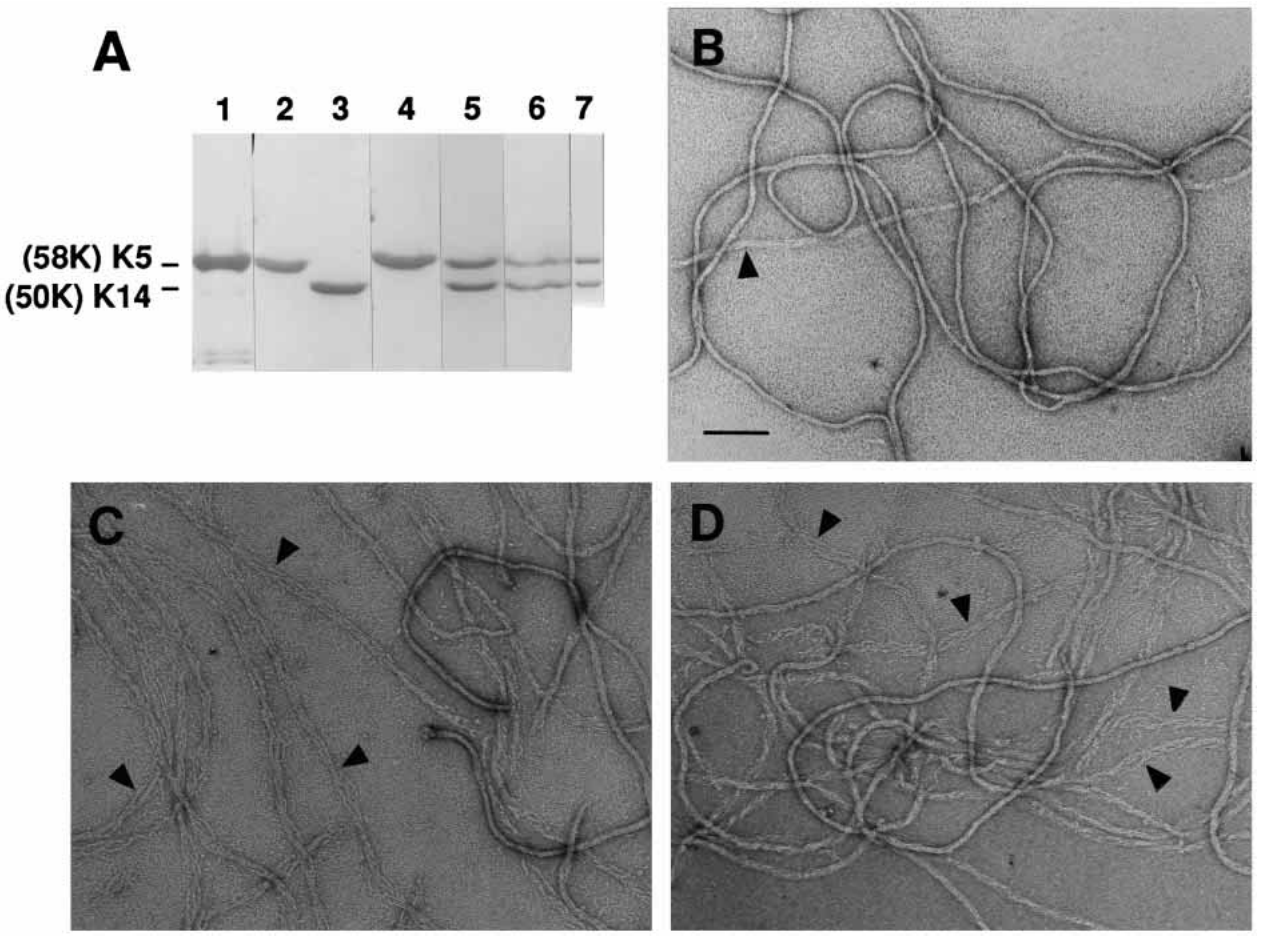


Fig. 7. The genetically engineered 327M:T and 329N:K linker point mutations perturb 10 nm filament formation in vitro. Site-directed mutagenesis was utilized to engineer the 327M:T and 329N:K point mutations in the coding sequence of a functional human K5 cDNA (Lersch et al., 1989). The pET8c bacterial expression vector was used to produce milligram quantities of wt human K14 (Marchuk et al., 1984) and mutant and wt K5. Following FPLC purification, proteins were subjected to type I-type II complex formation and then in vitro filament assembly (see Materials and Methods). Frame A shows a Coomassie Blue-stained SDS-polyacrylamide gel of: lane 1, wt K5; lane 2, 327M:T K5; lane 3, wt K14; lane 4, 329N:K K5; lane 5-7, purified complexes of wt K14 and: lane 5, wt K5, lane 6, 327M:T K5, lane 7, 329-N:K K5. Shown in addition are IFs assembled from wt K14 and: (B) wt K5; (C) 327M:T K5; (D) 329N:K K5. Note that filaments assembled from 327M:T K5 or 329N:K K5 are somewhat shorter, but most notably, they are more unraveled and less uniform than those assembled from wt K5 (arrowheads denote fraying). Bar, 200 nm.

(Humphries et al., 1993). Moreover, while our report was out for review, another study appeared, reporting two additional L1-2 linker mutations in W-C EBS families: one is R331:C in the K5 L1-2, and the other is V270:M in the K14 L1-2 (Fig. 8; Rugg et al., 1993). The fact that five different families with EBS have mutations in L1-2 of the keratin polypeptides lend strong support to the evidence that these basal keratin L1-2 linker mutations account for the EBS phenotype.

It is intriguing that the recently reported K14 272M:R L1-2 mutation produced a K-EBS phenotype (Humphries et al., 1993), while the similar K5 327M:T L1-2 mutation produced a clinically milder (W-C) case of EBS (this study). This variation in clinical severity generated by the mutated methionine residue could arise from either the specific mutation (M:R versus M:T), differences in the importance of the specific keratin in IF assembly (K14 versus K5) or in the polymorphic background of the two families. Indeed, even within a family with a specific keratin mutation, there seems to be some clinical heterogeneity, suggesting that there may be genetic factors in addition to the specific keratin mutation that can influence the clinical manifestations of these diseases.

A surprising finding emerging from the catalogue of keratin mutations in different EBS and EH patients is that mutations tend to be clustered, and sometimes even the exact same residue is mutated in different families with these diseases. Of the five EBS families with mutations in the L1-2 region, only one, the 331R:C K5 mutation (Rugg et al., 1993), involves a C to T transition at a CpG dinucleotide, i.e. a hotspot for mutagenesis (for review, see Fuchs and Coulombe, 1992). That L1-2 linker mutations have now been found in four of six analyzed incidences of W-C EBS suggests that mutations in the L1-2 linker may account for a significant proportion of W-C EBS cases.

The discovery that L1-2 residues are frequently mutated in mild to moderate EBS is especially intriguing in light of recent studies on a putative yeast IF protein, MDM1. An MDM1

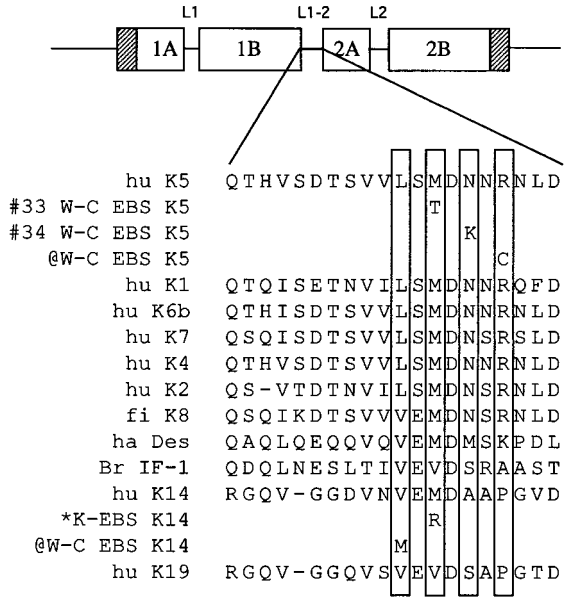


Fig. 8. K5 residues M327 and N329 in the L1-2 linker are conserved among type II keratins. Stick figure depicts secondary structure of human (hu) keratins. Large boxes encompass the central α-helical rod domain, separated into four segments by virtue of short non-helical linker segments, termed L1, L1-2 and L2. Hatched boxes denote the highly conserved end domains of the rod. Lines denote the non-helical head and tail domains, often conserved only for a single IF type. K5 327M:T and 329-N:K mutations are located in the L1-2 domain as is K14 272M:R, a mutation in a K-EBS family (Humphries et al., 1993), and the recently reported K5 331R:C and K14 270V:M in W-C EBS families (Rugg et al., 1993). Corresponding sequences are also provided from other type II IF proteins, including the fish (fi) keratin 8, and from the type III IF protein, hamster (ha) desmin, the cephalochordate *Branchiostoma lanceolatum* IF protein (Br-IF1) and the type I keratins, K14 and K19 (GenBank).

mutant fails to function in vivo or to assemble into IF-like structures in vitro when the temperature is shifted from 4°C to 37°C (McConnell and Yaffe, 1993). The ts MDM1 mutation is S257:N, at the residue equivalent to 331R of the K5 L1-2 linker. When this finding is coupled with our in vitro assembly studies on the K5 L1-2 linker mutations, it unveils the importance of a region of IF proteins that was hitherto poorly appreciated. How might the non-helical L1-2 linker be involved as a player in orchestrating 10 nm filament architecture? It is important to consider that the position of the L1-2 linker in the α-helical rod is conserved among all IFs (Hanukoglu and Fuchs, 1983), and mutations that remove the helix-destabilizing residues lead to filament aggregation in vitro (Letai et al., 1992). Other relevant points are that filaments assembled from K14 and either 327M:T or 329N:K K5 tend to unravel, with some overall shortening in length. Taken together, while the L1-2 linker may play some role in end-to-end interactions, it seems to play a prominent role in lateral associations. This is in quite striking contrast to the behavior of D-M EBS mutants (i.e. mutations in the rod ends), which seem to affect end-to-end interactions more prominently than lateral associations (Coulombe et al., 1991b).

Based on recent chemical cross-linking data (Geisler et al., 1992; Steinert et al., 1993), the carboxy end of the L1-2 linker region, i.e. the part of the linker where EBS mutations are clustered, is predicted to be in relatively close proximity to the region of putative overlap between two staggered, antiparallel keratin heterodimers. In a model of linear arrays of dimers, where the helix 1A ends of one dimer have been postulated to overlap with the helix 2B ends of the next dimer in line, the L1-2 linker segments of one row of dimers might be nearly opposite to the overlap segments of an adjacent row of dimers. If this model is correct, then the L1-2 linker region is in a position to play a major role in promoting appropriate staggered lateral associations. It may also play a role in stabilizing end-to-end interactions between dimers in an adjacent row. While further studies will be necessary to more fully elucidate the role of the L1-2 linker in IF assembly, our W-C EBS and filament assembly data presented here provide the first experimental evidence in support of this model, and add valuable new insights into our understanding of the role of this non-helical segment in IF structure.

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