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Genetics

The genetic basis of Weber–Cockayne epidermolysis bullosa simplex

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ABSTRACT **Epidermolysis bullosa simplex (EBS) is a group of autosomal dominant skin diseases characterized by blistering, due to mechanical-stress-induced degeneration of basal epidermal cells. Recently, it was discovered that the more severe types, Dowling–Meara and Koebner, are genetic disorders of the basal epidermal keratins, keratin 5 (K5) and keratin 14 (K14). Here, we show that the mildest type of EBS, Weber–Cockayne, is also a disorder of these keratins. Affected members of two unrelated families with Weber–Cockayne EBS had a T → G point mutation in the second base position of codon 161 of one of two K5 alleles, leading to an Ile → Ser mutation. This mutation was not present in unaffected members or in 156 alleles from normal individuals. Linkage analyses mapped the defect to the type II keratin gene cluster on chromosome 12q11–q13 (peak logarithm of odds score at θ = 0 of 3.0), providing strong additional evidence that this mutation is responsible for the Weber–Cockayne EBS phenotype. Conserved among type II keratins, Ile-161 is in the nonhelical head domain of K5, a region previously shown to be important for 10-nm filament assembly. The mutation generates a potential substrate site for protein kinase C, which could influence intermediate filament architecture, perhaps leading to the intrafilament association seen ultrastructurally in patients with the mutation.**

Epidermolysis bullosa simplex (EBS) has been subdivided into three major types (1, 2). Dowling–Meara (D–M) EBS is the most severe type, typified by blistering over whole body regions, keratin-filament clumping, and cytolysis in basal cells. Koebner EBS (K-EBS) is also characterized by generalized blistering and basal-cell cytolysis but with fewer abnormalities in basal-cell keratin networks. Weber–Cockayne (W–C) EBS is the mildest form, with blistering concentrated primarily in palmar and plantar regions, and very minor keratin-filament perturbations.

Anton-Lamprecht (1) originally suggested that D–M EBS might be a disorder of keratin, based on ultrastructural analysis of EBS skin. She noted that tonofilament clumping often preceded cell cytolysis, making it unlikely that cytolysis could be initiated by aberrant protease action. Experimental support for this hypothesis was not provided until years later, when it was discovered that transgenic mice expressing a keratin 14 (K14) mutant displayed abnormalities bearing a striking resemblance to D–M EBS (3). Subsequent analyses of the two known basal-specific keratin genes of human patients with D–M EBS or K-EBS led to the discovery of point mutations in either the K5 or K14 genes (refs. 4–6; see also ref. 7).

The known K14 and K5 point mutations of D–M and K-EBS reside in the α-helical rod domains involved in coiled-coil heterodimerization (8). D–M EBS mutations are clustered within the highly conserved rod ends (4, 6), whereas the K-EBS mutation is more internal (5). When engineered in wild-type K14 or K5 cDNA expression vectors, the D–M EBS mutations (*i*) perturbed keratin network formation in transfected keratinocytes and (*ii*) perturbed 10-nm intermediate filament (IF) assembly *in vitro* (4, 9). These aberrations were similar to those in cultured EBS keratinocytes (4, 10) and in EBS IFs (4), demonstrating that the mutations were functionally responsible for the disease. In contrast, the K-EBS mutation exhibited milder effects on IF assembly and network formation (9, 11), revealing a correlation between the mutation and disease severity.

The genetic basis for the milder type of EBS, W–C EBS, has not yet been determined. However, restriction fragment length polymorphism analyses have linked W–C EBS to either of the two major clusters of keratin genes on chromosomes 12 and 17 (5, 12). In addition, transgenic mice expressing a mildly disrupting K14 mutant exhibit basal-cell cytolysis in their paw skin (13). Thus it seems likely that the underlying genetic basis of W–C EBS is a mildly disrupting K14 or K5 mutation. We have now examined this possibility, using ultrastructural, chromosomal, and sequence analyses on nine families with W–C EBS.

MATERIALS AND METHODS

Biopsies and Blood Samples. One clinically affected member of each family donated skin biopsies for culturing keratinocytes and for ultrastructural studies (4). 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. DNAs were then resolved by gel electrophoresis, as described (14). Primers were as follows: D17S800 (AMF200zf4), GGTCTCATCCATCAGGTTTT and ATAGACTGTGTACTGGGCATTGA (14); D17S846 (GAS), TGCATACCTGTACTACTTCAG and TCCTTTGTTGCAGATTTCTTC (15); K1, ATAACTGAGCTTCCTCTTGC and GGATCCCCGGCCTCCTATGG. RNAs from cultured keratinocytes were primed with random hexamers and reverse-transcribed into cDNA (4). Reaction products were amplified by PCR and sequenced, using biotinylated primers and solid-phase DNA sequencing or CircumVent (New England Biolabs). K5 primers were chosen from the published sequence (16, 17).

RESULTS

Diagnosis of EBS Families. Affected family members exhibited mild blistering of palmar and plantar skin upon

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Abbreviations: EBS, epidermolysis bullosa simplex; D–M, Dowling–Meara; K-EBS, Koebner EBS; K5, K14, etc., keratin 5, keratin 14, etc.; W–C, Weber–Cockayne; IF, intermediate filament; lod, logarithm of odds.

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physical trauma. Ultrastructural analyses revealed features typical of EBS (Fig. 1*A*). Approximately 30–40% of basal epidermal cells displayed mild perturbations, including (*i*) signs of vacuolization, often adjacent to the nucleus, (*ii*) lateral aggregates of tonofilaments, and (*iii*) a sparcity of tonofilaments. Basal keratin was filamentous and often in wavy bundles (Fig. 1 *D* and *E*): None of the W–C EBS samples showed signs of basal tonofilament clumping typical of D–M EBS. Basal nuclei often appeared irregular and in two or more segments, suggestive of distortion or multinucleation (Fig. 1*C*). Endoplasmic reticulum vesiculation and cell degeneration were sometimes evident. None of the basal-cell aberrancies were typical of normal skin (Fig. 1 *B* and *F*). The mild aggregation and waviness of basal-cell keratin filaments and the apparent distortions in basal nuclei seemed more prominent than previously noted and provided a stronger link between keratin-filament abnormalities and the W–C EBS phenotype than had been appreciated.

Linkage Analyses of W–C EBS Families 37 and 26. Prior studies on a large W–C EBS family indicated linkage [peak logarithm of odds (lod) score at a θ of 0.001 = 7.43] to the keratin cluster on chromosome 12q11–q13 (5). Similar studies suggested linkage of one W–C EBS family to 17q12–q21 (peak lod score at a θ of 0.1 = 2.45) and linkage of another to 12q11–q13 (peak lod score at θ of 0.08 = 1.37) (12).

To explore the possibility that W–C EBS may be a genetic disorder of K14 and K5, we first tested for linkage of our families to probes recognizing highly polymorphic loci near the K14 gene (17q12–q21; ref. 18). AFM200zf4 at the *D17S800* locus adjacent to the keratin cluster on chromosome 17 has six allelic variations (14). Electrophoretic separation of genomic PCR fragments encompassing this polymorphic site revealed that no single *D17S800* allele cosegregated with the EBS disease in family 37, and moreover, both individuals 37.3 (unaffected) and 37.7 (affected) received allele A from their affected mother 37.2. Similarly, member 26.4 (unaffected) inherited allele B from individual 26.1 (affected), who received it from individual 26.5 (affected). With the AFM200zf4 marker, the lod score at θ = 0 for family 37 was −7.23 (two-point linkage analyses were performed using the MLINK program; the frequency of the W–C EBS allele was estimated at 1/50,000 with a penetrance value of 1.0). Studies with *D17S849*, adjacent to the keratin cluster on chromosome 17, were consistent with the notion that the genetic defect for these two families does not reside on chromosome 17 (Fig. 2).

To determine whether W–C EBS in families 37 and 26 might be linked to 12q11–q13, where the K5 gene resides (19), we used two polymorphic markers. 1G12A/CA has five allelic variations and was discovered in a cosmid that maps to 12q12 and contains a keratin-like sequence (R. Kucherlapati, personal communication). While this marker was not informative for family 26, allele B cosegregated with the disease in family 37 (Fig. 2). The other marker detects two allelic variations in K1 (20). Allele A, corresponding to the larger K1, cosegregated with the disease in both families. These data were suggestive that the defect in these families might be in the K5 gene.

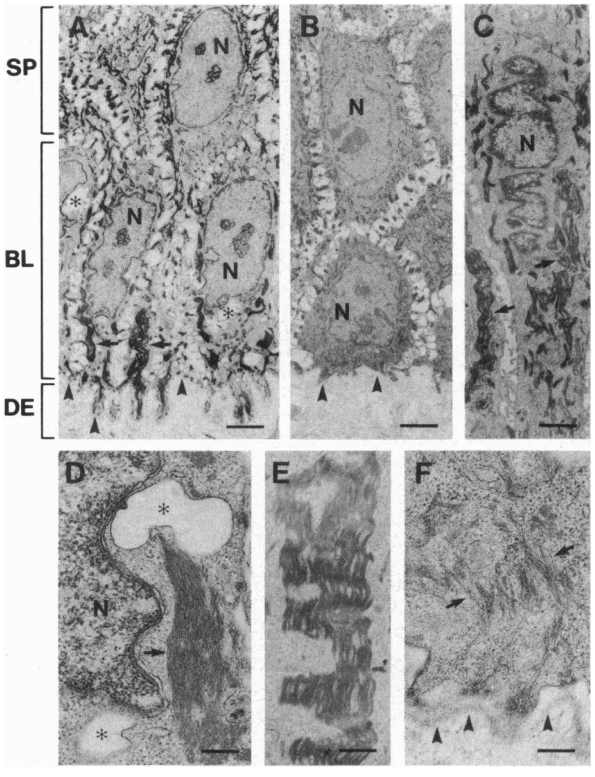

FIG. 1. Ultrastructure of W–C EBS. (*A*) Basal and spinous layers of epidermis from affected member of EBS family 37. Note vacuoles and wavy tonofilament aggregates in basal cells. (*B*) Normal skin. Note typically thin keratin tonofilaments in basal cells. (*C*) Extreme example of nuclear abnormalities in a basal cell from affected member of family 31. (*D*) High magnification of a tonofilament aggregate and vacuoles in basal cell of skin from individual 37.7. (*E*) High magnification of family-31 basal cell, showing bundles of wavy filaments. (*F*) High magnification of normal basal tonofilaments. N, nucleus; BL, basal layer; SP, spinous layer; DE, dermis; arrowheads, basement membrane; arrows, keratin filament bundles; asterisks, vacuoles. (Bars: *A*, 3 μm; *B*, 3 μm; *C*, 1.7 μm; *D*, 0.28 μm; *E*, 1.1 μm; *F*, 0.22 μm.)

A Point Mutation Encoding an Ile → Ser Substitution Near the Beginning of the K5 Rod Domain in W–C EBS Family 37. To ascertain whether the K5 gene was the source of the genetic defect in W–C EBS family 37, we isolated RNAs from keratinocytes cultured from a skin biopsy from a member of family 37 and PCR-amplified and sequenced the K5 cDNAs. Only a single-point substitution at nt 482 (A of the first ATG = 1; refs. 16 and 17) distinguished one of the K5 alleles from wild type. The transversion caused an Ile (ATC) → Ser (AGC) mutation at codon 161 (Fig. 3*A*). The PCR and sequencing were reproducible, indicating that the transversion was not due to a polymerase error during PCR. Both adenosine and cytidine occurred in the sequencing ladder of the noncoding strand (thymidine and guanosine in the coding strand). When subcloned, some cDNAs contained only the mutant guanosine (Fig. 3*B*), while others contained the wild-type thymidine (Fig. 3*C*), analogous to the control (Fig. 3*D*). This confirmed the presence of both mutant and wild-type sequences in the cDNA pool, as expected of an autosomal dominant disease.

Cosegregation of the Ile-161 → Ser Mutation with the Disease in Two Families in W–C EBS. The Ile-161 → Ser mutation obliterated a *Fok* I site, enabling a rapid assessment of whether the mutation cosegregated with the EBS disease (Fig. 4). When digested with *Fok* I, a control 555-bp genomic PCR DNA encompassing this site was cleaved to 466 bp. In contrast, PCR DNAs from seven affected members of family 37 were ≈50% (on a molar basis) undigested. These members were also heterozygous for an *Sfa*NI site, also obliterated by the T → G mutation (data not shown). Unaffected family members had alleles that were homozygous for the *Fok* I site.

Surprisingly, affected members from W–C EBS family 26 had DNAs that were also heterozygous for the *Fok* I site. The other seven families were homozygous as were 16 distinct incidences of D–M EBS and K-EBS. Genomic DNAs from 78 normal individuals were also homozygous verifying that the loss of this site was not merely reflective of a rare polymorphic variation. When the *Fok* I site was used as a genetic marker, a combined lod score at θ = 0 of 3.0 was obtained for families 37 and 26. This suggested strongly that the W–C EBS

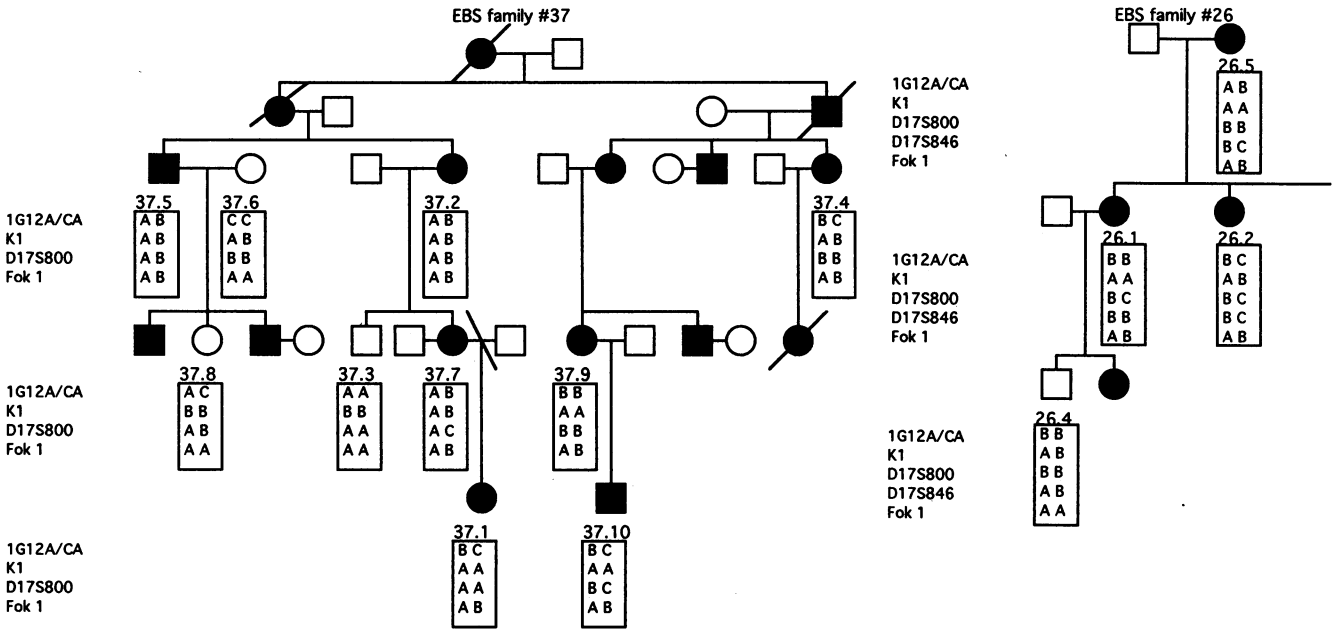

FIG. 2. Pedigrees of W–C EBS families 37 and 26 and the chromosomal segregation of four polymorphic loci. Numbers were assigned to members for which blood was obtained (skin biopsies and blood were obtained from members 37.7 and 26.5). The individual's alleles for each locus are boxed. Solid symbols, members clinically affected with W–C EBS; open symbols, unaffected individuals; cross bar to right, deceased; cross bar to left, divorced; square, male; circle, female.

lesion in these families resided within the keratin locus on chromosome 12q11–q13, specifically, within the K5 gene.

To verify that the heterozygous loss of the *Fok* I site from families 37 and 26 was due to the T → G transversion at codon 161, we sequenced their genomic DNAs (Fig. 5). Indeed, the exact same T → G transversion was present in both families and cosegregated with the disease. Moreover, the T → G transversion was present in ≈50% of the functional K5 alleles of affected, but not unaffected, family members.

Complete sequencing of the K5 cDNA from individual 37.7 did not uncover any additional mutations. However, there were a few discrepancies between published K5 sequences (16, 17) and our four wild-type K5 DNAs. The K5 residues Ser-9 (TCC), Gly-10 (GGA), Ala-11 (GCG), Ser-79 (AGT), Gly-80 (GGT), Ser-386 (TCT), and Ser-558 (AGC) were as reported by Lersch *et al.* (17), not Eckert and Rorke (16). Residues Arg-37 (CGG), Ser-38 (TCC), Glu-261 (GAA), and Glu-271 (GAG) were as reported by Eckert and Rorke (16), not Lersch *et al.* (17). In addition, there were two polymorphisms, one in the head [Gly or Glu at position 138; (GGA)/(GAA)] and one in the rod [Glu or Asp at position 197; (GAA)/(GAC)]. Polymorphisms in the variable head and tail domains of other keratins have been described (20, 21).

DISCUSSION

A number of lines of evidence indicates that the T → G transversion at nt 482 of human K5 underlies the genetic basis for W–C EBS in families 37 and 26: (*i*) The mutation was not found in 156 wild-type alleles. (*ii*) The mutation was carried only by affected family members. (*iii*) The mutation was inherited in an autosomal dominant fashion, characteristic of the pattern of inheritance in these families. (*iv*) The mutation

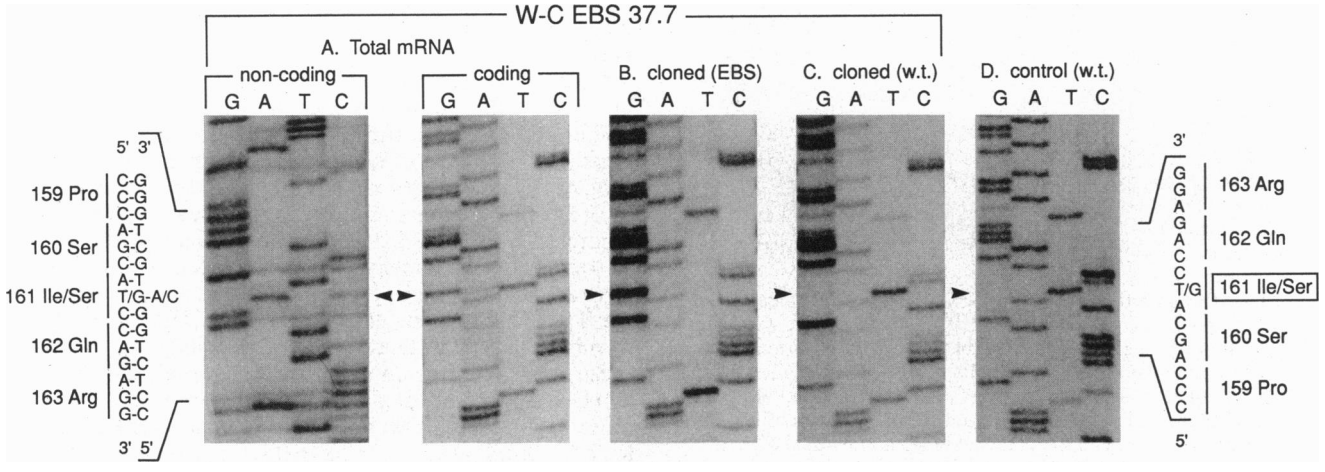

FIG. 3. G → T transversion at codon 161 in an affected W–C EBS individual. PCR was used to amplify K5 sequences from epidermal mRNA of keratinocytes cultured from patient 37.7. Amplified fragments from two rounds of PCR were subjected to DNA sequencing, either directly or after subcloning. Note the A → C transversion (T → G in the coding strand) at nt 482 (codon 161) in one of two 37.7 alleles (arrowhead). Sequences are from the following samples: (*A*) Total K5 PCR mixture of 37.7 mRNA. (*B*) Cloned cDNA from the mRNA of mutant 37.7 allele. (*C*) Cloned cDNA of the mRNA of the wild-type 37.7 allele. (*D*) Control DNA from normal individual.

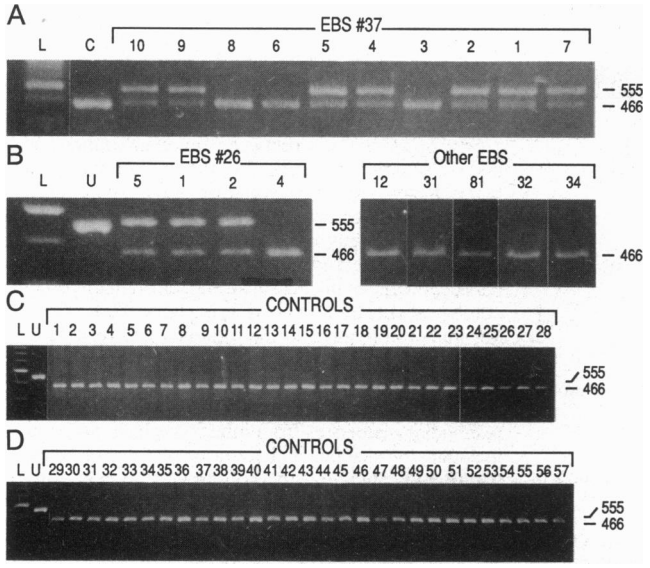

FIG. 4. Heterozygous obliteration of a *Fok* I site in affected family members of two W–C EBS families. Genomic DNAs were PCR-amplified to produce a 555-bp K5 DNA fragment encompassing the T → G transition at nt 482. DNAs were digested with *Fok* I, which cleaved wild-type DNA to 466 bp (and a small 89-bp fragment that is not shown). After digestion, DNAs were resolved by agarose gel electrophoresis and stained with ethidium bromide. (*A*) Lanes: L, DNA ladder; C, control DNA; remaining lanes, family 37 DNAs from affected members 10, 9, 5, 4, 2, 1, and 7 and unaffected members 8, 6, and 3. (*B*) (*Left*) Lanes: U, undigested PCR fragment; family 26 DNAs from affected members 5, 1, and 2 and unaffected member 4. (*Right*) Representative examples of affected members from W–C EBS families 12, 31, 18, 32, and 34. (*C* and *D*) Genomic DNAs from blood of first 57 of 78 normal individuals.

was in the K5 gene on chromosome 12 (18), and the peak lod score at $\theta = 0$ was 3.0 for chromosome 12 (−7.2 for chromosome 17).

Residue 161 of K5 is near or at the junction between the head and rod domains of the IF polypeptide. This residue is Ile in 87% of type II keratins (Fig. 6) and is in a region essential for 10-nm filament assembly (25). The other W–C EBS incidences did not contain the Ile-161 → Ser mutation. However, given (*i*) the degree of conservation of other IF residues, (*ii*) the importance of other residues in IF assembly, and (*iii*) the obligatory heteropolymeric nature of keratin IFs, it is probable that point mutations in other critical residues of either the K5 or K14 genes could generate the W–C EBS phenotype. It is also possible that mutations within a minor basal keratin or in an IF-associated protein might account for some W–C EBS cases.

It is interesting that the mildest cases of EBS (present study) and EH (24) have mutations in the type II keratin head, adjacent to the rod, whereas the moderate case of EBS has a mutation centrally in one of the α-helical domains of the type I rod (5), and more severe cases of EBS (4, 6) and EH (22, 23) have mutations within the highly conserved ends of the type I or type II rods. It is both relevant and revealing that none of the 16 incidences of K-EBS or D–M EBS had the Ile-161 → Ser W–C EBS K5 mutation, and none of the W–C EBS patients had the Arg-125 → Cys or His D–M EBS K14 mutation. Thus, a trend seems to be emerging relating location of mutations within the keratin polypeptide to disease severity.

We were surprised that the same residue was mutated in two of nine W–C EBS families. Although a high frequency of one other mutation, Arg → Cys or His, has been found in 6 of 11 incidences of EBS and EH (4, 22, 23), this site is also a hot spot for C → T transition mutagenesis by methylation and deamination (26). In contrast, the Ile → Ser mutation is a T → G transversion that is not a known hot spot for mutagenesis. It may be that this is one of only a few ways of generating the W–C EBS phenotype.

It is intriguing that the Ile → Ser mutation generates a potential phosphorylation site, Ser-Gln-Arg, for protein kinase C (27). While the possible significance of this observation remains to be determined, it is notable that cdc kinase-mediate phosphorylation of serines in the head regions of two other IF proteins, lamin A (type V) and vimentin (type III), are responsible for destabilizing these IF networks in a cell-cycle-dependent fashion (28–31). Moreover, phosphorylation of IFs by protein kinase C can trigger such diverse responses as diassembly of *in vitro*-formed polymers of vimentin (32) and nuclear import of lamin B *in vivo* (33). Thus phosphorylation might play a role in mediating the deleterious effects of the Ile → Ser mutation on IF network formation in W–C EBS basal cells.

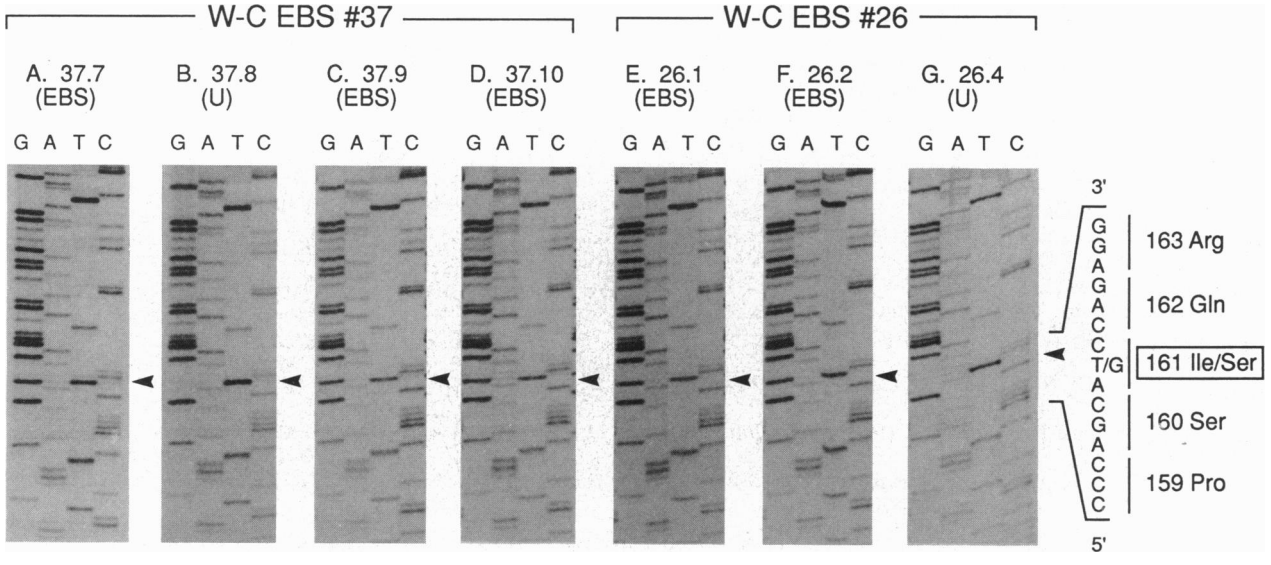

FIG. 5. T → G transversion in K5 codon 161 cosegregates with affected members of W–C EBS families 37 and 26. For genomic DNA from each available member of families 37 and 26, the region encompassing the putative T^{482} → G transition was amplified by PCR, and the fragment was sequenced. Representative examples of coding strand sequences from affected (EBS) and unaffected (U) individuals are shown.

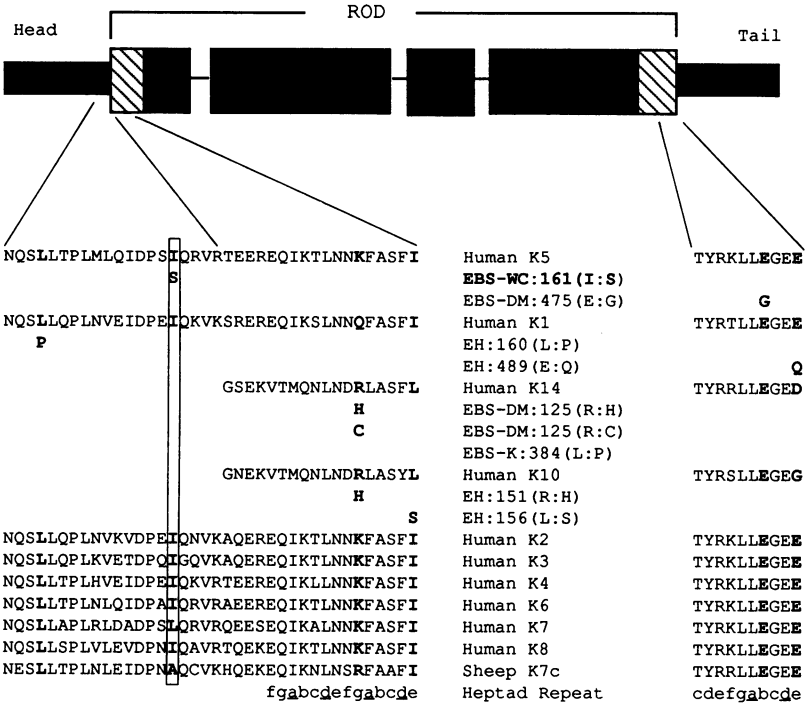

FIG. 6. Correlation between mutation location and disease severity in genetic disorders of keratin. Stick figure depicts secondary structure of human keratins. Large boxes encompass the central rod domain, predicted to be largely α-helical. Hatched boxes denote the highly conserved end domains of the rod. Solid bars denote the nonhelical head and tail domains, often conserved only for a single IF type. Note the K5 Ile → Ser mutation relative to the rod ends and relative to known keratin point mutations in more severe cases of EBS (4–6) and EH (22, 23). Note also type II head domain mutation in a family with mild EH (24). Corresponding sequences from other type II IF proteins, including the sheep wool keratin 7c, are also provided (GenBank). Shown at the bottom are the heptad repeats of hydrophobic residues, where most first (a) and fourth (d) residues of every seven throughout the α-helical rod are hydrophobic, enabling two IF polypeptides, in this case a type I and type II keratin, to intertwine in a coiled-coil fashion (8). The residues encoding more severe EBS and EH mutations are conserved among most IF proteins; the residues encoding W–C EBS and less-severe EH mutations are conserved among type II keratins.

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