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Genetics

The genetic basis of epidermolysis bullosa simplex with mottled pigmentation

(keratin filaments/cytoskeleton/genetic disease/intermediate filament function)

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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. It is now well-established that the three major subtypes of EBS are genetic disorders of the basal epidermal keratins, keratin 5 (K5) and keratin 14 (K14). Here we show that a rare subtype, referred to as EBS with mottled pigmentation (MP), is also a disorder of these keratins. Affected members of two seemingly unrelated families with EBS-MP had a C to T point mutation in the second base position of codon 24 of one of two K5 alleles, leading to a Pro:Leu mutation. This mutation was not present in unaffected members nor in 100 alleles from normal individuals. Linkage analyses mapped the defect to this type II keratin gene (peak logarithm of odds score at $\phi = 0$ of 3.9), which is located on chromosome 12q11-q13. This provides strong evidence that this mutation is responsible for the EBS-MP phenotype. Only conserved between K5 and K6, and not among any of the other type II keratins, Pro-24 is in the nonhelical head domain of K5, and only mildly perturbs the length of 10-nm keratin filaments assembled *in vitro*. However, this part of the K5 head domain is likely to protrude on the filament surface, perhaps leading to additional aberrations in intermediate filament architecture and/or in melanosome distribution that are seen ultrastructurally in patients with the mutation.**

Epidermolysis bullosa simplex (EBS) has been subdivided into three major types (1–3). (*i*) EBS Dowling-Meara (EBS-DM) is the most severe type, typified by blistering over whole body regions and keratin filament clumping and cytolysis in basal cells. (*ii*) EBS Köbner (EBS-K) is also characterized by generalized blistering and basal cell cytolysis, but with fewer abnormalities in basal cell keratin networks. (*iii*) EBS Weber-Cockayne (EBS-WC) is the mildest form, with blistering concentrated primarily in palmar and plantar regions, and very minor keratin filament perturbations. An unusual and rare form of EBS with generalized blistering and hyper- and hypopigmented spots over the body surface was first reported by Fischer and Gedde-Dahl (4). The unifying feature of all of these EBS subtypes is basal cell cytolysis, with a largely normal differentiation process in the suprabasal layers.

Anton-Lamprecht initially suggested that EBS-DM might be a disorder of keratin (1), and demonstrated that keratin filament clumping preceded cell cytolysis. Experimental support for this hypothesis was provided years later, when transgenic mice expressing a mutant keratin 14 (K14) gene displayed abnormalities bearing a striking resemblance to EBS-DM (5). Since K14 (type I) and its partner K5 (type II) is the keratin pair specifically expressed in the basal layer of epidermis (6), this led to the prediction that mutations in either one of these two keratins might be the underlying cause of EBS in humans. Subsequent analyses of human patients with EBS-DM or EBS-K led to the discovery of point mutations in either the K5 or K14 genes (7–9). Later studies on EBS-WC added this subtype to the list of genetic disorders of these basal keratins (10–12).

The classification according to subtype can also be made genetically and according to the ability of different K14 and K5 mutations to affect their assembly into 10 nm intermediate filaments (13, 14). Patients with EBS-DM frequently have point mutations located within 10 amino acid residues at either end of the central, α-helical rod domain involved in coiled-coil heterodimerization between type I and type II keratin polypeptide chains (Fig. 1*A*; refs. 7, 9, 18–20). EBS-K mutations are typically more internal within the rod (8, 21, 22). In contrast, EBS-WC mutations seem to localize to two specific regions: (*i*) an I161S hot spot found in one of two K5 alleles of a number of apparently unrelated cases of EBS-WC (10, 23) and (*ii*) a region within a nonhelical linker segment (L1-2) located in the middle of the α-helical rod segment of the K14 and K5 polypeptides (11, 12, 22).

These mutations affect intermediate filament assembly in different ways. The EBS-DM mutations affect filament elongation (7, 13, 14). The elongation process is thought to depend upon the head-to-tail alignment of heterodimers in a linear array (ref. 24; Fig. 1*B*). The importance of the rod ends is likely due to an interaction between the amino end of one heterodimer and the carboxy end of a connecting heterodimer (26, 27). In contrast, the L1-2 linker mutations in EBS-WC seem to have a greater effect on lateral associations, yielding filaments *in vitro* that are longer, but more unraveled, than DM mutant filaments (11). It has been postulated that the linker may be involved in aligning antiparallel arrays of dimers in a half-staggered position relative to each chain (24–28). Of the head and tail segments of K5 and K14, all but the portion of the K5 head domain adjacent to the rod domain could be deleted without grossly perturbing 10 nm filament assembly *in vitro* (29).

The genetic bases for the rarer subtypes of EBS, including EBS with mottled pigmentation (EBS-MP), have not yet been determined. EBS-MP is unusual in that patients have skin blistering similar to that of EBS, but in addition, they have striking MP (4, 30–33). In the initial Swedish Epidermolysis Bullosa simplex (SEB1) family described with this syndrome,

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Abbreviations: EBS, epidermolysis bullosa simplex; EBS-DM, EBS Dowling-Meara; EBS-K, EBS Köbner; EBS-WC, EBS Weber-Cockayne; EBS-MP, EBS with mottled pigmentation.

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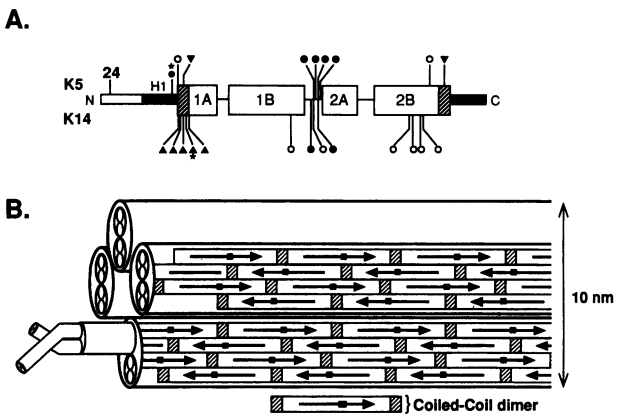

FIG. 1. Mutations in EBS and model of keratin filament assembly. (*A*) Stick Fig. depicts the secondary structure predicted for keratin (15, 16). Open boxes denote the four α-helices (1A, 1B, 2A, and 2B) constituting the coiled-coil rod domain. Lines between these segments are three short nonhelical linker segments (L1, L1-2, and L2, respectively). Hatched boxes denote highly conserved ends of the rod. Two black boxes denote the H1 portion of the nonhelical head domain (17) and the carboxyl-terminal tail segment. Remainder of the head domain is shown in white. Point mutations found thus far in patients with EBS are indicated by vertical bars. Top, K5 mutations; bottom, K14 mutations. Mutations are: solid circles, EBS-WC; open circles, EBS-K; triangles, EBS-DM; *, hot spots where mutations have been found repeatedly. Vertical bar marked 24 is discussed in text. N, amino terminus; C, carboxyl terminus. (Reprinted with permission from Blackwell Publishers.) (*B*) Putative arrangement of heterodimer keratin subunits in the 10 nm keratin filament. Model is adapted from that previously described (24). Arrow within each heterodimer indicates direction of the two parallely aligned polypeptides, from base (N terminus) to tip (C terminus). Hatched boxes denote highly conserved ends of the rod, which are predicted to overlap in the arrangement of linear arrays (26, 27). Small black box denotes region of L1-2 linker where EBS mutations are clustered (see *A*). [Reprinted with permission from The Rockefeller University Press (25).]

affected members had pigmented spots 2–5 mm in diameter that formed a mosaic pigmentation pattern over the body surface (4). The pigmentation anomalies in EBS-MP are contrasted from many other genetic disorders in that inflammation is not generally involved. It has been postulated that the pattern of MP might be dependent upon autosomal inactivation of an ectodermal gene, perhaps one involved in the uptake of melanosomes by epidermal cells (4). Melanocytes themselves, and melanosome production, appear normal (33).

In this EBS-MP family now spanning four generations, all 12 affected members showed clinical features of both EBS and MP. Whether the disorder represents a single mutant gene or mutations in two very closely linked genes has remained unanswered, and the etiology of the disorder remains unknown. Previously, we noted that the distribution of mitochondria is aberrant in some EBS cases (11, 34). In this context, we were intrigued by the possibility that certain keratin disorders might affect organelle distribution, perhaps including melanosomes. To explore this possibility further and to determine the underlying genetic basis of EBS-MP, we used ultrastructure, sequence, and functional analyses on two different families with this disorder.

MATERIALS AND METHODS

Biopsies and Blood Samples. One clinically affected member of family EBS 44 (EBS 44-3) and three of family SEB1 (SEB1-41, 1-42, and 1-44) donated skin biopsies for ultrastructural studies. Biopsies were taken from a nonblistered area of hypo- and hyperpigmented skin. Blood samples (5 ml) were taken for isolation of genomic DNA from four family members of EBS 44 and from 18 family members of SEB1. From the deceased SEB1-22, frozen fibroblasts were recovered for DNA isolation.

DNA Sequence Analysis. Four different pairs of oligonucleotide primers were selected to encompass the entire coding sequence of the K5 gene (35, 37), and polymerase chain reaction (PCR) amplification was performed to amplify K5 sequences from patients EBS 44-3 and SEB1-22. DNAs were then resolved by gel electrophoresis, and fragments were subcloned into the pCRII plasmid vector (TA Cloning System, Invitrogen). Twelve to 13 clones were pooled and purified using the Qiagen (Chatsworth, CA) Plasmid Maxi Kit. DNAs were sequenced using a standard dideoxy protocol (Sequenase 2.0, Amersham). To confirm the presence or absence of the identified mutation in the remaining family members, we amplified the appropriate region and sequenced the PCR product using a CircumVent Thermal Cycle dideoxy Sequencing Kit (New England Biolabs).

PCR Amplification of Specific Allele Analysis of the Mutation. PCR amplification of specific alleles was used to check for the absence of the EBS-MP mutation in unrelated wild-type individuals. Primers used were: wild-type allele, 5′-GCCTCTGCCATCACCCC-3′; mutant allele, 5′-GCCTCTGCCATCACCCT-3′; both, 5′-CTTGTCGATGAAGGAGG-3′.

Site-Directed Mutagenesis. The EBS-MP point mutation was engineered in (*i*) pETK5, a bacterial expression vector (38), and (*ii*) pJK5, an SV40 promoter-enhancer-based mammalian expression vector (13). All clones were verified by sequencing the genetically engineered segments.

RESULTS

Diagnosis of EBS-MP Families. EBS 44 is a small family of German origin, and has been followed clinically by one of us (J.-D.F.). There is no known prior history of EBS in the family, but the mother (EBS 44-3) displayed skin blisters predominantly over her palms and soles (see family tree in Fig. 2). These blisters appeared at birth and arose in response to mild physical trauma. Blisters often healed without scarring, and became less severe with age. At the histological and ultrastructural level (see below), blisters occurred as a consequence of basal cell cytolysis. On the basis of these clinical and ultrastructural features and, in addition, immunofluorescence antigenic mapping with basal and basal lamina markers (J.-D.F., unpublished work), a diagnosis of EBS-WC was made. In addition, a pigment anomaly was also present at birth. The trunk and extremities displayed a mosaic pattern of dark and light pigmented spots (2–5 mm diameter) with irregular borders. The MP was more prominent in early life, and became less visible with age. These clinical features were inherited by one of her sons (EBS 44-6). In addition, the boy, now 12, continues to develop painful erosions within the oral cavity, both buccal mucosa and tongue.

The features seen in EBS 44-6 were similar to those observed in affected members of family SEB1, the first family

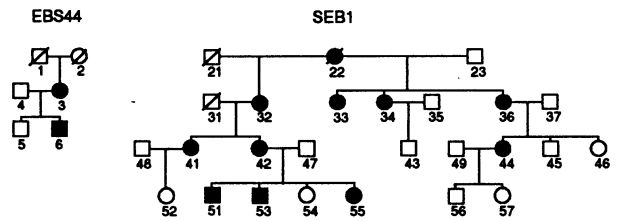

FIG. 2. Pedigrees of EBS 44 and SEB1 families. Numbers were assigned to members for which blood was obtained (skin biopsies in hyper- and hypopigmented areas were additionally obtained from EBS 44-3, SEB1-41, SEB1-42, and SEB1-44 for the purposes of the present study). Solid symbols, members clinically affected with EBS; open symbols, unaffected individuals; cross bar to right, deceased; squares, males; circles, females.

described with EBS-MP (see family tree in Fig. 2; ref. 4). This family originated in Scandinavia and was followed clinically through four subsequent generations by one of us (T.G.-D.). Generalized skin blisters appeared at birth in 7 of the 12 affected patients and by early childhood in the remainder of affected members. Patterns of MP in this family were similar to those seen in EBS 44-3 and 44-6 and have been described in detail previously (4). In combination with ultrastructural analyses showing basal cell cytolysis (see below), a diagnosis of EBS with MP was made. While both EBS-MP families are Caucasian and of European origin, there is no known ancestral relation between them. Moreover, EBS 44-3 is anamnestically a *de novo* mutant.

Ultrastructural Analyses. Ultrastructural analyses of EBS 44-3 and of SEB1-41, SEB1-42, and SEB1-44 revealed features typical of EBS (Fig. 3*A*). In EBS 44-3, ≈10% of basal epidermal cells displayed mild perturbations, including signs of vacuolization, often adjacent to the nucleus. In the basal layer, mild abnormalities in keratin cytoarchitecture were consistently observed, including disorganized keratin filaments and varying degrees of aggregation of keratin filaments (Fig. 3 *A* and *B*, EBS 44-3; Fig. 3*C*, control; Fig. 3*D*, SEB1-44). Most notably was a fine network of seemingly short keratin filaments in EBS 44-3 basal cells, even in cells displaying no signs of cytolysis. Intriguingly, some basal and suprabasal cells showed perinuclear shells of keratin filaments, leaving perinuclear gaps of as much as 100 nm or more of free cytoplasmic space with a paucity of organelles (double arrow in Fig. 3*B*). These shells are not typical of EBS, but have been described for ichthyosis hystrix Curth-Macklin type (1, 39) and for transgenic mice expressing a mutant keratin 10 gene (40). These structures appeared quite frequently in EBS 44-3 epidermis, but were infrequent in the three SEB1 patients. In the SEB1-44 sample, the keratin filaments organized in these shells had lost

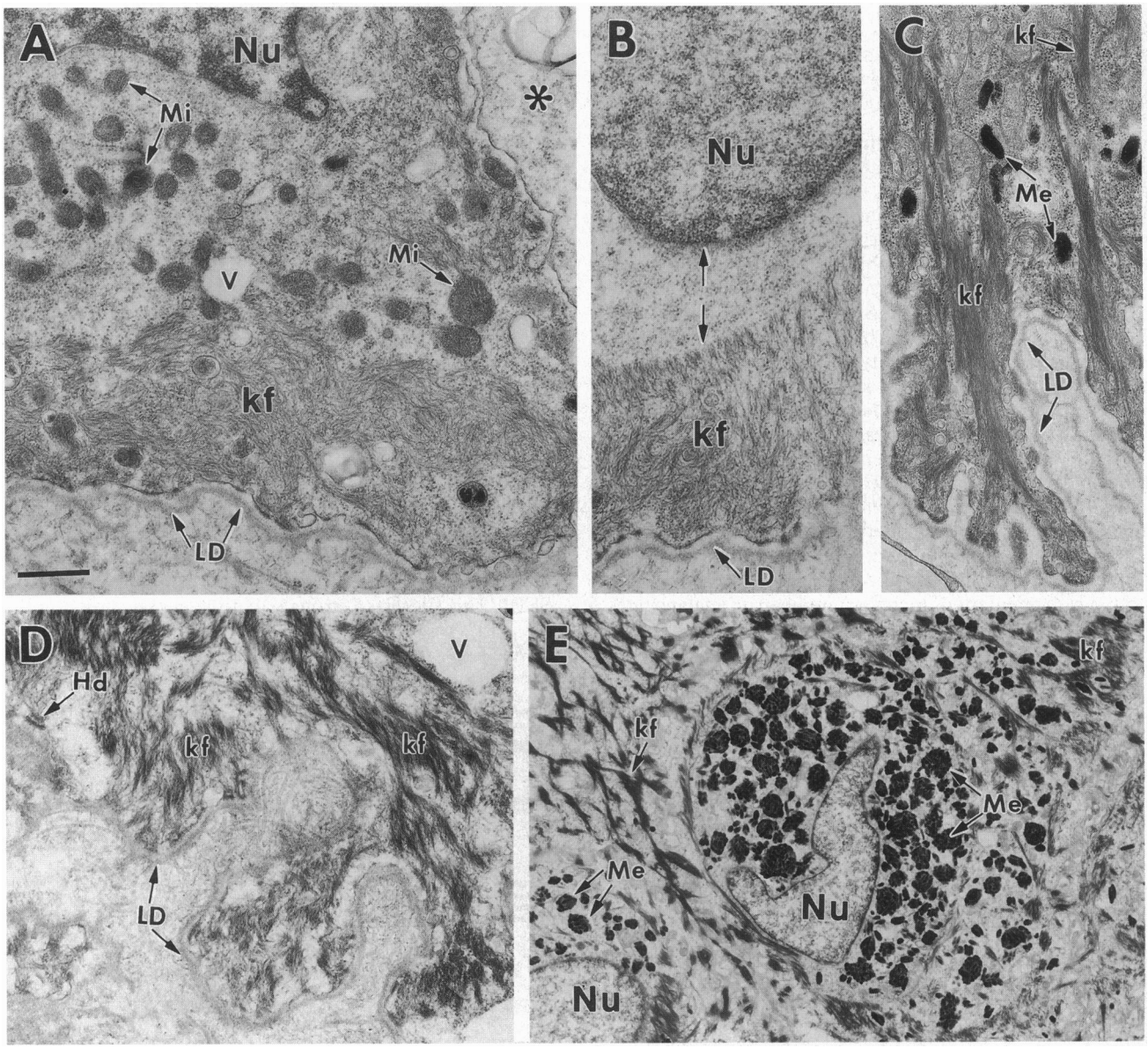

FIG. 3. Ultrastructure of EBS-MP. (*A*) Basal cell from the epidermis of EBS 44-3. Note keratin filaments, atypically in a thin, relatively dispersed array. Note cytolysis (asterisk) in basal cell at right. (*B*) Higher magnification of basal cell cytoplasm from EBS 44-3 showing disorganized network of seemingly short keratin filaments. The EBS 44 basal cells shown in *A* and *B* displayed a perinuclear shell of keratin filaments, leaving a gap (double arrow in *B*) with a paucity of cytoplasmic organelles. (*C*) Basal cell cytoplasm from control epidermis showing typical bundles of keratin filaments. (*D*) Sample of affected member of SEB1-44, showing initial, subclinical blistering. Note atypical disorganized aggregates of keratin filaments. (*E*) Basal and suprabasal epidermal cells from SEB1-44, revealing a cytoplasm densely packed with pigment granules. Nu, nucleus; LD, lamina densa; kf, keratin filaments; Mi, mitochondria; Me, melanin granules; Hd, hemidesmosome; V, vacuoles. (*A*, bar = 0.6 μm in *A–D*; *E*, bar = 2.1 μm.)

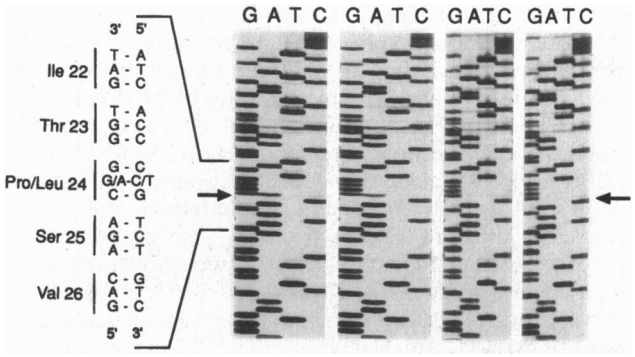

FIG. 4. C to T transition at codon 24 in affected family members of EBS 44. PCR was used to amplify K5 sequences from genomic blood DNAs of all family members of EBS 44. Amplified fragments from two independent rounds of PCR were subcloned and 12–13 independent clones were pooled and subjected to DNA sequencing. Note G to A transition (C to T in coding strand) at nucleotide 71 (codon 24) in one of two alleles (arrow). Sequences are from family members indicated across top. Note, sequences from the DNAs of unaffected members EBS 44–4 and 44–5 were indistinguishable from those of control DNAs (see ref. 10).

their desmosome/hemidesmosome contacts, whereas those cells that maintained these contacts displayed more normal appearing filament bundles.

There were no signs of clumping of amorphous keratin as is typical of EBS-DM. The abnormalities in keratin filament networks preceded cytolysis and blister formation, but increasing aggregation of keratin filaments were formed in areas with early signs of cytolysis in the SEB1 patients, especially in SEB1-44 (Fig. 3*D*). None of the basal cell aberrancies seen in EBS 44 or SEB1 patients were typical of normal skin. Thus, abnormalities in keratin filament networks existed in both EBS 44 and SEB1 epidermis, even though some variation seemed to exist between the two families.

In addition to keratin filament anomalies, abnormalities were seen in melanin granule distribution in EBS 44 and SEB1 epidermis, and this was especially prominent in SEB1-44 (frame E). Heavily loaded, hyperpigmented cells in the basal and suprabasal layers corresponded in their degree of pigmentation to that seen under conditions of postinflammatory and postbullous pigmentation. Increased numbers of melanocytes were present in both biopsy samples of SEB1-44, while their frequency was more normal in SEB1-41, SEB1-42, and EBS-44. Throughout the basal layer of EBS 44-MP skin, mitochondria were frequently clustered within the cytoplasm, an aberrancy previously noted for other types of EBS (Fig. 3*A*; see also ref. 11). Thus, the distribution of organelles appeared to be aberrant in EBS 44 and SEB1 basal epidermal cells, a feature that is likely to account for the MP seen in these patients.

Affected Members of EBS 44 and SEB1 Have the Same Point Mutation Encoding a Pro to Leu Substitution at Codon 24 in the Nonhelical Head Domain of K5. Given the diagnosis of EBS and the milder clinical features of EBS 44, we began by examining the K5 alleles of EBS 44-3 to determine whether mutations in K5 might be the source of the genetic defect in the EBS 44 family. PCR was used to amplify different segments of the K5 gene. Only a single point substitution at nucleotide 71 (A of the first ATG = 1; refs. 35 and 37) distinguished one of the K5 alleles from wild-type. The transition caused a proline (CCG) to leucine (CTG) mutation at codon 24 (Fig. 4). The PCR/sequencing was reproducible, indicating that the transition was not due to a polymerase error during PCR. Both G and A occurred in the sequencing ladder of the noncoding strand (C and T in the coding strand).

This same nucleotide substitution was also detected in the affected son, EBS 44-6 (Fig. 4). In contrast, only the wild-type G residue was detected in the DNAs of the two unaffected family members, EBS 44-4 and 44-5, analogous to that seen in control DNAs (35, 37). This confirmed the presence of both mutant and wild-type sequences in the genomic pool, as expected of an autosomal dominant disease.

Surprisingly, affected members from SEB1 had DNAs that were also heterozygous at nucleotide 71 (Fig. 5). This exact same nucleotide substitution, predicting a P24L mutation in one of the two K5 alleles, was found in affected family members 22, 32, 33, 34, 36, 41, 42, 44, 46, 51, and 55, and not in unaffected members 35, 37, 43, 47, 49, 52, and 54 (representative examples of these data are shown in Fig. 5). Genomic DNAs from 50 normal individuals were also homozygous for the wild-type sequence, verifying that the loss of this site was not merely reflective of a rare polymorphic variation. When nucleotide 71 was used as a genetic marker, a combined lod score at $\phi = 0$ of 3.9 was obtained for families EBS 44 and SEB1. This suggested strongly that the genetic lesion in these

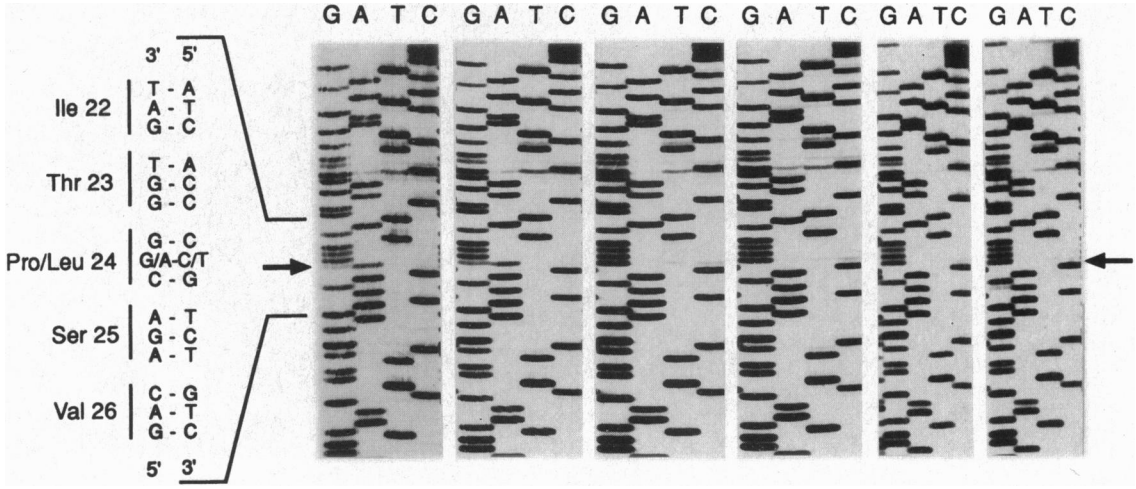

FIG. 5. C to T transition at codon 24 in affected family members of SEB1. PCR was used to amplify K5 sequences from genomic blood DNAs of the following SEB1 family members: 22, 32–37, 41–44, 46, 47, 49, 51, 52, 54, and 55 (see Fig. 2). Amplified fragments from two independent rounds of PCR were subcloned and 12–13 independent clones were pooled and subjected to DNA sequencing. Note G to A transition (C to T in coding strand) at nucleotide 71 (codon 24) in one of two alleles (arrow). Representative sequences are from family members indicated across top. Note, the heterozygous mutation found in SEB1-22 and SEB1-51 was present in DNAs of all affected family members, while unaffected members, including SEB1-37, SEB1-47, SEB1-52, and SEB1-54, had the wild-type nucleotide 71.

families resided within the keratin loci on chromosome 12q11-q13, specifically, within the K5 gene.

Sequencing of the EBS 44-3 K5 cDNA did not uncover any additional mutations. Discrepancies existed between our sequence and published K5 sequences (35, 37), but not with the sequence reported by Chan *et al.* (10). In addition to a K5 polymorphism previously described (196E/D GAA/GAC; ref. 10), we found three silent polymorphisms (116L/L CTC/CTT; 197T/T ACA/ACC; and 243L/L CTG/CTA). Other polymorphisms in the variable head and tail domains of other keratins have been described (41, 42).

The P24L Mutation Does Not Markedly Affect the Assembly of Keratin Filament Networks In Transfected Cultured Cells Nor the Assembly of Keratin Filaments *in Vitro*. Given that PtK2 cells have a keratin network that is more sensitive to subtle perturbations than epidermal keratinocytes (13), we used these cells to test for the effects of the P24L K5 mutation on filament network formation. The majority of PtK2 cells transfected with the P24L K5 expression vector showed a typical keratin network, indistinguishable from wild-type (Fig. 6A; see ref. 13 for examples of wild-type cells). Some of the cells contained a collapsed keratin network (Fig. 6*B*), but the percentage of these brightly anti-P stained cells was comparable to those seen in cells transfected with the wild-type K5 counterpart, and likely arise from transgene overexpression in some cells (see ref. 14).

We also examined the ability of bacterially expressed P24L K5 to assemble into keratin filaments when combined with wild-type K14 *in vitro*. Under the conditions used, wild-type K5 and K14 assembled into long 10 nm filaments (Fig. 6*C*). P24L K5 and K14 also assembled into 10 nm filaments, but there were some reproducible differences in their overall length and uniformity (Fig. 6*D*). Most notably, the mutant K5-containing filaments were ≈18% shorter than wild-type filaments (mutant, 1.12 μm mean length, 0.56 standard deviation; wild-type, 1.36 μm mean length, 0.42 standard deviation). No differences were seen in polymerization efficiency as judged by a filament centrifugation assay (data not shown; ref. 38). Thus, it seems that either (*i*) the mutation slightly enhances the nucleation of filament assembly, so that there are more assembled filaments with an overall shorter length, or (*ii*) the mutation slightly impairs the rate of elongation or the stability of the assembled filaments at steady state, leading to shorter filaments. These results were consistent with the ultrastructural data on EBS 44, revealing an overall shortened appearance to the keratin filaments in basal cells.

DISCUSSION

A number of lines of evidence indicate that the C to T transition at nucleotide 71 of human K5 underlies the genetic basis for EBS in families 44 and SEB1: (*i*) the mutation was not found in 100 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 peak logarithm of odds score at $\phi = 0$ was 3.9 for this mutation; and (*v*) point mutations in K5 and its partner K14 are known to account for all of the EBS cases reported thus far, and are therefore likely to account for the majority of EBS in the population (refs. 7–12, 25, and 34, and references therein). Interestingly, another family with EBS-MP was previously found to have a defect(s) mapping to chromosome 12, near or at the location of the K5 gene (45). However, after not detecting any mutation in the rod domains of keratins 5 and 14, these researchers erroneously concluded that EBS-MP is not a disorder of keratins 5 and 14 (45).

Residue 24 of K5 occurs early within the amino terminal head (Fig. 1), in a region that is not known to appreciably affect 10 nm filament assembly (17, 29). Moreover, the sequences within the amino half of the head domain are not highly evolutionarily conserved, even among the type II keratins (residue 24 is proline, however, in all K6 isoforms; ref. 46). Our filament network and *in vitro* assembly studies with P24L K5 demonstrate that this mutation does not grossly change the

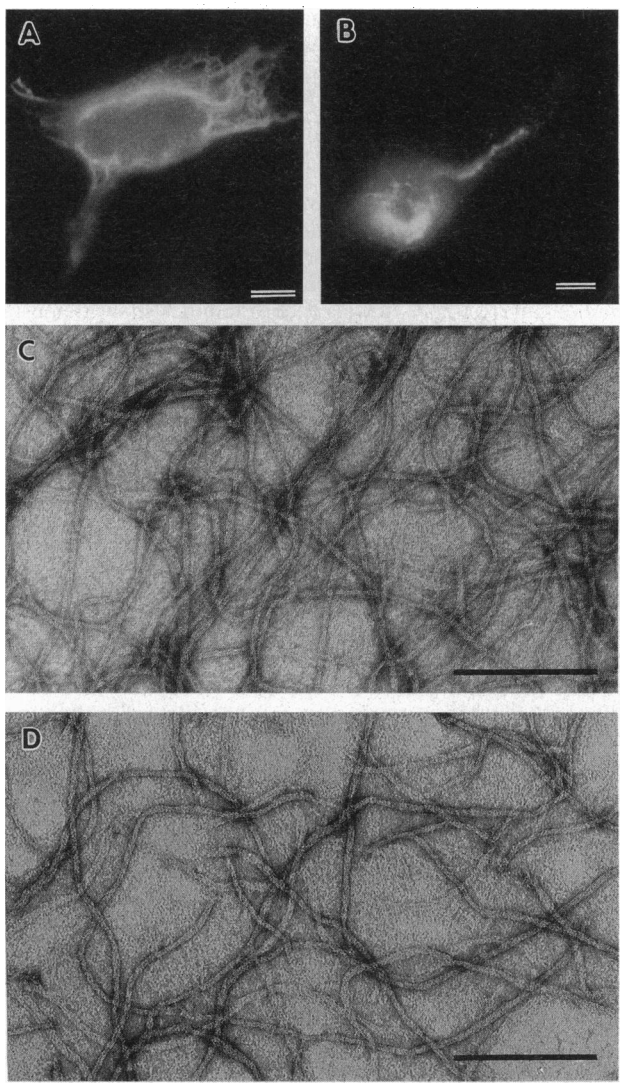

FIG. 6. Filament network formation and filament assembly are not appreciably affected in *in vitro* assays of the P24L mutation. (*A* and *B*) Transient transfections. Potoroo kidney epithelial (PtK2) cells were transiently transfected with epitope (neuropeptide substance P-tagged), wild-type, or mutant K5 cDNAs driven by the SV40 major early promoter and enhancer (13, 14). At 65 hr posttransfection, cells were fixed and examined by indirect double immunofluorescence, using a rabbit anti-P antiserum to recognize the neuropeptide substance P tag and an anti-K8 antibody (LE41) to recognize the endogenous keratin network. Shown are representative examples of cells transfected with the P24L K5 mutant and stained with anti-P. Data were indistinguishable from those obtained with wild-type K5 (data not shown; see ref. 13 for examples). The majority of the transfected cells displayed a wild-type network as shown in *A*. Approximately 13% of the transfected cells displayed a collapsed network as shown in *B*. (*C* and *D*) *In vitro* filament assembly studies. Wild-type K5 (*C*) and P24L K5 (*D*) were combined with wild-type K14 at a concentration of 300 μg/ml, and dialyzed against 3 mM Tris·HCl (pH 7.2) and 5 mM 2-mercaptoethanol (38, 43). Assembled filaments were diluted 1:10 in 3 mM Tris·HCl (pH 7.2) before negative staining and electron microscopy. Filament lengths were recorded from computer scanned negatives using the IMAGE software (National Institutes of Health, Bethesda). Assembled filaments from two separate experiments were analyzed, with comparable results. Note, a fraction of the assembled filaments was also subjected to centrifugation at 100,000 × *g* for 40 min at 4°C; both mutant and wild-type preparations yielded comparable amounts of keratin in the pellet fraction, indicating that the mutation does not impair the efficiency with which the protein polymerizes into filaments *in vitro*. (*A* and *B*, bar = 10 μm; *C* and *D*, bar = 200 nm.)

conformation of the head domain in a fashion that is incompatible with filament assembly, but that it does modestly affect the overall length and uniformity of the filaments. Thus, despite its position within the keratin polypeptide, the P24L mutation appears able to in part account for the perturbations seen in the basal keratin networks in EBS 44 and SEB1 families and for the fragility of these cells to mechanical stress.

We have not examined the possibility that polymorphic variations, either in the K5 and K14 themselves or in other associating proteins, might be responsible for the differences that exist in the keratin filament architectures within the two families. Interestingly, however, both families showed aberrations in the degree of the lateral association of keratin filaments into bundles, and both families displayed alterations in the overall keratin cytoskeletal network.

A striking and consistent feature of the two EBS-MP families was the variability in melanin granule distribution. *A priori* the high degree of subclinical blister formation in the EBS 44 and SEB1 patients together with signs of subsequent repair suggest the possibility that hyperpigmentation may arise from postbullous pigmentation. This notion is supported by a focal increase in the number of melanocytes in the SEB1-44 biopsies (data not shown). This said, all EBS subtypes are typified by basal cell cytolysis and tissue repair, and yet only a small fraction of these have been characterized as having EBS with MP. While we cannot unequivocally rule out the possibility that the MP arises from a second albeit closely linked gene defect, this seems unlikely. Rather, given that two seemingly unrelated EBS-MP families have the exact same keratin mutation, it is likely that this single mutation generates MP as well as EBS.

How might the P24L K5 mutation affect pigmentation in basal cells? Based on proteolysis experiments, it is likely that residue 24 is in a region that at least in part protrudes along the filament surface (47). Thus, the P24L K5 mutation could interfere with the association between keratin filaments and other elements in the basal cell cytoplasm. Based on our present studies, it seems most likely that the keratin defect causes a subtle perturbation in keratin cytoarchitecture that may influence either longevity of melanin granules in basal cells or alternatively the efficiency of melanin granule transfer from melanocytes. Alternatively, a perturbation in the association between the K5 head domain and desmosomes and/or hemidesmosomes (48) could contribute to cytolysis somewhat differently than defects in filament assembly, resulting in continual subclinical blistering and variability in epidermal pigmentation.

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