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Transgenic mice expressing a mutant keratin 10 gene reveal the likely genetic basis for epidermolytic hyperkeratosis

(skin disease/keratin/nuclear structure/epidermis)

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ABSTRACT **Epidermolytic hyperkeratosis (EH; previously called bullous congenital ichthyosiform erythroderma) is an autosomal dominant skin disease of unknown etiology, affecting ≈1 out of 300,000 people. It is typified by hyperkeratotic scaliness, blistering due to cytolysis within suprabasal epidermal cells, and hyperproliferation in basal cells. Histologically, EH epidermis exhibits a thickened stratum corneum and granular layer, with enlarged and irregular-shaped cells. Ultrastructurally, only suprabasal layers are affected, with three major aberrancies: (*i*) tonofilament clumping, (*ii*) nuclei and keratohyalin granules of irregular shape and size, and (*iii*) cell degeneration. We have discovered that transgenic mice expressing a mutant keratin 10 gene have the EH phenotype, thereby suggesting that a genetic basis for human EH resides in mutations in genes encoding suprabasal keratins K1 and K10. In addition, we show that (*i*) stimulation of basal cell proliferation can arise from a defect in suprabasal cells, and (*ii*) distortion of nuclear shape or aberrations in cytokinesis can occur when an intermediate filament network is perturbed.**

A possible clue to the genetic basis for epidermolytic hyperkeratosis (EH) is the similarity between tonofilament clumping in suprabasal cells of EH skin and in basal cells of skin from another blistering disease, epidermolysis bullosa simplex (EBS) (1–4). EH differs from EBS in that (*i*) suprabasal, rather than basal, cells are prone to cytolysis, and (*ii*) basal cell hyperproliferation is a hallmark of EH. Moreover, whereas terminal differentiation in EBS skin is normal, EH skin exhibits suprabasal irregularities in the shape and size of cells and their organelles and in the thickness of stratum corneum and granular layers.

In the past year, transgenic mouse technology was utilized to infer the genetic basis for EBS, which involves mutations in one of two genes encoding basal-specific epidermal keratins K5 and K14 (5, 6). Transgenic mice expressing mutant K14 genes exhibited EBS phenotypes (5, 6), and subsequent analyses of human EBS patients revealed mutations in the K5 and K14 genes (7–9). As epidermal cells commit to terminally differentiate, they switch off K5 and K14 expression and switch on a new set of keratins, K1 and K10 (10–14). In the fully differentiated squame, K1 and K10 (in slightly processed forms) constitute ≈85% of cellular protein (10). Given (*i*) the parallels between tonofilament clumping in EH and EBS, (*ii*) the knowledge that EBS is a keratin disorder, and (*iii*) the pattern of keratin expression during differentiation, we utilized transgenic technology to investigate the possibility that EH is a genetic disease involving K1 and K10 defects.

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RESULTS

Newborn Mutant K10 Mice Exhibit a Dry Skin Surface with Mild Blistering. For our studies, we isolated a K10 gene from a human genomic library. Sequence analysis indicated that this was equivalent to a previously reported human K10 gene (15). We prepared a truncated K10 transgene containing 7500 base pairs of 5′ upstream promoter/enhancer sequences coupled to sequences encoding (*i*) the amino-terminal nonhelical domain of K10, (*ii*) a hybrid truncated α-helical rod domain of K10 and K14, and (*iii*) the antigenic carboxyl-terminal portion of neuropeptide substance P as a tag (Fig. 1*A*). This transgene encoded the K10 equivalent of CΔ135K14P, previously shown to disrupt keratin filament assembly, even when present at only 0.5% of total keratins (5, 18).

Given that (*i*) sequence identity is high in the α-helical rod domain of K14 and K10 (15, 16), (*ii*) K1 can assemble into filaments with K14 or K10 (19), and (*iii*) the unique features of keratin pairs reside in their nonhelical ends (20), we expected that the hybrid would act in a dominant-negative fashion. Indeed, when expressed in keratinocytes, the truncated product, K10CΔ135, disrupted the endogenous keratin network, as judged by double immunofluorescence with anti-substance P (anti-P) and anti-endogenous keratin antibodies (Fig. 1*B*; anti-P staining shown). The behavior of K10CΔ135 (Fig. 1*B Middle*) was similar to K14CΔ135 (Fig. 1*B Right*), except that K10CΔ135 cells were fewer and often suprabasal. Thus, the mutant gene had two important prerequisites: *in vivo*, the mutant was likely (*i*) to be expressed suprabasally and (*ii*) to be a potent disrupter of keratin networks.

Transgenic mice were produced by using an outbred strain (CD-1) (6). Eight mice were transgenic, as judged by PCR analysis of skin DNAs. Founder mouse no. 29 exhibited an average phenotype (Fig. 1*C*). Most notably, front paws were scaly and blistered (*Inset*), and trunk skin was duller, with fewer surface lines. K10CΔ135 expression was detected by immunoblot analysis of epidermal intermediate filament (IF) proteins (Fig. 1*D*). Five animals produced anti-P hybridizing proteins of 40 and 37 kDa. Since the predicted size of K10CΔ135 is 39.3 kDa and since K1/K10 proteins are processed by 2–3 kDa during differentiation (10), the two bands are likely intact and processed transgene protein, respectively. It was estimated that the mice expressed K10CΔ135 at 2–10% of endogenous K10 levels (Fig. 1*D*; ref. 6).

Intraepidermal Shearing Arising from Cytolysis in Mutant K10 Mouse Skin. The histopathology of back skin from mutant K10 mice revealed abnormalities restricted to suprabasal epidermal layers (Fig. 2). In particular, the stratum corneum was unusu-

Abbreviations: IF, intermediate filament; EH, epidermolytic hyperkeratosis; EBS, epidermolysis bullosa simplex; anti-P, anti-substance P.

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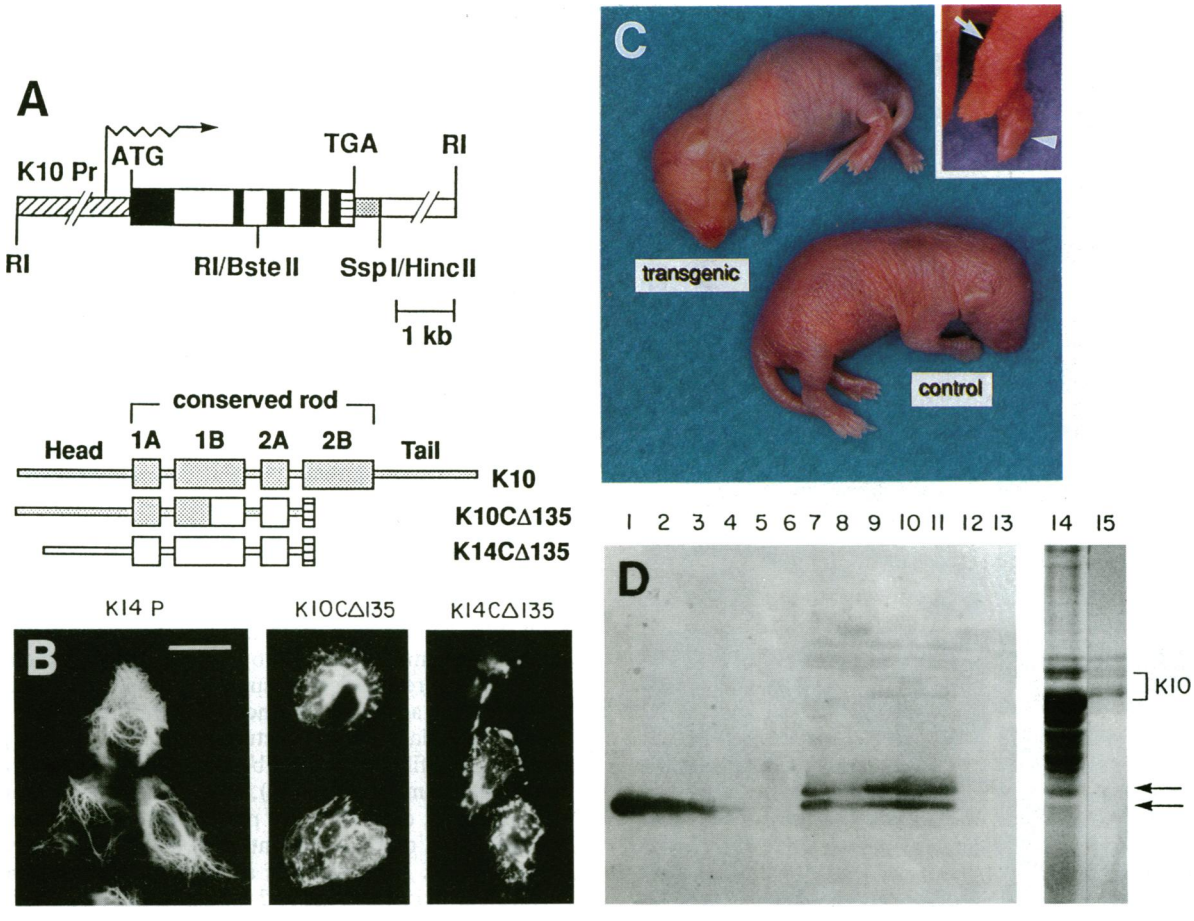

FIG. 1. K10 mutant gene and its phenotype in cultured keratinocytes and in mice. (*A Upper*) Transgene. Hatched bars, 5′ sequence of human K10 gene extending to an *Eco*RI (RI) site 7.5 kilobases 5′ to the TATA box; black boxes, coding sequences of exons I and II of human K10 and III, IV, and part of V of human K14 (up to amino acid 332; ref. 16); open boxes, K10 intron I, part of K10 intron II (up to the *Eco*RI site) and part of K14 intron II (beginning at the *Bst*EII site), K14 intron III, and K14 intron IV; horizontal hatched box, substance P tag (17); stippled box, K14 3′ sequence. (*A Lower*) Protein secondary structure (15, 16) of K10, K10CΔ135, and K14CΔ135. Boxes denote conserved α-helical-containing regions of K10 (in grey) and K14 (in white). Nonhelical head domains are from K10 (K10CΔ135) or K14 (K14CΔ135). (*B*) Transient transfections of human SCC-13 epidermal keratinocytes with plasmids containing genes encoding substance P-tagged wild-type human K14 (*Left*), K10CΔ135 (*Middle*), and K14CΔ135 (*Right*). Transgene protein was detected with anti-P. (*C*) Transgenic K10CΔ135 mouse no. 29 and its 28-hr-old control littermate. (*Inset*) Higher magnification of front paw. (*D*) Anti-P immunoblot and Coomassie blue staining of IF proteins from K10 mutant and control mice. IF proteins were resolved by SDS/PAGE. Purified NΔ117CΔ42K14P was used as a protein standard (18). Gels were subjected to immunoblot analysis by using anti-P (lanes 1–13) or Coomassie blue staining (lanes 14 and 15). Lanes 1–6, NΔ117CΔ42 protein at 1400, 400, 100, 25, 10, and 5 ng, respectively; lanes 7–11, five expressing K10CΔ135 mice (nos. 29, 36, 2, 1, and 3, respectively); lane 12, nonexpressing K10CΔ135 mouse; lane 13, control mouse; lane 14, overloaded lane (20 μg) of IF proteins from mouse no. 29 to illustrate relative levels of endogenous K10 and two transgene K10 bands (arrows); lane 15, 3 μg of control mouse skin IF proteins.

ally thick, and cell size and organization within the granular layer were perturbed (Fig. 2*A*, transgenic; *B*, control). In skin from the highest expressing mice, cytolysis and intraepidermal shearing were prevalent. Such suprabasal shearing was similar to basal shearing in K14CΔ135 transgenic mice (5); it was not seen in control skin. In contrast, basal cells, hair follicles, and dermis seemed unaffected.

A gradient of cell degeneration existed from the inner to the outer suprabasal layers. This was most evident in semithin sections (Fig. 2*C*) and was strikingly similar to human skin from patients with EH (Fig. 2*D*). Human skin has a thicker spinous layer than mouse skin, and hence it was not surprising that EH skin exhibited more layers of degenerating cells than transgenic skin. Despite this difference, both skins exhibited a gradient of cytolysis, with seemingly normal basal and inner spinous layers and degenerating upper spinous and granular layers. This gradient paralleled the natural pattern of K1/K10 accumulation, which begins in the first suprabasal layer and increases throughout differentiation (10–14).

Expression of the Transgene Suprabasally and Stimulation of Proliferation Basally. To confirm that human K10 was appropriately expressed, we stained skin sections with antibodies against the transgene product. As expected, anti-P staining was exclusive to suprabasal cells of transgenic skin (Fig. 2*E*, transgenic; *E′*, control) and paralleled anti-mouse K10 staining (Fig. 2*F* and *F′*). In contrast, anti-K14 stained only basal cells (Fig. 2*G* and *G′*). Transgenic anti-P and anti-mouse K10 stainings were patchy, suggesting that mutant K10 expression may have altered the organization and/or expression of keratin in suprabasal cells. Finally, anti-filaggrin staining revealed a thickened granular layer, with large filaggrin-containing granules (Fig. 2*H* and *H′*). Stainings provided a graphic illustration that degeneration and intraepidermal shearing occurred suprabasally.

Interestingly, K6 was induced in mutant K10 skin (Fig. 2*I* and *I′*). This keratin and its partner K16 are normally restricted to hair follicles, but they are expressed suprabasally in epidermis of hyperproliferative skin (21). Incubation of control and K10CΔ135 mouse skin in medium containing bromodeoxyuridine (22), followed by anti-bromodeoxyuridine staining of skin sections, confirmed that basal cell proliferation was elevated ≈3–5 times in the K10 mutant mice

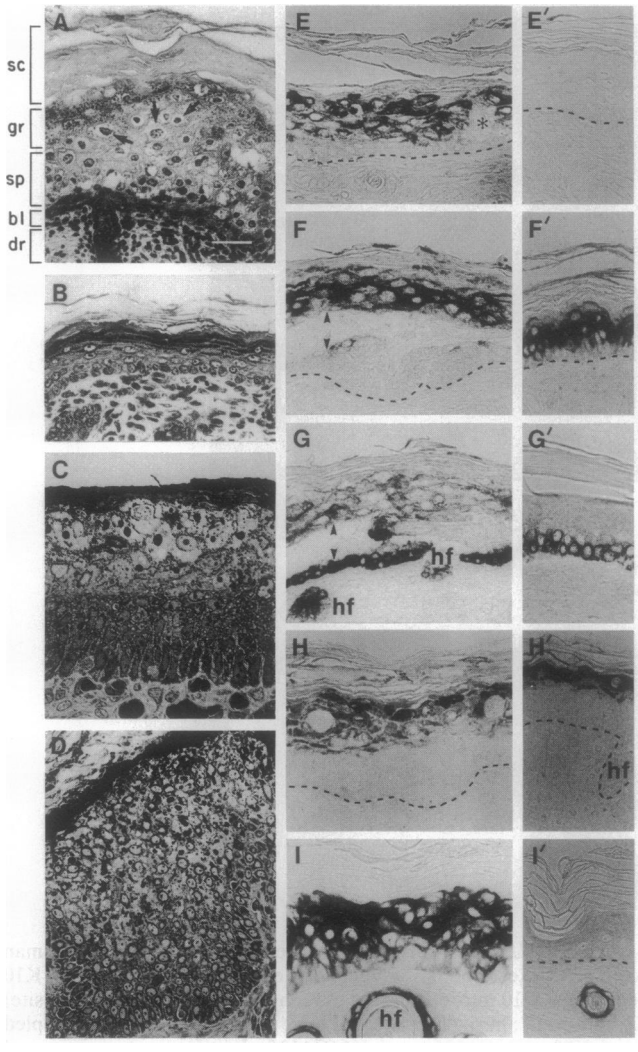

FIG. 2. Histochemistry of skin from K10 mutant mice. Skins from 28-hr-old control and K10 mutant mice were prepared for light microscopy (5). Sections (5 μm or 0.75 μm) were stained with hematoxylin/eosin (H/E), toluidine blue, or antibodies. (*A*) Transgenic back skin of mouse no. 1 (H/E). (*B*) Control back skin (H/E). (*C*) Semithin section of epoxy resin-embedded tissue of same animal as in *A*. (*D*) Semithin section of epoxy resin-embedded tissue of EH patient. (*E–I*) Transgenic skin (nos. 29 and 36) stained with anti-P [*E*; 1:100 dilution (17)], anti-mouse K10 (*F*; 1:100 dilution), anti-K14 (*G*; 1:200 dilution), anti-mouse filaggrin (*H*; 1:300 dilution), anti-mouse K6 [*I*; 1:300 dilution (13)]. (*E'–I'*) Control skin stained with same antibody series as in *E–I*. (Bar in *A* represents 75 μm for all panels except *D*, for which it represents 50 μm.) sc, stratum corneum; gr, granular layer; sp, spinous layers; bl, basal layer; hf, hair follicle; dr, dermis. The asterisk in *E* denotes patchy staining. Dashed lines denote basement membrane; arrows in *A* denote degenerated granular cells; opposing arrowheads in *F* and *G* denote suprabasal intraepidermal shearing.

(data not shown). Thus, the basal compartment seemed to be stimulated as a consequence of degeneration in the suprabasal compartment. The molecular mechanism by which this fascinating signaling takes place remains to be elucidated.

Aggregation of Suprabasal Keratin Filaments and Granular Cell Anomalies. The pathobiology and epidermal hyperproliferation of K10 mutant mice resembled that of the human disease EH (1–3). To explore further this relation, we compared the ultrastructure of transgenic and control mouse skin with that from EH patients. As expected, basal epidermal cells of mutant mouse and EH skin appeared normal, with the exception of a few signs of hyperproliferation (a greater than normal nuclear-to-cytoplasmic ratio, enlarged nucleoli, and poorly differentiated cytoplasm). In contrast, suprabasal layers of mutant K10 skin exhibited cytoskeletal aberrancies (Fig. 3). Tonofilaments were disorganized and often clumped (Fig. 3*A*, transgenic; *Inset*, control), a feature particularly prominent in upper spinous cells (Fig. 3*A'*). These clumps were labeled with both anti-P and anti-mK10 antibodies (Fig. 3*B*), were strikingly similar to those seen in EH skin (Fig. 3*C*), and were also present in granular layers (Fig. 3*D*). Moreover, keratohyalin granules were irregular in size and in shape, and they associated with filament clumps both in K10 mutant mouse (Fig. 3*E*) and in EH skin (Fig. 3*F*). Sometimes, the density of filament aggregates surrounding granules made distinction between the two structures difficult. This was in contrast to normal epidermis, where thin bundles of filaments associated with uniform granules (Fig. 3*G*). Finally, suprabasal cell degeneration was prevalent (see asterisks). Not all suprabasal cells with tonofilament clumps displayed signs of cytolysis, suggesting that cytolysis was subsequent to cytoskeletal abnormalities. These observations were consistent with our previous finding that keratinocytes become fragile without a proper keratin network (6).

Mutant-Induced Changes in Nuclear Shape and in Organization of IFs Around the Nucleus. Upper spinous cells of K10CΔ135 mouse skin often displayed perinuclear halos of clear cytoplasm, surrounded by shells of tonofilaments (Fig. 4*A*). These were also seen in suprabasal cells of EH skin (Fig. 4*B*; refs. 1–3) and were distinctly different from the normal nuclear-cytoplasmic architecture (Fig. 4*C*). While perinuclear rings of filaments have been observed in basal cells of mouse and human EBS (4–6), they are not as prominent as in suprabasal cells of EH, presumably because K1/K10 filaments are more abundant and bundled than K5/K14 filaments (23). Nevertheless, a correlation existed between perinuclear filament rings and mutant-induced perturbations of keratin networks in both basal and spinous cells.

Another characteristic of K10 mutant skin, previously described for EH (1, 4), was the presence of seemingly double nuclei in some suprabasal (mitotically inactive) cells (Fig. 4*D*). These lobes were sometimes uneven in size and shape, suggesting that these cells might have distorted rather than duplicated nuclei. Interestingly, in EBS, basal but not suprabasal cell nuclei were sometimes distorted and seemingly binucleate (Fig. 4*E*). The correlation between nuclear aberrations and defective keratin filament networks strongly suggested that the appearance of double nuclei in thin sections was induced by abnormalities in the cytoplasmic IF network.

DISCUSSION

Previously, we provided evidence to suggest that when the keratin network is perturbed, a basal epidermal cell becomes fragile and prone to lysis upon mild physical trauma (5, 6). *A priori*, since basal cells have a columnar shape and since mutant K14-expressing basal cells lysed in a defined zone beneath the nucleus and above the hemidesmosome, it seemed possible that fragility might be cell shape-dependent (6). While this may be true for columnar basal cells, our studies now extend the role of mechanical integrity to the K1/K10 cytoskeletal network and to the more flattened, suprabasal cells. Fragility was greatest in upper spinous layers and this can be attributed to several factors. First, K5/K14-containing filaments persist in inner spinous layers, even though anti-K14 staining is masked in these cells (10–14). Therefore, K5/K14 filaments in lower spinous layers will lessen destabilizing effects of K1/K10 mutants. Moreover, K1/K10 levels are higher in upper spinous layers, further increasing the ratio of suprabasal to basal keratins. Additional factors contributing to fragility of upper layers may be increased proximity of these cells to the skin surface and possible differentiation-dependent differences in cell shape and/or integrity of keratin networks. While additional

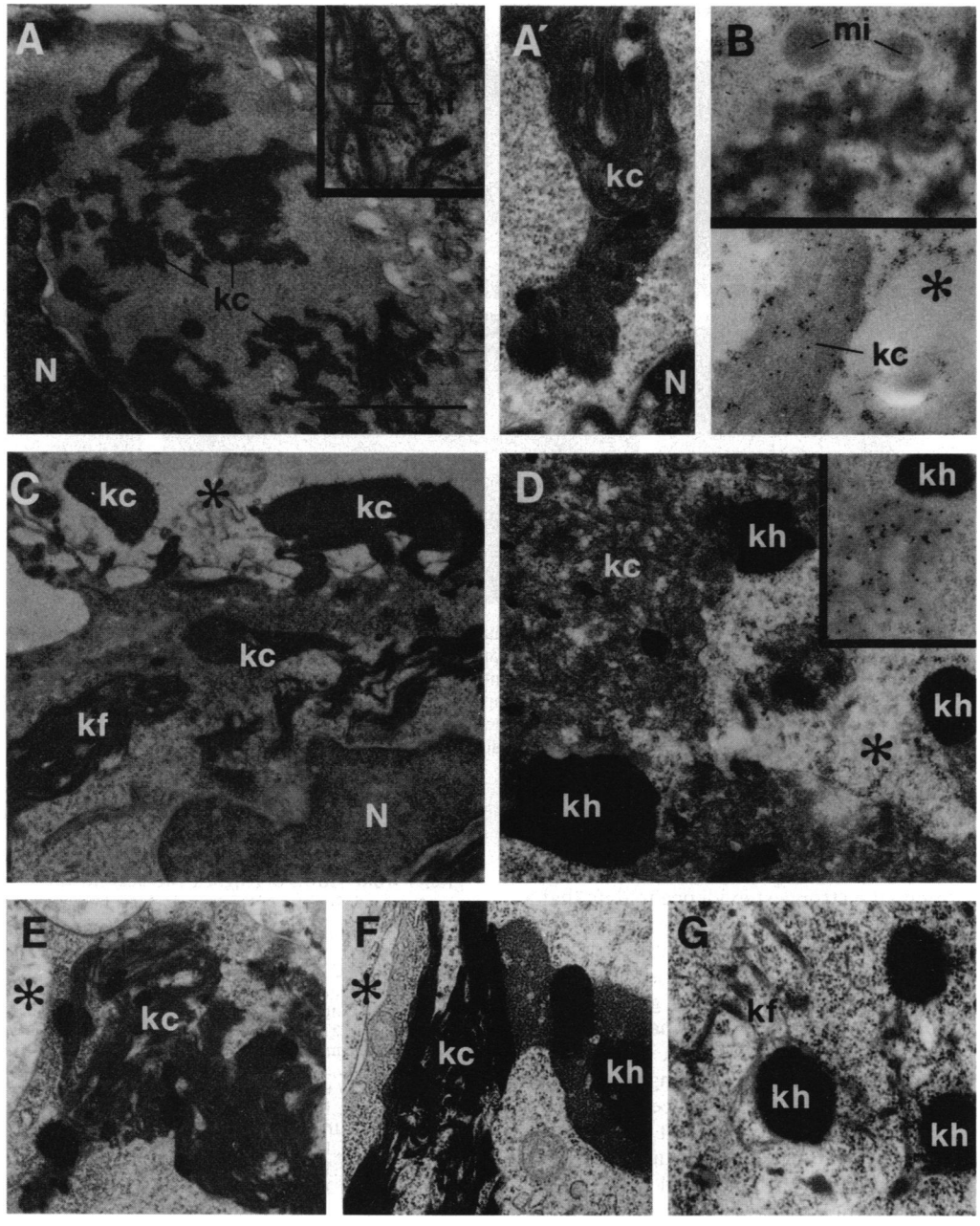

FIG. 3. Irregularities in the cytoskeletons of differentiating epidermal cells from a K10 mutant mouse and from a human EH patient. Back skins were from 28-hr-old transgenic and control mice and from an adult EH patient. Skins were fixed and sectioned for routine and immunoelectron microscopy (23). Antisera used were rabbit anti-P (1:300 dilution) and mouse anti-K10 (1:150 dilution), with a 15-nm gold particle-conjugated secondary antibody (23). Specificity of cytochemical labeling was confirmed by repeating with preimmune sera and by repeating with omission of the primary antisera step. Spinous cell from a transgenic mouse (*A*) or control mouse (*A Inset*) are shown. (*A'*) Keratin filament aggregates in an upper spinous cell. (*B*) Keratin clumps and tonofilaments in a transgenic spinous cell labeled with anti-P (*Upper*) or anti-mouse K10 antibodies (*Lower*). (*C*) Spinous cell from patient with EH. (*D*) Granular cell from a transgenic mouse. (*D Inset*) Anti-P staining of tonofilament clumps associated with keratohyalin granules. (*E*) Transgenic granular cell with aggregates of keratin filaments in association with keratohyalin granules. (*F*) Similar to *E*, but from EH epidermis. (*G*) Granular cell of control mouse. (Bar in *A* represents 1 μm in *A*, *A'*, *B*, *D*, *F*, and *G* and 0.5 μm in *C* and *E*.) N, nucleus; kc, keratin clumps; kf, keratin filaments; mi, mitochondria; kh, keratohyalin granules. Asterisks indicate cytolysis.

studies will be necessary to elucidate finer differences in cell fragility, our studies, first with K14/K5 and now with K1/K10, make it tempting to speculate that integrity of other cell types expressing other keratin pairs or other IF proteins may be compromised without a proper IF network. In Alzheimer's disease, for instance, disorganization of the neurofilament network may contribute to cell degeneration.

The progressively increasing ratio of mutant to wild-type keratins from lower to upper suprabasal layers enabled us to examine stages of cell degeneration. This revealed a parallel between mutant keratin expression, perinuclear filament shells, and nuclear aberrancies. Early EH reports also described binucleate suprabasal cells (1–4), a feature purportedly verified by the presence of double nucleoli (1). However, normal uninuclear epidermal cells frequently have double nucleoli (Q.-C. Yu and E.F., unpublished results), and hence this is not a reliable measure. It could be that perinuclear shells interfere with normal cell division both within the basal layer (K5/K14 defects) and as cells complete their final DNA replication upon commitment to terminally differentiate (K1/K10 defects). Alternatively, keratin filaments may play a vital role in providing a scaffold to support the nucleus and perhaps other cyto-

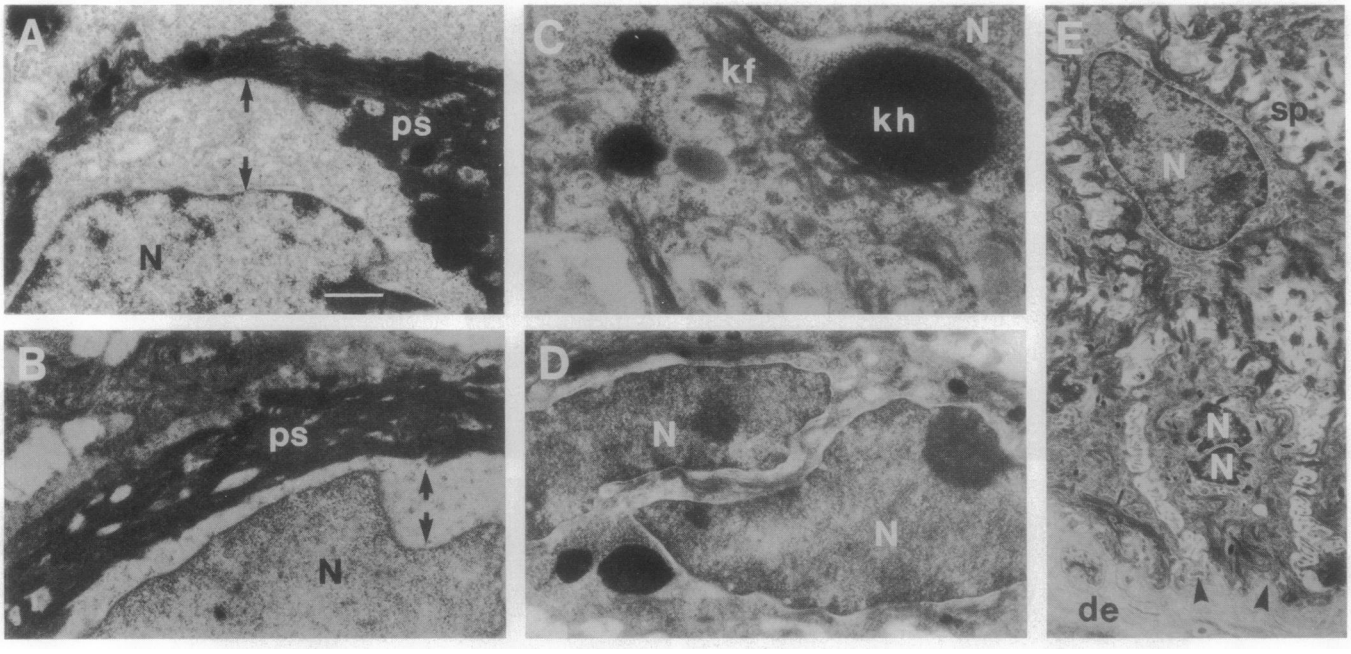

FIG. 4. Mutant keratin-induced irregularities in keratin filament–nuclear interactions and nuclear shape. Micrographs were prepared as described in the legend to Fig. 3. (*A*) Transgenic spinous cell with nuclear halo of clear cytoplasm (opposing arrows), surrounded by perinuclear shell of tonofilament aggregates. (*B*) Same as *A*, but from an EH patient. (*C*) Cytoplasm surrounding nucleus of a control mouse spinous cell. (*D*) Transgenic spinous cell showing two nuclei. (*E*) Skin from patient with EBS Weber–Cockayne, denoting aberrant nuclear shape and binucleate appearance in basal but not spinous cells. (Bar in *A* represents 0.5 μm for *A–D* and 3 μm for *E*.) N, nucleus; ps, perinuclear shell; kf, keratin filaments; kh, keratohyalin granules; sp, spinous cell; de, dermis.

plasmic organelles. In this case, keratin defects might weaken the scaffold, leading to nuclear distortion and giving the appearance of binucleation in ultrathin sections. Serial sectioning should distinguish between these possibilities.

Our data also provide the strongest evidence to date that a genetic basis for human EH resides in defective keratin networks within differentiating epidermal cells. We found that aberrancies in K1/K10 keratin networks affected filaggrin-containing keratohyalin granules and granular cell shape, perhaps explaining why some researchers have suggested that EH may be a filaggrin defect (24). Although we have not ruled out this possibility, the timing of K1/K10 synthesis more closely parallels the appearance of EH aberrancies than does filaggrin synthesis. Thus, it seems likely that, as first suggested by Anton-Lamprecht and Schnyder (3), the majority of EH cases will arise from keratin gene defects. Unequivocal verification must necessarily await analysis of K1 and K10 genes in EH patients.

Based on our studies with K14, those mutants that more severely disrupt filament assembly are those that generate a severe phenotype, both in mice (5, 6) and in humans (7–9; 25). Given the parallels between our K14 and K10 mutant transgenic animals and the parallels between EBS and EH, it seems likely that different mutations in the human K1/K10 genes will account for the variation in severity of the EH phenotype. A consideration for EH not relevant for EBS is that in palmar/plantar skin there is a unique keratin, K9, expressed suprabasally (10, 11). Thus, some cases of epidermolytic palmoplantar keratoderma, resembling EH (3, 4), may arise from K9 mutations. As further studies are conducted, the extent to which human EH arises from different mutations in suprabasal keratins should become apparent.

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