

Gene Targeting at the Mouse Cytokeratin 10 Locus: Severe Skin Fragility and Changes of Cytokeratin Expression in the Epidermis

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Abstract. Bullous congenital ichthyosiform erythroderma (BCIE) is a dominantly inherited blistering skin disorder caused by point mutations in the suprabasal cytokeratins 1 or 10. Targeting the murine *cytokeratin 10* gene in ES cells resulted in mice with different phenotypes in the homozygotes and heterozygotes; both of which exhibit similarities to specific clinical characteristics of BCIE. Homozygotes suffered from severe skin

fragility and died shortly after birth. Heterozygotes were apparently unaffected at birth, but developed hyperkeratosis with age. In both genotypes, aggregation of cytokeratin intermediate filaments, changes in cytokeratin expression, and alterations in the program of epidermal differentiation were observed. In addition we demonstrate, for the first time, the existence of the murine equivalent of human cytokeratin 16.

IN certain cultured cells and tissues like skin, cytokeratin intermediate filaments are among the most prominent cytoskeletal proteins (Fuchs and Green, 1978, 1980; Franke et al., 1981). Ironically this has not helped to unravel their functions, although many have been suggested during two decades of intense research (e.g.: Traub, 1985; Traub and Shoeman, 1994). Demonstration of a complete absence of cytoplasmic intermediate filaments in cultured cells (Venetianer et al., 1983) without a recognizable effect on basic cellular functions has hinted towards their involvement in development and differentiation but also stressed the requirement for more complex experimental systems, such as animal models, to study their function.

For structural reasons, the ~20 epithelial cytokeratins and ~10 hair keratins were grouped into the type I and II classes of intermediate filaments proteins (Moll et al., 1982; Schiller et al., 1982; Fuchs and Weber, 1994). Unlike most other intermediate filaments proteins, the expression of the two cytokeratin classes is highly regulated during development and in tissue differentiation, with anything between 2 and 11 cytokeratins in the same cell. They form obligatory copolymers of equimolar amounts of at least one member of each class (Moll et al., 1982; Sun et al., 1984). Whereas in vitro an unlimited promiscuity between any type I and II is possible (Hatzfeld and Franke, 1985), the patterns of cytokeratin expression in vivo are restricted (Moll et al., 1982; Sun et al., 1984), suggesting distinct functions for various cytokeratin pairs. All basal ke-

ratinocytes of stratified epithelia, for example, synthesize predominantly cytokeratins 5 and 14, with minor amounts of cytokeratin 15 (Moll et al., 1982; Leube et al., 1988; Lloyd et al., 1995), which upon terminal differentiation of skin are sequentially replaced by cytokeratins 1, 2e, and 10 (Fuchs and Green, 1980; Collin et al., 1992).

Like in other intermediate filament subunits the ends of the cytokeratin rod domains are highly conserved and believed to be essential for correct assembly (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1991, 1992). Mutations in these domains or their complete removal lead to a collapse of intermediate filaments (Albers and Fuchs, 1987, 1989; Hatzfeld and Weber, 1990; Wilson et al., 1992), whereas amino acid alterations along the rod seem to interfere less with intermediate filaments stability.

The logical extension of previous work on cytokeratin domain analysis (Albers and Fuchs, 1987, 1989) has resulted in transgenic mice that overexpress considerable amounts of truncated, peptide-tagged cytokeratin 14 subunits in the basal layer of epidermis (Coulombe et al., 1991b). Such truncated subunits collapse existing filaments, acting as dominant-negative chain terminators (Hershkowitz, 1987). The mice overexpressing truncated cytokeratin 14 developed fragile skin with extensive blistering and aggregation of cytokeratin filaments in the basal epidermal compartment, resembling epidermolysis bullosa simplex (EBS)¹, a human inherited skin disease with previously unknown aetiology (Anton-Lamprecht, 1978). This has stimulated the search for point mutations in epidermal cytokeratin genes of patients with mostly dominantly inherited skin disorders. These include EBS (cytokeratins 5

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1. *Abbreviations used in this paper.* BCIE, bullous congenital ichthyosiform erythroderma; EBS, epidermolysis bullosa simplex; PGK, phosphoglycerate-kinase.

or 14; Bonifas et al., 1991; Coulombe et al., 1991a; Lane et al., 1992), epidermolytic palmoplantar keratoderma (cytokeratin 9; Reis et al., 1994; Torchard et al., 1994), pachyonychia congenita (cytokeratins 6, 16, or 17; Bowden et al., 1995; McLean et al., 1995), ichthyosis bullosa of Siemens (cytokeratin 2e; Kremer et al., 1994; McLean et al., 1994; Rothnagel et al., 1994) and bullous congenital ichthyosiform erythroderma (BCIE; cytokeratins 1 or 10; Chipev et al., 1992; Rothnagel et al., 1992). The majority of mutations found so far in the different cytokeratins cluster around the conserved helical domains with a few in other domains except the tail (McLean and Lane, 1995).

Common to all these disorders is the aggregation/alteration of cytokeratin filaments that leads to cytolysis and blistering. The cytokeratin carrying the mutation clearly dictates the layer of the epidermis affected and determines some of the clinical features that distinguish these diseases. There is considerable variation, however, within these diseases in the severity, onset and the body sites involved (Anton-Lamprecht, 1994; DiGiovanna and Bale, 1994; McLean and Lane, 1995). It is not yet clear whether these differences can be correlated to the position of a given mutation in the relevant cytokeratin gene.

BCIE is caused by point mutations in either cytokeratin 1 or 10 (Cheng et al., 1992; Chipev et al., 1992; Rothnagel et al., 1992), which are expressed in the suprabasal epidermis. Histological features include cytokeratin filament aggregation, alteration of nuclear shape, vacuolization of spinous and granular layer keratinocytes and cytolysis. Clinically BCIE includes localized or extensive blistering with denudation in severe cases as well as acanthosis and hyperkeratosis (Traupe, 1989; Ishida-Yamamoto et al., 1992; Anton-Lamprecht, 1994). As in other cytokeratin-related disorders that show hyperkeratosis, the link between cytokeratin mutations and cell proliferation is unclear. Complicated by erythroderma and secondary infections of the upper epidermis this disorder is very debilitating and disfiguring.

So far a limited number of mouse models to study intermediate filaments functions have been generated including conventional transgenic mice overexpressing either intact or truncated cytokeratins in appropriate or ectopic sites (Coulombe et al., 1991b; Vassar et al., 1991; Fuchs et al., 1992; Blessing et al., 1993; Rothnagel et al., 1993; Albers et al., 1995). Although mice overexpressing cytokeratin 10/14 hybrid or cytokeratin 14 truncations developed phenotypes with some similarity to EBS or BCIE (Coulombe et al., 1991b; Vassar et al., 1991; Fuchs et al., 1992), they suffered from copy number and position effects of conventional transgenes. It remains difficult to decide whether unbalanced and/or overexpression of cytokeratins or cytokeratin fragments represent gain or loss of function phenotypes. Since recently reported knockout mice that lacked the type III intermediate filaments proteins vimentin (Colucci-Guyon et al., 1994) or GFAP (Gomi et al., 1995; Pekny et al., 1995) showed no recognizable phenotype, the discussion of intermediate filaments function has been reopened. However, the demonstration of mid-gestational lethality of cytokeratin 8 knockout mice (Baribault et al., 1993, 1994) and two extremely rare cases of EBS patients lacking cytokeratin 14 in their basal epidermis (Chan et al., 1994; Rugg et al., 1994) suggested that

cytokeratin intermediate filaments do play essential roles. Such patients, however, cannot provide reasonable models to study the biology of disease. Support for the requirement of cytokeratin also came from recently published cytokeratin 14 knockout mice (Lloyd et al., 1995).

In the present study, we have initiated a mouse model for BCIE by targeting the mouse *cytokeratin10* gene in ES cells. Homozygous and heterozygous cytokeratin 10 knockout mice develop a phenotype with histological and ultrastructural similarity to BCIE and reveal the previously uncertain existence of the murine cytokeratin 16, a marker for wound healing, repair, and hyperproliferation (Weiss et al., 1984).

Materials and Methods

Construction of the Targeting Vector

The targeting vector was constructed from a 6-kb BamHI fragment including *cytokeratin 10* sequences starting at bp 246 (these data available from GenBank under accession number M10081). This fragment was obtained from an 129/ola-derived genomic DNA library (constructed by T.M. Magin), by screening with a probe containing 330 bp of the 3' untranslated region of the human *cytokeratin 10* gene (kindly provided by W.W. Franke, German Cancer Research Centre, Heidelberg, Germany).

The targeting vector contains two arms of mouse *cytokeratin 10* genomic DNA; a 5'-1.2-kb SmaI-EcoRI fragment and a 3'-2.7-kb XhoI-BamHI fragment, separated by the phosphoglycerate-kinase (PGK) promoter-driven HPRT minigene (PGK/pDWM1) (Magin et al., 1992). The HPRT minigene replaces exons 3-7 and is in the antisense orientation with respect to the cytokeratin 10 transcription unit. This construct was inserted into a Bluescript II SK+ vector containing a Herpes simplex virus thymidine kinase gene. The targeting vector was linearized with XbaI, so that vector sequences were downstream of the thymidine kinase gene (see Fig. 1 a). For electroporation, DNA was purified by Qiagen columns (Qiagen, Inc., Chatsworth, CA), followed by phenol/chloroform extraction and ethanol precipitation.

Cell Culture and Electroporation

Culture conditions for the embryonic stem cell line HM-1, electroporation with linearized vector DNA, and selection of targeted colonies were described previously (Stacey et al., 1994).

Preparation and Analysis of Genomic DNA

Colonies surviving the HAT/ganciclovir selection regime were transferred into 24-well plates (Falcon Plastics, Cockeysville, MD) and approximately half of each was used for PCR analysis (Selfridge et al., 1992). The primers used were specific for the sequence upstream of the SmaI site of *cytokeratin 10*: ATGGCAACTCAAGCCAGCGAG (these sequence data available under GenBank accession number M10081 nucleotides 724-744) and for the HPRT minigene: AGCCTACCCTCTGGTAGATTGTCG (HPRT cDNA sequence, positions 960-983). Reaction conditions were as described before (Selfridge et al., 1992) with the following time/temperature parameters: 30 s 94°C, 30 s 65°C and 1 min 72°C. An Omnigene thermocycler (Hybaid, Teddington, UK) was used. Taq polymerase was purchased from GIBCO BRL (Gaithersburg, MD). Genomic DNA from colonies that produced a PCR product was isolated from confluent ES cell cultures by proteinase K and RNAase A digestion at 37°C overnight, followed by phenol/chloroform extraction and ethanol precipitation. Genomic Southern blotting was carried out under stringent conditions (washes at 68°C in 0.1 × SSC) to analyze the structure of the targeted allele by hybridization with three probes: (a) 3.5-kb downstream sequence outside the targeting vector; (b) the HPRT minigene, and (c) the 0.5-kb BamHI/SmaI fragment located upstream of the targeting vector.

Preparation and Identification of Transgenic Mice

Cells from colonies with the correct DNA structure as determined by PCR and genomic Southern analysis were injected into blastocysts from C57B16 mice and returned to CBA or MF-1 recipients. Chimeras pro-

duced were bred with C57Bl6, 129 or Balb/c mice and germline pups carrying the targeted allele were identified by coat color and PCR of tail DNA (as described for ES cell colonies). F1 pups carrying the altered allele were interbred. Pups resulting from these matings were genotyped using a PCR assay of mouse tail DNA that included the two primers described for identifying ES cell colonies and an additional primer downstream of the EcoRI site: ACAGGAGGATGACGGTATACC (these data are available from Genbank under accession number L00193; positions 461–441). This PCR reaction gave two products; one specific for the wild type (1.1 kb) and one for the altered allele (1.4 kb).

RNA Analysis of Litters

The skin was removed from decapitated neonates after a 30–40-s immersion in distilled water at 65°C and snap frozen at –70 °C. Total RNA was extracted as described by Chomczynski and Sacchi (1987) with minor modifications. Aliquots (30 µg) were loaded on 1.5% agarose gels in the presence of formamide/formaldehyde as denaturant and RNA was transferred to Genescreen Plus membrane (Du Pont Co., Wilmington, DE). The 5' BamHI-SmaI fragment and the 3' nontranslated region of mouse *cytokeratin 10* were used as probes for cytokeratin 10 message. Probes used to examine expression levels of other cytokeratins included the 3' nontranslated region of mouse cytokeratins 1, 17, and 14 (all donated by Dr. H. Winter, German Cancer Research Centre, Heidelberg). Cytokeratin 16 probes were prepared from a vector containing the human cytokeratin 16 cDNA (donated by Prof. E.B. Lane, University of Dundee) by digestion with MscI and XbaI. The cytokeratin 16 probe coded for 120 bp of the tail domain.

Protein Analysis of Skin

Protein extracts enriched in cytoskeletal proteins were prepared from frozen skins by homogenization as described previously (Magin et al., 1983) in the presence of 2 mM PMSF. One- and two-dimensional gel electrophoresis were carried out essentially as described (Magin et al., 1983). For separation by isoelectric focusing the ampholines (Sigma Chem. Co., St. Louis) pH 4–6 (0.6%), pH 5–8 (0.4%), pH 7–9 (0.6%), and 3.5–10 (0.4%) were used to allow better separation of basic cytokeratins. For nonequilibrium pH gradient electrophoresis, broad-range ampholines pH 3.5–10 (2%) were used. Proteins were transferred electrophoretically to nitrocellulose membranes (0.2-µm pore size; Schleicher and Schuell) in a semi-dry transfer cell (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. The membrane was blocked with 1% fetal calf serum in Tris-buffered saline with Tween 20 (TBST) (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% Tween 20) at room temperature and incubated with the primary antibody diluted in TBST for 2 h. Primary antibody dilutions were as follows: DEK-10 (cytokeratin 10; Dako Ltd., Hamburg, FRG) 1:1,000; KG 8.60 (cytokeratins 1, 10; Sigma Chem.) diluted 1:4,000; LH2 (cytokeratin 10; Lane et al., 1991) diluted 1:5; LL001 (cytokeratin 14; Purkis et al., 1990) diluted 1:5; LL025 (cytokeratin 16; Lane et al., 1991) diluted 1:5 (LH2, LL001, and LL025 were donated by Prof. I.M. Leigh, Royal London Hospital). For detection an anti-mouse IgG alkaline phosphatase conjugate (Promega Corp., Madison, WI) was diluted 1:7,500 and detected using NBT and BCIP as recommended by the manufacturer.

Immunofluorescence

Frozen sections (5 µm thick) of mouse skin were fixed in acetone at –20°C for 10 min and blocked with 5% goat serum. All antibodies were diluted in 1% BSA in PBS. The primary antibodies dilutions were as follows: EAB 904 (anti-cytokeratin 1; Ortho Diagnostics, Neckargemünd, FRG) undiluted, RKSE 60 (anti-cytokeratin 10, kindly provided by Dr. Ramaekers, Maastricht, The Netherlands) 1:20, LL001 (anti-cytokeratin 14) undiluted. A Texas-Red conjugated donkey anti-mouse IgG antibody diluted 1:200 (Jackson ImmunoResearch Labs., Inc., West Grove, PA), was used as the secondary antibody. Slides were mounted in Mowiol (Calbiochem Novabiochem, La Jolla, CA) containing 0.1% Dabco.

Transmission Electron Microscopy

Skin samples were processed for electron microscopy as described (Eady, 1985) with a few modifications. Briefly, primary fixation was for 48 h at 4°C in half strength Karnovsky fixative (Karnovsky, 1965). Specimens were post-fixed in 1.3% osmium tetroxide in distilled water for 2 h at 4°C. After dehydration in a graded ethanol series, specimens were embedded in epoxy resin (Taab 812) via propylene oxide. Semithin sections (0.5 µm)

and ultrathin sections (60–90 nm) were cut on a Reichert OMU-4 ultramicrotome. Semithin sections were stained with Richardson's stain (Richardson et al., 1960). Ultrathin section were collected on pioloform-coated copper grids and double stained with 5% uranyl acetate in ethanol and lead citrate (Reynolds, 1963). Sections were viewed using a Jeol 100 CX transmission electron microscope (JEOL U.S.A. Inc., Peabody, MA).

Results

Isolation and Targeting of the Mouse Cytokeratin 10 Gene

To achieve good gene targeting frequencies we reisolated the previously identified mouse *cytokeratin 10* gene (Krieg et al., 1985) from an isogenic 129/ola DNA genomic library. To introduce several independent point mutations by our two-step gene replacement procedure (Stacey et al., 1994) the targeting vector was designed to replace cytokeratin 10 exons 3–7 with an HPRT minigene (Fig. 1 a). Here we report on results after the first targeting step 3 out of 96 selected colonies resistant to HAT/ganciclovir yielded a PCR product diagnostic for gene targeting. Southern blotting confirmed the structure expected for a homologous recombination event (Fig. 1 b), and ES cells from one clone were used to generate chimeras that showed germline transmission at a good frequency. Most results were derived from intercrosses between 129/C57Bl6 founder animals containing the cytokeratin 10 targeted allele and C57Bl6 or Balb/c mice.

Homozygous Cytokeratin 10 Knockout Mice Show High Perinatal Lethality

Mice heterozygous for the targeted cytokeratin 10 allele were intercrossed to study the phenotype of homozygous cytokeratin 10 knockout mice. Generally homozygous mice exhibited very fragile skin with severe erosions and erythema. Skin loss and/or erythema were seen frequently on the ventral aspects surrounding the umbilicus, but also elsewhere on the body. Blisters appeared around the face, the forepaws and elsewhere on the trunk (Fig. 2 a). The extent of skin loss varied considerably, even within a single litter and in repeated litters from the same breeding pairs. These mice represented ~25% of all pups, arguing against intrauterine loss. Affected pups died a few hours after birth. When on rare occasions we noted pups being born, homozygotes appeared with little or no damage to their skins. However, persistent grooming by their mother or other mice led to skin loss, offering a possible explanation for the variation in the extent of skin loss when the mice were found. In contrast to normal or heterozygote animals, the stomachs of homozygotes did not contain milk. It may be significant that in mice, unlike in humans, the esophagus and forestomach have stratified squamous epithelia (Hummel et al., 1966) that express cytokeratins 1/10 (Schweizer et al., 1988).

Heterozygous Cytokeratin 10 Knockout Mice Develop Severe Hyperkeratosis but Survive to Adulthood

Pups with one targeted allele usually showed a normal phenotype at birth, although erythroderma was occasionally observed on the forepaws. Hyperkeratosis was first observed over the trunk region at the onset of hair growth

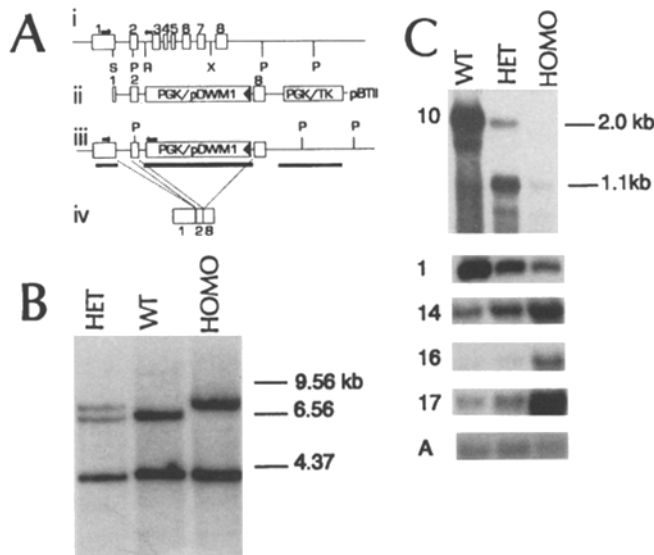


Figure 1. (A, i) Structure of the cytokeratin 10 locus. Arrows indicate the position of PCR primers used to identify the wild-type allele. Letters show positions of key restriction sites: S, SmaI; P, PstI; R, EcoRI; X, XhoI. (ii) The targeting vector consists of an HPRT minigene (PGK/DWM1) flanked by two arms of homology to mouse cytokeratin 10: at the 5' end a 1.2-kb SmaI-R1 fragment including exon 2; at the 3' end an XhoI-BamHI fragment including exon 8 and 1.5 kb of sequence downstream of cytokeratin 10. A thymidine kinase gene (PGK/TK) is also included for selection against random integration. The vector was linearized such that the vector (pBTII) sequences were downstream of the TK gene. (iii) The targeted locus; arrows indicate the position of the PCR primers used to identify the targeted allele. Probes used for Southern analysis are shown as thick lines underneath; (iv) the presumptive mRNA product, derived from exons 1, 2, and 8. (B) Southern analysis of wild-type, heterozygote, and homozygote mice. DNA was digested with PstI and labeled with the 3' probe shown in (iii). (C) Northern analysis. Cytokeratin 10 RNA was detected using the 5' and 3' probes. Identical bands were obtained with both probes (5' probe shown). The normal allele expresses a product of 2.0 kb and the altered allele one of 1.1 kb. Cytokeratin 16 RNA was detected with a human probe coding for the carboxy-tail domain. Other cytokeratins were detected with mouse probes to the 3' nontranslated region. An actin probe was used to compare loading of total RNA. Northern blots for cytokeratin 10 were exposed for shorter times and performed in a separate gel than those for other cytokeratins.

but improved shortly afterwards. From the third week on, most of the heterozygotes developed hyperkeratosis and scaling on the feet, the ears and the tail, the latter with a distinctive furrowed, verrucous skin (Fig. 2, b-d). Feet covered with yellow crusts that healed without scarring were a distinct feature of mice (this is also a characteristic of BCIE patients, see Frost and Van Scott, 1966). The degree of scaling and hyperkeratosis increased with age, again resembling the human phenotype. Examination of several animals revealed that the weight of heterozygous animals was only $\sim 2/3$ of normal littermates at three months of age and beyond. The older heterozygous animals were susceptible to loss of skin on the bristle pads. The expression of cytokeratins 1 and 10 is first detected in the embryo in the nasal region (embryonic day 13.5) and

the whisker pads (embryonic day 14.5) before general expression in other regions of the skin (embryonic day 15.5) (Byrne et al., 1994). The nose and bristle pads may therefore be particularly sensitive to loss of cytokeratin 10 in the adult. Feeding through the bars of the cage and grooming would potentially exacerbate a weakness. Eye and skin infections were also more common in heterozygous adults than other adult animals which is probably due to bacterial or fungal growth in dead scaling skin as observed in patients with BCIE (Baden, 1991).

Changes in Skin Morphology and Aggregation of Cytokeratin Filaments in Cytokeratin 10 Knockout Mice

To examine the effect of the cytokeratin 10 knockout on the distribution and structure of cytokeratin filaments and their consequence for cell integrity, light and electron microscopy of forepaw epidermis were carried out. Compared to the wild-type the nonlesional suprabasal epidermis of heterozygote animals was slightly acanthotic and hyperkeratotic (Fig. 3, a and b) with an increase of oedematous cells. The intercellular space in the upper spinous and the granular layer was markedly widened. Ultrastructurally spinous and granular cells showed alterations in the distribution of cytokeratin filament bundles. Areas of filament-free cytoplasm were seen next to abnormal cytokeratin aggregates (Fig. 4 c). These cytokeratin aggregates were mostly located in the cell periphery, either close to the membrane or attached to desmosomes (Fig. 5, d and e). Remnants of intermediate filaments were also found associated with keratohyalin granules. The distribution of mitochondria and other organelles was not obviously altered in the heterozygous cytokeratin 10 knockout mice. The size of the cytokeratin aggregates was generally smaller in mice than those in affected human tissue. We did not find perinuclear shells in any of the sections, in contrast to those described in various forms of BCIE (Traupe, 1989). As in BCIE patients the basal layer was unaffected in knockout mice.

Lesional skin of homozygote animals was markedly acanthotic and hyperkeratotic and the upper layers were abnormally translucent (Fig. 3 c). By electron microscopy many spinous and granular cells showed intracellular oedema formation and were lysed. The number of cytokeratin filament bundles appeared to be markedly reduced (Fig. 4 d), a finding confirmed by our biochemical analyses (see below). The aggregates were distinctly smaller in homozygous than in heterozygous mice and had a tendency to round up (Fig. 5 e). Some of the remaining cytokeratin bundles were ultrastructurally abnormal with short wire-like filaments, others were morphologically inconspicuous and tended to be associated with desmosomes (Fig. 4 d) or close to the plasma membrane. Overall, the continuity of cytoplasmic intermediate filaments connecting neighboring cells through desmosomes was severely disrupted. The transition zone between granular and cornified layers was broadened with many incompletely cornified cells in the lower most stratum corneum (Fig. 5, a and b). The cytoplasm of these cells contained remnant organelles, indicating a disruption of the normal differentiation pathway.

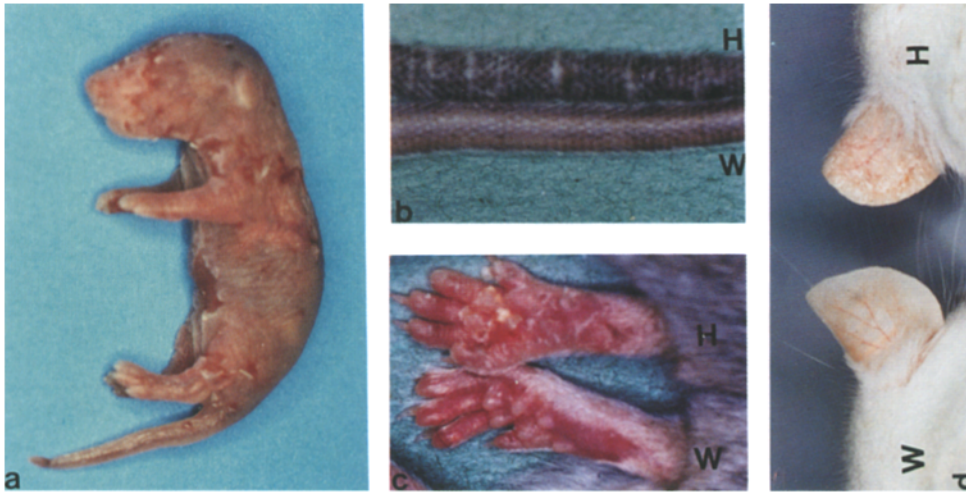


Figure 2. (a) 1-d-old homozygous mouse. Mice homozygous for the targeted allele exhibit large areas of skin loss. Heterozygous mice (H) showed extensive hyperkeratotic scaling of the skin on (b) tail, (c) paws, and (d) ears. A wild-type (W) litter mate is shown on the left of each picture for comparison. Both mice were ~2 mo old.

Immunofluorescence Analysis of Homo- and Heterozygous Cytokeratin 10 Knockout Mice Indicates Changes in Suprabasal Cytokeratin Patterns

During the preparation of frozen sections it was noted that the suprabasal epidermis of heterozygous and even more of homozygous mice did split off from the basal layer, thus confirming the skin fragility of live mice. When frozen sections of back skin (Fig. 6), tail, or forepaw (not shown) of homozygous cytokeratin 10 knockout mice were incubated with several cytokeratin 10 antibodies, no staining was observed. A reduced, but clearly persistent, level of staining with cytokeratin 1 antibodies was seen in sections stained in parallel, in agreement with several reports demonstrating reduced stability of unbalanced type I or II cytokeratins (e.g., Kulesh et al., 1989; Lu and Lane, 1990; Magin et al., 1990; Blessing et al., 1993). Although immunofluorescence analysis is not a quantitative technique, with other explanations like epitope masking as a formal possibility, our biochemical analysis confirmed a decrease in cytokeratin 1 (see below). This is in contrast to the unaltered expression of cytokeratin 5 in cytokeratin 14 knockout mice (Lloyd et al., 1995). As the terminal domains of cytokeratins 1/10 are sequentially removed during terminal

differentiation of the epidermis (Fuchs and Green, 1980; Banks-Schlegel, 1982), they might naturally be more susceptible to proteolysis than those of cytokeratins 5 or 14 when present in unequal amounts.

In BCIE patients, the cytokeratin filament system of the basal cell layer is generally unaffected by changes in the suprabasal layer (Ishida-Yamamoto et al., 1992). Our studies showed a comparable level of cytokeratin 14 staining in the basal layer of back, tail, and paw skin (not shown) in all genotypes with some suprabasal cell staining, in accordance with published data (Schweizer, 1993). There was however no increase in cytokeratin 14 in suprabasal cells of the knockout mice as a potential consequence of the targeted disruption of cytokeratin 10.

Injection of newborn control and cytokeratin 10 knockout mice with bromodeoxyuridine and detection by anti-bromodeoxyuridine antibodies provided evidence for an increase (~1.5–2-fold) in basal cell proliferation in the knockout mice (not shown). Similar observations were made in conventional transgenic mice expressing a truncated human cytokeratin 10/14 hybrid in the suprabasal epidermis (Fuchs et al., 1992) and in BCIE patients. Taken together with our own findings in the cytokeratin 10

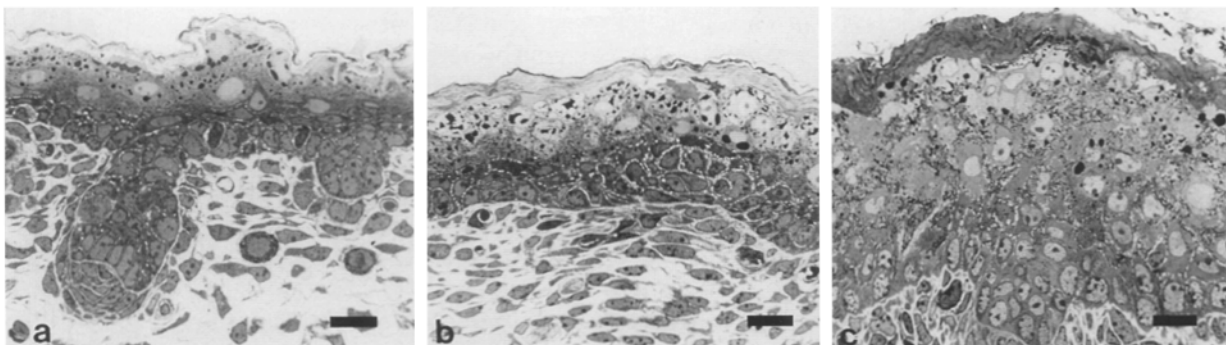


Figure 3. Light micrographs of semithin (1 μm) resin sections of murine paw skin showing epidermal changes in animals heterozygous (b) and homozygous (c) for the truncated cytokeratin 10 gene, in comparison to normal ridged skin (a) of wild-type animals. (a) Ridged epidermis comprises of 6–8 nucleated cell layers and a thin orthokeratotic stratum corneum. Epidermal buds are indicative of forming eccrine glands. (b) Lesional ridged skin is slightly acanthotic and hyperkeratotic with signs of incomplete cornification in places. (c) Perilesional ridged epidermis is markedly acanthotic and hyperkeratotic. Numerous suprabasal cells contain granular or fibrillar material. All, Richardson stain. Bars, 20 μm .

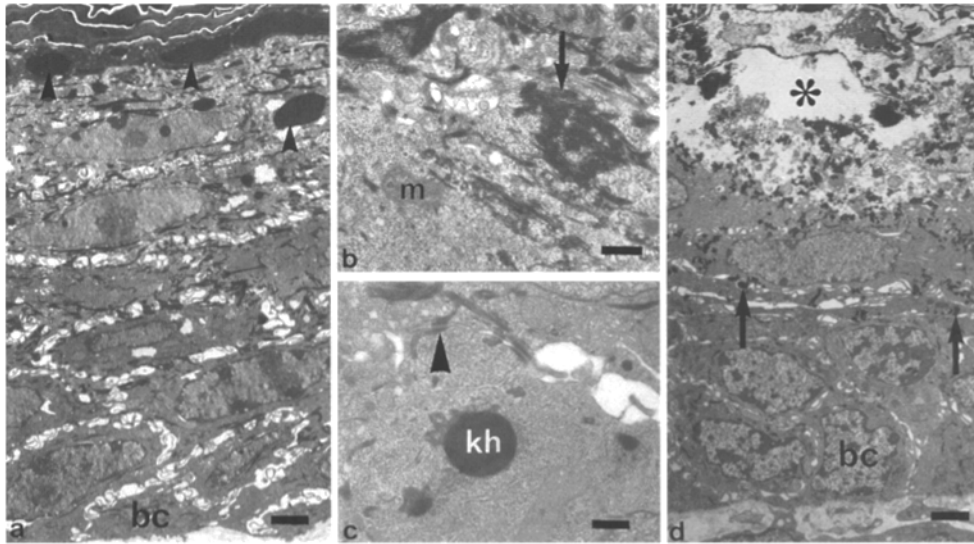


Figure 4. Ultrastructural analysis of paw epidermis from wild-type (a), heterozygous (b), and homozygous (c and d) mice. (a) Normal ridged epidermis. Cytokeratin filament bundles are arranged in bundles of varying length. Note the prominent keratohyalin granules (arrowheads). bc, basal cell. (b) Spinous cell containing areas of filament-free cytoplasm as well as abnormal cytokeratin aggregates (arrow). m, mitochondrion. (c) Granular cell. Note the overall sparsity of cytokeratin filaments. Remaining filaments are associated with desmosomes (arrowhead) and keratohyalin (kh). (d) Overview. Basal cells are normal. Suprabasal cells show

electron dense irregularly shaped aggregates located near the cell membrane (arrows). Note the highly electron-lucent cytoplasm (asterisk) of granular cells. Bars: (a and d) 2 μm ; (b and c) 0.5 μm .

knockout mice on the expression of the hyperproliferation marker cytokeratin 16 (see below) and those of Fuchs et al. (1992) regarding cytokeratin 6, this suggests that alterations in the organisation or expression of the hallmark suprabasal cytokeratins 1, 2e, or 10 are the key events to trigger hyperproliferation. Understanding of the pathway by which this is controlled remains to be worked out and might provide a basis for therapy approaches.

Biochemical Analysis Suggests a Basis for Different Phenotypes in Homo- and Heterozygous Cytokeratin 10 Knockout Mice and Reveals the Existence of Mouse Cytokeratin 16

To examine the cytokeratin pattern in homo- and heterozygote cytokeratin 10 knockout mice we isolated total RNA from these mice and wild-type littermates for Northern blotting and cytoskeletal proteins for two-dimensional gel analysis. Hybridization of RNA from all three genotypes with highly specific cytokeratin 10 probes showed the predicted 2-kb mRNA from the normal allele in wild-type and heterozygote mice. In the latter, this mRNA was reduced significantly below the predicted level of 50%. Homozygotes showed no wild-type sized cytokeratin 10 RNA, even upon prolonged exposure time. Instead, an additional mRNA of 1.1 kb from the altered allele was detected with both the cytokeratin 10 5' probe (Fig. 1 c) and a probe to the 3' nontranslated region. Hybridization of the 1.1-kb transcript with this 3' probe suggested the presence of exon 8. This is possible only if the HPRT minigene has been spliced out (Fig. 1 a). Indeed, an HPRT probe did not bind to the 1.1-kb RNA upon reprobing (not shown). The size of this transcript is consistent with the predicted size of exons 1, 2, and 8 (1.08 kb).

When we examined the levels of cytokeratin 1 RNA in skin samples from our mice we were surprised to find its level reduced in heterozygotes and to a greater extent in homozygotes (see Fig. 1 c). So far, there are no indications for crossregulatory mechanisms acting at the level of tran-

scription between expression pairs for any of the many cytokeratins (Oshima, 1992). One possible explanation for a decrease in cytokeratin 1 mRNA is a reduction in the number of intact suprabasal keratinocytes due to the cytolysis (see Fig. 3). Alternatively cytokeratin 1 may be down-regulated as part of a response to stimulation by growth factors released during wound healing (Mansbridge and Hanawalt, 1988; Choi and Fuchs, 1990).

In contrast, there is an increase in the level of cytokeratin 14 mRNA (Fig. 1 c) that is easily explained by the increase in mitotic activity in the basal cell layer detected by bromodeoxyuridine staining.

Changes are known to occur in cytokeratin 6/16 expression in response to wound healing (Weiss et al., 1984; Mansbridge and Knapp, 1987) and in hyperproliferative diseases including BCIE (Weiss et al., 1984; Finzi et al., 1991; Ishida-Yamamoto et al., 1992; Wilson et al., 1994). Although it has been suggested that there is no mouse cytokeratin 16 equivalent (e.g., Schweizer, 1993), we probed RNA from all genotypes with probes specific to cytokeratins 16 and 17, which is usually expressed along with cytokeratins 6/16 (Moll et al., 1982). Weak signals for both cytokeratins were detected in skin RNA from wild-type mice, most likely from hair follicles where they are expressed. A dramatic increase for both cytokeratins 16 and 17 was detected in homozygous cytokeratin 10 knockout mice. Although the increase of cytokeratin 17 RNA seems even stronger than that of 16, one has to keep in mind that cytokeratin 16 was hybridized to a human probe.

Next we assessed whether the changes in cytokeratin RNA profiles were translated into the corresponding proteins. Staining of cytoskeletal proteins by Coomassie Blue showed that suprabasal cytokeratins were among the most prominent proteins in wild-type mice (Figs. 7, a-c and 8). There was a marked decrease in cytokeratin 10 in heterozygous and a complete absence in homozygous mice, in agreement with the Northern blot results. Another noticeable change was the reduction of cytokeratin 1 in hetero- and homozygous knockout mice, reflecting the decrease in

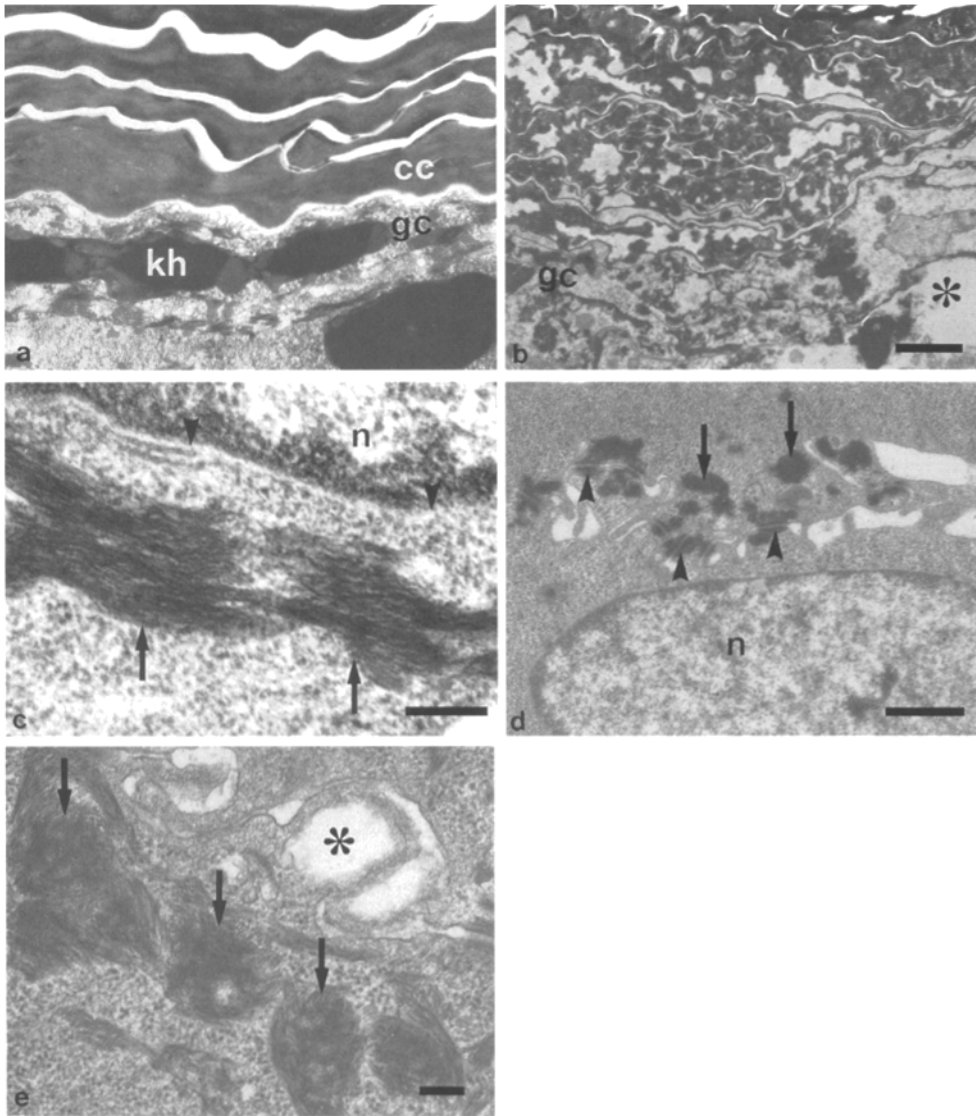


Figure 5. Ultrastructural analysis of the upper epidermis and cytokeratin filaments in wild-type (*a* and *c*) and homozygous (*b*, *d*, *e*) animals. (*a*) Note the marked morphological changes between the uppermost granular cell (*gc*) and the lowermost cornified cell (*cc*). The latter is a flattened anucleated cell with homogenous electron-dense cytoplasm. Organelles and typical type I/II keratohyalin (*kh*) granules as present in the cell layer below have disappeared completely. (*b*) In contrast homozygous animals show a broad transition zone of multiple layers of incompletely cornified cells. Their cytoplasm contains remnant organelles and shows a dappled pattern of electron-lucent and electron-dense areas. (*c*) Normal cytokeratin filament bundles (*arrowheads*) and nuclear membrane (*n*) are arranged in parallel, separated by a small rim of cytoplasm. The bundle is composed of individual cytokeratin aggregates resulting in a characteristic fibrillar pattern. (*d*) Abnormal cytokeratin aggregates in suprabasal keratinocytes. Cytokeratin clumps (*arrows*) are located next to the cell membrane and in association with desmosomes (*arrowheads*). Note the lack of normal cytokeratin filament bundles in both cells. *n*, nucleus. (*e*) Higher magnification reveals that abnormal cytokeratin aggregates (*arrows*)

consist of short cytokeratin filaments in a whorl-like configuration. Note the fibrillar pattern in the periphery and the granular appearance in the center of the cytokeratin aggregates. Asterisk, intercellular space. Bars: (*b*, *c*, and *e*) 2 μm ; (*d*) 1 μm .

its mRNA. It remains to be seen how the still considerable amounts of cytokeratin 1 are stabilized in the suprabasal epidermis. The level of basal cytokeratins was slightly increased (Figs. 7, *g-i* and 8).

Therefore we examined changes in cytokeratin expression profiles further by the more sensitive Western blot technique. Blotting with a specific cytokeratin 10 antibody showed that cytokeratin 10 was reduced in samples from heterozygote mouse skins (Fig. 7 *e*) as in our Northern blot results. In accordance with our Northern blot results homozygote samples showed a complete absence of cytokeratin 10 protein using monospecific or broad-range monoclonal antibodies (Fig. 7 *f*). We then searched for a potential cytokeratin 10 polypeptide (cytokeratin 10T) coded by exons 1, 2, and 8 in the knockout mice. It would include the head domain, coil 1A, part of coil 1B, and the ultimate cytokeratin 10 amino acid encoded by exon 8. The predicted polypeptide should have an isoelectric point of 9.1 and a

molecular mass of 27.5 kD. This very basic polypeptide was detected in NEpHGE gels of heterozygote and homozygote cytoskeletal proteins (see Fig. 8). Unfortunately, the cytokeratin 10 antibody LH 2 (Lane et al., 1991) reacts with the carboxyterminus so we could not unequivocally characterize cytokeratin 10T by Western blotting. Several other cytokeratin 10 antibodies showed no reaction with 10T either. It is however detectable among proteins translated in vitro from RNA of cytokeratin 10 knockout mice but not from wild-type littermates (not shown). Apart from the disappearance of intact cytokeratin 10 the most dramatic changes occurred in the expression of cytokeratin 16. We applied an antibody specific to human cytokeratin 16 to see whether the increase in its RNA was reflected into proteins as well. This antibody reacted very specifically with a cytokeratin migrating in a position expected for cytokeratin 16, which is increased dramatically in heterozygotes and homozygotes (Fig. 7, *l*

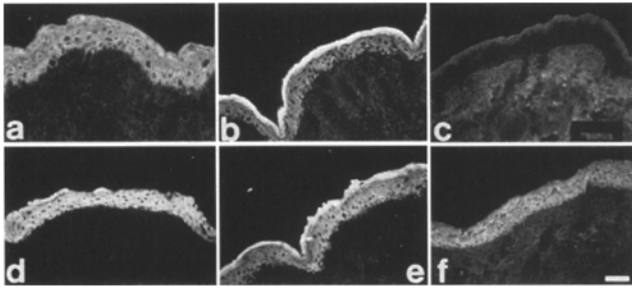


Figure 6. Immunofluorescence analysis of back skin from wild-type (*a* and *d*), hetero- (*b* and *e*), and homozygous (*c* and *f*) mice. Fixed cryosections were stained with monoclonal antibodies to cytokeratin 10 (*a*–*c*) or cytokeratin 1 (*d*–*f*). Note the complete absence of cytokeratin 10 in homozygous mice and the persistent, albeit, decreased staining for cytokeratin 1 in heterozygous (*e*) and homozygous (*f*) mice. Bar, 50 μ m.

and *m*) and present as a minor protein in wild-type mice. Taken together with our RNA data, this provides strong evidence for the presence of cytokeratin 16 in the mouse. Nevertheless, in comparison to the massive amounts of suprabasal cytokeratins, the total amounts of cytokeratins 16 and 17 were small in cytokeratin 10 knockout mice. It is clear from the phenotype of cytokeratin 10 knockout mice and the electron microscopy data that neither these two nor any other cytokeratins present in the suprabasal epidermis were able to compensate for the lack of cytokeratin 10. This might have important implications for therapy approaches of dominant cytokeratin disorders based on overexpression of cytokeratins.

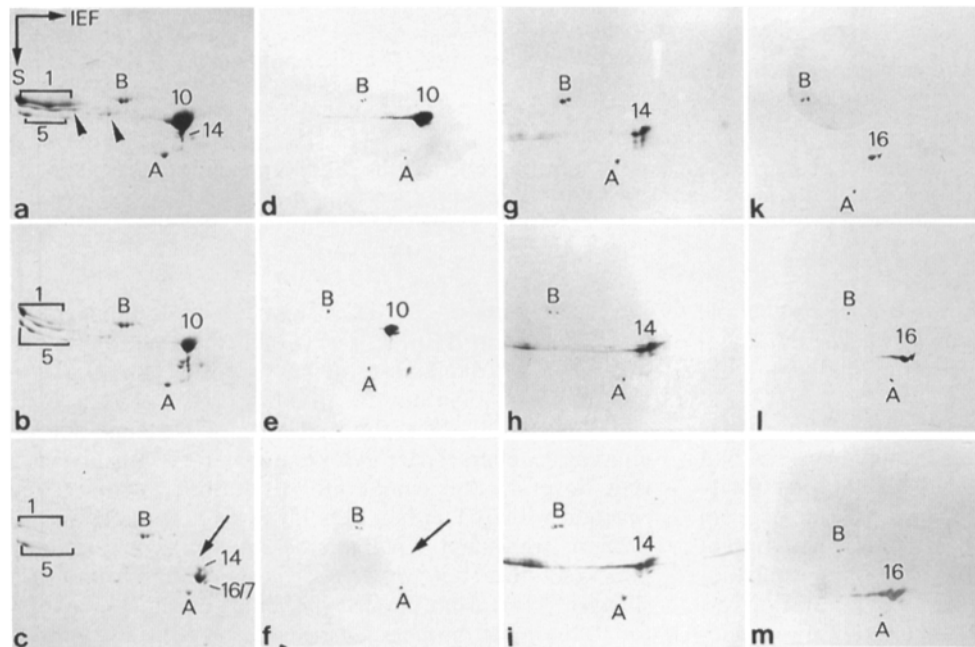


Figure 7. Analysis of cytoskeletal proteins by 2-D gel electrophoresis (isoelectric focusing, IEF, for separation in the first dimension and SDS-PAGE in the second dimension). (*a*, *d*, *g*, *k*) Cytoskeletal extracts from wild-type, (*b*, *e*, *h*, *l*) hetero-, and (*c*, *f*, *i*, *m*) homozygous mice. (*a*–*c*) Coomassie-blue stained proteins. Note the complete absence of cytokeratin 10 in *c* (position indicated by arrow) and the concomitant reduction of cytokeratin 1 in *b* and *c*. Arrowheads indicate the typical processed products of cytokeratin 1. (*d*–*m*) Proteins after Western blotting with specific antibodies, detected with alkaline phosphatase and chromogenic substrates. Individual cytokeratins are numbered according to Moll et al. (1982). (*d*–*f*)

Detection of cytokeratin 10. Note the absence of cytokeratin 10 in homozygous mice (*f*, arrow). (*g*–*i*) Detection of cytokeratin 14. There is an increase in immunoreactive material in heterozygous and homozygous samples. The banding in *i* results from undissolved cytokeratin 14 complexes in the first dimension electrophoresis. (*k*–*m*) Identification of murine cytokeratin 16. Low but detectable expression in wild-type mice (*k*), most likely from hair follicles. Note a considerable increase in homozygous (*m*) animals. *B* and *A* represent bovine serum albumin and actin, respectively, added as reference markers. In the Western blots the position of *B* and *A* was marked by pencil after Ponceau S staining of total proteins.

Discussion

The targeted disruption of the *cytokeratin 10* gene generated mice in which the program of epidermal differentiation is severely and permanently altered in homo- as well as in heterozygous animals. These mice represent a very good model for BCIE, demonstrating for the first time that the absence of a functional cytokeratin 10 and not the overexpression of normal or engineered cytokeratin subunits is the genetic basis for the disease. In conjunction with mice carrying knockouts for cytokeratins 8 (Baribault et al., 1993; Baribault et al., 1994) and 14 (Lloyd et al., 1995), our mice clearly demonstrate that the cytokeratin intermediate filaments fulfil essential functions during development and tissue homeostasis. It will be a challenging task to see whether all the members of the complex cytokeratin gene family are functionally important and/or interchangeable and whether vimentin (Colucci-Guyon et al., 1994) or GFAP knockout mice (Gomi et al., 1995; Pekny et al., 1995) really lack a phenotype. Moreover, the hyperkeratosis in BCIE and in our cytokeratin 10 knockout mice suggests that cytokeratins are involved in functions beyond maintaining structural integrity in epithelia.

Similarity of the Mouse Model to BCIE

Clinically BCIE patients have extremely fragile skin at birth with blisters and ichthyotic erythroderma. During later life skin blistering and erythema tend to subside and acanthosis and hyperkeratosis become common features (Traupe, 1989; Anton-Lamprecht, 1994; DiGiovanna and Bale, 1994). Ultrastructurally, abnormal clumps or aggre-

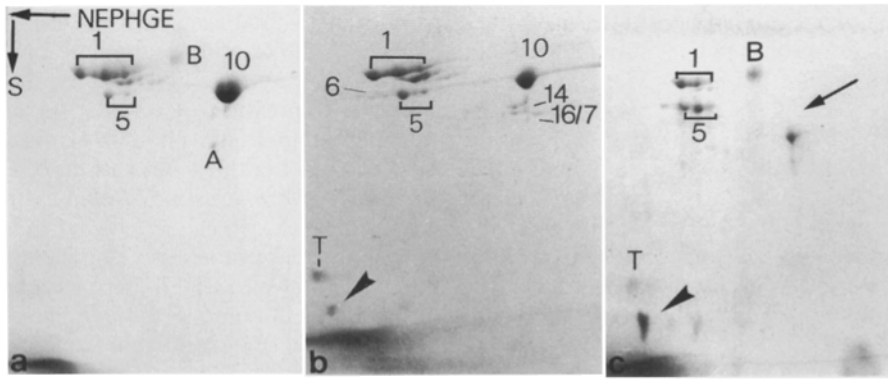


Figure 8. Detection of truncated cytokeratin 10 (cytokeratin 10T) in cytoskeletal extracts from hetero- and homozygous mice (*b* and *c*) in comparison to wild-type mice (*a*). NEPHGE, nonequilibrium gel electrophoresis for separation of proteins in the first dimension. The position of cytokeratin 10T is indicated (arrow) in *b* and *c*. Trypsinogen (T, IEP 9.3; ~24 kD MW) was added as an additional marker. Note the increase in cytokeratins 6 and 16 and the reduction of cytokeratin 1 in the knockout mice (*b-c*). For other labeling see Fig. 7.

gates of cytokeratin filaments, often forming perinuclear shells in otherwise translucent cells, are restricted to the upper spinous and the granular layer. Morphologically our cytokeratin 10 knockout mice exhibited two different phenotypes in homo- and heterozygotes that complement each other to resemble BCIE (e.g., DiGiovanna and Bale, 1994). The former had very fragile skin with small blisters and large areas of skin loss, particularly on the ventral surface and the forepaws and died soon after birth. The latter showed no clinical phenotype at birth but developed hyperkeratosis and flaking of tail, ear and foot epidermis. The restriction of hyperkeratosis to less hairy body sites and the observed transient hyperkeratosis of the body skin prior to the onset of fur growth suggest that hair follicles strengthen the skin. This was also observed in the cytokeratin 14 knockout mice (Lloyd et al., 1995). Alternatively, it is possible that the intermediate filament cytoskeleton is more susceptible to constant mechanical strain in epithelia with an increased rate of cell turnover like the foot sole epidermis.

The suprabasal cytolysis, oedema formation and hyperkeratosis typical of BCIE are clearly reproducible in mice. As judged by EM, there is only a quantitative difference between the hetero- and homozygous cytokeratin 10 knockout mice. Cytokeratin clumps are present in both but they are smaller and fewer in homozygotes. Residual filamentous material was frequently attached to desmosomes, close to the plasma membrane or associated with keratohyalin granules. The intracellular distribution of cytokeratin aggregates was somewhat different to findings in BCIE patients (e.g., Anton-Lamprecht and Schnyder, 1974) and to transgenic mice overexpressing a truncated human cytokeratin 10/14 hybrid (Fuchs et al., 1992). The reason behind this difference could be one of the following: first, it could be an intrinsic property of human cytokeratins or their interaction with associated proteins. Human cytokeratins could function inappropriately when expressed in mouse tissue. Second, it may be due to the nature of mutations in humans, conventional transgenes and our cytokeratin 10 knockout mice being different. Although a number of cytokeratin mutations have been compared in cultured cells or in vitro (Letai et al., 1992, 1993), there are not yet sufficient data from animal models to conclude on their effects on cytoskeletal architecture. Thirdly, the overall amount of cytokeratins in our homozygous mice was drastically reduced. This in itself might alter the intracellular organization of the cytoskeleton. Resid-

ual cytokeratins, whether bona fide intermediate filaments or assembled in an unknown fashion, have a tendency to attach to desmosomes as shown in our cytokeratin 10 and also in cytokeratin 14 (Lloyd et al., 1995) or 8 (Baribault et al., 1993; Baribault et al., 1994) knockout mice but also in two rare cases of EBS, lacking functional cytokeratin 14 (Chan et al., 1994; Rugg et al., 1994). This is strikingly similar to the first appearance of desmosomes and cytokeratin filaments in the early mouse embryo (Jackson et al., 1980). Whether this primarily reflects a high affinity between these structures or whether desmosomes represent some kind of cytokeratin organizing center is presently unknown.

The Genetic and Biochemical Basis for the Phenotype of Cytokeratin 10 Knockout Mice

We have disrupted the *cytokeratin 10* gene in mice by replacing exons 3–7 with an HPRT minigene (Magin et al., 1992; Selfridge et al., 1992) to prepare for the introduction of point mutations in a later second targeting step (for the procedure, see Stacey et al., 1994). Instead of generating a complete *cytokeratin 10* null allele, this has led to a drastically shortened cytokeratin 10 RNA coded by exons 1, 2, and 8 and an independent HPRT transcript that confers HAT resistance to targeted ES cells. In several independent RNA preparations from heterozygous mice there was more cytokeratin 10 RNA from the targeted than from the wild-type cytokeratin 10 allele. This could reflect more efficient processing of the short RNA or its greater stability. It is possible on the other hand that the a priori assumption of equal expression from both alleles is not correct for all cytokeratin genes. In the light of known polymorphisms in some cytokeratin genes (Korge et al., 1992a,b; Wanner et al., 1992) it seems possible that mutations in regulatory sequences exist that could modulate allelic expression. This can be analyzed in patient samples present in several labs.

We have identified a shortened cytokeratin 10 polypeptide (cytokeratin 10T) putatively comprising the head, 1A and 39 amino acids of the 1B domain that is probably encoded by the short RNA. A very similarly sized predicted cytokeratin 14 polypeptide, generated by an in-frame stop codon in a recently described EBS patient (Chan et al., 1994), could not be found among total or cytoskeletal proteins, probably due to the very low expression level of this protein and the relative instability of the mRNA. Although the presence of cytokeratin 10T in the cytoskeletal frac-

tion is biochemically different from BCIE cases analyzed so far, the resulting changes in cytokeratin expression and epidermal differentiation in cytokeratin 10 knockout mice are remarkably similar to those occurring in the human disease.

Does the cytokeratin 10T polypeptide act as a dominant-negative in heterozygous mice? Without additional biochemical data it is difficult to say. At first glance its presence in the cytoskeletal fraction seems to favor this view since it suggests formation of insoluble cytokeratin complexes. Either it is involved in heteromeric complexes with cytokeratin 1 (or another type II cytokeratin) as do corresponding fragments of cytokeratins 18/8 (Hatzfeld et al., 1987; Magin et al., 1987) which are deficient in further assembly steps or it forms homomeric complexes that prevent filament formation (Hatzfeld and Weber, 1991). Previous experiments (Kouklis et al., 1991; Chipev et al., 1992; Hatzfeld and Weber, 1992) have shown that short peptides derived either from the H1 or the rod end domain can disassemble intact intermediate filaments or interfere with filament assembly. Unfortunately these experiments were only carried out *in vitro* using an excess of peptide that were much shorter than cytokeratin 10T. The structures observed *in vitro* were clearly different from those in our mice. Therefore an alternative mechanism might be considered, addressing a fundamental issue of cytokeratin biology, namely the importance of the balance of natural cytokeratin pairs. Could the phenotype be due to the unbalanced expression of cytokeratin 1, particularly in the homozygotes where we have detected significant amounts of cytokeratin 1 in the absence of intact cytokeratin 10? This is very reminiscent of the ectopic expression of cytokeratin 1 in the pancreas (Blessing et al., 1993) that led to diabetes in transgenic mice, whereas similar mice expressing cytokeratin 10 in the same tissue were apparently normal. Taken together it seems possible that an excess of cytokeratin 1 is toxic to cells if not in heterotypic complexes with cytokeratin 10. Future experiments will also address the question whether any of the other type I cytokeratins present in the upper epidermis (14, 15, 16, 17) might be involved in complex formation with cytokeratin 1, leading to filament aggregation.

Finally it seems worthwhile to screen patients with severe forms of BCIE for truncations similar to the one we generated in our mice.

The Cytokeratin 10 Knockout Changes Cytokeratin Expression Patterns and Epidermal Differentiation

The overall amount of cytokeratin filaments and aggregates, as shown by EM and biochemical analyses, is clearly reduced in hetero- and even more in homozygous mice. Nevertheless the homozygous mice developed a far more severe phenotype indicating an inverse correlation between severity of disease and size/number of cytokeratin aggregates. This would argue that the residual amount of functional cytokeratins rather than the size of cytokeratin aggregates (Coulombe et al., 1991b) determines the severity of disease.

We found increased expression of cytokeratins 16 and 17 in the knockout mice. The detection of cytokeratin 16 is remarkable as it had been thought not to exist in mice be-

fore (Schweizer, 1993; for a different observation see Takahashi et al., 1994). Although final confirmation has to await the cloning of mouse cytokeratin 16 our data provide evidence for the conservation of cytokeratin expression patterns in mammals. Expression of cytokeratin 16 has been documented before in BCIE patients (Ishida-Yamamoto et al., 1992) and cytokeratin 6 was shown to be expressed in transgenic mice overexpressing human cytokeratin 10/14 hybrids (Fuchs et al., 1992).

Hyperproliferation and hyperkeratosis develop in cytokeratin 10 knockout mice as well as in BCIE patients. Again this is more pronounced in homozygous animals in which the balance between epidermal and hyperproliferative cytokeratins is shifted towards the latter. The relationship between cytokeratin expression and hyperproliferation is not completely clear. Even though cytokeratin 6/cytokeratin 16 expression is normally associated with hyperproliferation, this cytokeratin pair is also expressed in non-proliferating tissue (Moll et al., 1982; McLean et al., 1995; and references therein). One possible explanation that has been suggested is that cytokeratin 6/cytokeratin 16 represents an alternative differentiation pathway when it is not permissive for the normal differentiation to occur (Schermer et al., 1989). The molecular basis for hyperproliferation is presently unknown. Among the large number of growth factors and cytokines involved in wound healing (McKay and Leigh, 1991) TGF alpha, that acts through the EGF receptor, is an attractive candidate. It appears to induce cytokeratin 6 and cytokeratin 16 expression through EGF response elements in the promoters of the genes for these cytokeratins (Jiang et al., 1993). Interestingly, TGF alpha is elevated in the hyperproliferative skin of BCIE, squamous cell carcinoma, and psoriasis (Finzi et al., 1991).

Implications for Therapy

The cytokeratin 10 knockout mice provide convincing evidence that neither residual basal cytokeratins 5/14 nor cytokeratins 6/16/17 could rescue the phenotype. Similarly in conventional transgenic mice that expressed quite large amounts of truncated cytokeratin 10/14 hybrids (Fuchs et al., 1992) the expression of cytokeratin 6 (and probably 16/17 as well), could not compensate for the phenotype caused by the dominant-negative effect of the mutant protein. This might be due to cell type-specific functions not catered for by cytokeratins 6/16/17. It is possible that these hyperproliferative cytokeratins are tailored to suit a transient state like the one found in wound healing but not the needs of a stress-exposed epithelium like epidermis. Which options (leaving aside the issue of epidermal stem cells and transfection efficiency) does that leave to treat BCIE or EBS? First, it still remains a possibility that overexpression of the same cytokeratin subunit as the one mutated allows formation of normal intermediate filaments. This route would include overexpression of cytokeratin 1 or 10 in BCIE. It is possible that rare, recessive cases of BCIE or mutations leading to a mild phenotype can be rescued in this way. Secondly, suppression of the mutant cytokeratin subunit expression by anti-sense technology seems feasible in the light of recent transgenic experiments demonstrating rescue of a lethal pro-alpha1(I) procollagen by expression of a corresponding anti-sense construct (Khillan

et al., 1994). As this approach is nonselective for the mutant and the wild-type allele an additional normal subunit of a different cytokeratin or the same cytokeratin from a different species needs to be expressed. Thirdly, it might be possible to complement the function of mutant cytokeratins by expression of a different intermediate filaments, e.g., class III subunit, as has been indicated by Lloyd et al. (1995). Based on the knowledge that cytokeratins do not copolymerize with other intermediate filaments subunits this might be an effective strategy.

Finally, with recent improvements in gene targeting frequencies (Melton, 1994), correction by this technology remains a potential option. All these approaches still need considerable experimental improvements, particularly regarding techniques to introduce DNA efficiently into living epithelia. Our knockout mice should provide an attractive system to explore some of these aspects.

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