Abnormal Epidermal Keratinization in the Repeated Epilation Mutant Mouse

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ABSTRACT Repeated epilation (Er) is a radiation-induced, autosomal, incomplete dominant mutation in mice which is expressed in heterozygotes but is lethal in the homozygous condition. Many effects of the mutation occur in skin: the epidermis in Er/Er mice is adhesive (oral and nasal orifices fuse, limbs adhere to the body wall), hyperplastic, and fails to undergo terminal differentiation. Skin from fetal +/+, Er/+ and Er/Er mice at ages pre- and postkeratinization examined by light, scanning, and transmission electron microscopy showed marked abnormalities in tissue architecture, differentiation, and cell structure; light and dark basal epidermal cells were separated by wide intercellular spaces, joined by few desmosomes, and contained phagolysosomes. The numbers of spinous, granular, and superficial layers were highly variable within any given region and among various regions of the body. In some areas, 2-8 layers of granular cells, containing large or diminutive keratohyalin granules, extended to the epidermal surface; in others, the granular layers were covered by several layers of partially keratinized or nonkeratinized cells. In rare instances, a single or small group of cornified cells was present among the granular layers but was not associated with the epidermal surface. Both the granular and nonkeratinized/partially keratinized upper epidermal layers in Er/Er skin gave positive immunofluorescence with antisera to the histidine-rich, basic protein, filaggrin. Proteins in epidermal extracts from +/+, Er/+ and Er/Er mice were separated and identified by radio- and immunolabeling techniques. The Er/Er extract was missing a 26.5-kdalton protein and had an altered ratio of bands in the keratin region. The 26.5-kdalton band was histidine-rich and cross-reacted with the antisera to rat filaggrin. Several high molecular weight bands present in both Er/Er and +/+ extracts also reacted with the antisera. These are presumed to be the precursors of filaggrin and to account for the immunofluorescence in Er/Er epidermis even though the product protein is absent. The morphologic and biochemical data indicated that the genetic defect has a general and profound influence on epidermal differentiation, including alteration of two proteins (filaggrin and keratin) important in normal terminal differentiation, tissue architecture, and cytology. Identification of epidermal abnormalities at early stages of development (prekeratinization) and defective structure of other tissues and gross anatomy suggest that the mutation is responsible for a defect in some regulatory step important in many processes of differentiation and development.

The repeated epilation mutation is thought to have been induced by \( \gamma \)-irradiation (19) of the Er gene on chromosome 4 (12, 16). The condition is inherited as an autosomal, incomplete dominant and is named for the pattern of hair loss and regrowth that occurs in viable, fertile Er/+ adults (19). The mutant Er/Er animals die in utero or moments after birth. The oral cavity is fused, giving the impression of no mouth (17) and, although they make respiratory movements at birth, the external nares are sealed or are limited to pinhole-sized openings. There are marked skeletal abnormalities of the limbs and in the facial region; the paws and tail are stunted and all appendages adhere to the body. The palate is cleft and fused with the tongue. The...
Er/Er mice have an unusual, tight, thickened skin that was originally described as lacking granular and cornified layers (17).

In contrast with the observations of Guenet et al. (17), our preliminary histologic examination of Er/Er epidermis revealed hyperplastic (instead of absent) granular layers and prominent keratohyalin granules in granular cells, yet an almost complete absence of keratinization in more superficial cells, cells which normally form the keratinized stratum corneum. The purpose of the present investigation was to extend these observations and to define the morphologic and biochemical properties of the epidermis in fetal and newborn +/+ , Er/+ and Er/Er mice, specifically, to determine (a) what effect the mutation has on epidermal architecture, cytology, and pattern of differentiation, (b) whether the defect is expressed before keratinization or simultaneous with the onset of keratinization, and (c) whether, and how, either or both of the two major proteins (filagrin and keratin) associated with keratinization are affected (3, 7, 15, 33).

MATERIALS AND METHODS

Animals

Er/+ breeding stock was obtained from Jackson Laboratories, Bar Harbor, Maine. The background genotype was C57/B6J. Animals were maintained in an air-conditioned, windowless room with 12-12 light cycle and fed NIH-007 diet and water ad libitum. At age 10 wk, the Er/+ animals were distinguished from normal by sparse hair and regions of hairless skin. Matings of two Er/+ animals were confirmed by observation of a vaginal plug on the morning of day zero of gestation. The mothers were killed on the designated day of gestation and the uterus was quickly excised. The embryos or fetuses were dissected into saline under a microscope and classified as to age. At day 13, only two phenotypes were separable, Er/Er and others combining Er/+ and +/+ genotypes. By day 18 or 19, three phenotypes were clearly recognizable; Er/+ animals had edema of the feet and a hemorrhagic tail tip; the latter persists one day after birth. Most Er/Er mice were alive with active reflex movements and good membrane circulation on day 18, whereas on day 19 many were dead with partial placental separation. The amniotic fluid from Er/Er fetal mice was bloody. Births of timed and untimed Er/+ x Er/+ matings were observed and live newborn animals of all three genotypes were identified.

Histology and Ultrastructure

Animals removed from the uterus of 13-, 18-, and 19-d pregnant females were immediately immersion-fixed in half-strength Karnovsky’s fixative (22) buffered in 0.1 M cacodylate buffer and fixed for several hours. Small segments of the head and dorsal and lateral body wall from one-half of several animals of each genetic type were dissected for histologic and transmission electron microscopy (TEM) studies. The remaining half of each animal was photographed from internal and external aspects, then processed for scanning electron microscopy (SEM). All samples were washed several times in buffer, postfixed in 2% OsO₄ in distilled water, and dehydrated through a graded series of alcohol into 100% ethanol (EtOH). Samples for light microscopy (LM) and TEM were embedded in Epon containing 1.5% SDS and 1.5% 2-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue, destained, and photographed. Protein concentration was determined by the Bio-Rad technique (Bio-Rad Laboratories, Richmond, Calif.) using cyctrometatin as a standard.

Immunologic Detection of Antigen

Detection of antigens (histidine-rich proteins: filagrin) on SDS gels was done by the method of Renart et al. (31). Samples of epidermal extracts were electrophoresed on gels containing N,N'-diallyltartardiamide (DATD) cross-linker. The gel was then sandwiched between two sheets of diazobenzylxosymethyl (DBM) paper (prepared according to manufacturer’s instructions [Schleicher & Schuell, Inc., Keene, NH]) for transfer of proteins. The paper containing the transferred proteins was incubated with antisemir to rat epidermal filagrin or preimmune control serum, each diluted 1:50 in buffer 1 (31), washed with two changes of buffer 1, incubated with 125I-protein A (~0.25 μCi/gel slot) for 2 h, washed, dried, and fluorographed. Fluorographs were scanned in white light at a densitometer (Helena Laboratories, Beaumont, Tex.).

RESULTS

Morphology

SURFACE MORPHOLOGY—SEM: The skin from the normal mouse was loose, with puckered wrinkles in a regular pattern and cells that were similar in size and bended in surface appearance (Fig. 1a). The Er/+ mouse skin showed dorsoventrally oriented wrinkles (Fig. 1b) while the skin of the Er/Er mouse appeared smooth, taut, and ragged in appearance (Fig. 1c). Individual cells of both Er/+ and Er/Er mice were variable in size, irregular in shape, and had a smooth membrane surface. These variations from normal were more marked in Er/Er epidermis than in Er/+ tissue.

HISTOLOGY OF THE EPIDERMIS: The normal 19-d fetal mouse trunk epidermis was a 50-μm-thick epithelium with one basal, two spinous, three to four granular and six to eight cornified layers (Fig. 2a). Hair follicles extended from the basal layer at regular intervals into the dermis. The epidermis of Er/+ animals was indistinguishable from the +/+ epidermis. Small amounts of glycogen were detected in spinous cells and in developing follicles of +/+ and Er/+ epidermis. Marked abnormalities in epidermal thickness and organization were seen in skin from the Er/Er littermates (Figs. 2b and 3a-c). Overall, the epidermis was hyperplastic, yet highly inconsistent in thickness in any given region. Basal cells often stained more darkly compared with those of the +/+ and Er/+
epidermis and with cells from other layers of the Er/Er epidermis (Figs. 2b and 3a). In the thickened areas, increases in one or more of the spinous, granular, or superficial regions were seen. A true cornified zone was not seen, although isolated areas of cornified cells (Fig. 3b) were present within the epidermis, surrounded by cells which were morphologically less differentiated (Fig. 3b). Cells of the granular and superficial layers, in particular, were irregular in shape and oriented at random (Fig. 2b and 3a and b). Large amounts of glycogen were seen as PAS-positive deposits in spinous and granular cells (data not shown).

ULTRASTRUCTURE OF THE EPIDERMIS: By TEM, marked abnormalities were seen in cells of all layers of the Er/Er mouse epidermis in comparison with the +/+ (Fig. 4) or Er/+ animals. Normal mouse basal cells were joined tightly together and with cells of the spinous layer. In Er/Er mice, the number of desmosomes among basal cells was sparse. The basal lamina was intact and bound to basal cells by typical hemidesmosomes. Basal cells contained phagolysosomes (Fig. 5a) and mitochondria with large, electron-opaque, membrane-bounded granules (Fig. 5a, inset). All cell layers above the basal layer in the Er/Er mouse were variable in number and morphology (Figs. 2b, 3, and 5). In some regions a single spinous layer separated basal and granular layers (Fig. 5a); in others, even adjacent regions, there were several layers of spinous cells (Fig. 5b). The only notable difference in the fine structure of spinous cells compared with those of +/+ or Er/+ animals was in the mitochondria which, like those in the basal cells, contained large, electron-opaque spheres.

Granular layers in the Er/Er epidermis varied in number (2–8), in content of keratohyalin granules, and in position relative to the epidermal surface (Figs. 2b, 3, and 5). In the +/+ mouse (Figs. 2a and 4), keratohyalin granules were present in cells of each of three to four granular layers and increased in size progressively toward the skin surface. They were typically associated with keratin filaments. By contrast, granular cells in the Er/Er epidermis were variable in size, shape, and association with filaments regardless of the position of the cell within the epidermis (Figs. 2b, 3, and 5a). In a few regions, kerato-

FIGURE 2 Epidermis from the trunk of +/+ (a) and Er/Er (b) mice. The epidermis is constant in thickness and hair follicles form at regular intervals in +/+ mice. The Er/Er epidermis is hyperplastic and variable in thickness. Dark basal cells (b), thickened spinous cell zone, a granular zone of variable thickness, and noncornified superficial cells are apparent. × 200.

FIGURE 1 The surface of trunk skin from 18-d-gestation +/+ (a), Er/+ (b), and Er/Er (c) mice. The skin in the +/+ mouse is loose and falls into a series of puckered folds (a), is arranged in longitudinal folds in Er/+ animals (b), and is smooth and taut in the Er/Er mouse (c). × 36.
Hyalin granules were not evident histologically (Fig. 3c) but at the ultrastructural level were observed as small, round bodies (Fig. 5b). Granular cells were generally located subjacent to one or more layers of nongranular, surface-related cells (Fig. 3a and b), but in a few instances they were totally superficial or covered by only a single cell layer (Figs. 3b and c, 5a, and 6a). Thus, in contrast with an earlier report (17), the numbers of granular cell layers appeared exaggerated in the mutant rather than lacking.

Fully cornified cells were identified only as isolated cells or multiple layers of keratinized cells within deeper epidermal strata (Figs. 2b, 3, and 6a), some of these "embedded" squames were nearly normal in morphology (Fig. 6a); the cell contents were electron-dense, nuclear and cytoplasmic remnants were not evident, and a cornified envelope (32) formed the cell boundary. Contents of lamellar granules were abundant in the extracellular space surrounding such cells (Fig. 6, inset).

Cells with a transitional morphology ("T" cells) were occasionally identified among upper strata cells. These cells showed partial nuclear disintegration, loss of organelles, condensation of filaments with keratohyalin, and a cornified cell envelope. Other surface cells had only some of the cornified features of "T" cells (Fig. 6b), while still others were completely noncornified, having an unmodified plasma membrane and a full complement of cytoplasmic organelles (Fig. 5).

Samples of skin from the head of all animals were compared with body epidermis to determine whether there was regional...
variation in epidermal structure or pattern of differentiation. Only in the +/+ animal was the epidermis of the head identical with that of the trunk. Head epidermis from Er/+ animals was thickened as a consequence of increases in all suprabasal cell layers, whereas in the Er/Er animals thickening was due primarily to a greater number of cell layers in granular and cornified regions (Fig. 7). Granular cells of the Er/Er epidermis were flattened, electron-dense (Fig. 7a), and demonstrated a gradual morphologic transition into superficial cells that retained a nucleus, cellular organelles, vacuoles, and bundles of electron-dense filaments, embedded in an electron-opaque matrix (Fig. 7b). Such cells lacked a cornified cell envelope. A broader spectrum of superficial cell types occurred in Er/+ animals (data not shown) but, like the Er/Er superficial cells, they contained a variety of organelles and often a nucleus.

Observations of the head by SEM revealed alterations in the surface properties and in the development of epidermal appendages of Er/Er and Er/+ animals when compared with the +/+ mice. Vibrissae were fully exposed in the +/+ mice, reduced in Er/+ mice, and absent from Er/Er animals (data not shown). It is characteristic of the adult Er/+ mice to have fewer vibrissae than normal.

Fetal mice from 13-d-gestation litters were examined to determine whether the epidermis of Er/Er animals is normal before keratinization, in which case, the genetic defect would be a primary keratinization defect and would be expressed only with this event, or whether the mutation affects epidermal development more generally before keratinization is initiated. 13-d Er/Er mice were identified by their blunt limbs and stumpy tail. The Er/+ mice, however, could not be distinguished from the genetically normal animals. As in the older fetus, epidermal thickness was irregular (two to seven layers) in the Er/Er mutant compared with the normal (Figs. 8a and b). Cells of the lowermost layers were widely separated (Fig. 8b) and attached by few desmosomes, as compared ultrastructurally with tissue from the normal 13-d fetal three-layered epidermis (Figs. 9a and b). In addition, a diffuse matrixlike material was observed in the intracellular spaces among Er/Er
epidermal cells (Fig. 9b); morphologically similar material was associated with the basal lamina in greater amounts in Er/Er animals than in the normal (Fig. 9b). Cell shapes were highly irregular in the Er/Er epidermis but the cytologic characteristics of basal, intermediate, and peridermal cells were equivalent in Er/Er and +/+ animals. The irregularity in cell size, shape, and organization was also recognized at the epidermal surface by the aberrant shape, arrangement, and distribution of microvilli of peridermal cells (Fig. 10).

**Biochemistry**

The proteins in epidermal extracts from newborn +/+, Er/+
+ and Er/Er mice were compared by SDS PAGE. The +/+ and Er/+ extracts had almost identical protein profiles (Fig. 11, lanes a and b). The keratin region contained prominent bands of 65, 62.5, 59, and 56 kdaltons, minor bands of 62, 57, and 53 kdaltons, and several additional bands between 53 and 44 kdaltons. A prominent band was seen at 26.5 kdaltons but was reduced in intensity in the heterozygote. The extract of the Er/Er epidermis (Fig. 11, lane c) had multiple protein differences from the normal (Fig. 11, lanes a and b). The 26.5-kdalton protein was absent from the Er/Er animal. In the keratin region, the band at 62.5 kdaltons was missing; several bands (53, 49, 46, and 44 kdaltons) were much stronger in the Er/Er extract than in the +/+ and occasionally appeared as

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**Figure 6**

Upper epidermal layers from Er/Er mice showing various states of partial keratinization (a). A normally differentiated keratinized squame (Sq) is embedded among granular cells (G). Expelled lamellar granules (LG and arrows) completely surround this cell. Two cells (A and B) at the surface show certain features of keratinization including a cornified cell envelope and a depletion of most organelles. × 6,600. Inset, × 22,500. (b) Surface keratinocytes showing varying states of terminal differentiation. All three cells (C, D, and E) have a cornified cell envelope (CCE). Cell E still has keratohyalin granules (KHG), a pyknotic nucleus (N), and large dense bundles of keratin filaments (KF); the cytoplasm is dense. Extruded lamellar granules (arrows) fill the extracellular space around cell E. Cells C and D contain primarily keratin filaments. The remnant of an organelle is seen in cell D. × 13,200.
doublets (Fig. 12, lane c; compare high molecular weight bands). There were additional alterations in proteins of a wide range of molecular weights.

Incorporation of radiolabeled histidine was performed in vivo in the +/+ and Er/+ animals to determine whether any of these proteins could be characterized as histidine-rich. In extracts from mice which had been labeled for 2 h, multiple high molecular weight bands were labeled (Fig. 12, lanes a and a'). In contrast, labeling for 24 h (Fig. 12, lanes b and b') resulted in the incorporation of histidine into the 26.5-kdalton band as well as the high molecular weight bands. Since the 26.5-kdalton band was strongly labeled after 24 h (Fig. 12, lanes b and b'), it was considered a histidine-rich protein. This band corresponded to the band which was absent from the Er/Er extract (Fig. 11, lane c).

Detection of Antigens

Epidermal proteins which are immunologically related to rat epidermal filaggrin were identified in the gels by reaction with antiserum, followed by 125I-protein A. A densitometric scan of the fluorograph is shown in Fig. 13. The 26.5-kdalton band previously identified as a histidine-containing protein in the +/+ and Er/+ samples (peak f) was cross-reactive. This band was missing from the Er/Er extract. Additional cross-reactive bands in all three extracts were found at the origin (peak a) and at approximately 145 (peak b), 116 (peak c), 90 (peak d), and 59 kdaltons (peak e). The high molecular weight cross-reactive bands were most intense in the Er/Er and were broader (compare peaks d and e) when compared with the heterozygote and normal extracts.

Immunofluorescence

An indirect immunofluorescent reaction using antibody to rat epidermal filaggrin is shown in Fig. 14. Skin from both the +/+ and the Er/Er mice gave a strong positive reaction. In the normal, the positive reaction was found in the stratum corneum, and was associated with granules in the granular layers (Fig. 14a). In the Er/Er, a both diffuse and granular immunofluorescent reaction was seen throughout the cytoplasm of granular and superficial cells (Fig. 14b).

DISCUSSION

The present study has shown abnormalities in epidermal mor-

![Image 7a](image7a.png)  ![Image 7b](image7b.png)

**Figure 7** Cells in basal (a) and superficial (b) regions of Er/Er head epidermis. (a) Basal (B) and spinous (S) layers are similar to trunk layers. Spinous cells include poorly preserved glycogen deposits (arrows). Granular cells (G) have keratohyalin granules and a marked density of the cytoplasm. ×2,100. (b) Three zones of superficial cells (A–B–C). The lower cell (C) retains the nucleus (N). Seven cells in the midzone (B) have keratohyalin granules (KRG), dense bundles of keratin filaments (KF), and abundant ribosomes (arrows). Cells of the most superficial zone (A) have an even greater density and indistinguishable contents. Extruded lamellar granules (circled) can be seen between cells. ×8,500.
FIGURE 8  Trunk epidermis from normal (a) and Er/Er (b) mice at 13 d of gestation. (a) Normal epidermis is regular in thickness. (b) The Er/Er epidermis is thickened to seven cell layers in some regions and narrows to two layers in others. Cells are separated by wide intercellular spaces. X 300.

FIGURE 9  Full-thickness epidermis from the 13-d-gestation normal (a) and Er/Er (b) mouse. (a) Cells of basal (B), intermediate (I) and peridermal (P) layers are closely associated and joined by desmosomes (D). Some glycogen (Gl) deposits are present in the cytoplasm. (b) Cells are widely separated and joined by few desmosomes. A diffuse matrix material is present in the intercellular spaces (arrows). Similar material is associated with the basal lamina. X 325.

The epidermis of the Er/Er mouse is hyperplastic, has a variable and aberrant pattern of organization, fails to synthesize the histidine-rich product, filaggrin, produces abnormal keratins, and does not carry out the normal events of terminal differentiation.

Many of the morphologic findings of the present study differ from those of Guenet et al. (17) in their original report of the Er/Er mouse. They described an epidermis with only basal and spinous layers and rare development of hair follicles. In contrast, we have observed an epidermis which is hyperplastic and has exaggerated numbers of granular layers, superficial noncornified cells, isolated foci of partially cornified cells, and hair follicles spaced at irregular intervals. The discrepancies between our findings and those of Guenet et al. (17) may be due to differences in sampling and/or in breeding stock. Our mice were bred on a uniform genetic background, whereas Guenet et al. used two genetic types of matings to produce the homozygous embryos.

Epidermal hyperplasia in the Er mutant mouse is associated with tissue edema, hence widened intercellular spaces, increased accumulations of glycogen, large intramitochondrial spheres, phagolysosomes in keratinocytes, the presence of dark keratinocytes, and increased numbers of keratohyalin granules in granular cells (2, 4, 13, 20, 30). Such features are also characteristics of epidermal hyperplasia associated with pathologic conditions (e.g., benign keratoses and neoplasias), can be induced by mechanical, chemical, thermal, or radiation assault (2), and are believed to be associated with a general state of...
cellular injury and altered metabolism. The intramitochondrial granules, for example, have been observed not only in hyperplastic mouse epidermis (14) but also in fetal mouse epidermal cells (35), cultured mouse epidermal cells (our unpublished observation), and in normal, in metabolically deficient and in transformed epithelial cells from a wide variety of organs (bladder [23], stomach, colon, uterus [reviewed in reference 21] and in healing wounds [34]). In many of these instances there is an undifferentiated or dedifferentiated state of the tissue as in the Er/Er mouse.

The histologic and cytologic similarities between pathologic, induced, and Er/Er hyperplastic epidermis are probably the consequence of very different underlying defects. While some of the induced hyperplastic conditions are combined with a subterminally differentiated or dedifferentiated state of the tissue (e.g., chemically induced neoplasia [30]), there seem to be no examples where terminal differentiation fails to occur as it does, almost uniformly, in the Er/Er mouse. It may not be possible to find such a comparison because the full effects of the mutation in the Er/Er mouse are fatal.

In normal epidermis, differentiation progresses uniformly throughout the tissue in an orderly sequence of basal-spinous-granular-cornified cells. A "pattern" of epidermal differentiation in Er/Er tissue cannot be described because of marked variability in the presence and numbers of granular layers, and presence and position of cornified, partially cornified, and noncornified cells among cell strata. The variability of the products of terminal differentiation raises the question of whether there is differing ability of cells derived from different epidermal stem cells (different epidermal proliferating units [1, 28, 29]) to carry out the events of differentiation or whether some local, even microenvironmental, factors influence differentiation.

Assuming that the granular cells differentiate into the overlying (more superficial) noncornified cells, then the fate of the keratohyalin granules during the transition must be explained. During normal keratinization, a high molecular weight, phos-
phorylated, histidine-rich precursor protein (10, 26) in kerato-
hyalin granules is converted via a multienzyme-regulated se-
quence to a lower molecular weight product, filaggrin. Filag-
grin is believed to be the electron-dense matrix protein (8)
which embeds electron-lucent keratin filaments in the cornified 
cell to form a filament-matrix assembly described ultrastruc-
turally as the keratin pattern (5). Although filaggrin was absent
from Er/Er epidermal extracts (Figs. 12 and 13) and present in
diminished amounts in Er/+ extracts (Figs. 11 and 13), the
immunofluorescence studies (Fig. 14b) showed that some form
of a histidine-rich protein was present in the noncornified
surface cells. For the following reasons, we suspect that this
protein is the precursor of filaggrin and that the mutation
blocks its normal conversion to the product. First, a series of
high molecular weight, histidine-rich proteins was identified
on gels of epidermal extracts from all three genotypes; secondly,
the precursor protein in keratohyalin granules cross-reacts with
antibody against the product (filaggrin) (9); and, finally, the
precursor has been shown to be incapable of combining with
keratin filaments in vitro to form macrofibrils (11).

Thus we conclude that the epidermis of the Er/Er mutant
mouse contains precursor histidine-rich matrix protein which
cannot undergo the posttranslational modifications necessary
to generate the 26.5-kdalton functional protein found in normal
stratum corneum. This could be due to an alteration in the
primary structure of the histidine-rich protein gene product or
to an alteration in an enzyme responsible for some posttrans-
lational modification.

We favor the latter possibility for the reason that, although
the alterations in both the histidine-rich and keratin proteins
might explain the abnormal keratinization, they are insufficient
to explain the organizational defects in the tissue, the structural
changes in the prekeratinized embryonic epidermis, and the
various other phenotypic changes in face and appendages of
the mutant animal. The alterations in filaggrin and keratin are
probably only two examples of many proteins which may be
modified by a defect in a generalized regulatory step in protein
processing. The defect at the Er locus has been shown to have
a widespread effect on epidermal differentiation, tissue archi-
tecture, and gross structure of the mutant mouse, but continued
study of one of the effected proteins (filaggrin) may lead to the
discovery of the general alteration, which then can be tested
on other tissue proteins.

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