Abnormal Expression and Processing of Keratins in Pupoid Fetus (pf/pf) and Repeated Epilation (Er/Er) Mutant Mice

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Abstract. The pupoid fetus (pf) and repeated epilation (Er) mutations of mice result in a failure of epidermal differentiation in homozygotes. Expression of the epidermal keratins has been followed in pf/pf and Er/Er mice by two-dimensional gel electrophoresis, and by immunohistochemistry and Western blotting using polyclonal antibodies that are monospecific for individual keratin polypeptides. Our results show that expression of the differentiation-specific keratins (K1 and K10) is delayed in both the pf/pf and Er/Er mutants and that, when these keratins do appear later in development, they are localized in the deeper layers of the thickened mutant epidermis. Conversely, K6 and K16, two keratins found in low abundance in normal epidermis, are abundant in mutant epidermis. In newborn mutant epidermis, K6 and K16 are found to be most abundant in the outermost epidermal cells, a distribution opposite to that of K1 and K10. These findings suggest that the expression of these hyperplastic keratins in mutant mice may occur to the exclusion of the differentiation-specific keratins both during development and in newborn animals. Differentiation, and an apparently normal pattern of keratin expression, occur when whole pf/pf or Er/Er skin is grafted to normal mice. These results suggest that the pf and Er genes may be expressed systemically and that transfer of the mutant skin to a "normal" environment results in the recovery of a normal phenotype.

THE epidermis, the epithelial component of skin, undergoes an orderly sequence of histogenesis and differentiation during the course of development. These processes are well defined by both morphologic and biochemical markers and are regulated at different interacting levels of order including dermal–epidermal interactions (Sengel, 1976; Sawyer, 1983), epidermal cell interactions (Hennings et al., 1980; Fisher and Holbrook, 1987) and gene expression (Fuchs and Green, 1980; Roop et al., 1983). One potentially valuable approach to study the regulation of development of any organ system is to identify genetic variants in which the process under study is interrupted. Two mutations causing a failure of epidermal differentiation in mice, have now been identified (Guenet et al., 1979; Holbrook et al., 1982; Fisher et al., 1984; Fisher and Kollar, 1985; Anderson et al., 1985). The pupoid fetus (pf)[1] and repeated epilation (Er) mutations are similar in several respects: both affect surface epithelia of ectodermal origin causing hyperplasia and failure of keratinization, both are first expressed at similar times during development, both result in grossly similar phenotypes including stumpy limbs and snouts, and both mutations are lethal at birth in homozygotes. Since pf and Er have been mapped to the same region of chromosome 4 (Meredith, 1964; Guenet, 1977; Eicher and Fox, 1978; Green, 1982), a close genetic relationship between these mutations has been suggested as well. In spite of these similarities, a striking difference between pf/pf and Er/Er epidermis occurs during development when a variety of cells from the dermis penetrate into and through the pf/pf epidermis (Fisher et al., 1984; Fisher and Kollar, 1985; Anderson and Ede, 1985) so that by birth the epidermis is permeated by a network of dermal cells. Such a disruption does not occur in the Er/Er mutant.

In an effort to better characterize and compare the abnormal development of pf/pf and Er/Er epidermis, we have examined keratin expression in the epidermis of mutants and normal littermates. The keratins are a family of approximately 30 polypeptides (Moll et al., 1982*a*; Steinert et al., 1985) that are differentially expressed among various epithelia as well as within different layers of stratified epithelia such as the epidermis (Fuchs and Green, 1980; Franke et al., 1981; Tseng et al., 1982). Epidermal keratin expression is regulated so that, as cells move from a basal to a suprabasal position, a switch to expression of genes specifying higher molecular mass forms occurs (Fuchs and Green, 1980; Schweizer et al., 1984). In the mouse these high molecular mass or differentiation-specific keratins have molecular masses of 67 (K1) and 59 (K10) kD whereas the keratins of the proliferating basal cells have molecular masses of 60 (K5) and 55 (K14) kD. The expression of another keratin subset with molecular masses of 59.5 (K6) and 50 (K16) kD in the mouse has been associated with conditions of hyperplasia

1. *Abbreviations used in this paper*: 1- and 2-D, one and two dimensional; Er, repeated epilation; pf, pupoid fetus.

in human (Moll et al., 1982*a*; Weiss et al., 1984) and murine (Roop et al., 1984*b*; Knapp et al., 1987) epidermis. Keratin expression is also posttranslationally regulated during epidermal cell differentiation. With formation of the stratum corneum the high molecular mass keratins are proteolytically modified (Fuchs and Green, 1980); in mice this is evidenced by the processing of the 67-kD keratin into the 64- and 62-kD keratins (Bowden et al., 1984).

Preliminary observations of pf/pf mice suggested that differences in the synthesis and processing of epidermal keratins exist between mutants and normal littermates (Fisher, 1987). The present study uses antibodies monospecific for keratin polypeptides (Roop et al., 1984*a*; Roop et al., 1985) to follow expression of individual keratin gene products in both pf/pf and Er/Er mutant skin and correlates these observations with one- and two-dimensional gel electrophoresis and Western blots of epidermal extracts. In addition, keratin expression in grafted mutant skin is compared with expression in grafted normal skin. Our observations document that the pf and Er mutations have similar effects on keratin expression including delayed onset of expression of the high molecular mass (differentiation-specific) keratins, failure to process the differentiation-specific keratins, and expression of the hyperplastic keratins. Furthermore, our data suggest that these abnormalities of keratin expression and keratinization occur secondary to expression of the mutant gene.

Materials and Methods

Animals

The pf and Er mouse colonies are maintained at the University of Washington in the Division of Animal Medicine. Both the pf and Er mutations have been previously described (Holbrook et al., 1982; Fisher et al., 1984; Fisher, 1987). Er mouse breeding stock was obtained from The Jackson Laboratory, Bar Harbor, ME. In some cases Er/Er skin was obtained through the courtesy of Dr. Kenneth Brown of the National Institutes of Health.

Histology and Light Microscopy

Normal and mutant skin was fixed in one-half strength Karnovsky's fixative (Karnovsky, 1965) in 0.1 M cacodylate buffer, postfixed in 2% OsO_4 in distilled water, rinsed in distilled water, and dehydrated through a graded ethanol series. After two changes of propylene oxide, and an extensive change in 1:1 propylene oxide:Epon, the specimens were embedded in Epon (Luft, 1961). Semithin sections (1 μm) were cut with a diamond knife and stained according to the technique of Richardson et al. (1960).

Antibodies

The preparation of antibodies against synthetic peptides corresponding to keratin subunits has been described in detail elsewhere (Roop et al., 1984*a*). Briefly, unique amino acid sequences deduced from nucleotide sequences of cDNA clones were identified at the carboxy terminus of various epidermal keratins of mice. Synthetic peptides corresponding to these unique sequences were prepared, coupled to bovine serum albumin (BSA), and used to immunize rabbits. The resultant antibodies were found to be monospecific for individual keratin peptides (Roop et al., 1984*a*; Roop et al., 1985). The antibodies used in the present study are directed against the 67-kD (K1), 59-kD (K10), 59.5-kD (K6), and 55-kD (K14) keratins. In addition antibodies were prepared against a synthetic peptide corresponding to the last 15 amino acids of the carboxy terminus of a mouse 50-kD keratin (K16) specified by the nucleotide sequence for pKSCC 50 recently published by Knapp et al. (1987). These antibodies were found to be highly specific for this protein.

Immunohistochemistry

Pregnant female mice were killed by cervical fracture. Mutants and normal littermates were dissected from the uterus in Hank's balanced salt solution and immediately fixed in Carnoy's fixative for 30–60 min at 4°C. Skin was removed, rinsed in 70% ethanol, dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin by routine procedures. Sections (6–8 μm) were mounted on albumin-coated slides and allowed to adhere overnight on a slide warmer at 42°C. Sections were deparaffinized in two changes of xylene, rehydrated through a graded ethanol series to water, and rinsed in 0.01 Tris-HCl (pH 7.6). Endogenous peroxidase was inactivated in 0.75% H_2O_2 (30 min) and nonspecific binding sites were blocked with 2% goat serum for 30 min. Antikeratin antibodies were applied for 1 h at room temperature in the following dilutions: anti-67 kD (K1)–1:300; anti-59 kD (K10)–1:800; anti-59.5 kD (K6)–1:1,000; anti-55 kD (K14)–1:1,000; anti-50 kD (K16)–1:400. After three rinses with buffer, localization of antikeratin binding was accomplished according to the avidin-biotin-peroxidase complex (ABC) technique of Hsu et al. (1981). Biotinylated goat anti-rabbit antibodies (Vector Laboratories, Burlingame, CA; diluted 1:200) were applied for 30 min, sections were rinsed with three changes of buffer (5 min each), and avidin-biotin-peroxidase conjugate (Vector Laboratories) was applied for 30 min. After another extensive series of buffer rinses, peroxidase was visualized in 0.05 M Tris-HCl (pH 7.6) containing 0.06 (wt/vol) diaminobenzidine-HCl and 0.03% (vol/vol) H_2O_2. Sections were rinsed extensively in tap water, dehydrated through a graded ethanol series, cleared in xylene, and mounted in Permount.

Keratin Extraction

Epidermis was easily separated from dermis after a 1-min bath in 55°C H_2O followed by immersion in 4°C Hank's balanced salt solution. Keratins were extracted according to a modification of the technique of Schweizer and Winter (1982) for examination of keratin patterns by one-dimensional (1-D) gel electrophoresis. Briefly, epidermis was homogenized in 10 mM Tris-HCl (pH 8.0) containing 1.5 M KCl, 10 mM NaCl, 2 mM dithiothreitol, 0.5% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride. The keratins remain in the insoluble pellet following centrifugation for 5 min in an Eppendorf centrifuge (model 5414, Brinkmann Instruments Co., Westbury, NY). The pellet was then collected and rehomogenized in the extraction buffer. This process was repeated four to five times for examination of keratins by 1-D gel electrophoresis, and two times for Western blot analysis. The keratins were dissolved from the remaining aqueous insoluble pellet in a phosphate buffer containing 5% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol.

1- and 2-D Polyacrylamide Gel Electrophoresis (PAGE)

Keratins were separated by 1-D SDS-PAGE in either 12% gels or 7.5–12% gradient gels according to the technique of Laemmli (1970). 2-D gel electrophoresis of keratins was performed by the method of O'Farrell et al. (1977), as modified in the first dimension by Harding and Scott (1983). Second-dimension gradients were 7.5 to 12% acrylamide.

Western Blotting

1- and 2-D gels were electrophoretically transferred overnight to nitrocellulose. Nitrocellulose was rinsed in 10 mM Tris-saline and blocked in 10% crude hemoglobin, and keratins were detected with monospecific polyclonal antibodies and visualized according to the technique of Sternberger (1979). Goat anti-rabbit IgG was purchased from Cooper Biomedical, Inc. (Malvern, PA) and rabbit peroxidase antiperoxidase was purchased from Miles Laboratories, Inc. (Naperville, IL).

Results

Light Microscopy

Newborn pf/pf mutant epidermis remains nonkeratinized at birth with a reduced granular layer and no stratum corneum (Fig. 1 *A*). In addition, because of the developmental history of the pf/pf epidermis (Fisher, 1987), a network of dermis extends throughout the mutant epidermis (see *arrows*, Fig. 1 *A*). The Er mutant, although it may have a hyperplastic granular layer, fails to form a stratum corneum (Fig. 1 *B*). On the other hand, normal epidermis keratinizes at 17–18 d of development so that by birth both granular and cornified cell

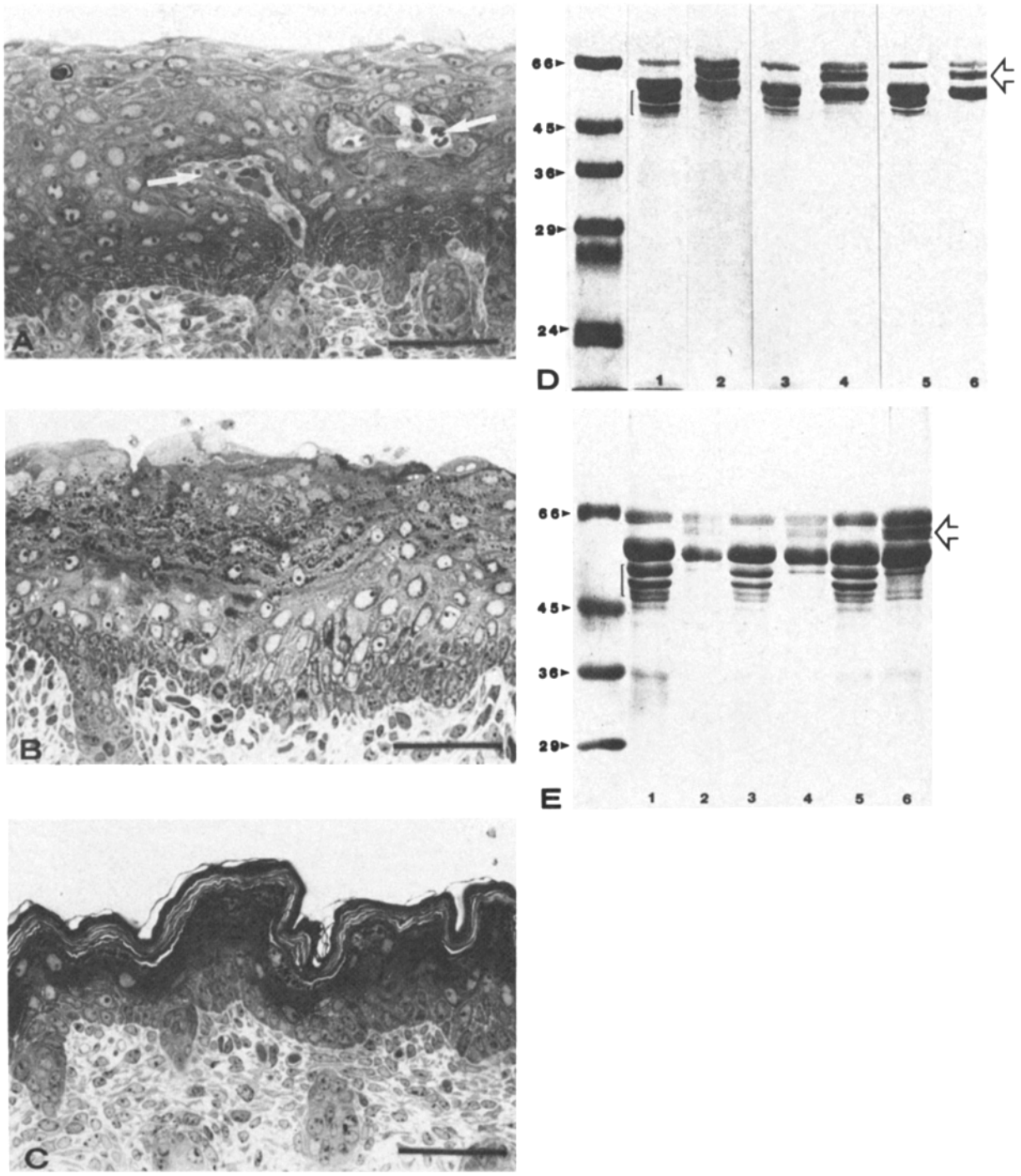


Figure 1. Light microscopy and 1-D gel electrophoresis of keratins. (*A*) Newborn pf/pf epidermis is hyperplastic and remains undifferentiated having no stratum corneum and few keratohyalin granules. A network of dermal cells (*arrows*) is found throughout the pf/pf epidermis. Bar, 50 μm. (*B*) Er/Er epidermis also lacks a stratum corneum although it may have a hyperplastic granular layer. Bar, 50 μm. (*C*) Newborn normal epidermis is highly differentiated having a well-developed stratum corneum and stratum granulosum. Bar, 50 μm. (*D*) The epidermal keratins of newborn pf/pf (lanes *1*, *3*, and *5*) and normal (lanes *2*, *4*, and *6*) littermates exhibit distinctly different electrophoretic patterns. The 64- and 62-kD keratins (*open arrow*) are missing from pf/pf epidermis, suggesting that keratin processing does not occur in the mutants. In addition, several lower molecular mass bands between 49 and 55 kD (*bracket*) are found in mutant epidermis that are either missing from or reduced in normal epidermis. Lanes *1* and *2*, head epidermis: lanes *3* and *4*, ventral epidermis; lanes *5* and *6*, dorsal epidermis. (*E*) The epidermal keratins of newborn Er/Er mutants (lanes *1*, *3*, and *5*) and normal (lanes *2*, *4*, and *6*) littermates. The Er/Er keratin profiles are very similar to those of pf/pf mutants. The 64- and 62-kD keratins are missing from the mutants (*open arrow*) and several lower molecular mass keratins accumulate in the mutant epidermis that are missing from or reduced in normal epidermis (bracket). Lanes *1* and *2*, head epidermis; lanes *3* and *4*, ventral epidermis; lanes *5* and *6*, dorsal epidermis.

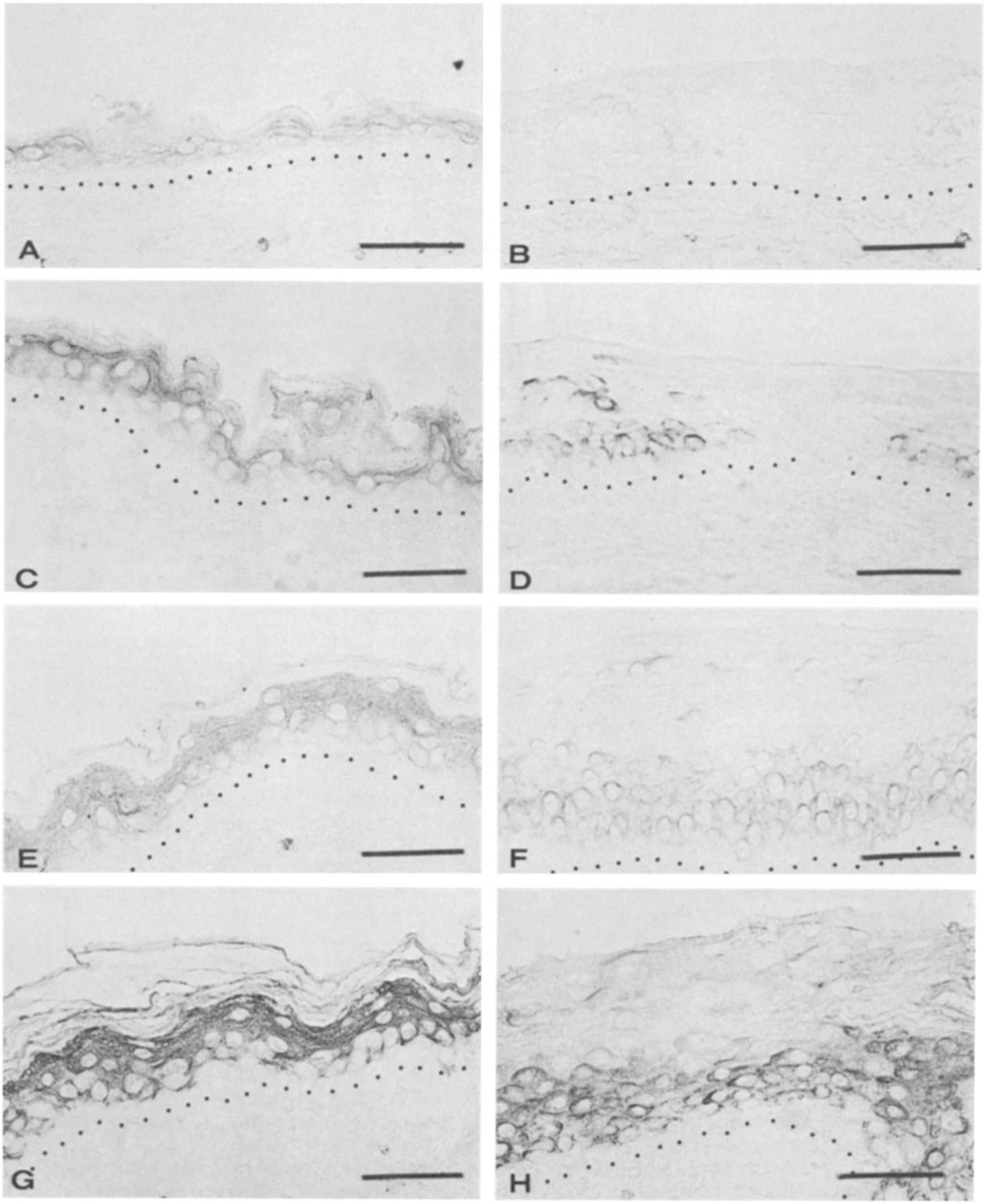


Figure 2. Localization of the 67- (K1) and 59- (K10) kD keratins (differentiation-specific keratins). (*A*) The suprabasal cell population of normal 15-d gestation epidermis is positive for the 67-kD keratin. Dotted line indicates basement membrane. Bar, 50 μm. (*B*) The hyperplastic 15-d gestation pf/pf epidermis is negative for the 67-kD (K1) keratin. Dotted line indicates basement membrane. Bar, 50 μm. (*C*) By 17 d of development, suprabasal cells of normal epidermis are strongly positive for the 59-kD keratin. Dotted line indicates basement membrane. Bar, 50 μm. (*D*) In the 17-d pf/pf mutant, a discontinuous subpopulation of suprabasal cells located in the deeper layers of the thickened epidermis are positive for the 59-kD keratin. Dotted line indicates basement membrane. Bar, 50 μm. (*E*) Suprabasal cells of normal newborn epidermis are positive for the 67-kD keratin with the exception of the stratum corneum which is negative indicating the onset of processing. Dotted line indicates basement membrane. Bar, 50 μm. (*F*) The newborn pf/pf mutant epidermis has a continuous population of suprabasal cells positive for the differentiation-specific keratins that are located in the deeper layers of the thickened pf/pf mutant epidermis. The superficial cells of the mutant epidermis remain negative; this result is not due to processing in that the 64- and

layers are easily identified by light microscopy (Fig. 1 *C*). It was anticipated that the undifferentiated state of the mutant epidermis may be reflected by the keratin composition.

1-D Gel Electrophoresis of Newborn Epidermal Keratins

The keratins of normal newborn animals from the pf stock (Fig. 1 *D*, lanes *2*, *4*, and *6*) and Er stock (Fig. 1 *E*, lanes *2*, *4*, and *6*) produced similar electrophoretic patterns and were consistent with previous descriptions of similar preparations (Schweizer and Winter, 1982). On the other hand, newborn pf/pf (Fig. 1 *D*, lanes *1*, *3*, and *5*) and Er/Er (Fig. 1 *E*, lanes *1*, *3*, and *5*) mutants produce keratin electrophoretic profiles that are very similar to each other and distinct from those of normal animals. These differences hold up for keratins from various anatomic areas including the head (Fig. 1, *D* and *E*, lanes *1* and *2*), ventrum (Fig. 1, *D* and *E*, lanes *3* and *4*) and dorsum (Fig. 1, *D* and *E*, lanes *5* and *6*). The mutants are deficient for two keratins that migrate at 64 and 62 kD. In addition, both pf/pf and Er/Er mutants have abundant amounts of several low molecular mass keratins that are either missing from or reduced in normal mice. Thus, the pf and Er genes produce similar defects in homozygotes as reflected in the electrophoretic profiles of the epidermal keratins. These differences between keratin 1-D electrophoretic profiles of normal and mutant mice are supported and extended by immunohistochemical observations, Western blotting, and 2-D gel electrophoresis.

Immunohistochemical Observations

67- (K1) and 59- (K10) kD (Differentiation-specific) Keratins. K1 and K10 are members of a keratin pair that is coexpressed in normal and mutant skin. The suprabasal cell population of normal 15-d epidermis is positive for K1 (Fig. 2 *A*) whereas 15-d pf/pf epidermis is negative (Fig. 2 *B*). By 17 d of development, suprabasal cells of normal epidermis are strongly positive for the 59-kD keratin (Fig. 2 *C*). In the 17-d mutant a discontinuous subpopulation of suprabasal cells located in the deeper layers of the thickened epidermis is positive for the 59-kD keratin (Fig. 2 *D*). Suprabasal cells of normal newborn epidermis are positive for the 67-kD keratin with the exception of the stratum corneum which is negative (Fig. 2 *E*). These data suggest that the processing of the high molecular mass keratins occurs at least in part at the carboxy terminus recognized by these antibodies (Roop et al., 1984*a*). The newborn mutant epidermis has a continuous population of suprabasal cells positive for the differentiation-specific keratins that are located in the deeper layers of the thickened pf/pf mutant epidermis (Fig. 2 *F*). The superficial cells of the mutant epidermis remain negative; this negative result is likely not due to processing because the 64- and 62-kD keratins, which are derived from processing of the 67-kD keratin, are not present in newborn mutant epidermis (see Fig. 1, *D* and *E*). The Er/Er mutant shows a similar pattern of staining; normal 18-d epidermis (Fig. 2 *G*) stains in a pattern similar to newborn epidermis, whereas 18-d Er/Er mutant epidermis (Fig. 2 *H*) has a pattern of staining similar to newborn pf/pf epidermis.

59.5-kD Keratin (K6) Localization. At 17 d of development the 59.5-kD keratin (K6) is undetectable in normal epidermis by immunohistochemical methods (Fig. 3 *A*). However, 17-d pf/pf mutant epidermis is strongly positive for K6 with a gradual increase in staining intensity from the negative basal cell layer to the intensely stained superficial cell layers (Fig. 3, *B* and *C*). This pattern of staining is evident in older specimens as well. Newborn, normal epidermis is negative (Fig. 3 *D*) whereas the hyperplastic epidermis of the newborn pf/pf mutant is strongly positive for K6 (Fig. 3, *E* and *F*). The epidermal cells surrounding the dermal inclusions high in the newborn mutant epidermis are negative (Fig. 3 *F*; *arrows*) just as the basal cells at the normal dermal–epidermal junction; however, the epidermal cells adjacent to the superficial-most dermal cells on the surface of the pf/pf epidermis are strongly positive for K6 (Fig. 3 *F*; *arrowheads*). The 18-d Er/Er mutant epidermis shows a pattern of distribution for K6 that is very similar to the pf/pf mutant (Fig. 3, *H* and *I*) whereas the epidermis of normal littermates is negative (Fig. 3 *G*).

55-kD Keratin (K14) Localization. The basal cells and hair buds of normal 15-d epidermis are stained with antibodies directed against K14 (Fig. 4, *A* and *C*). In contrast, the entire epidermis of the 15-d pf/pf mutant is strongly stained with the same antibodies (Fig. 4, *B* and *D*). Similar results are attained with newborn skin. The basal cells as well as the hair follicles of normal, newborn epidermis stain positively for K14 (Fig. 4, *E* and *G*), while newborn pf/pf (Fig. 4 *F*) and 18-d Er/Er (Fig. 4 *H*) epidermis are completely and intensely stained with the antibody.

Western Blotting for K6 and K14. Epidermal keratins of normal, and pf/pf and Er/Er mutant mice were analyzed by Western blotting and 2-D gel electrophoresis in an effort to confirm and extend the immunohistochemical studies. Coomassie Blue-stained gels of normal and pf/pf mutant littermates from 15- and 17-d gestation litters (Fig. 5 *A*, lane *2*) confirmed the absence of the differentiation-specific keratins (note that the 67-kD band is missing) from the 15-d mutants. Western blotting for the 59.5-kD keratin (K6) (Fig. 5 *B*) failed to detect this polypeptide in normal 15-d (lane *1*) or 17-d (lane *3*) epidermal extracts whereas extracts of the corresponding mutant littermates (lanes *2* and *4*) were positive. Antibodies directed against the 55-kD keratin (K14) (Fig. 5 *C*) recognized a single, prominent band in extracts of 15-d normal (lane *1*) and mutant (lane *2*) epidermis. Likewise, extracts of 17-d normal (lane *3*) and mutant (lane *4*) epidermis also yielded a prominent band of 55-kD in Western blot with anti-K14. The 17-d pf/pf mutant extract had multiple lower molecular mass bands that also reacted with the antibodies, suggesting that degradation of keratins that are immunoreactive with antibodies directed against K14 oc-

62-kD keratins are not present in newborn mutant epidermis (see Fig. 1). Dotted line indicates basement membrane. Bar, 50 μm. (*G*) Normal 18-d epidermis stains in a pattern similar to newborn epidermis. Dotted line indicates basement membrane. Bar, 50 μm. (*H*) 18-d Er/Er mutant epidermis exhibits a pattern of staining similar to newborn pf/pf epidermis. Dotted line indicates basement membrane. Bar, 50 μm.

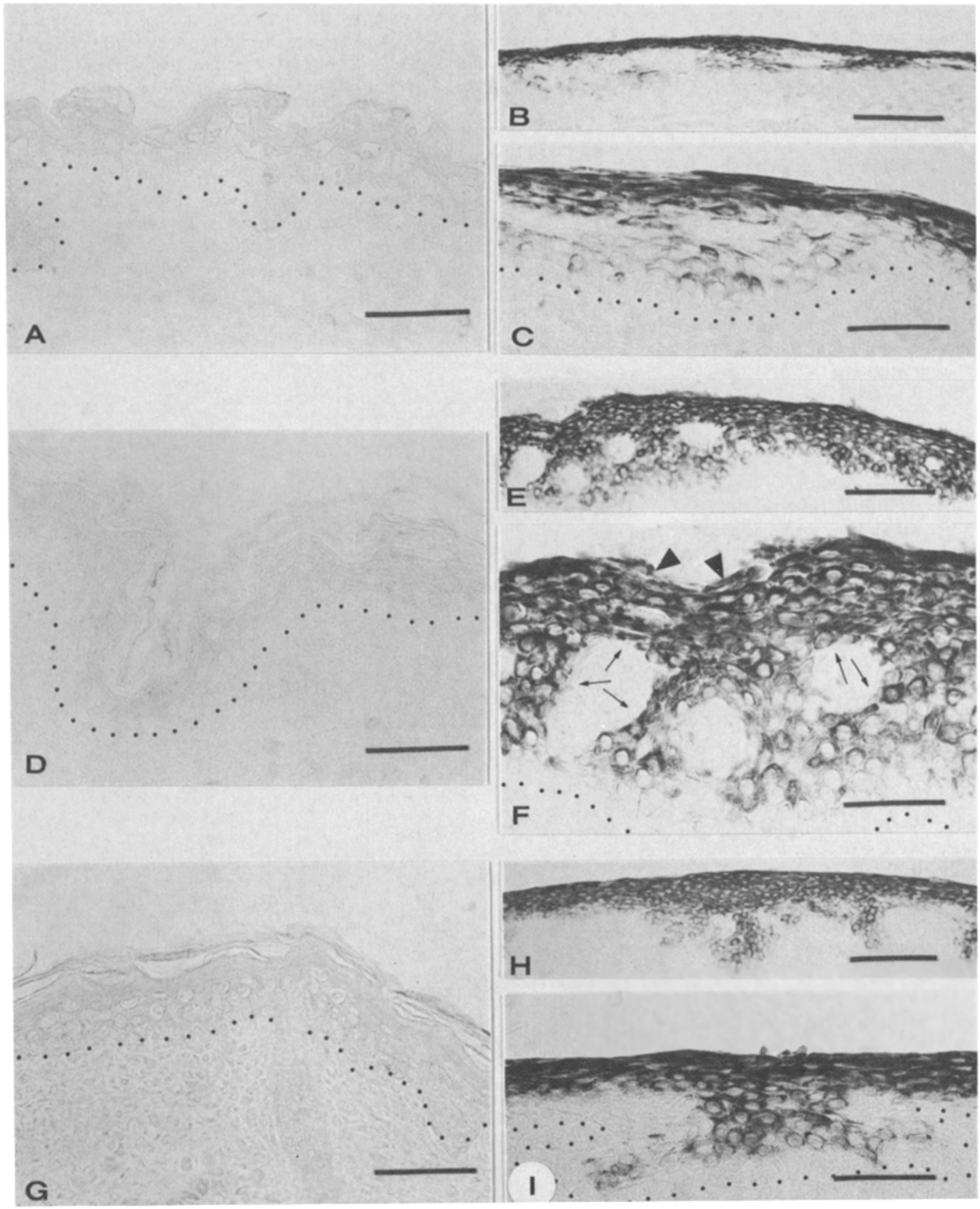


Figure 3. Localization of the 59.5-kD keratin (K6). (*A*) At 17 d of development the 59.5-kD keratin (K6) is undetectable in normal epidermis by immunohistochemical methods. Dotted line indicates basement membrane. Bar, 50 μm. (*B* and *C*) The 17-d pf/pf mutant epidermis is strongly positive for K6 with a gradual increase in staining intensity from the negative basal cell layer to the intensely stained superficial cell layers. Dotted line indicates the basement membrane. Bars, 100 μm (*B*) and 50 μm (*C*). (*D*) Newborn, normal epidermis is negative for K6. Dotted line indicates the basement membrane. Bar, 50 μm. (*E* and *F*) The hyperplastic epidermis of the newborn pf/pf mutant is strongly positive for K6, exhibiting a gradual increase in staining intensity from the negative basal cells to the strongly positive superficial keratinocytes. The epidermal cells surrounding the dermal inclusions high in the newborn mutant epidermis are negative (*arrows*) just as the basal cells at the normal dermal–epidermal junction; however, the epidermal cells adjacent to the superficial-most dermal cells on the surface of the pf/pf epidermis are positive for K6 (*arrowheads*). Dotted line indicates the basement membrane. Bars, 100 μm (*E*) and

curs in the mutant epidermis. Analysis of other samples indicates that the amount of proteolysis is variable with the preparation and type of keratin examined. The 17-d pf/pf mutant extract also had a band of ~50 kD (lane *4*, *arrow*) that was immunoreactive with anti-K14 antibodies but Western blots of 2-D gels have failed to demonstrate immunoreactivity of this antibody with the major 50-kD, acidic component of mutant epidermis (data not shown).

Western Blotting and Localization of 50-kD Keratin (K16). Antibodies directed against K16 were found to be monospecific for the 50-kD keratin (Fig. 6 *B*). K16 was present as a faint band in extracts of normal epidermis (Fig. 6 *B*, lane *b*) but was present in abundance in the extracts of pf/pf and Er/Er mutant epidermis (Fig. 6 *B*, lanes *c* and *d*). Immunolabeling of normal epidermis demonstrated immunoreactivity in hair follicles of normal skin, whereas interfollicular epidermis was negative (Fig. 6 *C*). Mutant epidermis, on the other hand, had immunoreactive cells in the hair follicles as well as in the outer cell layers of the thickened interfollicular epidermis (Fig. 6 *D*). The expression of K16 in the outer layers of the mutant epidermis occurred as expected for a keratin functioning as a keratin pair of K6 (see Fig. 3). Control sections demonstrated that the positive cells found in normal and mutant dermis (Fig. 6, *E* and *F*) were due to endogenous peroxidase activity.

2-D Gel Electrophoresis. Several differences between normal and mutant epidermal keratin composition were detected by 2-D gel electrophoresis. As expected from the 1-D gels, the 64- and 62-kD keratins were present in newborn normal epidermis (Fig. 7 *A*) but missing from newborn Er/Er epidermis (Fig. 7 *B*) indicating a failure to process the mutant 67-kD keratin. The 59.5-kD keratin (K6) is not detected in normal epidermis but is abundant in mutant epidermis as detected in Coomassie Blue-stained 2-D gels (Fig. 7 *B*) and confirmed by Western blots of 2-D gels (Fig. 7 *B*, *inset*). In normal epidermis the acidic keratins of 59 kD (K10) and 55 kD (K14) are easily detected. These same acidic keratins are abundant in the mutant epidermis along with the third acidic keratin of 50 kD (K16). Thus a pair of keratins identified as K6 and K16 that are not commonly expressed in normal epidermis are found in abundance in the epidermis of newborn mutants.

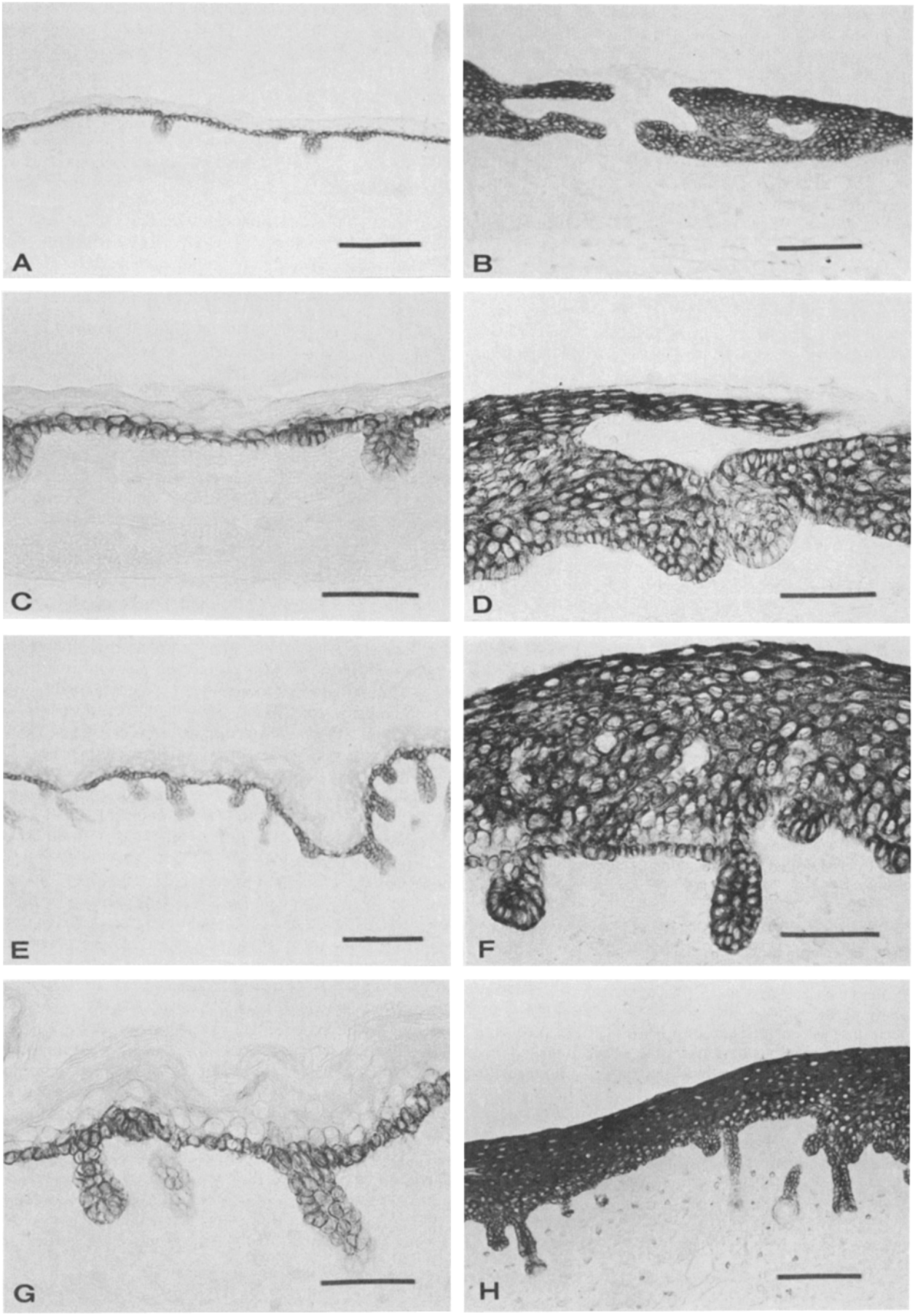
Grafting Experiments. Previous data indicated that pf/pf (Fisher et al., 1984) and Er/Er (Fisher, 1987) epidermis recovers a normal phenotype when whole skin is grafted to normal animals. The pf/pf and Er/Er epidermis keratinizes normally with morphologically normal granular and cornified layers and is indistinguishable from the epidermis of grafted skin from normal littermates (Fig. 8, *A–C*). Immunohistochemical examination of grafted mutant skin with antibodies directed against the 67-kD keratin (K1) (Fig. 8 *D*) results in a normal pattern of staining including negative basal cell staining, positive suprabasal cell staining, and negative stratum corneum staining indicating the onset of processing of the 67-kD keratin in the stratum corneum. Grafted Er/Er mutant skin recovers a keratin electrophoretic profile that is similar to the keratin profile of grafted skin from normal littermates (Fig. 8 *E*). The presence of the 64- and 62-kD keratins confirms that keratin processing occurs normally in grafted mutant skin. Similar results have been attained for the pf/pf mutant mouse (data not shown).

Discussion

The pf/pf and Er/Er mutations cause related, yet distinct, developmental abnormalities in ectodermally derived surface epithelia of mice. In addition, both mutations cause grossly similar defects including stumpy limbs and snouts, and both mutations result in hyperplasia and failure of differentiation in the epidermis as well as in oral epithelia (Guenet et al., 1979; Holbrook et al., 1982; Tassin et al., 1983; Kollar, 1983; Fisher et al., 1984; Fisher, 1987). Furthermore, both mutations have been mapped to the same region of chromosome 4 (Meredith, 1964; Guenet, 1977; Eicher and Fox, 1978). The rationale for the present investigation of keratin expression in pf/pf and Er/Er epidermis was twofold. Our goal was to gain insight into the failure of epidermal differentiation that affects both animals and, specifically, to study expression in these mutants of a family of proteins, the keratins, that are sensitive indicators of epithelial differentiation.

The onset of expression of both the pf and Er mutations correlates with epidermal stratification at 13 d of gestation. At this time the epidermis of animals homozygous for pf or Er stratifies in a disorderly fashion having variable numbers of cell layers, while the epidermis of normal littermates stratifies normally with an evenly thick intermediate cell layer. As development proceeds the hyperplastic epidermis of both mutants grows increasingly thick so that by birth it is many times thicker than normal but is not keratinized. During the course of normal development in mice (Schweizer and Winter, 1982; Roop et al., 1983; Ouellet et al., 1986), in rabbits (Banks-Schlegel, 1982) and in humans (Moll et al., 1982*b*; Dale et al., 1985; Fisher and Holbrook, 1987), the differentiation-specific keratins appear in the intermediate cell layers and correlate with the stratification of the simple, embryonic epidermis into a complex, multilayered epithelium. By 15 d of development in the mouse, these keratins are expressed in abundance in the normal intermediate cell layer (Fig. 2 *A*) but are absent from the 15-d pf/pf epidermis (Fig. 2 *B*). By 17 d the pf/pf epidermis is producing the differentiation-specific keratins in some deep, suprabasal cells (Fig. 2 *D*), and by birth a continuous layer of K1- and K10-positive cells can be identified in the deeper portions of the pf/pf epidermis (Fig. 2 *F*). Our biochemical data confirm this. Thus, the data suggest a delay in expression of the differentiation-specific keratins in pf/pf epidermis (Fig. 5, *A*). In addition, it would appear that a subpopulation of mutant keratinocytes fails to express K1 and K10 throughout the course of development. At 15 d of gestation these cells represent the entire embryonic keratinocyte population (Fig. 2 *B*).

50 μm (*F*). (*G*) Normal 18-d gestation epidermis is negative for K6. Dotted line indicates the basement membrane. Bar, 50 μm. (*H* and *I*) The 18-d Er/Er mutant epidermis shows a pattern of distribution for K6 that is very similar to the pf/pf mutant. Dotted line indicates the basement membrane. Bars, 100 μm (*B*) and 50 μm (*C*).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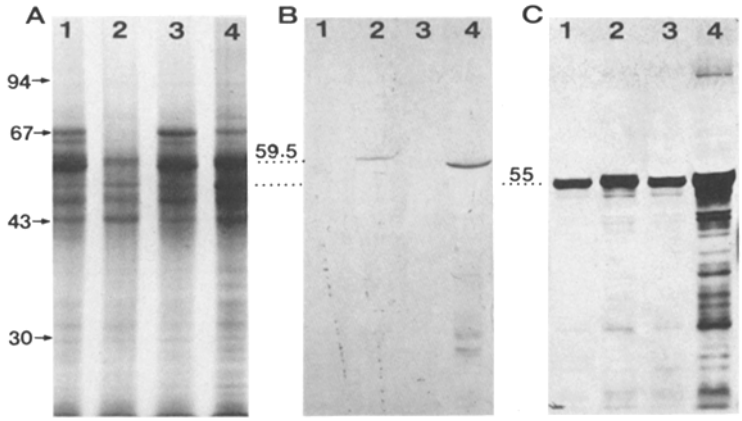

Figure 5. Western blotting. (*A*) Coomassie Blue-stained gel of 15- and 17-d normal and mutant keratin extracts. The keratins migrate between 70 and 45 kD. Note the absence of the 67-kD keratin (K1) from the 15-d gestation extract supporting the immunohistochemical findings. Lane *1*, 15-d gestation normal epidermis; lane *2*, 15-d gestation pf/pf epidermis; lane *3*, 17-d gestation normal epidermis; lane *4*, 17-d gestation pf/pf epidermis. (*B*) Western blot identical to that in *A* reacted with the 59.5 kD keratin (K6). K6 is missing from normal epidermis but present in both 15- and 17-d gestation pf/pf mutant epidermis. Lane *1*, 15-d gestation normal epidermis; lane *2*, 15-d gestation pf/pf epidermis; lane *3*, 17-d gestation normal epidermis; lane *4*, 17-d gestation pf/pf epidermis. (*C*) Western blot identical to that in *A* reacted with antibodies to the 55-kD keratin (K14). More immunoreactive material appears to be present in extracts of mutant epidermis (lanes *2* and *4*) than in normal (lanes *1* and *3*). The arrow indicates some immunoreactivity with a 50-kD component that is probably due to proteolysis. Lane *1*, 15-d gestation normal epidermis; lane *2*, 15-d gestation pf/pf epidermis; lane *3*, 17-d gestation normal epidermis; lane *4*, 17-d gestation pf/pf epidermis.

At 17 d of gestation cells that are competent for production of K1 and K10 arise from the basal cell population (Fig. 2 *D*) so that by birth a layer of cells exist that are positive for the differentiation-specific keratins (Fig. 2 *F*). The more superficial cells of the newborn mutant epidermis, presumably the same cells that were negative in the 15-d animal, fail to express K1 and K10 throughout the course of their development. These observations suggest that expression of the differentiation-specific keratins is due to a shift in gene expression which is delayed in pf/pf and Er/Er mutant mice. Furthermore, cells undergoing this shift may arise from clonal expansion of competent basal cells.

In contrast, the expression of the K6 precedes that of the differentiation-specific (K1 and K10) keratins during development of the pf/pf and Er/Er epidermis (Fig. 5 *B*) and later in development has a distribution that is opposite that of the differentiation-specific keratins; the basal-most cell layers are negative or weakly positive and staining increases through the cell layers so that the superficial-most cell layers are intensely positive for K6. The keratin counterpart of K6, K16, is also present in mutant epidermis in a distribution opposite that of K1 and K10 (Fig. 6). These data suggest that expression of these two subsets of keratins is mutually exclusive in mutant epidermis; a cell dedicated to the expression of one pair of keratins fails to synthesize significant amounts of the other pair. The hyperplastic keratins are not expressed in the normal epidermis in which the shift in gene expression to K1 and K10 has occurred at the appropriate developmental age. Similar observations have been made of human epidermis (Weiss et al., 1984) and rabbit cornea (Schermer et al., 1987).

The keratins contain protease-sensitive terminal peptide sequences that project from a protease-resistant filament core (Steinert et al., 1983). It could be argued that the absence of reactivity of antibodies directed against K1 and K10 to the superficial cell layers of the older mutant epidermis (pf/pf or Er/Er) is due to proteolytic modification of these keratins in that the antibodies employed in these studies are directed against unique carboxy-terminal peptides. However, this is unlikely since evidence of proteolytic modification of the differentiation-specific keratins (i.e., the 64- and 62-kD keratins) is not detected in the newborn mutant epidermis by 1- and 2-D gel electrophoresis (Figs. 1 and 7).

The carboxy terminus of K14 is identical to that of a 50-kD keratin (Roop et al., 1985). Although there is cross-reactivity of antibodies directed against the K14 carboxy terminus with a 50-kD component in our 1-D blots of 17-d mutant epidermis (Fig. 5 *C*), this sample shows considerable evidence of proteolysis making it difficult to draw conclusions regarding the source of this protein. Our blots of 2-D gels have failed to demonstrate immunoreactivity of the antibodies with a 50-kD component of mutant epidermis. Recent sequence data for K16 (Knapp et al., 1987) has demonstrated that K14 and K16 have completely different carboxy termini. Antibodies directed against the K16 carboxy terminus react with a single keratin of 50 kD that is present in much higher levels in pf/pf and Er/Er mutant epidermis over normal epidermis (Fig. 6) indicating that the major 50-kD component of mutant epidermis is K16.

Dramatic differences in immunolabeling with the anti-K14 antibody exist between normal and mutant epidermis (Fig. 4). K14 is detected primarily in the basal cells of normal epidermis but in all cell layers of mutant epidermis at all gestational ages tested. We believe that these differences are

Figure 4. Localization of the 55-kD keratin (K14). (*A* and *C*) The basal cells and hair buds of normal 15-d epidermis are stained with antibodies directed against K14. Bars, 100 μm (*A*) and 50 μm (*C*). (*B* and *D*) The entire epidermis of the 15-d pf/pf mutant is intensely stained with the same antibodies. Bars, 100 μm (*B*) and 50 μm (*D*). (*E* and *G*) The basal cells as well as the hair follicles of normal, newborn epidermis stain positively for K14. Bars, 100 μm (*E*) and 50 μm (*G*). (*F*) Newborn pf/pf epidermis, on the other hand, is completely and intensely stained with the antibody. Bar, 50 μm. (*H*) 18-d Er/Er epidermis is intensely stained in the same manner as pf/pf epidermis. Bar, 100 μm.

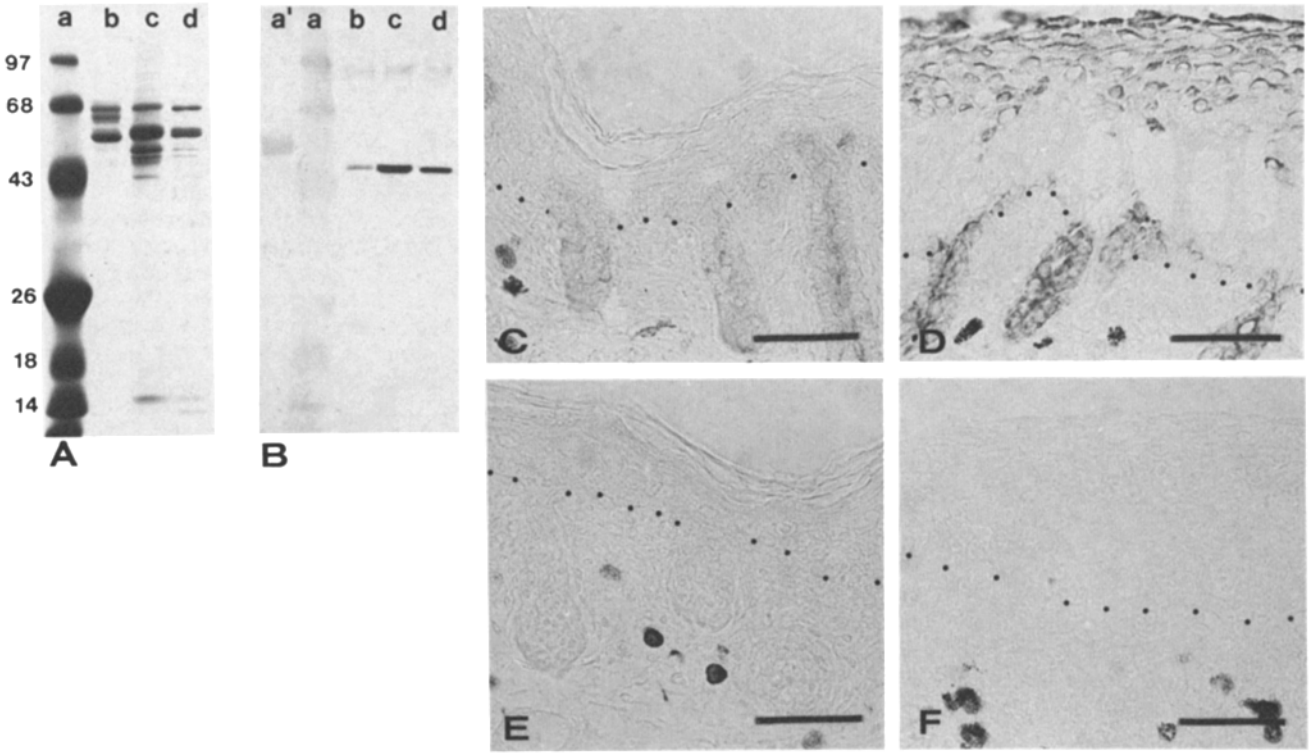

Figure 6. Western blotting and localization of 50 kD keratin (K16). (*A*) Coomassie Blue-stained SDS-polyacrylamide gel showing position of standards and profiles of extracts from normal, pf/pf mutant, and Er/Er mutant mouse epidermis. Lane *a*, standards; lane *b*, normal epidermis; lane *c*, pf/pf mutant epidermis; lane *d*, Er/Er epidermis. (*B*) Corresponding Western blot for K16 of gel shown in *A*. Lane *a'*, 55-kD standard; lane *a*, standards; lane *b*, normal epidermis; lane *c*, pf/pf mutant epidermis; lane *d*, Er/Er epidermis. Note the intensely positive bands at 50 kD in both pf/pf and Er/Er mutant extracts (lanes *c* and *d*). (*C*) Immunohistochemical localization of K16 in 18-d normal keratinized epidermis. K16-positive cells are present in the hair-follicles. Dots indicate the position of the basement membrane. Bar, 50 μm. (*D*) Immunohistochemical localization of K16 in pf/pf mutant epidermis. Note reactivity of antibody with hair follicles as well as with suprabasal cell layers. Dots indicate the position of the basement membrane. Bar, 50 μm. (*E*) Control for *C* demonstrating endogenous peroxidase activity. The positive cells in dermis are probably mast cells. Dots indicate the position of the basement membrane. Bar, 50 μm. (*F*) Control for *D* demonstrating endogenous peroxidase activity. The positive cells in dermis are probably mast cells. Dots indicate the position of the basement membrane. Bar, 50 μm.

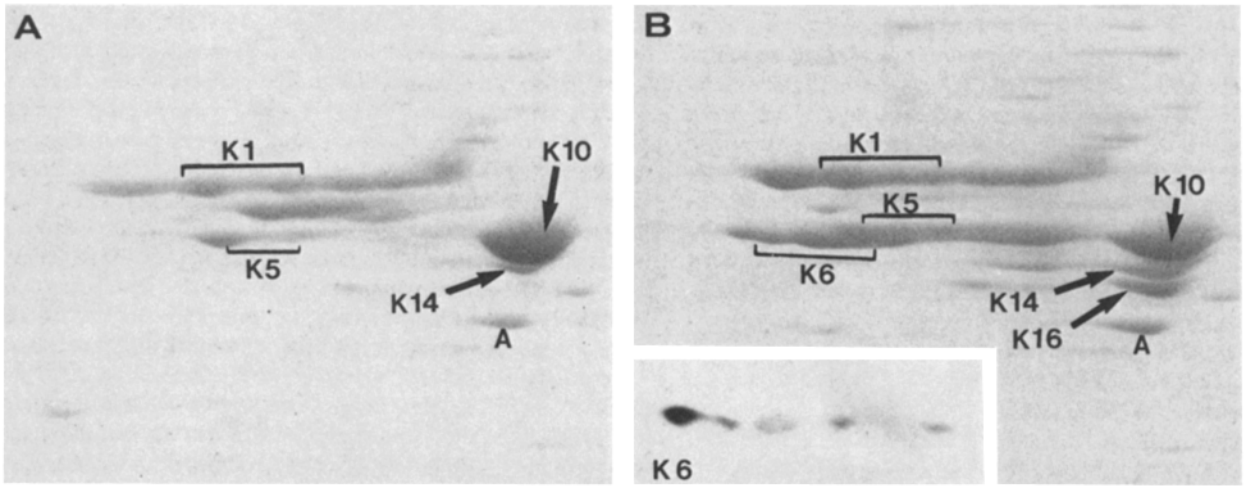

Figure 7. 2-D gel electrophoresis of newborn normal and Er/Er mutant epidermal extracts. (*A*) The prominent keratins of normal newborn epidermis are the 67- (K1), 60- (K5), 59- (K10), and 55- (K14) kD keratins. Note the position of the 64- and 62-kD keratins that are processed from K1. (*B*) Newborn Er/Er epidermis also expresses K1, K5, K10, and K14. In addition, two other prominent keratins of 59.5 (K6) and 50 (K16) kD are present. Immunoblot analysis of the 2-D gel with antibodies directed against the 59.5-kD keratin (K6) has allowed us to distinguish between K5 and K6 (*inset*). Similar findings have been attained for pf/pf epidermis.

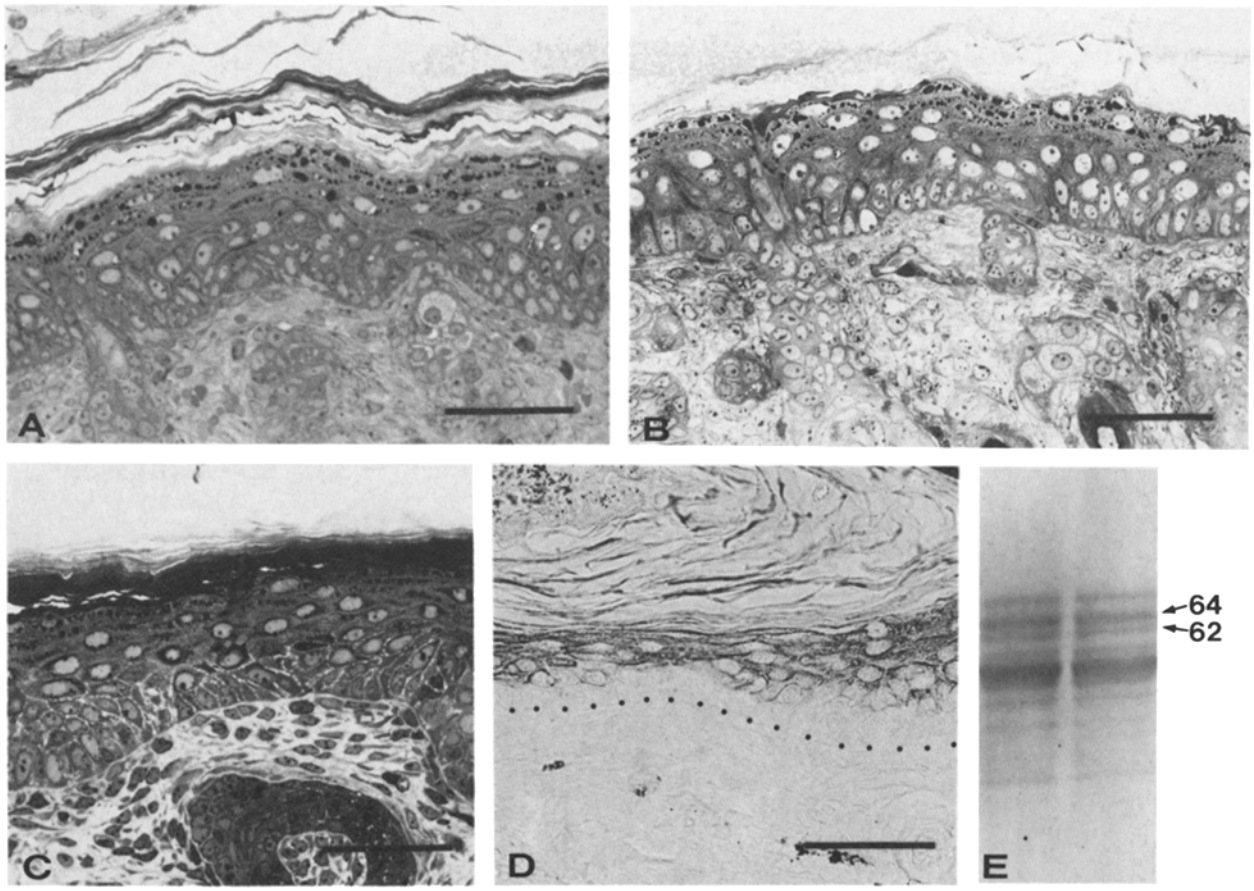

Figure 8. Recovery of a normal phenotype in mutant skin after grafting. (*A*) Normal 18-d skin grafted for 5 d. Bar, 50 μm. (*B*) 18-d pf/pf mutant skin grafted for 5 d recovers a normal phenotype. The grafted mutant skin keratinizes in a normal fashion with normal granular (stratum granulosum) and cornified (stratum corneum) cell layers. It is indistinguishable from grafted normal skin (*A*). Bar, 50 μm. (*C*) Grafted Er/Er skin also recovers a morphologically normal phenotype that is indistinguishable from grafted normal skin. 17-d Er/Er skin grafted for 6 d is shown here. Bar, 50 μm. (*D*) Grafted Er/Er skin reacted with antibodies against the 67-kD keratin (K1) exhibits a normal staining pattern with negative basal cells, positive suprabasal cells, and negative stratum corneum indicating processing of the keratin. Dotted line indicates basement membrane. Bar, 50 μm. (*E*) Keratin extracts of 17-d Er/Er mutant skin (lane *1*) and 17-d normal skin (lane *2*) grafted for 6 d each are identical and indicate recovery of a normal phenotype in grafted mutant skin. The presence of the 64- and 62-kD keratins indicates that keratin processing is now occurring.

probably due to an increase in K14 expression in the mutant epidermis or to a failure to suppress K14 gene expression with movement of the basal cells to a suprabasal position. On the other hand, the striking difference in labeling between mutant and normal epidermis may be attributed to an absence of masking of the K14 carboxy terminus in the mutant epidermis due, at least in part, to a reduction in expression of the differentiation-specific keratins.

During the course of normal epidermal differentiation most keratins arise from expression of unique genes whereas others are derived from processing of unique gene products (Fuchs and Green, 1980). The 64- and 62-kD keratins of mice, two keratin subunits known to be major stratum corneum components and to arise from the proteolytic processing of the 67-kD keratin (K1) (Bowden et al., 1984), appear at 18 d of development coincident with the first evidence of keratinization (Schweizer and Winter, 1982). Newborn pf/pf and Er/Er mutants fail to express these keratins even though they are abundant in normal littermates (Figs. 1 and 7), suggesting that the mutant epidermis is deficient for the necessary protease(s) or that K1 is altered so that it is unsusceptible to proteolytic action. It was previously demonstrated that profilaggrin, the major keratohyalin protein, is synthesized but not proteolytically processed to filaggrin in Er/Er mice (Holbrook et al., 1982). Thus there may be a general deficiency in protease activity in pf/pf and Er/Er mice that results in the failure to process the major constituents of the differentiated epidermal cell, the differentiation-specific keratins and/or profilaggrin, into the lower molecular mass forms that are associated with formation of the stratum corneum. At present it remains unclear whether the proteolytic processing of keratins and filaggrin represents a specific regulatory step important for formation of the terminally differentiated cell of the stratum corneum, or if these events occur secondarily to a general hydrolytic environment associated with cell death and cornification. It should be noted, though, that the paucity of morphologic markers of differentiation such as lamellar granules and thickened cellular envelopes from pf/pf and Er/Er skin (Holbrook et al., 1982; Fisher et al., 1985; Fisher, 1987) suggests a general failure of epidermal

differentiation not limited to keratin and filaggrin expression; hypotheses concerning the nature of action of the pf and Er genes should account for these observations.

Grafting of whole skin from pf/pf (Fisher et al., 1985) and Er/Er (Fisher, 1987) mutants results in the recovery of a phenotype that is indistinguishable from normal grafted tissue. Grafting results in the rapid recovery of normal keratin synthesis, as determined by comparison with grafted normal skin, in both pf/pf and Er/Er skin. These and other similar results have been interpreted to indicate that pf/pf and Er/Er mutant skin are capable of keratinizing in a normal fashion when provided with the proper environment (Fisher et al., 1985; Fisher, 1987). Not only does the grafted mutant epidermis synthesize normal keratins indicating that expression and processing of the keratins is normal, but it also produces the normal morphologic indicators of keratinization including lamellar granules and thickened cell envelopes (Fisher et al., 1985; Fisher, 1987). This phenomenon of recovery of a normal phenotype in mutant epidermis has also been observed in organ cultures of Er/Er (Salaun et al., 1986; Stewart and Fisher, unpublished observations) and pf/pf (Fisher, unpublished observation). These results indicate that removal of either pf/pf or Er/Er skin from the mutant environment by grafting or organ culture allows the epidermis to pursue its normal course of development and differentiation. For these reasons it has been proposed that the pf and Er genes are not primarily expressed in the mutant skin but are expressed systemically (Fisher et al., 1985; Fisher, 1987). The results presented in this article support this hypothesis. It is clear from cell culture and grafting studies that the keratinocyte environment has a profound influence on keratin expression (Doran et al., 1980; Breitkreutz et al., 1984). Our studies present evidence for a model in which epidermal keratin expression is powerfully but reversibly altered by the pf/pf and Er/Er mutant environment.

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