

Keratin Alterations during Embryonic Epidermal Differentiation: A Presage of Adult Epidermal Maturation

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ABSTRACT Differentiation of the epidermis during embryonic rabbit development was found to be accompanied by dramatic changes in keratin proteins. Immunofluorescent labeling with keratin antiserum revealed that the undifferentiated epithelium of 12-d embryos was already committed to making keratin proteins. At 18 d of embryogenesis, the epithelium contained keratin proteins in the molecular weight range of 40,000–59,000. The stratification of the epithelium into two cell layers at 20 d of development coincided with the appearance of a 65-kdalton keratin. When a thick stratum corneum developed at 29 d, several additional keratins became prominent, most notably the large keratins (61- and 64-kdalton) and a 54-kdalton keratin. In addition, the 40-kdalton keratin, which had been present in earlier embryonic epidermis, disappeared. Newborn epidermis resembled that of a 29-d embryonic epidermis, with the exception of the appearance or increase in concentration of two more keratin species (46- and 50-kdalton). In vitro culturing of keratinocytes from 12- and 14-d embryonic skin demonstrated that these cells contained essentially the same keratin profiles as the undifferentiated epithelium of 18-d embryos (40–59 kdalton). Keratinocytes grown from older embryos contained increased amounts of keratin, similar to the in vivo situation, but did not synthesize the high molecular weight keratins. The changes observed during embryonic epidermal differentiation appear to be recapitulated during the sequential maturation steps of adult epidermis.

During the course of embryonic development, the keratinocyte undergoes a defined program of differentiation reminiscent of that of the adult epidermis. The epithelium is transposed from a single layer of cells into a highly organized stratified squamous epithelium. Although the morphological changes which characterize this maturation process have been well documented (3, 5, 8, 10–12, 15, 19, 20, 24), the biochemical events involved are poorly understood.

In an earlier study, we examined the development of the keratinocyte as a differentiated cell type during embryonic development, using the cross-linked envelope as marker (5). In the present study we sought to examine the development of the keratinocyte in the embryonic epidermis in terms of the appearance of its other principal product of differentiation, the keratins. Because of the ability to grow epidermal keratinocytes by co-cultivation with lethally irradiated 3T3 cells (25), we also used this cell culture system to analyze events involved in the process of growth and differentiation of the epidermal keratinocyte during embryonic development.

Here we show that the undifferentiated epithelium of 12-d

rabbit embryos contains keratin proteins, in apparent contrast to the rat embryo (14). During the course of terminal differentiation of the keratinocyte during embryonic development, dramatic changes in keratin proteins occur. These changes in keratin expression during embryonic development closely resemble those observed during the postnatal maturation of the epidermis.

MATERIALS AND METHODS

Preparation of Embryonic Tissue and Cell Culture

On the appropriate day of gestation denoted in the experiment, embryos were removed from pregnant New Zealand white rabbits (Margaret's Home Farm, Greenfield, MA) after being killed by a lethal injection of 1 ml of somlethal (6 g) (J. A. Webster Veterinary Supply Co., North Billerica, MA) into the ear vein. The embryos were killed and the dorsal skin was excised. In the case of 12- and 14-d embryos, skin from the ventral surface was also frequently taken to obtain a reasonable amount of material.

With the exception of 12- and 14-d embryos, the subcutaneous tissue and as much dermis as possible was removed from the skin of rabbit embryos obtained

at different stages of development. The remaining epithelium and dermis was then minced and disaggregated into single cells with a solution of 0.25% trypsin (Trypsin 1-300; ICN Nutritional Biochemicals, Cleveland, OH) and 0.02% collagenase (Cl. histolyticum, CLSPA, Worthington Diagnostics, Freehold, NJ), both dissolved in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (Gibco Laboratories, Grand Island Biologicals Co., Grand Island, NY) (5). Cells were added to dishes containing irradiated 3T3 cells (25) and grown in fortified Eagle's Medium supplemented with 20% fetal calf serum (FCS) (M. A. Bioproducts, Walkersville, MD), 0.4 $\mu\text{g}/\text{ml}$ hydrocortisone (25), and 15 ng/ml epidermal growth factor (26, 27).

Epidermal keratinocyte colonies could not be grown from trypsinized cells suspensions of 12- or 14-d embryonic skin. However, small pieces of skin (explants) isolated from 12- and 14-d embryos when allowed to attach to the surface of a tissue culture dish would give rise to epithelial colonies. After attachment of the explants to the surface of the dish (by air-drying at room temperature for brief periods of time), culture medium containing 4×10^5 irradiated 3T3 cells was added to the dish, and the cells were incubated at 37°C. Under these conditions, epidermal keratinocytes could be seen radiating out from the explants within only a few hours.

Analysis of Keratin Proteins from Embryonic Rabbit Epidermis and Newborn Epidermis

Skin was removed from rabbit embryos at 18, 20, 23, 25, and 29 d of development and from newborns. Pieces of skin were then stirred in a flask containing 2 M sodium bromide for 1 to 2 h at 37°C, essentially as described by Huang et al. (21). After incubation, the skin was removed to a tissue culture dish containing PBS, and the epidermis was cleanly lifted from the dermis as a pure sheet of epithelial cells with the aid of forceps and a dissecting microscope. After several rinses with PBS to remove the sodium bromide, equivalent amounts of epithelium were either solubilized directly in a solution containing 20 mM Tris-HCl, pH 7.4, 5% SDS, and 10 mM dithiothreitol or first extensively extracted with 20 mM Tris-HCl, pH 7.4, before remaining proteins were solubilized in the same volume of 20 mM Tris-HCl, pH 7.4, 5% SDS, and 10 mM dithiothreitol as the first part. Equal aliquots were subjected to PAGE. Protein concentrations were determined using the method of Lowry et al. (23).

Analysis of Keratin Proteins in Cultured Embryonic Keratinocytes

When keratinocyte colonies derived from embryonic skin of embryos of different ages had grown to confluence, the fibroblasts were selectively removed with EDTA (31). The remaining keratinocytes were trypsinized, divided into two equal parts, and washed once with PBS. Proteins were extracted from these keratinocytes according to the same protocol described for the intact epithelium and were analyzed by PAGE.

PAGE

Proteins were separated by electrophoresis using SDS in the discontinuous vertical slab gel system described by Laemmli (22). Polyacrylamide gels (8.5% acrylamide:bisacrylamide 30:0.8 by weight) were used for the protein separation. Electrophoresis was carried out at 30 mA per gel until the tracking dye reached the bottom of the gel. The gels were fixed and stained overnight in a solution of 0.25% Coomassie Blue in 50% methanol and 10% acetic acid. The gels were then diffusion-destained by repeated changes of a solution of 50% methanol and 10% acetic acid and dried on a Hoefer Scientific Gel Drying Apparatus (Hoefer Scientific Instruments, San Francisco, CA).

Immunofluorescent Detection of Keratin in Intact Rabbit Skin

Either the whole embryo (12, 14, and 16 d of embryonic development) or excised skin (20 d and older) was isolated and frozen in isopentane chilled in liquid N_2 . Once the embryo or skin was completely frozen, it was removed from the isopentane and stored in a freezer at -70°C . Next, 5- μm samples were sectioned on a cytotostat and then stored at -70°C . When all of the samples were ready for immunofluorescence, the slides containing the tissue sections were allowed to equilibrate to room temperature for 45 min. Subsequently, the sections were soaked in PBS for 10 min before treatment with a 1:40 dilution of antiserum prepared against whole keratins purified from human stratum corneum (28, 33) using indirect immunofluorescence (6). The sections were viewed through a Zeiss Universal Microscope using epifluorescence illumination. Photographs were taken with Tri-X film.

Immunofluorescent Detection of Keratin in Cultured Embryonic Keratinocytes

Colonies of keratinocytes were grown either from explants (in the case of 12- and 14-d embryonic skin) or from trypsin-disaggregated suspensions of cells (16 d or older) as described earlier. Because of the poor adhesive properties of keratinocytes grown from embryonic skin of early stage embryos, the cells were grown on tissue culture dishes rather than on glass cover slips. When the colonies had reached a suitable size, they were rinsed once with PBS, fixed for 30 min at room temperature in 3.7% formaldehyde, and stored at 4°C in PBS.

For immunofluorescent staining, the keratinocytes were permeabilized for 10 min with freezer-cold methanol (-20°C). The cells were stained with keratin antiserum as described previously for intact tissue.

RESULTS

Keratin Proteins from Embryonic Epidermis

Skin from rabbit embryos of various ages was removed and exposed to a solution of 2 M sodium bromide for 1 to 2 h at 37°C to cleanly separate the embryonic epidermis from the dermis. The separation took place cleanly at the basement membrane, as shown in Fig. 1. Whether the epithelium was only a single layer of cells, as in the case of 18-d embryos (Fig. 1a), or a fully stratified squamous epithelium, as observed with 29-d embryonic skin (Fig. 1c), the epithelium could be easily lifted away from the dermis as a pure sheet of keratinocytes. This method of separation worked equally well for skin preparations of all ages. Fig. 1b and d demonstrate that the epithelium was entirely removed from the dermis of the preparation of either age.

Nonkeratin proteins can be removed selectively from the epithelium by extraction with dilute aqueous buffer, leaving primarily only the keratin proteins to be extracted with SDS and reducing agent (33). When an amount of epithelium equivalent to that used for the total protein analysis was first extracted extensively with dilute buffer before treatment with SDS and reducing agent, the keratin protein profile shown in Fig. 2 resulted. As expected for epidermal keratins, one finds a predominance of water-insoluble proteins in the molecular weight range extending from 40 to 65 kdalton. Because the epidermis developed in defined morphological stages (5), we could clearly demonstrate that the basal cells themselves, represented by 18-d embryos, contained keratin proteins in the molecular weight range of 40–59 kdalton. Only the highest molecular weight keratins (61, 64, and 65 kdalton) were absent from the undifferentiated epithelium of 18-d embryos. At 20 d of development when the embryonic epidermis morphologically differentiated into two-cell layers, the 65-kdalton keratin protein (labeled *a* in Fig. 2) appeared and paralleled the commitment of the cells to terminal differentiation. The 64- and 61-kdalton keratins (labeled *b* and *c*, respectively) were not observed until 29 d of embryonic life when the most obvious morphological change in the epidermis was the development of a well-defined stratum corneum. Two minor protein bands can be seen in the array of proteins extracted from 18-d embryonic rabbit epidermis which comigrate with the 64- and 65-kdalton keratin proteins. It is not known whether these proteins are, in fact, the same proteins which are prominent at later stages of development at much higher concentrations. In addition, another keratin protein, labeled *d* (Fig. 2), which is 54 kdalton, was noted to appear or greatly increase in concentration at 29 d of embryonic development. During development, most of the keratin proteins in the 40- to 65-kdalton molecular weight range were found to increase markedly in concentration. The keratin proteins extracted from newborn

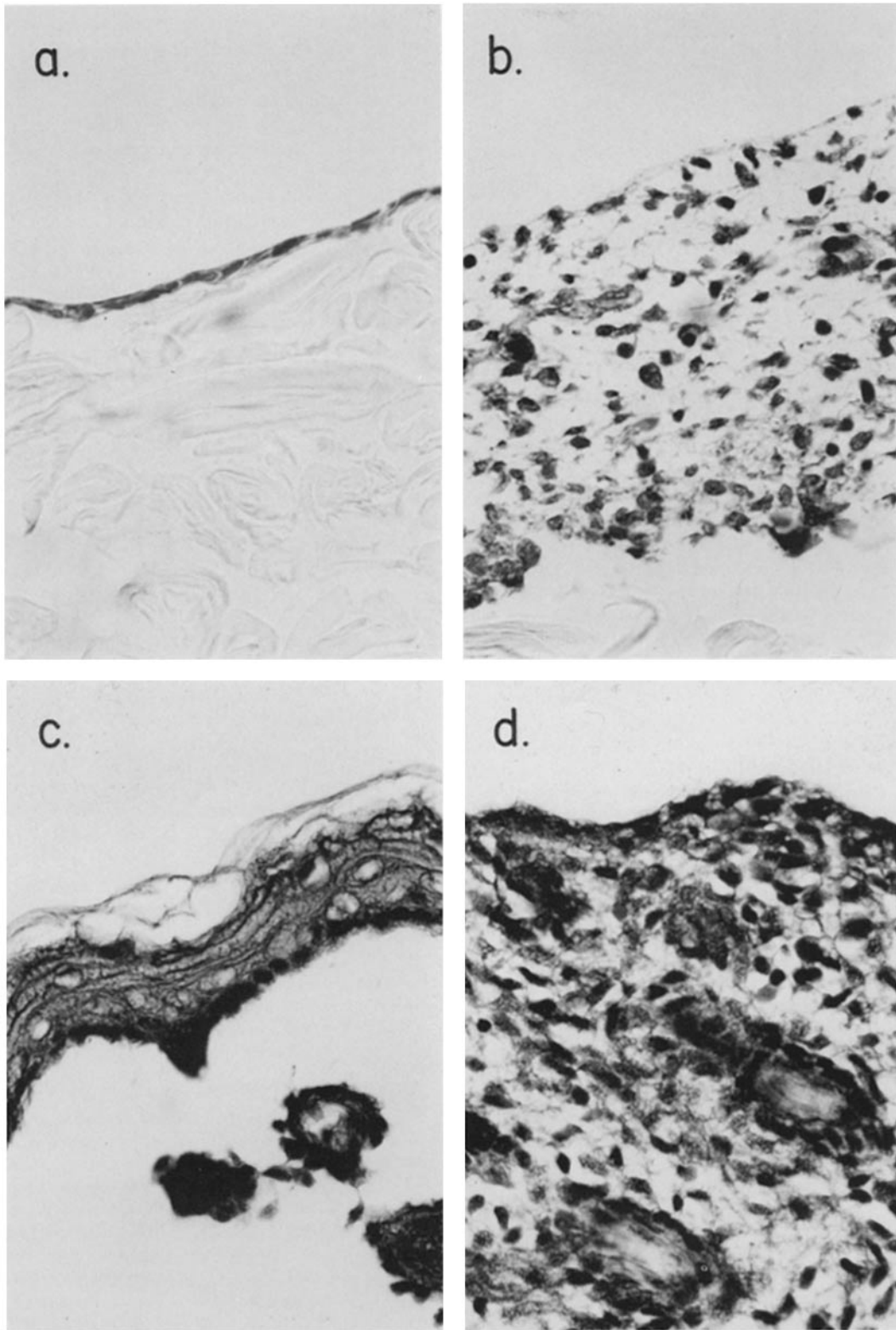


FIGURE 1 Separation of the embryonic epidermis from the dermis of intact skin of 18- and 29-d rabbit embryos. The embryonic epidermis and dermis were separated as described in the Materials and Methods and then fixed overnight at 4°C in 3.7% formaldehyde, dehydrated, embedded, sectioned, and stained. Since the embryonic epidermis from 18-d skin was only a single layer of cells and therefore delicate, it was supported on filter paper throughout all of the histologic procedures. The dermis was handled similarly at this stage of development. (a) Embryonic epidermis from skin of 18-d rabbit embryos. (b) Dermis from skin of 18-d rabbit embryos. (c) Embryonic epidermis from skin of 29-d rabbit embryos. (d) Dermis from skin of 29-d rabbit embryos. $\times 400$.

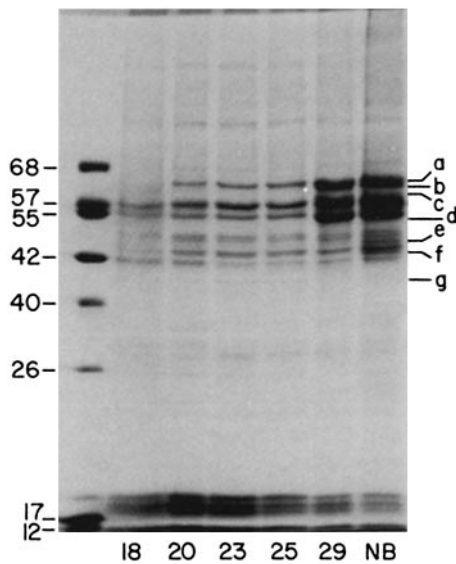


FIGURE 2 Water-insoluble proteins extracted from embryonic epidermis at different stages of development and from the epidermis of newborns. Embryonic epidermis was isolated from the skin of rabbits of different ages. An amount of tissue equivalent to that used for analysis of total proteins was taken and first exhaustively extracted several times with large volumes of 20 mM Tris-HCl, pH 7.4, at 4°C. After the final extraction, the water-insoluble proteins were then dissolved in the same volume of detergent and reducing agent as had been used for the analogous sample being analyzed for total protein content. An aliquot equal to that used for total protein analysis was analyzed by SDS slab gel electrophoresis. Changes in keratin protein species during development were designated by letters included on the right-hand side of the gel. The molecular weight standards ($\times 10^3$) are shown on the left-hand side of the gel. The molecular weight assigned to the different epidermal keratins was derived from the best fitting curve for the standards. Therefore, the molecular weight for a particular protein may not correspond exactly to that of an individual standard. The 40-kdalton molecular weight standard was found to run anomalously.

epidermis were found to resemble 29-d patterns with the exception of the appearance, or dramatic increase in the concentration of, two more keratin protein species of molecular weight 50 and 46 kdalton, labeled *e* and *f*, respectively. Of importance also was a 40-kdalton protein, labeled *g*, which was present in the epidermis up until 25 d of embryonic life but not at later stages of development and presumably represents a keratin protein.

Keratin Proteins in Cultured Epidermal Keratinocytes

The ability of cultured embryonic keratinocytes to synthesize keratin proteins was investigated. The earliest age at which keratinocyte colonies could be grown from trypsin-disaggregated cell suspensions of embryonic skin was 16 d (data not shown). While the epithelial colonies which grew from 16-d embryonic epidermis resembled keratinocyte colonies in appearance, they were fewer in number, less adherent to the surface of the dish, and did not stratify to the same extent as keratinocyte colonies grown from older epithelia (5). While epithelial colonies could not be grown from trypsinized cell suspensions of 12- and 14-d embryonic rabbit skin, explants from these embryos were able to give rise to keratinocyte colonies. When the keratinocyte colonies were confluent, the

3T3 cells and contaminating rabbit fibroblasts were removed with EDTA. The remained keratinocytes were extracted with dilute Tris buffer containing detergent and reducing agent, either directly to analyze for total cell proteins, or after extensive extraction with dilute Tris buffer to examine for the presence of epidermal keratins. Analysis of the proteins extracted from cultured keratinocytes of 12-, 14-, and 29-d embryonic skin is shown in Fig. 3 (lanes 1-6).

The developmental alterations in the keratin proteins extracted from these cultured keratinocytes closely resembled the changes in keratins observed in the intact epithelium (Fig. 2), with the exception of the absence of the high molecular weight keratins (61, 64, and 65 kdaltons) in the cultured keratinocytes. Similar to the *in vivo* situation, keratin proteins in the molec-

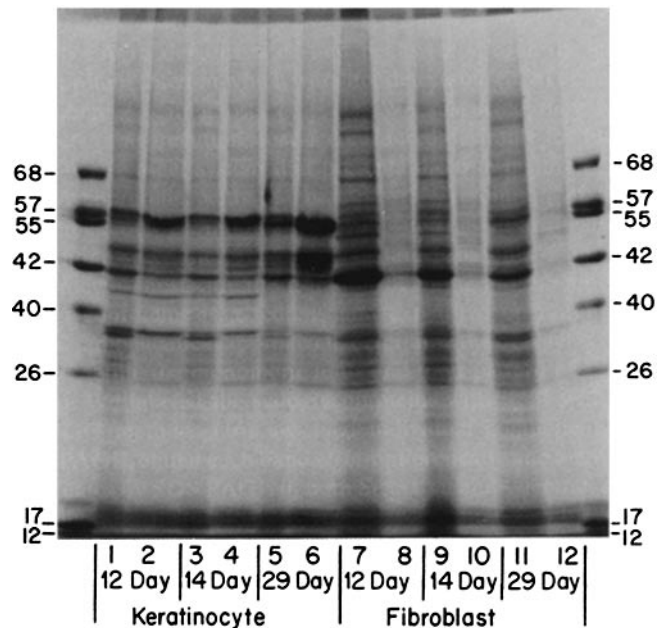


FIGURE 3 Total cell proteins and water-insoluble proteins extracted from cultured epidermal keratinocytes and fibroblasts grown from 12-, 14-, and 29-d embryonic skin. In the case of 12- and 14-d embryos, keratinocyte colonies were initiated from 1-mm² explants which were allowed to attach to the surface of the tissue culture dish. In the case of 29-d embryos, keratinocyte colonies developed from trypsinized cell suspensions of embryonic skin. Cultures of fibroblasts were obtained by plating trypsin-disaggregated cell suspensions of embryonic skin from embryos of different ages into tissue culture dishes containing no irradiated 3T3 cells, conditions favoring the growth of fibroblasts only. Usually, fibroblasts were passaged once to insure complete elimination of keratinocytes. Cells from confluent cultures were trypsinized, divided equally into two parts, and washed once with PBS. One part was extracted directly with 20 mM Tris-HCl, pH 7.4, 5% SDS, and 10 mM dithiothreitol and analyzed by gel electrophoresis to study total cell proteins (odd numbered tracks). The other part was first extracted several times at 4°C with large volumes of 20 mM Tris-HCl, pH 7.4, to remove the water-soluble proteins. Then, the remaining water-insoluble proteins were solubilized in the same volume of detergent and reducing agent as the first part, and an equivalent aliquot was analyzed by gel electrophoresis to study water-insoluble proteins (even numbered tracks). Tracks 1-6: proteins extracted from keratinocytes grown from 12-, 14-, and 29-d embryonic skin. Tracks 7-12: proteins extracted from fibroblasts grown from 12-, 14-, and 29-d embryonic skin. The molecular weight standards are shown in the outermost lanes on either side of the gel. They are the same as those described in Fig. 2. The 40-kdalton standard was found to run anomalously.

ular-weight range of 40–59 kdalton were found early in development (8 d before the onset of the morphological differentiation of the epithelium) and these proteins increased markedly in concentration during embryonic development. Analogous to the *in vivo* keratin protein changes, keratinocytes grown from embryonic skin at early stages of development contained an apparent 40-kdalton keratin. This protein was absent in keratinocytes grown from 29-d embryonic skin. Significant changes in the protein profiles of these keratinocytes were also found in the 35,000 molecular weight range. These changes involved two proteins. The largest protein (35 kdalton) was water insoluble and the other (34 kdalton) was water soluble. Both proteins were found to decrease in concentration during development. Comparisons of these protein changes in the cultured keratinocyte and in the intact epithelium are difficult because these proteins account for only a small proportion of the total cell protein in the intact epithelium.

Examination of the proteins extracted from fibroblasts grown from 12-, 14-, and 29-d embryonic skin (Fig. 3, lanes 7–12) showed no abundance of proteins in the region of 40–59 kdalton. The majority of these proteins, especially in the molecular weight range of 40–59 kdalton, were water soluble.

Immunofluorescent Detection of Keratin in Intact Rabbit Skin

Analysis of proteins extracted from the embryonic epidermis or cultured keratinocytes at various stages of development by polyacrylamide vertical slab gel electrophoresis indicated that keratin proteins were present in epidermal keratinocytes at all stages of development examined, even as early as 12 d of embryonic life. To pursue this observation further, we employed indirect immunofluorescence using antiserum to whole keratins purified from human stratum corneum (28, 33) to examine when the embryonic epidermis first becomes committed to making keratin proteins. The results are shown in Fig. 4. Fluorescence was found in the epidermis even at the earliest stage of development examined (i.e., 12 d of gestation). In addition, total epidermal fluorescence dramatically increased during development. A higher magnification of the embryonic epidermis of 12-d rabbit embryos revealed a fine array of fibrous filaments in the cytoplasm, characteristic of keratin (data not shown). When the epithelium was reacted with pre-immune serum or antiserum to keratin which had been adsorbed with keratin purified from human stratum corneum, no staining of the epithelium was observed. When the epidermis had begun to stratify at 20 d, a stronger fluorescence was noted in the more differentiated cells of the upper cell layers in contrast to the basal layer of cells which showed a less intense fluorescence (Fig. 5). Whether the embryonic epithelium was only two-cell layers thick (Fig. 5a) or a multi-layered stratified squamous epithelium (Fig. 5b), stronger fluorescence was noted in the more differentiated cells of the upper cell layers. In no case was the antiserum to keratin found to stain the dermis at any stage of development. Control experiments testing the immunofluorescent staining of embryonic skin obtained from various stages of development using pre-immune serum or antiserum to keratin which had been adsorbed with keratin were negative.

It is not known whether the increased fluorescence of the more differentiated cells is due to an increase in the concentration of existing keratin species, the appearance of new keratin species, or both of these possibilities. Analysis of the keratin

protein changes during the development of the epithelium would seem to suggest the latter possibility.

Detection of Keratin in Cultured Embryonic Keratinocytes

Keratinocytes were grown from embryonic skin isolated from rabbit embryos at different stages of development and reacted with antiserum to keratin using the technique of indirect immunofluorescence. Keratinocytes derived from epithelia at all stages of development were found to stain with antiserum to keratin. Keratinocytes grown from skin isolated from rabbit embryos at very early stages of development (12 and 14 d) (Fig. 6a) were found to contain a cytoplasmic network of fibrous filaments identical to those found in keratinocytes cultured from 29-d embryonic skin (Fig. 6b). These filaments could be seen in the keratinocytes of 12- and 14-d embryonic skin as soon as the outgrowth from the explant was initiated (usually within 1 d) and hence did not result from *in vitro* maturation of the epithelium. The presence of keratin filaments in the keratinocytes of 12- and 14-d epithelium is consistent with earlier experiments (gel protein profiles and immunofluorescent staining of intact skin), suggesting the presence of keratin in these cells at very early stages of development. 3T3 cells did not stain with the antiserum to keratin, and staining of keratinocytes at all stages of development with pre-immune serum or antiserum to keratin which had been adsorbed with keratin was negative.

Colonies of keratinocytes grown from embryonic skin of rabbits at different stages of development stratify in culture, similar to results with adult skin (25, 31, 32). They consist of a basal layer of smaller, dividing cells (Fig. 7a), giving rise to the large, differentiated cells in the upper cell layers (Fig. 7b). Earlier studies involving the immunofluorescent staining of the intact epithelium showed that as the epithelium stratified the more differentiated cells of the upper cell layers stained more strongly with antiserum to keratin. Therefore, we investigated whether keratinocyte colonies grown from embryonic skin at different stages of development exhibited this same capability. We found that the larger, more differentiated cells in the upper cell layers stained more strongly with the antiserum to keratin than did the smaller basal cells (Fig. 8), analogous to the result with intact epithelium. Since no new keratin protein species were found to appear in the cultured keratinocytes during the course of embryonic development (Fig. 3), the increased fluorescence in these differentiated cells probably reflects an increased amount of keratin.

DISCUSSION

Several studies have indicated that adult epidermis undergoes a defined program of differentiation involving many changes in keratin proteins as the epidermal cells progress from the basal to the outermost layers (1, 7, 13, 16, 29, 30, 34). Our knowledge of this differentiative process during the course of embryonic development is still very limited (2–4, 9, 14). We chose to use rabbit epidermis because it resembles human epidermal differentiation and permits analysis of embryonic events.

This investigation demonstrates that the differentiation of the epidermis during the course of embryonic development is accompanied by changes in the pattern of keratin proteins, analogous to those observed during the terminal differentiation

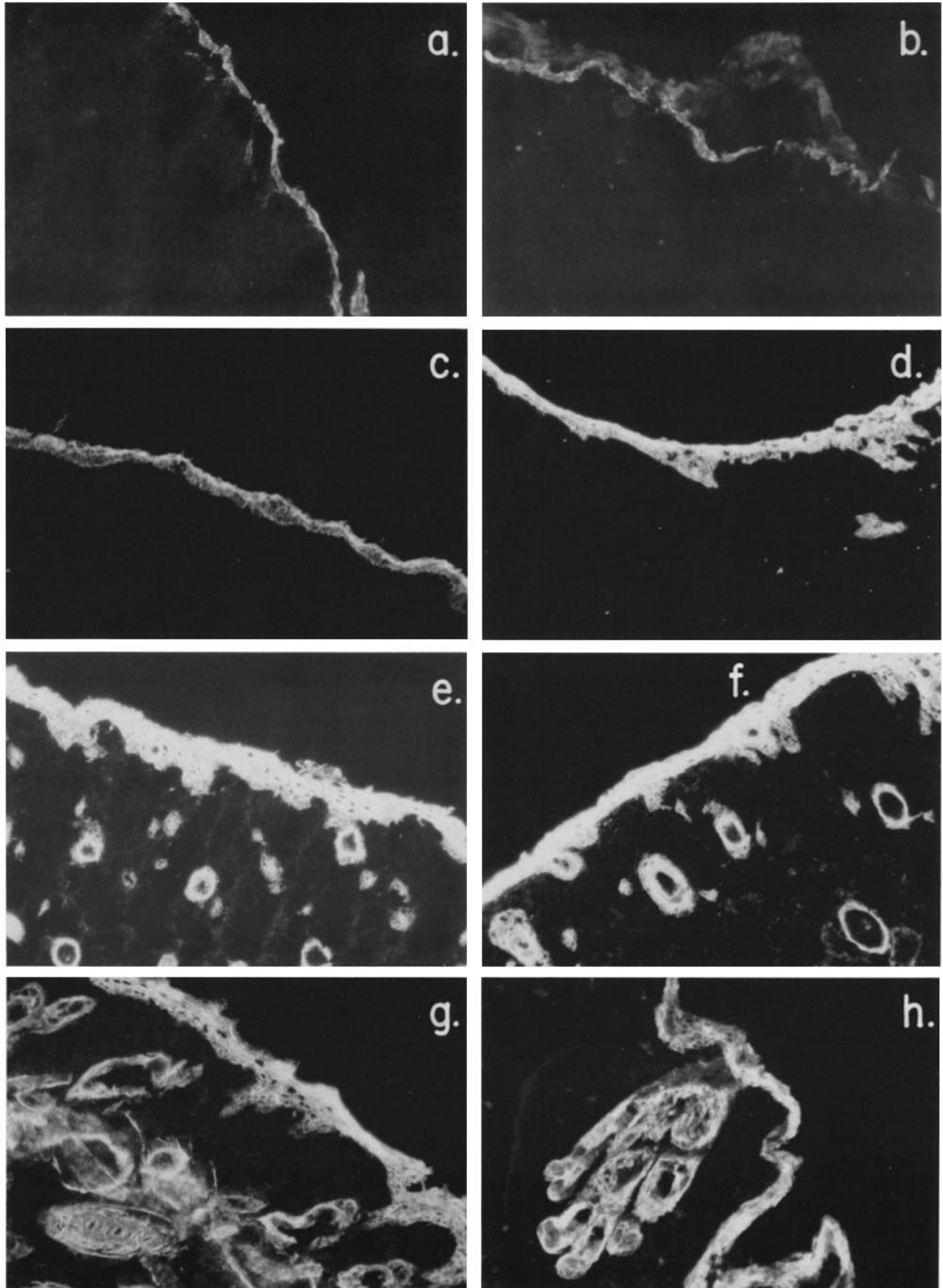


FIGURE 4 Immunofluorescent staining of intact skin isolated from rabbits at different stages of embryonic and postnatal development using antiserum to keratin. Either the whole embryo (12 and 16 d) or excised skin (20 d of embryonic development through adult) were isolated, frozen in isopentane chilled in liquid N_2 , and sectioned on a cryostat (5- μ m section). The tissue was then reacted with antiserum prepared against whole keratin purified from human stratum corneum using indirect immunofluorescence. (a-h) Immunofluorescent staining of 12-, 16-, 20-, 23-, 25-, and 29-d embryonic skin and the skin of newborn and adult, respectively. $\times 125$.

FIGURE 5. Immunofluorescent staining of 20- and 23-d embryonic skin using antiserum to keratin. (a) 20-d embryonic skin reacted with antiserum to keratin. (b) 23-d embryonic skin reacted with antiserum to keratin. As the epithelium stratifies, a stronger fluorescence is noted in the more differentiated cells of the upper cell layers than in the basal cells. $\times 160$.

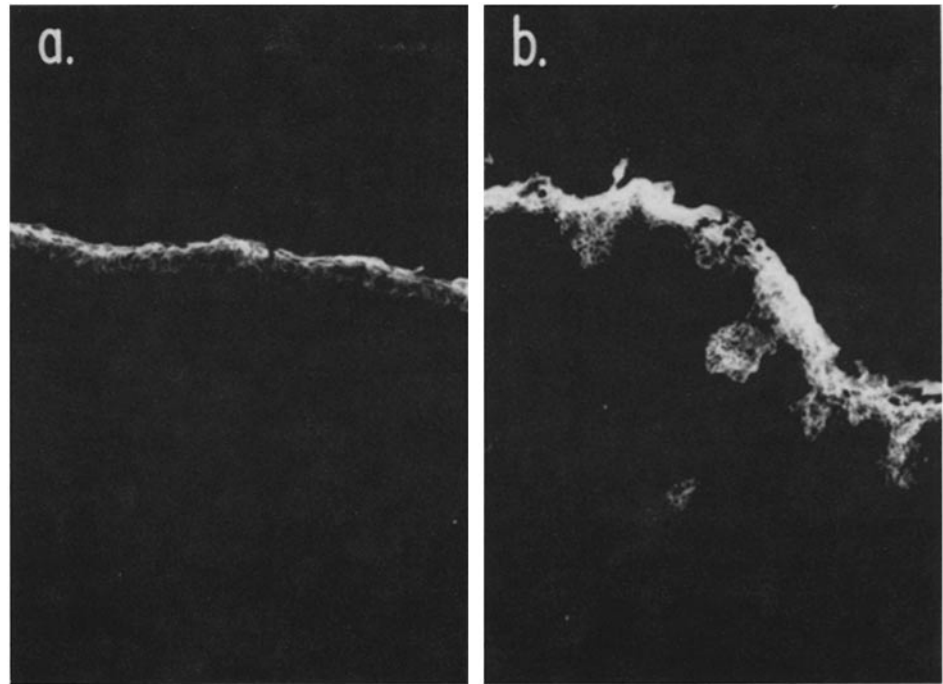
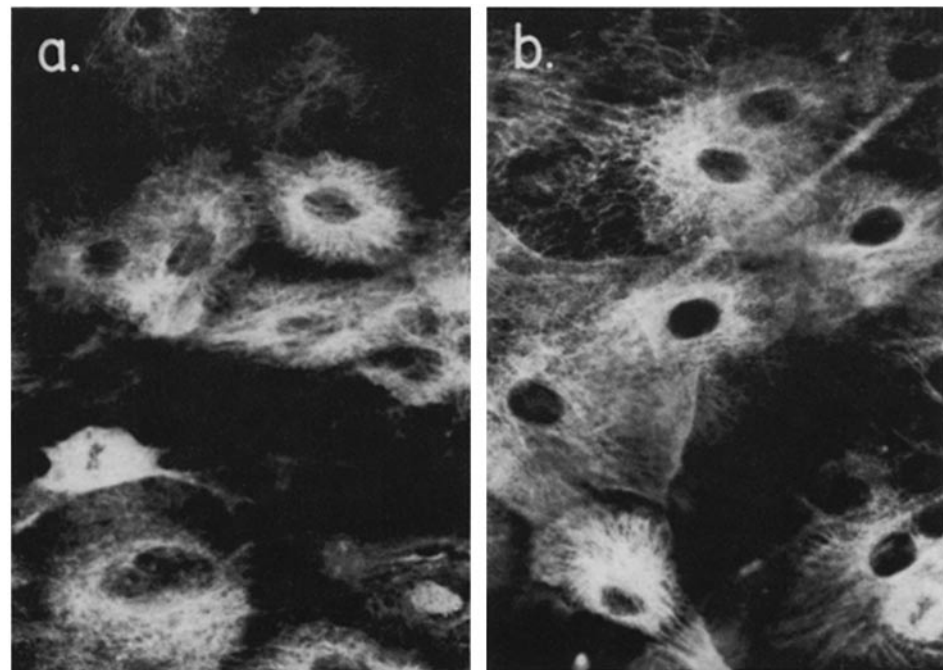


FIGURE 6. Immunofluorescent staining of keratinocytes grown from 14- and 29-d embryonic skin and stained with antiserum to keratin. Keratinocytes grown from embryonic skin of embryos of different ages were reacted with antiserum to keratin using the technique of indirect immunofluorescence. (a) Keratinocytes grown from 14-d embryonic skin and stained with antiserum to keratin. (b) Keratinocytes grown from 29-d embryonic skin and stained with antiserum to keratin. Note the dense array of fibrous filaments in the cytoplasm of keratinocytes grown from both 14- and 29-d embryonic skin, characteristic of keratin. $\times 400$.



of the adult epidermis (7, 16). The most significant finding of the present study was the presence of keratin proteins in the embryonic epidermis at 12 d of gestation (8 d before morphological differentiation of the epidermis). At this stage of development, the external epithelium of the rabbit lacks cells capable of forming cross-linked envelopes and by that criterion is a "primitive or precursor epithelium" (5). Comparisons of these results and the work of Fuchs and Green (16) indicate that the cells of this primitive epithelium seem to be analogous to the cells of the basal layer of the adult epidermis, both synthesizing essentially the same molecular weight keratins (46–59 kdalton). In addition, we observed an apparent 40-kdalton keratin protein in the basal layer of the embryonic epidermis which was

not present in the basal-spinous layers of the adult epidermis, consistent with these findings showing the lack of expression of this keratin at 29 d of embryonic life and later. The progression of the epidermal cells from the basal layer to the outermost layers, during embryonic development or during the postnatal maturation, seems to be accompanied by similar but not identical changes in the patterns of keratin proteins. As reported in previous research (7, 16), we observed that significant changes in keratin proteins were initiated in the spinous layers. In contrast to some reports (16), however, where the presence of large keratins (61, 64, and 65 kdalton) was believed to be correlated with the development of the stratum corneum, we found that the 65-kdalton keratin appeared very early in

the program of terminal differentiation. The appearance of this keratin species at 20 d of gestation (when the epidermis first stratifies into a two-cell layered structure) paralleled the commitment of the basal cells to terminal differentiation. Similar to the results of Fuchs and Green (16), the presence of the 61- and 64-kdalton keratins in the embryonic epidermis did correlate with the formation of a stratum corneum.

The basal layer of the undifferentiated epithelium of 18-d embryos contained essentially the same molecular weight keratins as cultured keratinocytes grown from embryonic skin at very early stages of development (12 and 14 d). While the commitment of the basal cells to terminal differentiation in the epidermis was found to be accompanied both by increases in the amount of keratins and by the appearance of new keratins, the cultured keratinocytes showed only increased amounts of keratins. Previously, it has been shown that cultured keratinocytes derived from neonatal and adult human and rabbit epidermis do not synthesize large keratins (16, 32, 33). The lack of synthesis of some large keratins (>60 kdalton) may be due to the lack of *in vitro* stratum corneum formation (18) or vice versa. The cultured keratinocytes did synthesize an apparent 40-kdalton keratin at early stages of development which was not present in keratinocytes grown from 29-d embryonic skin, consistent with the *in vivo* findings.

Our studies have revealed the presence of an apparent 40-kdalton keratin in the embryonic epidermis of rabbits. The

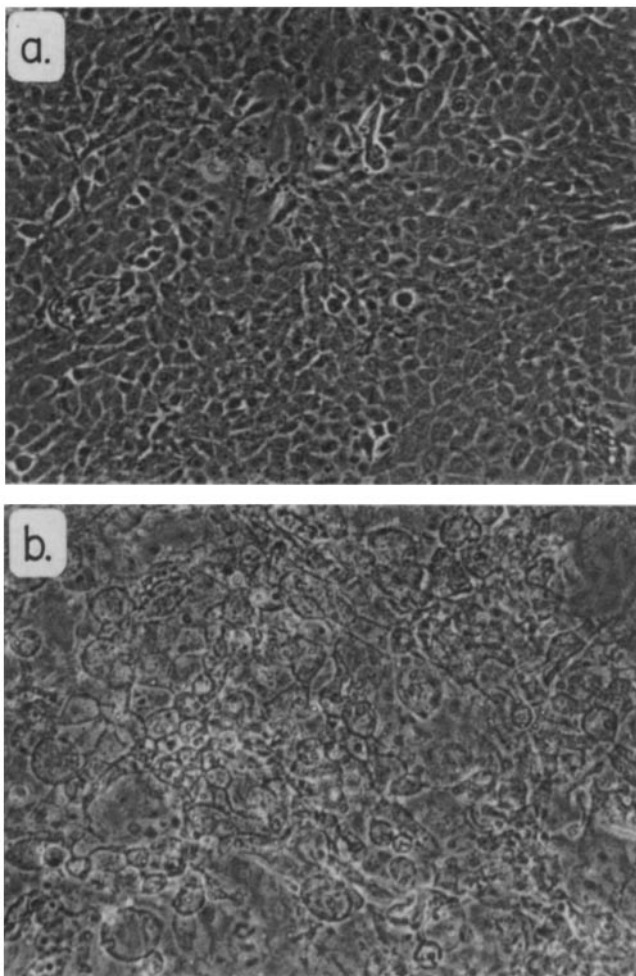


FIGURE 7 Typical epidermal keratinocyte colony grown from 29-d embryonic skin and examined by phase contrast microscopy. (a) Basal cell layer. (b) Upper cell layers. $\times 160$.

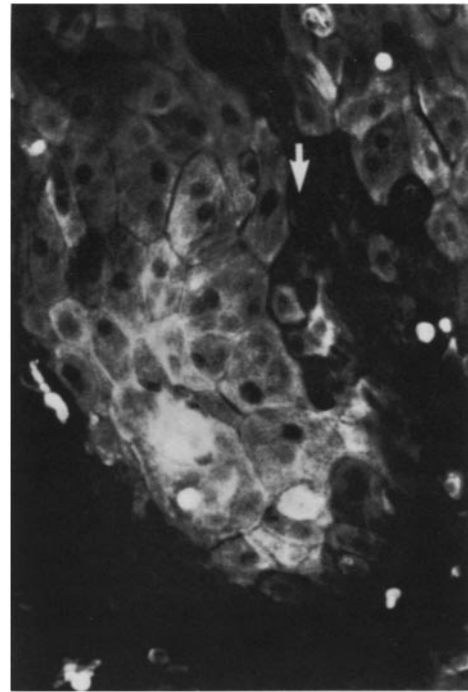


FIGURE 8 Immunofluorescent staining of a typical keratinocyte colony grown from 29-d embryonic skin. Trypsinized cell suspensions of embryonic skin obtained from embryos of different ages were inoculated into dishes containing lethally irradiated 3T3 cells. The keratinocyte colonies which developed were stained with antiserum to keratin using indirect immunofluorescence. The larger, more differentiated cells stained more strongly with the keratin antibody than the smaller, basal cells. Arrow points to one of the smaller basal cells which are most easily seen at the edge of the colony. $\times 250$.

identical solubility characteristics and molecular weight of this protein with respect to 40-kdalton keratin (17, 35) suggest that they are indeed the same, although we have not yet performed definitive biochemical analysis. This 40-kdalton protein was present in the embryonic epidermis from very early stages of development (i.e., in the undifferentiated epithelium) until 25 d of embryonic life. Its disappearance at 29 d of gestation correlated with the appearance of large keratins (61 and 64 kdalton) in the epidermis and the development of a thick stratum corneum. Interestingly, inhibition of keratinization in cultured keratinocytes (by the addition of Vitamin A to the medium) results in suppression of synthesis of the 67-kdalton keratin and stimulation of the synthesis of a 40-kdalton keratin (17). Vitamin A seemed to be affecting the program of differentiation in these epidermal cells in a manner analogous to that observed during embryogenesis.

Adult human conjunctiva (17) and cultured conjunctival keratinocytes (17, 32) have also been found to synthesize the 40-kdalton keratin but not the 67-kdalton keratin typical of epidermis. It is possible that at early stages of development conjunctival and epidermal keratinocytes embryologically share the 40-kdalton keratin. At later stages of maturation, however, the tissues diverge developmentally. The epidermis forms a granular layer and a stratum corneum, during which time the 40-kdalton protein is lost. The apparent concerted regulation of the 40-kdalton keratin with the large keratins in embryonic rabbit epidermis supports this premise.

Recently, Wu and Rheinwald (35) detected a 40-kdalton in three out of nine cell lines cultured from human squamous cell

carcinomas. It is interesting that when these cultures were transplanted to nude mice, they formed a stratum corneum containing large keratins, and the synthesis of the 40-kdalton keratin was suppressed. These results are consistent with our studies which demonstrate an inverse relationship between the expression of 40-kdalton keratin and the larger keratins.

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Note Added in Proof: Identification of the water-insoluble proteins from embryonic epidermis (18 and 29 d) and cultured keratinocytes (14 and 29 d) as keratin proteins has been confirmed by immunoprecipitation studies using whole keratin antiserum.

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