Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 716–720, February 1983
Biochemistry

Keratin gene expression in mouse epidermis and cultured epidermal cells

(cDNA clones/RNA blot analysis/differential gene expression)

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Communicated by Howard A. Bern, November 8, 1982

ABSTRACT The major differentiation products of mouse epidermis are keratins of 40–70 kilodaltons (kDal). We have prepared a library of cDNA clones from total poly(A)$^+$ RNA from newborn mouse epidermis. Clones corresponding to the major *in vivo* keratins of 55, 59, and 67 kDal have been isolated and characterized. By RNA blot analysis of poly(A)$^+$ RNA from newborn mouse epidermis, we have identified RNA species that are approximately 1,600, 2,000, and 2,400 nucleotides in length and are complementary to the cDNAs for the 55-, 59-, and 67-kDal keratins, respectively. Analysis of RNA from primary cultures of newborn mouse epidermis by this same technique shows greatly reduced levels of these RNAs. Transcripts complementary to all three cloned cDNAs are abundant in 14- to 16-day embryonic and adult mouse skin. Thus, altered expression in culture does not appear to be due to induction of a developmentally programmed switch by placing the cells in culture but instead is due to factors modulating expression within the culture system. Because the 55-, 59-, and 67-kDal keratins are the major proteins in epidermis they probably represent keratin associated with terminal differentiation. The expression data suggest that cultured cells are blocked in expression of differentiation keratins but instead synthesize other keratin family members probably related to cytoskeletal functions.

Keratins constitute a family of at least 10 related α-helix-rich structural proteins of 40–70 kilodaltons (kDal) and make up the subunits of the intermediate filaments (keratin filaments) present in large amounts in mammalian epidermis (1). Changes in keratin synthesis have been observed for differentiating epidermis during embryonic development (2) and during the terminal differentiation of the adult epidermis (3–6). Different keratin polypeptide patterns are also seen for keratinocytes in intact epidermis and keratinocytes growing in culture (6–9). When cultured keratinocytes are transplanted to animals, they begin to synthesize keratins typical of intact epidermis (9). A somewhat similar observation has been made for cell lines cultured from human squamous cell carcinoma in that the keratins synthesized by these cell lines change when they are grown as tumors in *nude* mice (10). Thus there is considerable evidence that keratin synthesis can be altered by changing the environment of the cell, either normally during differentiation as the epidermal cells progress from the basal to the outer layers, or artificially by growing the epidermal cells in culture. These changes in keratin synthesis probably occur at the transcriptional level because individual keratins are translated from different mRNAs in human epidermis (11) and mouse epidermis (this study).

Defining the mechanism by which keratin gene expression is regulated has considerable importance because members of this family of proteins are the major components of the cytoskeleton of most epithelial cells (12) as well as the major differentiation product of the epidermis. Expression of these proteins is altered during experimental skin carcinogenesis (13) and in a variety of pathological processes in the skin (14) and in other target organs. Altered patterns of keratin expression are being used diagnostically to characterize certain forms of cancer (15–18) and to identify the epithelial origin of cultured cells (12). We felt that the most direct approach toward elucidating factors that regulate keratin gene expression was to construct cDNA clones for keratin mRNAs and to utilize these cloned cDNAs as specific probes for keratin gene transcripts. This report describes the results of this effort as well as presenting direct evidence for the modulation of keratin gene expression by cell culture.

MATERIALS AND METHODS

Preparation of Epidermis and Cultivation of Cells. The preparation of epidermis from newborn BALB/c mice and the isolation and cultivation of epidermal cells have been described (19). Adult back and tail epidermis was prepared as described by Santoianni and Rothman (20) except that 200 mM vanadylribonucleoside complex (Bethesda Research Laboratories), a ribonuclease inhibitor (21), was added to the saline solution to obtain intact RNA.

Isolation of RNA. Newborn mouse epidermis was placed in liquid nitrogen immediately after separation and ground in a mortar. RNA was isolated from the ground epidermis with guanidine·HCl and was enriched for poly(A)$^+$ RNA as described by Fuchs and Green (11). The extraction of RNA from adult back and tail epidermis was as for newborn epidermis except that 20 mM vanadylribonucleoside complex was present in the first 8 M guanidine·HCl extraction. RNA was extracted from cell culture by adding the 8 M guanidine·HCl directly onto the cells after rinsing with saline. The procedure used for newborn epidermis was then followed.

Fractionation of Epidermal Poly(A)$^+$ RNA. Epidermal poly(A)$^+$ RNA (150 μg) was loaded into three 8-mm slots and separated by electrophoresis in a 1.5% agarose gel containing 10 mM methylmercury hydroxide as described (22). The gel was sliced into 2-mm fractions and the RNA was isolated from each slice as described by Fuchs and Green (11). The RNA fractions were then analyzed in the reticulocyte system (see below). ^{32}P-Labeled DNA complementary to selected fractions was synthesized as described (23).

Analysis of Translation Products. Poly(A)$^+$ RNAs were translated in a cell-free rabbit reticulocyte lysate system containing [^{35}S]methionine (New England Nuclear). Translation products were analyzed by polyacrylamide/NaDodSO$_4$ gel electrophoresis as described by Laemmli (24). The gels were subjected to fluorography using EN^{3}HANCE (New England

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Abbreviations: kDal, kilodaltons; NaCl/Cit, 0.15 M NaCl/0.015 M trisodium citrate (standard saline citrate).

Nuclear) and exposed to x-ray film (Kodak, XAR-5) at −70°C. Immunoprecipitation was performed as described by Stanley *et al.* (25).

Cloning of Keratin cDNAs. Methods used in the construction of the cDNA library have been described (26). Briefly, double-stranded cDNA was synthesized with reverse transcriptase from poly(A)$^+$ RNA isolated from newborn mouse epidermis and the hairpin loop was cleaved with S1 nuclease. Approximately 16 dCMP residues were added to the 3′ ends of the double-stranded cDNA with terminal transferase. The tailed cDNA was annealed with plasmid vector pBR322 that had been linearized by cleavage with *Pst* I restriction endonuclease and tailed with an average of 14 dGMP residues per 3′ terminus. An aliquot of this mixture was used to transform *Escherichia coli* K-12 strain RRI. Transformants containing recombinant plasmids with keratin cDNA inserts were identified by colony hybridization with [^{32}P]cDNA complementary to partially purified keratin mRNAs. Plasmid DNA was prepared from positive recombinants by the method of Birnboim and Doly (27).

Hybridization-Selection Assay. The identity of recombinants selected in the initial screening was confirmed by the hybrid-selection assay essentially as described by Cleveland *et al.* (28). Plasmid DNAs were linearized with *Eco*RI and 10 μg was bound to (13-mm) nitrocellulose filters (Schleicher and Schuell, BA85). The filter-bound DNA was prehybridized for 2 hr at 41°C in 50% (vol/vol) formamide (Fluka)/0.4 M NaCl/10 mM 1,4-piperazinediethanesulfonic acid, pH 6.4/5 mM EDTA/250 μg of poly(A) per ml/250 μg of yeast tRNA per ml/0.2% NaDodSO$_4$. Hybridization was for 20 hr at 41°C in the same buffer (150 μl per filter) containing 15 μg of epidermal poly(A)$^+$ RNA. The filters were washed two times (5 min each) with 1× NaCl/Cit/0.1% NaDodSO$_4$ at room temperature [1× NaCl/Cit is 0.15 M NaCl/0.015 M trisodium citrate (standard saline citrate)], three times (5 min each) with 0.1× NaCl/Cit/0.1% NaDodSO$_4$ at room temperature, and two times (5 min each) with 0.1× NaCl/Cit/0.1% NaDodSO$_4$ at 60°C for filters containing pK335 and pK1005 DNA and at 68°C for the filter containing pK276 DNA. RNA was eluted from the filters in 300 μl of water at 100°C for 2 min. The RNA was collected by precipitation in the presence of 10 μg of yeast tRNA and analyzed by translation in the reticulocyte system.

RNA Blot Analysis. RNA was denatured with glyoxal as described by McMaster and Carmichael (29). The RNA was fractionated on a 1.1% agarose gel and transferred by blotting to nitrocellulose paper as described by Thomas (30). The filter-bound RNA was prehybridized in 50% formamide/5× NaCl/Cit/20 mM sodium phosphate, pH 6.5/500 μg of yeast tRNA per ml/0.1% NaDodSO$_4$/0.05% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone. The RNA blots were prehybridized for 20 hr at 42°C. Hybridization was performed at 42°C for 20 hr in the same buffer with 1–2 × 10^7 cpm of nick-translated ^{32}P-labeled DNA prepared as described (31). The filters were washed twice with 2× NaCl/Cit/0.1% NaDodSO$_4$ for 5 min each at room temperature, once with 1× NaCl/Cit/0.1% NaDodSO$_4$ for 1 hr at 68°C, and twice with 0.1× NaCl/Cit/0.1% NaDodSO$_4$ for 30 min each at 68°C. The blots were wrapped in Saran Wrap and exposed to x-ray film (Kodak, XAR-5) in the presence of a DuPont intensifying screen (Cronex, Lightning Plus) at −20°C.

RESULTS

Cloning DNAs Complementary to Keratin mRNAs. The translation products of total poly(A)$^+$ RNA isolated from newborn mouse epidermis are shown in Fig. 1, lane 1. Immunoprecipitation of these products with antiserum prepared against keratins present in mouse stratum corneum (32) demonstrates that the most abundant mRNAs present in this tissue code for keratins that are 55, 59, and 67 kDal (Fig. 1, lane 2). Previous studies have shown that this multivalent rabbit antiserum is able to react with most if not all of the keratins synthesized in intact epidermis and cultured epidermal cells (32). The band below T in lane 1 is actin. Because the concentration of keratin mRNAs was quite high in mouse epidermis, a cDNA library was constructed with cDNA synthesized from total epidermal poly(A)$^+$ RNA. Recombinant plasmids were screened with cDNA synthesized from partially purified mRNA coding for the 59- and 67-kDal keratins. These mRNAs were obtained by fractionating epidermal poly(A)$^+$ RNA in a 1.5% agarose gel containing 10 mM methylmercury hydroxide. The gel was sliced into 2.0-mm fractions and the RNA was isolated from each slice. Translation products of these RNA fractions are shown in Fig. 2. Fractions 11 and 14 were enriched in mRNA coding for the 67- and 59-kDal keratins and these were used for the synthesis of [^{32}P]cDNA. In the process of screening the cDNA library with these cDNAs, recombinants that contained sequences complementary to the mRNA coding for the 55-kDal keratin were also isolated.

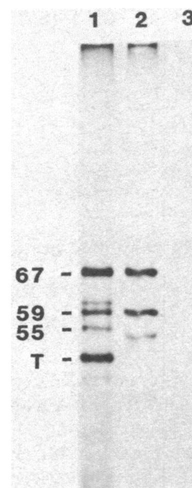

FIG. 1. Translation products of mouse epidermal poly(A)$^+$ RNA. Poly(A)$^+$ RNA was extracted from newborn mouse epidermis and translated in an *in vitro* reticulocyte system containing [^{35}S]methionine. The labeled proteins were separated electrophoretically in an 8.5% polyacrylamide/NaDodSO$_4$ gel and visualized by fluorography. Lane 1, translation products of mouse epidermal poly(A)$^+$ RNA. Lane 2, translation products immunoprecipitated with keratin antiserum. Lane 3, translation products that had been allowed to react with control serum. The 55-kDal band is distorted by unlabeled IgG in the immunoprecipitate. T indicates an mRNA-independent artifact of the translation system (which also appears in Figs. 2, 3, and 5).

The identity of recombinants detected by screening was confirmed by hybridization-selection. Plasmid DNAs were bound to nitrocellulose filters and hybridized to epidermal poly(A)$^+$ RNA. The specifically bound RNA was eluted and translated *in vitro*. Translation products resulting from this selection are shown in Fig. 3. Lanes 3–5 contain immunoprecipitated translation products of RNA hybridizing to plasmid DNAs pK1005 (55 kDal), pK276 (59 kDal), and pK335 (67 kDal), respectively. Total and immunoprecipitable translation products of epidermal poly(A)$^+$ RNA are shown for comparison in lanes 1 and 2. The faint band seen in lane 5 is probably an incomplete translation product, because it is also seen in translation products obtained with the RNA fraction enriched in the mRNA coding for the 67-kDal keratin (Fig. 2, lane 11). However it is possible that this band is a keratin polypeptide coded for by a separate mRNA that cross-hybridizes with the cloned cDNA correspond-

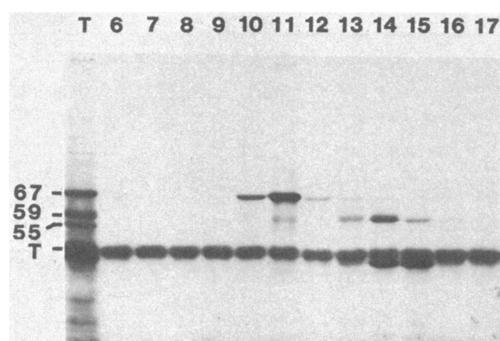

FIG. 2. Partial purification of mRNAs coding for the 67- and 59-kDal keratins. Epidermal poly(A)$^+$ RNA was fractionated by electrophoresis in a 1.5% agarose gel containing 10 mM methylmercury hydroxide. The gel was sliced into 2.0-mm fractions and the RNA was isolated from each slice. An aliquot of each fraction was translated in the *in vitro* system and analyzed by electrophoresis and fluorography. Lane T contains translation products of poly(A)$^+$ RNA. Lanes 6–17 contain translation products of RNA isolated from slices 6–17. RNA fractions 11 and 14, which were enriched in RNA coding for the 67- and 59-kDal keratins, were used for the synthesis of [^{32}P]cDNA.

ing to the 67-kDal keratin (pK335). The presence of keratin sequences within these cDNA clones has been confirmed by DNA sequence analysis (33).

It should be noted that short cDNA clones, which consisted primarily of 3′ noncoding sequences (approximately 350–450 nucleotides in length), were used for the hybridization selection assay. This was necessary because plasmids containing essentially full-length inserts (thus, most of the coding sequence) hybridized to all three mRNAs. As observed for other multigene families (28), the 3′ noncoding regions of these genes are not conserved evolutionarily. Short cDNA clones containing the 3′ noncoding sequences have been useful as specific hybridization probes to detect transcripts of the individual genes (to be shown below).

Identification of mRNAs Complementary to Keratin cDNAs. The cloned keratin cDNAs have permitted the identification of their corresponding mRNAs by RNA blot analysis. The mRNAs coding for the 55-, 59-, and 67-kDal keratins are approximately 1.6, 2.0, and 2.4 kilobases (kb) in length, respectively (Fig. 4). Recombinant plasmids have been isolated for all three keratins that have cDNA inserts large enough to contain greater than 90% of the sequence present in these mRNAs.

Differential Expression of Keratin Genes in Epidermal Cell Culture. A comparison of translation products of poly(A)$^+$ RNA isolated from primary cultures of newborn mouse epidermal cells with those of newborn mouse epidermis reveals several differences (compare lanes 4 and 5 with lanes 7 and 8 in Fig. 5). Synthesis of the 67- and 59-kDal keratins is greatly reduced in the cultured epidermal cells. This is evident from an analysis of translation products from cultured cell RNA or proteins synthesized by intact cells (Fig. 5, lanes 1 and 2). The major keratins synthesized by cultured cells are approximately 60, 55, and 50 kDal, whereas the major keratins synthesized in newborn epidermis are 67, 59, and 55 kDal. Keratins that are 60 and 50 kDal are also synthesized in newborn epidermis but to a lesser extent.

To determine whether the mRNAs coding for the 67- and 59-kDal keratins were reduced in concentration in epidermal cell culture or present but not efficiently translated, cell culture poly(A)$^+$ RNA was subjected to blot analysis as shown in Fig. 6. As shown previously, specific mRNAs were detected for the 55-, 59-, and 67-kDal keratins in newborn epidermal RNA (lanes 1, 3, and 5); however, at this exposure mRNAs corresponding to these cDNAs were not detected in RNA prepared from cell culture (lanes 2, 4, and 6). Faint bands could be seen in the cell culture lanes after long exposure. The low level of transcripts for the 67- and 59-kDal keratin genes was not surprising, because cells in culture synthesize little of these proteins. The failure to detect comparable amounts of the 55-kDal transcript in both RNA preparations was surprising, because cells in culture synthesize a keratin that is approximately 55 kDal. This suggests that the mRNA coding for the 55-kDal keratin synthesized by cultured epidermal cells is sufficiently different in sequence to prevent its detection by the cDNA corresponding to the newborn epidermal 55-kDal keratin. These cell culture results were unchanged at all medium Ca^{2+} concentrations.

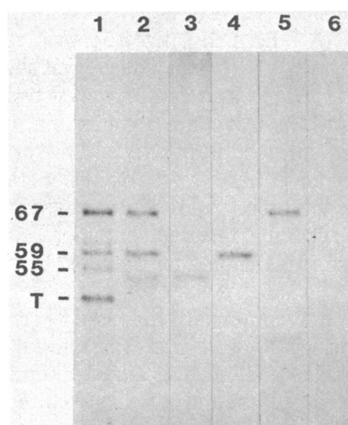

FIG. 3. Identification of recombinant plasmids containing keratin cDNA sequences. Nitrocellulose filters containing plasmid DNA were hybridized with epidermal poly(A)$^+$ RNA. Bound RNA was eluted and translated *in vitro*. Translation products were analyzed by electrophoresis and fluorography. Lane 1, translation products of total epidermal poly(A)$^+$ RNA. Lane 2, translation products immunoprecipitated with keratin antiserum. Lanes 3–5 immunoprecipitated translation products of RNA hybridizing to plasmid DNAs pK1005 (55 kDal), pK276 (59 kDal), and pK335 (67 kDal), respectively. Lane 6, translation products in lane 1 allowed to react with control serum. The 55-kDal band in lanes 2 and 3 is distorted due to the presence of unlabeled IgG in the immunoprecipitate.

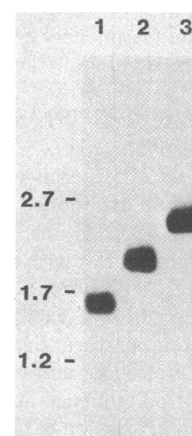

FIG. 4. Identification of mRNAs complementary to keratin cDNAs. Five micrograms of epidermal poly(A)$^+$ RNA was treated with glyoxal and separated by electrophoresis in a 1.1% agarose gel. The RNA was blotted onto nitrocellulose paper. Individual strips were hybridized to ^{32}P-labeled cDNAs corresponding to the 55-kDal (lane 1), 59-kDal (lane 2), and 67-kDal (lane 3) keratins and autoradiographed. The position of markers (3′ end-labeled restriction fragments generated by digestion of phage ϕX174 replicative form DNA with *Hpa* II and *Taq* I; the length is shown in kilobases) is indicated.

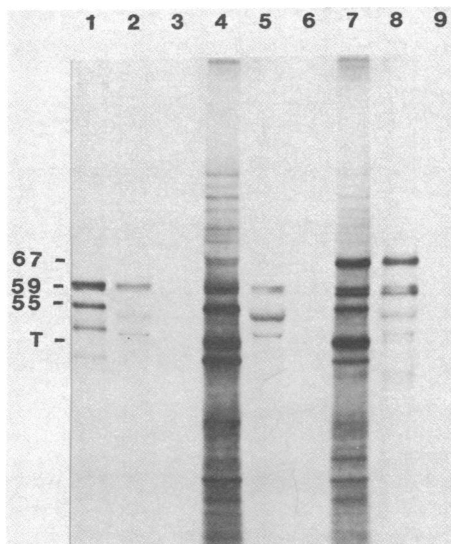

FIG. 5. Comparison of keratins synthesized by mouse epidermal cell culture and newborn mouse epidermis. [^{35}S]Methionine-labeled proteins were separated by electrophoresis and visualized by fluorography. A cytoskeletal extract prepared from primary epidermal cell culture labeled for 12 hr with [^{35}S]methionine is shown in lanes 1–3. Lanes 4–6 contain translation products of poly(A)$^+$ RNA isolated from primary epidermal cultures and lanes 7–9 contain translation products of poly(A)$^+$ RNA isolated from newborn epidermis. Total translation products are shown in lanes 4 and 7 and immunoprecipitated products in lanes 2, 5, and 8 (keratin antiserum) and lanes 3, 6, and 9 (control serum).

Previously we have shown that Ca^{2+} concentration regulates certain aspects of epidermal differentiation but not keratin synthesis (34).

As previously discussed, the 55-, 59-, and 67-kDal keratin mRNAs do contain sequences that will cross-hybridize with the cloned cDNAs. Cross-hybridization was avoided in this experiment by using stringent hybridization conditions and the short

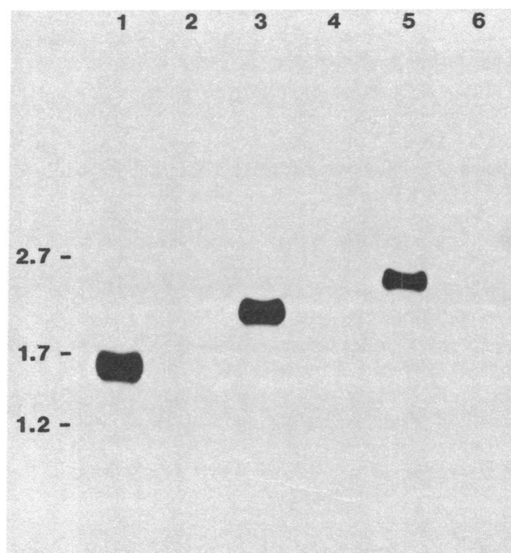

FIG. 6. Analysis of primary epidermal cell RNA with keratin cDNAs. Five micrograms of poly(A)$^+$ RNA from newborn mouse epidermis (lanes 1, 3, and 5) or primary cultures of mouse epidermal cells (lanes 2, 4, and 6) was analyzed as described in the legend to Fig. 4. Lanes 1 and 2 were hybridized to the 55-kDal keratin cDNA, lanes 3 and 4 to the 59-kDal keratin cDNA, and lanes 5 and 6 to the 67-kDal keratin cDNA.

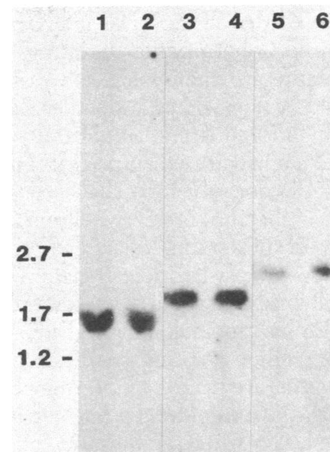

FIG. 7. Detection of keratin mRNAs in adult mouse epidermis. Ten micrograms of total RNA isolated from adult back (lanes 1, 3, and 5) or tail (lanes 2, 4, and 6) epidermis was analyzed as described in the legend to Fig. 4. Lanes 1 and 2 were hybridized to the 55-kDal keratin cDNA, lanes 3 and 4 to the 59-kDal keratin cDNA, and lanes 5 and 6 to the 67-kDal keratin cDNA.

cDNA probes described above. When longer cDNA probes are employed, cross-hybridization is observed (data not shown). However, even these longer probes fail to hybridize with RNA isolated from cultured cells.

Detection of mRNAs for the 55-, 59-, and 67-kDal Keratins in Adult Epidermis. Because changes in expression during development have been described for other multigene families (35–38) it was of interest to determine if the changes in keratin gene expression in cultured epidermal cells were due to a programmed switch that occurs normally during development. It seemed possible that culturing newborn epidermal cells may have caused them to express keratin genes expressed in adult mice. Total RNA was isolated from adult back and tail epidermis and analyzed by blotting (Fig. 7). Transcripts complementary to the 55-, 59-, and 67-kDal keratin cDNAs were detected in RNA from both adult tissues. We have also detected transcripts for these genes in 14- to 16-day embryonic skin (unpublished results). Therefore, the failure of cultured epidermal cells to synthesize these mRNAs appears to be due to factors within the culture system and is not due to a developmentally programmed switch.

DISCUSSION

Previous studies have shown that the program of keratin synthesis observed for keratinocytes in intact epidermis is altered when keratinocytes are grown in culture. Specifically, synthesis of the higher molecular weight keratins, the 65- to 67-kDal keratins (6–9) and in one case the 56-kDal keratin (9) is decreased in cultured keratinocytes. Cultured epidermal cells can regain their *in vivo* keratin phenotype when injected subcutaneously into *nude* mice (9). These results suggest that the program of keratin synthesis in epidermal cells can be modulated by factors within their environment. Fuchs and Green (39) have recently shown that cultured epidermal cells regain their ability to synthesize the 67-kDal keratin when they are grown in culture medium containing serum depleted of vitamin A. They also observed that the addition of retinyl acetate to the vitamin A-depleted medium stimulated the synthesis of 40- and 52-kDal keratins. It remains to be determined whether these effects of vitamin A are direct or indirect and if they occur at the level of transcription.

Different programs of keratin synthesis may correspond to the differentiation state of epidermal cells. Recently, Tseng *et*

al. (40) have proposed that the 56-kDal and 65- to 67-kDal keratins are markers for keratinization (terminal differentiation) because these keratins are immunologically distinct from other keratins, as judged by monoclonal antibodies, and are detected only in terminally differentiating, suprabasally located cells of the epidermis and not in cultured epidermal cells. The 55-, 59-, and 67-kDal keratins for which we have constructed cDNA clones are unique in that they are derived from intact epidermis and probably represent keratins related to terminal differentiation. On the basis of cross-hybridization (i.e., each full-length cDNA clone will hybridize with all three keratin mRNAs), these three keratins also have homology with each other but differ substantially from other keratins synthesized by epidermal cells. If these keratins are markers for terminal differentiation, their expression in intact epidermis but not in cultured cells could explain the lack of orthokeratinization observed by ultrastructure criteria in cultured epidermis (41), where a normal stratum corneum does not form.

The modulation of synthesis of these keratins in cultured epidermal cells appears to occur at the level of transcription because the concentration of mRNAs coding for the 55-, 59-, and 67-kDal keratins is greatly reduced in cultured cells and precursors for keratin mRNAs are not present in nuclear RNA isolated from these cells (data not shown). However, from these results we cannot rule out the possibility that RNA is transcribed from these genes and then rapidly degraded. Some of the keratins synthesized by cultured cells appear to be synthesized at low levels in intact epidermis as judged on the basis of similar electrophoretic mobilities; however, a more definite answer will be obtained when epidermal RNA is analyzed with cDNA probes corresponding to the keratins synthesized in cultured cells.

Individual keratin genes appear to be present in multiple copies in mammalian cells as determined by Southern blot analysis of genomic DNA (ref. 42; unpublished data). It is possible that different genes coding for structurally similar keratins may be expressed at different times during development, as has been observed for other multigene families, such as human globin genes (35), silkmoth chorion genes (36), *Dictyostelium* actin genes (37), and *Drosophila* tubulin genes (38). This does not appear to be the explanation for the altered expression observed in cultured cells because transcripts for the 55-, 59-, and 67-kDal keratin genes, which are expressed in newborn epidermis, are detected in RNA prepared from both embryonic and adult mouse skin. We assume that the failure of cultured cells to synthesize these keratins and the activation of expression of other genes in the keratin family are due to factors within the culture system. We have been able to detect transcripts of the 55-, 59-, and 67-kDal keratin genes in a malignantly transformed mouse cell line, Pam 212 (43). This cell line developed spontaneously in long-term culture of neonatal mouse epidermal cells (44). The overall pattern of keratin synthesis in these cells is similar to that of newborn epidermis. This cell line may be useful in elucidating factors that influence keratin gene expression in normal epidermal cells and may provide insight into the mechanism of altered regulation of these proteins in malignant cells.

The genomic sequences homologous to these keratin cDNAs should be isolated in order to define in detail the structure of these genes and surrounding sequences. These cDNA clones provide an essential tool for the analysis of the structure and regulation of this family of genes. We anticipate that these probes will facilitate our understanding of the function of keratin in the growth and differentiation of epithelial cells.

We thank Dr. Miriam Poirier for the keratin antiserum, Drs. Peter Steinert and Susan Banks-Schlegel for comments on the manuscript, and Margaret Green and Maxine Bellman for typing the manuscript.

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