Expression of Unusually Large Keratins during Terminal Differentiation: Balance of Type I and Type II Keratins Is Not Disrupted

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ABSTRACT When a basal epidermal cell undergoes a commitment to terminally differentiate, it ceases to divide and begins to migrate outward towards the surface of the skin. Dramatic changes in its cytoskeletal architecture take place, accompanied by numerous changes in the expression of keratins, a family of related polypeptides that form 8-nm filaments in these cells. We show here that a shift to the synthesis of unusually large keratins occurs that does not seem to disrupt the ratio of two distinct subfamilies of keratins. Preliminary studies indicate that this differentiation-specific shift may be at the level of transcriptional rather than post-trancriptional regulation. The striking similarities between these large keratins and the type I and type II keratins of basal epidermal cells suggests the important role that both classes of large keratin sequences must play in the assembly of the intermediate filaments within the differentiating keratinocyte.

Several laboratories have reported differences between the keratins of stratum corneum and those of the living layers of epidermis (1–4). During the course of terminal differentiation, changes occur in the expression of the keratin polypeptides that comprise the 8-nm tonofilaments (5–10). For the human, keratins of size 46, 48.5, 50, 52, 56, and 58 kd are synthesized by the basal epidermal cells (Fig. 1, lane *1*), whereas additional keratins of size 67, 65.5, and 56.5 kd are produced by the terminally differentiating epidermis (lane *3*). As the cells pass through the granular layer to the stratum corneum, a slight reduction in the size of the keratins takes place. As judged by in vitro translation of mRNAs isolated from basal (lane *2*) and differentiating (lane *4*) keratinocytes, the changes in keratin pattern that occur early in the course of terminal differentiation are clearly at the level of mRNA biosynthesis (5).

Recently, it was demonstrated that the keratins produced by basal epidermal cells can be divided into two distinct groups based on the ability of their mRNAs to cross-hybridize with two different cloned keratin cDNAs (11). Other epithelia express different subsets of keratins, but most if not all of these seem to be similar to one or the other of the two epidermal keratin subfamilies (12). The small (40–52 kd) and relatively acidic keratins have been named the type I class, and the large (53–58 kd) and more basic keratins have been named the type II class (13, 14). At least one member of each of the two keratin types seem to be expressed in all cells at all times, suggesting their combined importance in filament assembly (14). Sequence analyses (13, 15–18) have shown that while the two types of keratins share only low (<30%) homology, their predicted secondary structures are strikingly similar and are compatible with their playing an essential role in forming the coiled-coil backbone of the protofilament of the 8-nm keratin filament (13, 18).

The keratins typical of terminally differentiating keratinocytes seem to be unusually large and are not found in other epithelial cells (for review, see reference 19). Whether these keratins are members of the same two subfamilies of sequences already described for basal epidermal cells and other epithelial cells has not yet been determined. In this paper, we explore the relation of the differentiation-specific keratins to other epithelial keratins and we investigate the balance of the ratio of type I and type II keratins during terminal differentiation in human epidermis.

MATERIALS AND METHODS

Extraction of Keratins and Poly(A)+ RNAs from Cultured Human Basal Epidermal Cells and from Human Skin: Human epidermal cell strains were derived from newborn foreskin and used in their second to fourth subculture. They were grown according to the procedure of Rheinwald and Green (20, 21). When vitamin A is present in the cell culture medium, terminal differentiation is largely inhibited, and the cells resemble basal epidermal cells (22).

Whole human epidermis was obtained fresh as discarded material from surgical operations and used immediately. After the subcutaneous fat and dermis were clipped away, the epidermis was minced in the presence of vanadyl ribonucleoside complex and then frozen in liquid nitrogen prior to mRNA and

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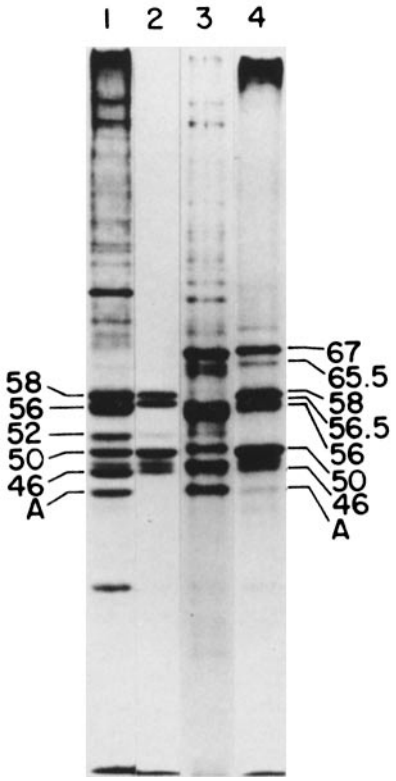


FIGURE 1 Synthesis of human epidermal keratins from Poly(A)+ RNA of cultured human basal epidermal cells and differentiating human epidermis. Poly(A)+ RNAs from cultured basal epidermal cells and from adolescent human foreskin epidermis were isolated and translated in vitro and the synthesized products were immunoprecipitated (5). The keratins were separated electrophoretically and the gel was fluorographed. Lane *1*, [^{35}S]methionine-labeled keratin extract from cultured cells; lane *2*, radiolabeled immunoprecipitated translation products from cultured cell RNA; lane *3*, radiolabeled keratin extract from epidermis; lane *4*, radiolabeled immunoprecipitated translation products from epidermis RNA. Molecular weight values are in kilodaltons. *A*, actin.

keratin extractions. Keratins and poly(A)+ RNAs were isolated as previously described (5).

Northern Blot Analysis: RNAs were first subjected to electrophoresis through formaldehyde agarose gels and were then transferred to nitrocellulose paper according to the procedure of Thomas (23). After a 24-h transfer, blots were heated to 80°C for 3 h and then prehybridized at 41°C in 50% deionized formamide, 5 × SSC (750 mM NaCl, 75 mM sodium citrate), 20 mM HEPES (pH 7.4), 1 mM EDTA, 100 μg/ml yeast tRNA, 0.01% BSA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone 360, 10 μg/ml sonicated, denatured *Escherichia coli* DNA and 1 μg/ml sonicated, denatured pBR322 DNA. The prehybridization solution was removed and replaced with a fresh aliquot of the same solution containing 1–5 × 10^5 dpm/ml of ^{32}P-labeled cDNA probe. Probe was prepared using sheared calf thymus DNA as primer and reverse transcriptase to copy the purified cloned cDNA inserts of either the 50-kd type I (KB-2) or the 56-kd type II (KA-1) keratins (11). After a 24-h incubation at 41°C, filters were washed in 0.1 × SSC, 0.1% SDS at various temperatures depending on the desired range of detection of homology. Whenever possible, the melting temperature (T_m) of a desired hybrid was calculated and wash conditions were adjusted to elute all hybrids with a T_m of 5°C less than this value (24).

Preparation of 3′ Noncoding Region Subclones: Based on the cDNA sequences of the human 50-kd type I (pKB-2; 15) and the human 56-kd type II (pKA-1; 13) keratins, restriction endonuclease fragments containing the 3′ noncoding region segments of the two hybrid plasmids were isolated and subcloned into the plasmid pUC-8. For pKB-2, a 70 bp *StuI/AluI* sequence located 20 nucleotide residues 3′ downstream from the TGA stop codon was subcloned. For stringent hybridization studies, a larger 140 bp *StuI/PstI* fragment encompassing not only the *StuI/AluI* fragment but also the remaining 70 noncoding nucleotide residues 3′ downstream was also utilized. For pKA-1, a 285 bp *AluI/PstI* fragment beginning at 235 nucleotide residues 3′ downstream from the TAA stop codon and ending at the polyA addition site was subcloned.

Isolation and Characterization of the Gene Encoding the Type I (50 kd) Human Keratin: Purification of the BglI Fragment Containing the 3′ Noncoding Portion of the Gene: A *HaeIII/AluI* human genomic library (Ed Fritsch, Genetics Institute, Boston, MA) has been screened with the human epidermal keratin cDNA probes KA-1 and KB-2 (11), and the genes encoding the type I and type II keratins have been isolated and partially characterized. The gene encoding the 50-kd type I keratin has been identified, and its sequence and characterization will be reported elsewhere. The 3′ noncoding region of the 50-kd keratin mRNA hybridized with a single 800 bp *BglI* fragment of the gene. The 5′ end of this *BglI* fragment contains the 130 bp noncoding portion of the 50 kd keratin mRNA, while the remainder of the sequence includes a 670 bp stretch that is 3′ downstream from the polyadenylation site. This fragment was isolated and purified according to the procedure of Birnboim and Doily (25), and then radiolabeled as described previously (11).

Immunoblot Analysis: Duplicate samples containing 5 μg of total protein were resolved by polyacrylamide gel electrophoresis, and unstained gels were then transferred electrophoretically to nitrocellulose paper. Each blot was first incubated in 10 ml of BSA-saline solution containing 100 μl of antiserum specific for either the type I or the type II keratins, and later placed in a solution containing 10^5 cpm per ml of ^{125}I-labeled *Staphylococcus aureus* protein A (26). Antisera were prepared by injecting male New Zealand white rabbits with gel-purified 50 (type I) or 56 kd (type II) basal epidermal keratins. These antisera were shown to cross-react with a number of other type I and type II keratins, respectively (14).

RESULTS

Keratin mRNAs Produced by Differentiating Epidermis Are of Two Distinct Types

Gel-fractionated poly(A)+ RNAs isolated from cultured human epidermal cells and from human epidermis were hybridized with ^{32}P-labeled probe to either the type I (50 kd) keratin cDNA or the type II (56 kd) keratin cDNA (Fig. 2). These probes have previously been shown to cross-hybridize

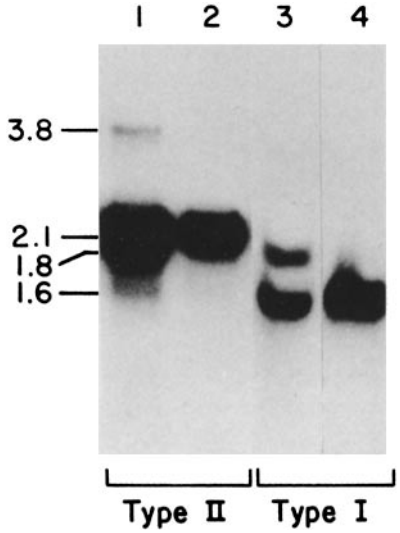


FIGURE 2 New RNAs of both keratin types are synthesized during terminal differentiation. Cloned cDNAs complementary to the mRNAs for the 56-kd type II (*left*) and the 50-kd type I (*right*) epidermal keratins were radiolabeled and used as probes to detect the presence of keratin mRNAs in basal and differentiating epidermal cells. Poly(A)+ RNAs were isolated from these cells as previously described (5) and resolved by formaldehyde agarose gel electrophoresis (11). Lanes *1* and *3*, poly(A)+ RNA from whole human epidermis; lanes *2* and *4*, poly(A)+ RNA from cultured human basal epidermal cells. Sizes (in kilobase pairs) of hybridizing RNAs were determined from the electrophoretic mobilities of eucaryotic and procaryotic rRNA markers.

with most if not all keratin mRNAs belonging to their respective type (12). Whereas mRNAs from differentiating epidermis contain two sets of sequences of 2.1 and 3.8 kb that hybridized with the type II probe (lane *1*), mRNAs from cultured human basal epidermal cells contain only the single set of sequences of 2.1 kb that had previously been shown to include the mRNAs for the 56- and 58-kd keratins (lane *2*) (11). Similarly, mRNAs from differentiating epidermis contain two sets of sequences of 1.6 and 1.8 kb that hybridized with the type I probe (lane *3*), while mRNAs from basal cells contain only one band of 1.6 kb that had previously been shown to include the mRNAs for the 46- and 50-kd keratins (lane *4*) (11).

Thus, the new RNAs that we have detected in Fig. 2 are of two distinct types. These RNAs are likely candidates to encode the keratins of 67, 65.5, and 56.5 kd that appear upon commitment of a cell to terminally differentiate (Fig. 1, lane *3*). We do not yet know whether the 3.8-kb RNA homologous to the type II keratin probe is in fact the mRNA for the 65.5–67-kd keratins, and if so, whether it is truly unusually large even for the 67-kd keratin (27, 28). It is possible that its migration through the gel system is anomalous. Quantitation of the new keratin RNAs could not be estimated because the extent of homology between these new RNAs and the 50 and 56 kd cloned keratin cDNAs was not known.

Differentiation-specific Keratins Are Similar to Type I and Type II Keratins of Basal Epidermal Cells

To explore the relation between the differentiation-specific keratins and the basal epidermal keratins, we first tested their ability to cross-react with antisera specific for either the type I 50-kd keratin or the type II 56-kd keratin of basal epidermal cells. Fig. 3 (*left*) shows that for differentiating epidermis, a new band of 56.5-kd specifically cross-reacted with antiserum against the 50-kd type I keratin (lane *1*). In the same sample, an additional band of 67 kd was found to cross-react with antiserum against the 56-kd type II keratin (Fig. 3, *right*, lane *1*). Thus, the shift to the synthesis of large keratins coincident with the commitment of an epidermal cell to undergo terminal differentiation clearly results in the appearance of unusually large keratins of both types.

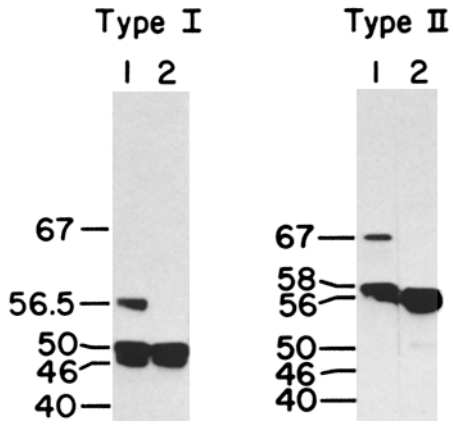


FIGURE 3 The large keratins of differentiating epidermis can be distinguished by their differential cross-reactivity with antibodies to type I (*left*) and type II (*right*) keratins. Antibodies were raised against electrophoretically purified 50-kd (*Type I*) and 56-kd (*Type II*) human keratins and have been shown to have general cross-reactivity with other keratins of the same type (14). These antisera were used in an immunoblot analysis to detect the presence of immunoreactive forms of both classes of keratins in differentiating (lane *1*) and basal (lane *2*) keratinocytes. Note that additional large keratins of both types are found in terminally differentiating cells. Molecular weight values are in kilodaltons.

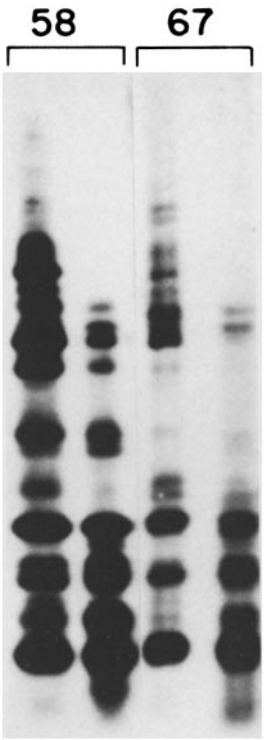


FIGURE 4 As judged by one-dimensional polypeptide mapping, the 67-kd keratin (*67*) of differentiating epidermis is similar to the 58-kd keratin (*58*) of basal epidermal cells. [^{35}S]-Methionine-labeled keratins from basal and differentiating keratinocytes were resolved by SDS PAGE. Individual bands were excised, and each gel slice was placed in duplicate into adjacent sample wells of a 15% gel. *Staphylococcus aureus* V8 protease was overlaid at amounts of 20 and 300 ng, respectively, and the protease and protein were electrophoresed through the stacking and running gels (41). Proteolytic fragments were visualized by fluorography and autoradiography (42).

To investigate the degree of similarity between the large and small keratins of the same type, we compared the polypeptide pattern generated by limited *S. aureus* V8 protease digestion of the type II 67-kd differentiation-specific keratin with that generated by the type II 58-kd basal keratin. (The molecular weight of the 56.5-kd keratin precluded clean separation from the 56- and 58-kd keratins by one dimensional gel electrophoresis, and peptide mapping of protein spots from two dimensional gels was not attempted.) Fig. 4 shows that the two keratins produced peptide fragments that are extremely similar (see also reference 22). These patterns also bear strong resemblance to the one observed previously for the s. corneum 65-kd keratin (29). These results confirm that a close relation exists between the large type II keratins of differentiating epidermis and the smaller type II keratins of basal epidermal cells.

The type I keratins are typically acidic in their isoelectric pH (4.5–5.5), whereas the type II keratins are more basic (6.5–7.5) (19). When keratins isolated from human epidermis and from cultured basal epidermal cells were resolved by two-dimensional gel electrophoresis, it could readily be seen that the three differentiation-specific keratins have very different isoelectric points (Fig. 5). Similar to the 58- and 56-kd keratins, the 67- and 65.5-kd keratins have very basic isoelectric focusing points (~7.5). In contrast, the 56.5-kd keratin resolved into two distinct polypeptides, each having an acidic isoelectric focusing point (~5.3), more similar to the 46- and 50-kd keratins.

By all criteria, the two largest keratins (65.5 and 67 kd) of differentiating keratinocytes belong to the type II class of keratins, whereas the other two keratins (isotypes, 56.5 kd)

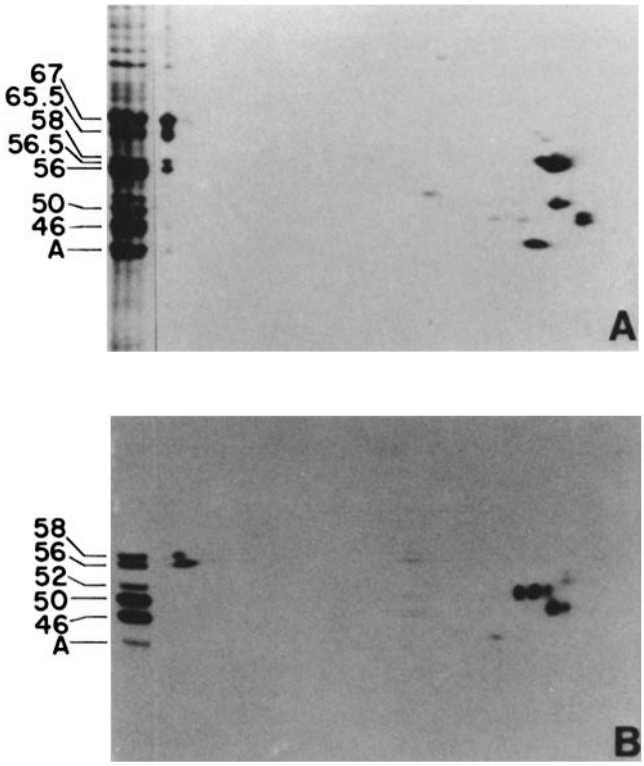
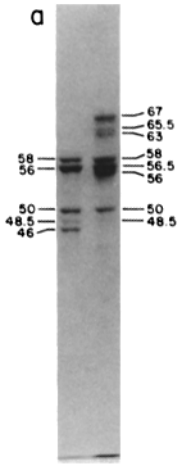


FIGURE 5 Two-dimensional gel analysis of keratin proteins from human epidermis (*A*) and cultured human basal epidermal cells (*B*). Proteins were radiolabeled for 8 h in vivo with [^{35}S]methionine. Epidermis was separated from dermis by treatment with 2 M NaBr. Epidermal cells were first extracted five times with 10 mM Tris HCl pH 7.4, 2 mM EDTA, 10 mM NaCl in the presence of protease inhibitor phenylmethylsulfonyl fluoride. After a subsequent series of extractions with 10 mM Tris·HCl, pH 7.4, 2 mM EDTA, 2% Triton X-100, the remaining residue was solubilized in 8 M urea, 10% 2-mercaptoethanol and analyzed electrophoretically. Two-dimensional gels were run as described by O'Farrell (43). Isoelectric focusing in the first dimension was with a 4.5–7.5 pH gradient from right to left; SDS PAGE in the second dimension was from top to bottom. An aliquot of unfocused protein was run as a marker in the second dimension. Molecular weight values are in kilodaltons. *A*, actin.



		Keratin M.W. ($\times 10^3$)	% of Total Keratin	
			Cultured	Tissue
Type II	large	67	0	16
		65.5	0	5
		63	0	7
	small	58	23	16
		56	41	16
	Type II combined		64	60
Type I	large	56.5	0	30
	small	50	18	9
		48.5	5	1
		46	13	0
	Type I combined		36	40

FIGURE 6 Intensity comparison of Coomassie Blue-detected Type II and Type I keratins in epidermal tissue and in cultured epidermal cells. (*a*) Keratins were extracted from back epidermal tissue (*right*) and from cultured epidermal cells derived from foreskin (*left*) and resolved by electrophoresis through an 8.5% SDS polyacrylamide gel. The bromophenol blue dye front was allowed to move 16 cm through the separating portion of the gel at 150 V such that the 56.5-kd keratin band could be separated from the 56-kd keratin band. The gel was stained with Coomassie Blue and scanned using a laser densitometer at 633 nm. (*b*) The intensity values expressed as percent of total keratins for individual keratins and for the combined type II and type I keratins are presented. Values were derived from four to five scannings of a track at different positions. Molecular weight values are in kilodaltons. Note that in the right lane of *a* the 56.5 and 56-kd keratins showed slightly faster mobility than usual. Molecular weight values are in kilodaltons. *A*, actin.

are more similar to the type I class. Although previous reports combining immunological and isoelectric focusing techniques initially made the assignment of the large, differentiation-specific keratins difficult (30), more recent studies have provided analagous findings to those that we describe here (31).

The Balance of Type I and Type II Keratins Is Not Disrupted during Terminal Differentiation

To measure the relative amounts of the type I and type II keratins in basal and differentiating epidermal cells, keratins were resolved by electrophoresis through a long (16 cm) polyacrylamide gel. Intensity scanning of the Coomassie Blue-stained gel revealed that the ratio of type II to type I keratins in basal epidermal cells is slightly more than 1:1 (Fig. 6). Upon commitment of a cell to undergo terminal differentiation, substantial levels of large keratins of both types accumulate. Surprisingly, even with these pronounced changes in keratin synthesis, the ratio of type I to type II keratins remains essentially unchanged. Whether this ratio is maintained in other keratinocytes was not determined.

Are Large Keratins of Differentiating Epidermis Expressed from the Same Genes as the Smaller Keratins of Basal Cells?

It has not yet been determined whether the new RNAs encoding the large type I and type II keratins originate from newly transcribed genes or alternatively from differential processing of heteronuclear RNAs for a single gene of each type. It is likely that at least some of the multiple genes for the type I and type II keratins are expressed, since hybridization studies have shown that the two basal type I mRNAs and the two basal type II mRNAs share substantial, but certainly not perfect, homology with each other (12).

To begin to investigate the relation between the RNAs encoding the large keratins and those of the basal keratins, we hybridized the Northern Blots shown in Fig. 2 with ^{32}P-labeled probes containing the 3′ noncoding regions (140 and 285 bp, respectively) of the type I and type II keratin cDNAs. Under conditions where an RNA-DNA duplex should have formed for a hybrid sharing sequence identity stretch of 140 by with a G-C content of 61% (24), only the 1.6-kb RNA and not the 1.8-kb RNA hybridized with the type I probe (data not shown). Under these conditions, the 3.8-kb RNA showed a detectable but very low homology with the 3′ noncoding portion of the 56-kd type II keratin cDNA. These results suggest that the noncoding segments of the large type I and type II keratin mRNAs are different from the noncoding segments of the small type I and type II keratin mRNAs.

To test the possibility that differential processing at the 3′ end of a single hnRNA transcript might give rise to two mRNAs with different 3′ noncoding regions, we isolated an

800 bp *BglI* fragment from the gene encoding the 50-kd human keratin. This fragment contains the 3′ untranslated sequence of the 50-kd keratin cDNA and in addition, ~670 nucleotides that are 3′ downstream from this sequence. Whereas strong hybridization of this fragment with the 1.6-kb RNA band occurred, no hybridization could be visualized with the 1.8-kb RNA band. Thus, if the two RNAs arise from the same gene, differential processing of the heteronuclear (hn)RNA hnRNA would have to generate different 3′ noncoding region segments that are not tightly linked in the genome.

DISCUSSION

The coordinate expression and coordinate evolutionary conservation of two distinct types of keratins in all vertebrate epithelia make it likely that the type I and type II keratins are, in some as yet unidentified way, essential in the formation of the coiled-coil protofilament backbone of the intermediate filament of epithelial cells (12–14, 18). In this study, we have shown that during terminal differentiation of the keratinocyte, when numerous changes in the keratin pattern take place, the ratio of the type I and type II keratins is not disrupted. Upon commitment of an epidermal cell to terminally differentiate, two additional type II keratins (65.5 and 67 kd) and two additional type I keratins (isotypes, 56.5 kd) are synthesized in roughly equal molar ratios (see also 30, 31). Thus it appears that not only is the ratio of type I and type II keratins constant for the epidermal cell, but in addition, different pairs of type I and type II keratins seem to be coordinately regulated and coordinately expressed at specific times in the differentiative pathway of the epidermal keratinocyte.

At this point, we have no evidence to suggest that this regulation is at the level of post-transcriptional processing, but rather it seems more likely to arise from the coordinate expression of newly transcribed genes. In this regard, the appearance of multiple mRNAs of both keratin types during epidermal terminal differentiation seems to differ significantly from the synthesis of multiple mRNAs for another intermediate filament subunit, vimentin (27). During erythroid development, there is a switch from the synthesis of a 2.3- to a 2.0-kb vimentin mRNA, due to the differential utilization of two polyadenylation signals at the 3′ end of the gene (27, 32). The same vimentin protein is produced from either transcript. In contrast, during keratinocyte differentiation, at least some of the multiple mRNAs of the same keratin type do not seem to arise from differential usage of polyadenylation signals, nor does it seem that they are generated from differential splicing at the 3′ noncoding end of a single gene. Although it has not yet been unequivocally demonstrated that these mRNAs are expressed from different genes, it is clear that the RNAs give rise to related polypeptides of markedly different size.

The functional significance of the unusually large keratins synthesized by terminally differentiating cells remains largely undetermined. Although morphological changes in the keratin filament network have been associated with terminal differentiation, they have not been positively linked with the appearance of these large keratins. Such changes include the interaction between keratin filaments and desmosomal plaques (33–38), and the formation of bundles (macrofibrils) of keratin filaments (39, 40). Nonethless, the finding that large keratins are expressed in all vertebrate epidermis and primarily in epidermis rather than internal epithelia, suggests that these keratins play a unique and important role in terminal differentiation (14). Precisely what this role might be must await the sequencing and characterization of these proteins.

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