Evidence for Posttranscriptional Regulation of the Keratins Expressed during Hyperproliferation and Malignant Transformation in Human Epidermis

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Abstract. Keratin K6 is a protein that is expressed in human skin under conditions of hyperproliferation (e.g., wound-healing, psoriasis, and cell culture) and malignant transformation (e.g., squamous cell carcinomas). When induced, the appearance of K6 is rapid: if skin tissue is placed in radiolabeled culture medium, this protein can be detected within an hour. The regulation of K6 seems to be controlled partly by a posttranscriptional mechanism: At least two K6 genes are actively transcribed both in vivo, when the protein is not made, as well as in vitro, when abundant levels of the protein are expressed. Substantial levels of K6a and K6b RNAs can be detected in skin by Northern Blot analysis, and these RNAs are largely, if not fully translatable in vitro. In situ hybridizations reveal that the RNAs are distributed throughout the living layers of the epidermis. The rapid induction of K6 expression through a posttranscriptional regulatory mechanism suggests that this keratin may play an important role in designing a cytoskeletal architecture that is compatible with the hyperproliferative state.

Epithelial cells manifest their common protective role by constructing an extensive cytoskeletal network comprised of three filamentous structures. Two of these, the 6-nm actin microfilaments and the 23-nm microtubules, are ubiquitous throughout the eukaryotic kingdom. The third, the 8-nm keratin filaments, are unique to epithelial cells and seem to be specifically tailored to suit the particular structural needs of each higher eukaryotic epithelial cell. Whereas the actins and tubulins have been highly evolutionarily conserved, the keratins form a highly divergent family of proteins (40-70 kD) which are differentially expressed in different epithelia and at different stages of differentiation and development (for review, see references 13, 35, and 44).

The keratins can be subdivided into two distinct groups by sequence homologies (21). Type I keratins are acidic (pKs 4.5-5.5) and generally small (40-56.5 kD), whereas type II keratins are basic (pKs 6.5-7.5) and larger (53-67 kD) (35). At least one member of each type is expressed at all times, suggesting the importance of both sequences in filament assembly (4). Indeed, no single keratin seems competent for filament formation (5, 22, 31, 43).

The epidermis of the skin serves the greatest protective role of all epithelia, and thus, it is not surprising that keratin synthesis is especially abundant in this tissue. Even in the basal layer, much of the total protein is keratin (8). When a basal cell undergoes a commitment to terminally differentiate and begins to migrate towards the skin surface, keratin synthesis increases as other metabolic processes come to a halt (16, 23). The fully differentiated cells of the outermost stratum corneum layer are merely cellular skeletons that are packed with bundles of keratin filaments (30).

Keratin synthesis in the epidermis is complex. In the basal epidermal layer, keratins of 58 and 50 kD are produced (11, 36, 45). These keratins have been designated K5 (type II) and K14 (type I) according to the nomenclature of Moll et al. (35). In response to an as yet unidentified trigger of terminal differentiation, a suprabasal epidermal cell begins to make two additional type II keratins, K1 (67 kD) and K2 (65 kD), and two type I keratins, K10 (56.5 kD) and K11 (56 kD) (4, 11, 49, 53). Throughout the suprabasal layers of the skin, these keratins are the predominant proteins that are synthesized.

When skin is injured, or if it otherwise undergoes hyperproliferation or malignant transformation, it induces the production of a type II keratin, K6 (56 kD), and a type I keratin, K16 (48 kD) (35, 51). Normally, however, these keratins are not expressed (4, 35). Whether the synthesis of these hyperproliferation-associated keratins may in some way be important for producing a cytoskeleton that is more compatible with either (a) a faster rate of cell division or (b) an increase in the number of cell cycles an epidermal cell undergoes during its lifetime, has yet to be determined.

The biochemical mechanisms underlying the differential expression of keratins in epidermis and during hyperproliferation remain largely unexplored. It is known that there are different mRNAs for most if not all of these keratins (2, 10, II), and these mRNAs are encoded largely if not solely by separate genes (15, 24, 27, 32, 33, 39, 41, 48). However, studies to investigate changes in keratin expression at the...
transcriptional and transcript-processing levels have not yet been conducted, and studies at the translational level have been hampered by the complexity of the epidermis and by the lack of monospecific monoclonal antibodies and cDNA probes.

In this paper, we begin to examine the regulation of epidermal keratin expression during differentiation and hyperproliferation. In particular, we focus on the differences in the mechanisms that control the expression of the epidermal keratin K14 and the hyperproliferation-associated keratin K6.

Materials and Methods

Source and Handling of Tissue

Human skin was obtained from material discarded during surgery or circumcision. Thigh skin was removed with a Padgett electric dermatome set at 0.008-0.015-in. thickness. Foreskin was shaved finely with a razor blade to remove subcutaneous fat and blood vessels. For mRNA preparations, tissue was frozen in liquid nitrogen within 3 min after its removal, and mRNA was extracted as described previously (10). For radiolabeling, tissue was incubated at 37°C in medium containing a mixture of \( [35S] \)methionine (100 \( \mu C/\)ml) and unlabeled methionine at 100 the normal levels. Sometimes as indicated in the text, tissue was first incubated in unlabeled nutrient medium before radiolabeling. For cell culture, trypsinized suspensions of epidermal cells were cultured using a fibroblast feeder layer and medium containing cholera toxin, epidermal growth factor, serum, and hydrocortisone as described previously (40).

Keratin Extraction

In the presence of a protease inhibitor (phenylmethylsulfonyl fluoride), water- and membrane-soluble proteins were removed from the tissue with low ionic strength buffer as described previously (9). The keratins were then extracted with 8 M urea and 10% \( \beta \)-mercaptoethanol (10).

Gel Electrophoresis and Immunoblot Analysis

One-dimensional SDS PAGE was conducted according to the procedure of Laemmli (28). Two-dimensional gel electrophoresis was performed so that the first dimensional separation was nonequilibrium-\( \phi \) gradient electrophoresis (37). Proteins were visualized by silver staining or by autoradiography. For immunoblot analysis, proteins from unstained two-dimensional gels were transferred electrophoretically to nitrocellulose paper, and the paper was hybridized with anti-keratin antisera (47) followed by the peroxidase-antiperoxidase technique (53). A general anti-keratin antisera was prepared by mixing polyclonal antisera against (a) s. corueum keratins (9) and (b) cultured cell keratins (14).

Subcloning of Coding and 3' Noncoding Keratin cDNA Segments into Riboprobe Vectors

K6 cDNA Subclones. The coding portions of a gene encoding K6b (48) share 97% homology with a 1,680-bp cDNA (KA-1) encoding K6a (21). A 1,415-bp Taq I/Xmn I fragment of pKA-1 (residues 20-1,435) containing predominantly the coding region of K6 was subcloned into E. coli plasmid SP64 (Promega Biotec, Madison, WI).

For specific probes to K6a and K6b, 3' noncoding segment subclones were prepared. Since the K6a and K6b 3' noncoding segments share significant homology (92%) within the first 125 nucleotides past their stop codons, it was necessary to subclone portions of the 400 bp of divergent sequence located immediately 5' to the polyadenylation signal. For the K6b gene, a 283-bp Sac I-Rsa I fragment extending from 169 bp 3' past the TGA stop codon to 68 bp 5' upstream from the polyadenylation signal was subcloned in the 3'-to-5' direction into pSP64 as described previously (48). For the K6a cDNA KA-1 (21), a 220-bp Alu I/Spe I fragment extending from 235 bp 3' past the TAA stop codon to 75 bp 5' from the polyadenylation signal was subcloned in the 3'-to-5' direction into plasmid PGEM 1 (Promega Biotec). The K6b probe did not cross-hybridize with KA-1, and the K6a probe did not cross-hybridize with the K6b gene.

K14 cDNA Subclones. KB-2 is a 1,410-bp cDNA clone encoding keratin K14 (45). It has a single TGA stop codon positioned at residue 1,230 (20). A 1,080-bp BstE II/Stu I fragment (nucleotide residues 170-1,250) encompassed a major portion of the coding region of K14 and was subcloned into pSP65. A 3' noncoding probe specific for the K14 mRNA was prepared from KB-2 by subcloning a 70-bp Stu I-Alu I fragment (nucleotide residues 1,228-1,298) into pSP64.

Radiolabeling of cRNA Probes

Salmonella SP6 RNA polymerase was used to prepare \(^{32}P\)- or \(^3H\)-labeled cRNA probes. The method of Cox et al. (3) was followed as described by the manufacturer, Promega Biotec.

In Situ Hybridizations

In situ hybridizations were conducted essentially according to the method of Cox et al. (3). Briefly, samples of facial tissue and foreskin were placed in 1% glutaraldehyde, 2.5% NaCl, and 50 mM sodium phosphate, pH 7.5 to fix the tissue immediately upon surgical removal. After paraffin embedding and sectioning (5 \( \mu \)m), tissue was treated with 0.5 \( \mu \)g/ml proteinase K for 30 min at 37°C and hybridized with \(^3H\)-UTP-labeled cRNA probes that had been hydrolyzed to an average size of 150 bp by sodium carbonate treatment at 60°C. Hybridization conditions were: 50% recrystallized formalde-}

Results

The Pattern of Epidermal Keratin Expression Changes When Human Skin Is Placed into Serum-containing Medium

When keratins were extracted from human skin and resolved by two-dimensional gel electrophoresis, three type II keratins, K1 (67 kD), K2 (65 kD), and K5 (58 kD), and three type I keratins, K10 (56.5 kD), KII (56.5 kD), and K14 (50 kD), were identified (Fig. 1; see also references 4 and 35). Depending on the urea concentration in the first dimension, some tetrameric complexes of keratins remained associated until the second dimension, when they were resolved into individual keratins (7). This caused several keratin spots with aberrant isoelectric mobilities (marked by overhead arrows in Fig. 1). The identification of all of the spots in Fig. 1 was verified by immunoblot analysis with a general anti-keratin antisera (44). Since glandular epithelium and hair follicles produce keratins (35) not present in the normal epidermis and not seen even upon overloading of the silver-stained gels, their contribution, if any, was judged to be minimal. These results are consistent with those that have been observed earlier (11, 35, 45).

Previous studies showed that when skin was sectioned (5 \( \mu \)m) parallel to its surface and the keratins were extracted, the pattern of keratins produced in the inner layers differed from that of the outer s. corneum layers (2, 11). The larger keratins of both types (K1, K2, K10, and KII) were produced only in the terminally differentiating cells of the suprabasal layers, and immunological studies confirmed this notion (49, 53). The smaller keratins, K5 and K14, seemed to be the only keratins present in the basal layer. Whether their expression is confined to this layer was not determined.

When human skin was placed in serum-containing me-
Keratins were assigned numbers according to their molecular weights and isoelectric mobilities as described by Moll et al. (35). α denotes the small amount of contaminating actin in 8-nm filament preparations. The overhead arrows mark the isoelectric mobilities of tetrameric complexes of type I and type II keratins that were not dissociated in the first dimensional gel.

dium in the presence of [35S]methionine for 5-12 h before extraction, the pattern of radiolabeled keratins in whole epidermis revealed that three additional keratins, K6 (56 kD), K16 (48 kD), and K17 (46 kD), were newly synthesized (Fig. 1 B). Two of these keratins, K6 and K16, have been associated with epidermal cells that undergo extensive growth either in vivo or in vitro, and they have been designated as molecular markers for hyperproliferative keratinocytes (51). Keratin K17 is also expressed in some hyperproliferating epidermal cells and is especially abundant in basal cell carcinomas (35, 51).

The synthesis of K6, K16, and K17 appeared to be induced rapidly: at the earliest time that radiolabel could be detected in the keratins (∼1 h), these keratins were seen. Once synthesized, the three keratins were stable: radiolabeled skin that was transferred to unlabeled medium for 48 h before extraction showed little difference in the pattern of keratin expression.

As skin was incubated in culture medium for longer time periods, additional changes in keratin pattern took place. These changes were readily observed by placing the tissue in unlabeled medium for 48 h before radiolabeling. After 2 d in culture medium, the differentiation-specific keratins (K1, K2, K10, and K11) were all markedly reduced (not shown). Thus, although the epidermal cells retained their protein-synthesizing capacity when placed into culture medium, two major changes occurred: one was rapid and involved the induction of the hyperproliferation-associated keratins; the other was slower, and involved the repression of the terminal differentiation-specific keratins.

Once these changes occurred, the pattern of keratins was nearly identical to that of epidermal cells that had been cloned and passaged in vitro for several hundred generations (Fig. 1, C and D). About 30% of the total protein synthesized by cultured epidermal cells is keratin (45). Remarkably, about half of this keratin is K6, K16, and K17, proteins that are not normally produced in skin.

Translation of Skin mRNA Yields Proteins That Co-migrate on Two-dimensional Gels with Keratins K6, K16, and K17

Poly(A)+ RNA was isolated from human foreskin and from cultured human epidermal cells. These mRNAs were then translated in vitro in a rabbit reticulocyte lysate system containing [35S]methionine, and the radiolabeled proteins were resolved by two-dimensional gel electrophoresis (Fig. 2).

Although the keratin patterns produced in vitro were very similar to those produced in vivo, several major differences were observed: (a) radiolabeled proteins that migrated with similar isoelectric mobilities to keratins K6, K16, and K17 were translated from foreskin mRNA (A), even though these proteins did not appear to be synthesized in the corresponding tissue extract, and (b) little (if any) translated K2 was detected from skin mRNA (A), even though the protein was readily detected in the keratin extracts of skin (compare with Fig. 1, A and B).

Although the two translated K6 spots always migrated at the same position on different two-dimensional gels, the putative K16 and K17 spots (46-48 kD) did not always reproducibly show the same isoelectric and SDS gel electrophoretic mobilities (Kim, K. H., unpublished observations). Sometimes they migrated at a more acidic pH, than seen in the two gels in Fig. 2. It is possible that for the gels shown, these keratins might have formed a complex with a more basic protein, perhaps a type II keratin (7); also com-

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Figure 2. In vitro translations of mRNAs isolated from human skin and cultured epidermal cells. Poly(A)+ RNAs from human foreskin (A) and cultured epidermal cells (B) were isolated and translated in a reticulocyte lysate system in the presence of [35S]methionine. Translated products were resolved by two-dimensional gel electrophoresis as described in the text. The gels were fluorographed and subjected to autoradiography. Keratins were identified by number according to their molecular weights and isoelectric mobilities.

Figure 3. Two distinct genes encode keratin K6, and the probes to the 3' noncoding portions of the K6a and K6b sequences are specific for these genes. Human genomic DNAs from nine random individuals were digested with the restriction endonuclease Eco RI. Human genomic DNA from one of these individuals was also digested with restriction endonucleases Bam HI, Hind III, or Bgl II. The fragments were separated by agarose gel electrophoresis. DNAs were transferred to nitrocellulose paper by blotting (42), and the paper was hybridized with radiolabeled 3' noncoding cRNA probes specific for either K6a or K6b mRNA. Lanes 1 and 2, control lanes showing that GK2 DNA, containing the 8.8-kb Eco RI fragment encompassing the human K6b gene (48), hybridizes only with the specific probe for K6b and not the probe for K6a. Lanes 3–II. Eco RI-digested human DNAs hybridized with probe to K6a; lanes 12–17, sequentially the same DNAs as lanes 3–8 hybridized with probe to K6b; lanes 18–20, sequentially the same DNAs as lanes 9–II probed with an equal mixture of both probes (demonstrating that the 8.8-kb fragments selected with these probes co-migrate); lanes 21–26, the same DNA as in lanes 3 and 12, but digested with either Bam HI (lanes 21 and 24), Hind III (lanes 22 and 25), or Bgl II (lanes 23 and 26) and hybridized with probes to either K6a (lanes 21–23) or K6b (lanes 24–26).

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ments, which indicate that in vivo, K2 appears at a later stage of differentiation than K1 (11).

**Preparation of cRNA Probes Specific for the RNAs Encoding Keratin K6: The Existence of Two Distinct Genes for K6**

To investigate in more detail the apparent presence in skin of mRNAs encoding the hyperproliferation-associated keratins, we prepared specific cRNA probes for K6, a keratin that we have studied extensively at the mRNA and gene level (48). Our previous studies on K6 had suggested that there might be at least two separate genes encoding this keratin, since (a) a cloned cDNA encoding K6α (21) shared only ~97% homology with the coding portion of a gene encoding K6β, and (b) the 3’ noncoding segments diverged after the first 125 nucleotides (48). To determine unequivocally whether these genes were not simply alleles, we prepared subcloned probes to the divergent portions of their 3’ noncoding sequences. In a Southern Blot analysis (42), the specific K6α probe did not cross-hybridize with a cloned 8.8-kb Eco RI fragment containing the human K6β gene (Fig. 3, lane 1), whereas the specific K6β probe hybridized strongly (lane 2). To verify that the probes were indeed selecting two different 8.8-kb Eco RI fragments, one of the samples of human genomic DNA was digested with either Bam HI, Hind III, or Bgl II, and the hybridizations were repeated (lanes 21–26). In this case, different sized bands were selected with each of the two probes. These results indicate clearly that there are two distinct genes encoding keratin K6, and these genes are present throughout the human population.

**Positive Hybridization and Northern Blot Analyses Demonstrate That the Two K6 mRNAs Are Present, But Not Translated in Human Skin**

To verify the specificity of our cRNA probes, we conducted positive hybridization and translation analyses with poly-(A)+ RNA from cultured human epidermal cells (Fig. 4). Under reduced stringencies, a total probe to K6 selected the mRNA to the closely related K5 in addition to K6 (lane 3), whereas the 3’ noncoding probes to K6α and K6β selected only the mRNA encoding K6 (lanes 4 and 5). Since many studies in this report involve the comparison of the expression of K6α and K6β mRNAs with the expression of the mRNA encoding K14, we prepared a specific 3’ noncoding probe to K14 mRNA (33). While the probe for the coding portion of K14 showed weak cross-hybridization with K17 mRNA (lane 6), the 3’ noncoding probe was specific for K14 mRNA (lane 7). Under the conditions used, none of our probes showed any selection of the mRNAs encoding the terminal differentiation-specific keratins of skin (not shown). The lack of cross-hybridization of our probes with the larger keratin mRNAs of differentiating cells was also evident from Northern Blot analyses (see below). To estimate the relative levels of the two K6 mRNAs in skin and cultured epidermal cells, total RNAs from both sources were fractionated by gel electrophoresis, transferred by blotting to nitrocellulose paper, and hybridized with radiolabeled cRNA probes complementary to K6α, K6β, and K14 mRNA (Fig. 5). For RNA of human skin, as well as cultured cells, a band corresponding in size to K6 mRNA (2.1 kb) hybridized strongly with the K6α probe (lanes 1 and 2). To verify that the probe was detecting bona fide K6 RNA, rather than homologous but not identical sequences, skin and cultured cell RNAs were hybridized in solution with the single stranded radiolabeled 3’ noncoding cRNA probe for K6α, and S1 endonuclease was added to digest any single stranded or imperfectly hybridized RNAs (data not shown). A single radiolabeled fragment of identical size was protected by both the skin and the cultured cell mRNAs, indicating that the hybridization visualized by Northern Blot analysis is due to the presence of K6α mRNA in human skin. K6β RNA was also detected in skin and cultured epidermal mRNA (lanes 3 and 4). Although this probe was of comparable size and specific activity to the K6a probe, extended exposure times were required to obtain a similar signal. This suggested that the levels of K6β RNA were significantly lower than the levels of K6α RNA. In addition, it seemed that both K6α and K6β RNAs were more abundant in cultured epidermal cells than in skin.

Quantitation of the relative levels of K6α and K6β mRNAs in the two samples was difficult. To obtain an estimate of the levels of K6α or K6β mRNA relative to an mRNA known to
Figure 5. Northern blot hybridization of epidermal mRNAs with specific K6 and K14 keratin probes. Alternating samples (0.5 μg) of poly(A)+ RNAs from epidermis (odd-numbered lanes) and cultured human epidermal cells (even-numbered lanes) were fractionated by electrophoresis on 0.8% agarose formaldehyde gels and then transferred to nitrocellulose paper by blotting (46). Blots were hybridized with the following 32P-labeled 3' noncoding region cRNA probes: lanes 1 and 2, K6a; lanes 3 and 4, K6b; lanes 5 and 6, K14; lanes 7 and 8, a mixture K6a and K14; lanes 9 and 10, a mixture of K6b and K14. Hybridized blots were washed thoroughly and subjected to autoradiography. For lanes 1-6, blots were exposed for variable times. For lanes 7 and 8 and 9 and 10, respectively, the blots for the skin and cultured cell mRNAs were developed such that the amounts of K14 hybridization in the two samples were approximately equal. This enables the reader to examine the difference in levels of either K6a or K6b mRNAs in skin as compared with cultured cells, relative to an equal amount of an mRNA (K14) known to be expressed in both RNA samples. The sizes of hybridizing RNA bands are indicated in kilobase pairs at left.

To examine the localization of the mRNAs encoding K6a, K6b, and K14 in human skin, we hybridized 3H-labeled cRNA probes in situ to cross-sections of tissue, and subjected the sections to autoradiography (Fig. 6). A cRNA probe to the coding portion of K6 hybridized fairly evenly throughout the living (protein synthesizing) layers of the epidermis (ep) of facial skin (A). Similar labeling was seen for the keratinized surface (B) and the unkeratinized surface (not shown) of foreskin. Silver grains were concentrated most heavily in the cytoplasmic regions of the epidermal cells. Very few grains were seen over the dermis (de), and these were usually concentrated over the few strata epithelial cells in the tissue. Only background labeling was found in the s. corneum layer (sc). In contrast to the distribution of the probe for the coding portion of K6, a probe for the K14 mRNA seemed to be predominantly in the basal epidermal layer of facial skin and foreskin (C and D). Although the silver grain density was very high in the basal epidermal cells, the grains were by no means exclusive to this layer. Since the total K6 probe did not show a hybridization pattern similar to K14, as would have been expected if there was cross-hybridization with K5 mRNA, it is likely that under the conditions used, the total probes were specific for their respective mRNAs.

To investigate the relative levels of K6a and K6b mRNAs in skin, cRNA probes specific for (a) the 3' noncoding portion of the K6a mRNA and (b) the 3' noncoding portion of the K6b mRNA, were hybridized with cross-sections of human skin (Fig. 7, A and B, respectively). These probes showed similar localization to the probe for the coding portion of K6. However, the exposure times for comparable grain density were much longer for the K6b than for the K6a probe. These results are consistent with our Northern Blot analyses, indicating that the K6b gene is expressed at lower levels than the K6a gene in epidermis.

To determine whether the localization of a cRNA probe specific for the 3' noncoding portion of the K14 mRNA was identical to that of the probe for the coding portion of K14, hybridizations were conducted with the 3H-labeled cRNA complementary to a 70-bp 3' noncoding segment of the K14 mRNA (C). Although hybridization still seemed to be preferentially over the basal layer, the differential labeling was not as striking as it was with the probe for the coding portion of K14. Since the noncoding probe is quite short and since the signal-to-noise ratio is much lower than for the coding probe, it may be that the basal-predominant labeling is obscured by the background hybridization. Alternatively, however, we cannot rule out the possibility that more than one gene encodes K14: one which is expressed primarily if not exclusively in the basal epidermal layer, and one which is still expressed predominantly in the basal layer but also shows significant hybridization throughout all of the living layers of the epidermis.

The Localization of K6 and K14 mRNAs in the Stratified Layers of Cultured Epidermal Cells Is Similar to That in Skin Epidermis

Under normal conditions (10⁻⁷ M vitamin A), cultured epidermal cells stratify, although they do not express the keratins characteristic of the terminal differentiative process (12). To investigate the localization of K6 and K14 mRNAs in the cultured cell colonies, we carefully dislodged the stratified cells from the culture dish with the enzyme dispase (17), and fixed and embedded the “tissue” as we did for human skin (3). With most of the radiolabeled probes, in situ hybridizations revealed similar mRNA distribution to that observed for skin (Fig. 8). The K6 coding region probe and...
Figure 6. In situ hybridization of 3H-labeled keratin cRNAs to cross-sections of epidermis. Tissues from human facial skin (A and C) and foreskin (B and D) were fixed in glutaraldehyde and sectioned (5 μm) as described in Materials and Methods. Hybridizations of radiolabeled cRNA probes were followed by RNAse treatment and autoradiography. Exposures were for 11 d. Probes were to: A and B, the 1,415-bp Taq I/Xmn I fragment of KA-1 encompassing a large portion of the K6a coding region; C and D, the 1,080-bp BstE II/Stu I fragment of KB-2 encompassing a large portion of the K14 coding region. Only bright-field (not dark-field) microscopy could be conducted, since fluorescing melanin granules were present in the epidermal sections. ep, epidermis; de, dermis; sc, stratum corneum. The innermost basal epidermal layer of the skin is indicated in A. Bar, 30 μm.

the K6a and K6b noncoding probes all showed fairly uniform distribution with only slight preference for the basal layer (A, C, and D, respectively). The K14 coding region probe showed prominent labeling in the basal layer, with substantial hybridization throughout the suprabasal cells as well (B). Similar to the results obtained with skin sections, the 3' non-coding probe of K14 did not show as predominant a basal layer localization as was observed with the probe to the coding portion of K14 (E). Possible reasons for this were discussed earlier.

Discussion

Multiple Levels of Regulation Seem to Be Involved in Differential Keratin Expression

In this study, we began to explore the molecular basis for the
Figure 7. In situ hybridizations of specific 3' noncoding keratin cRNAs to cross-sections of epidermis. Tissues from human foreskin were fixed in glutaraldehyde and sectioned (5 μm) as described in Materials and Methods. Hybridizations of radiolabeled cRNA probes were followed by RNAse treatment and autoradiography. Probes were to: A, the 220-bp Alu I/Spe I 3' noncoding fragment of K6α; B, the 283-bp Sac I/Rsa I 3' noncoding fragment of K6β; C, the 70-bp Stu I/Alu I 3' noncoding fragment of K14. Exposure time for A was 8 d; exposure times for B and C were 28 d. Bar, 30 μm.

Figure 8. In situ hybridizations of keratin cRNAs to cross-sections of cultured human epidermal colonies. Confluent cultures of human epidermal cells were removed with the enzyme dispase as described by Green et al. (17). After fixing and sectioning, the cells were hybridized with 3H-labeled keratin cRNA probes. Hybridized cells were treated with RNAse to remove unhybridized label, and then subjected to autoradiography. Probes were to: A, the 1,415-bp Taq I/Xmn I fragment of KA-1 encompassing a large portion of the K6α coding region; B, the 1,080-bp BstE II/Stu I fragment of KB-2 encompassing a large portion of the K14 coding region; C, the 220-bp Alu I/Spe I 3' noncoding fragment of K6α; D, the 283-bp Sac I/Rsa I 3' noncoding fragment of K6β; E, the 70-bp Stu I/Alu I 3' noncoding fragment of K14. Exposure times for A–C were 8–10 d; exposure times for D and E were 28 d. Bar, 30 μm.
differential expression of keratins in human epidermis and cultured epidermal cells. Our results have shown that the expression of these keratins may not always be controlled exclusively at the gene level. Indeed for the keratins K6 and K16, there seem to be posttranscriptional as well as transcriptional controls that operate to alter the expression of these proteins.

The expression of K14 and K5 seems to be regulated at least in part at the level of transcription, and the genes encoding these keratins are transcribed at high levels in the basal layer of epidermis and in the lower layer of epidermal colonies grown in culture. In these cells, the level of processed K14 mRNA is high, and it is actively translated. In the basal layer, the expression of K14 protein seems to mirror the level of K14 mRNA in a cell, as evidenced by the strong basal layer staining of a general type I keratin antibody, AE1 (53). In the suprabasal layers, however, the AE1 antibody shows no staining, even though some (albeit a lower level of) K14 mRNA is clearly present. One possibility is that the mRNAs for K14 and its partner K5 are not translated in the suprabasal layers. Alternatively, K14 mRNA may be effectively translated throughout the living layers of the epidermis, but in the suprabasal layers, its AE1-specific antigenic determinant might be masked. In fact, it is already known that the AE1 cross-reactivity of other type I keratins, namely K10 and K11, is masked in keratinizing cells (53). Thus, the decline in the expression of the basal epidermal keratins during terminal differentiation may not be as abrupt as was originally presumed.

In cultured epidermal cells, the mRNAs encoding the large keratinization-specific keratins are expressed only when the cells are induced to terminally differentiate (12). If the epidermis behaves in a similar fashion, then the induction of keratins K1, K10, and K11 in skin is also likely to be at the level of transcription. An unusual exception to the transcriptional regulation of the large keratins occurs in squamous cell carcinomas of mouse skin, where the mRNAs encoding the differentiation-specific keratins seem to be expressed even though they are not translated (52). Whether this regulatory control mechanism also operates in vivo in human skin cancers is not yet known, although it does not seem to play a role in keratin expression in cells cultured from human squamous cell carcinomas (25, 26).

Interestingly, whereas in epidermis, the transcription of the genes for the differentiation-specific keratins seems to coincide with the concomitant reduction of basal-specific transcription, in cultured cells, these processes can be separated. Thus, in stratified cultures, transcription of the K14 gene is still predominantly in the basal layer, but unless the keratinizing process is specifically induced, the large keratins are not synthesized.

In contrast to the normal program of epidermal differentiation, the regulation of the hyperproliferative state in skin may begin at the posttranscriptional level. Our results demonstrate convincingly that even though no K6 protein is made until epidermal cells are induced to hyperproliferate, there are two distinct genes encoding K6 that are both transcribed in all metabolically active layers of epidermis. The K6 RNA produced by the epidermis corresponds in size to the K6 mRNA expressed in cultured human epidermal cells, and it is largely, if not fully, translatable in vitro. Thus, unless K6, K16, and K17 are highly unstable in skin and specifically subject to degradation, the inhibition of their expression is likely to be at the translational level. Once the rapid posttranscriptional induction of these keratins has occurred during the course of hyperproliferation, an increase in the level of K6 mRNA occurs, suggesting that transcriptional enhancement or mRNA stability may contribute to the later stages of elevated K6 expression.

What Are the Molecular Mechanisms Underlying the Posttranscriptional Regulation of the Hyperproliferation-associated Keratins?

Posttranscriptional controls have been recognized for quite some time in many different systems. In oocytes, two major control elements seem to be operative: (a) ribonuclease complexes may make some mRNAs unavailable for translation (38); and (b) different mRNAs, perhaps by variations in their 5' untranslated leader sequences (34) or 3' untranslated sequences (54), may compete with different efficiencies for a limited translational capacity, which is then increased after fertilization (29). In ribosomal protein synthesis, the cell uses other posttranscriptional control mechanisms to ensure that each protein component is made in the proper amounts (50): Some gene products are subjected to translational regulation, apparently at the level of hnRNA processing; other genes overproduce their respective proteins, and the excess products are rapidly degraded.

We do not yet know the molecular details of the posttranscriptional process that limits the expression of certain epidermal keratins to conditions of hyperproliferation or malignant transformation. The rapidity with which the keratins K6, K16, and K17 are induced when skin undergoes a hyperproliferative response suggests that extracellular growth factors might play a role in the regulatory process. Growth factors can elicit extremely rapid intracellular responses (18, 55), and they are present under all of the conditions where the hyperproliferation-associated keratins are known to be expressed.

Many growth factors exert their action through a membrane receptor that subsequently acquires tyrosine kinase activity (for review, see reference 1). Changes in phosphorylations of serine and threonine residues are also mediated by growth factors (19). Interestingly, some changes in protein phosphorylation are known to have a profound effect on the translational machinery of a cell (6). Further studies will be necessary to determine whether a growth factor–induced phosphorylation might play a direct or indirect role in the differential translation of hyperproliferation-associated keratin mRNAs in the epidermis.

What Is the Biological Significance Underlying Posttranscriptional Regulation of Keratin K6?

Most cases of posttranscriptional regulation involve transcripts whose products must be made rapidly during development or in response to some external stimulus. In skin, hyperproliferation is induced quickly upon wounding or injury. The finding that the mRNAs for K6 (and presumably K16) are stored in all of the living layers of the epidermis suggests that these keratins may play an active, rather than a passive, role in the hyperproliferative response.

Little is known about how an epithelial cell manipulates its keratin filaments during the course of cell division, or...
how filaments comprised of different pairs of keratins might influence this process. It is possible that the hyperproliferation-associated keratins produce filaments that have properties compatible with either more rapid or more numerous cell divisions. If these filaments yield a more flexible cytoskeleton, then they may be important for the transient changes in cell-cell interactions that occur during the healing process. Thus, in normal epidermis, the induction of K6 and K16 synthesis may be a necessary prerequisite to a controlled hyperproliferative response. In the course of human disease, the regulatory process may become abnormal, and perhaps even irreversible, giving rise to uncontrolled hyperproliferation.

The precise molecular pathway leading to a specific and possibly reversible block in the translation of the hyperproliferation-associated mRNAs in normal epidermis remains to be elucidated. As we begin to analyze the regulatory sequences necessary for (a) the transcription of the K14 and K6 genes, and (b) the processing, stability, and translation of their transcripts, we hope to unravel some of the mysteries of this complex regulatory process.

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