Expression of Keratin K14 in the Epidermis and Hair Follicle: Insights into Complex Programs of Differentiation

Pierre A. Coulombe, Raphael Kopan, and Elaine Fuchs

Howard Hughes Medical Institute, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637

Abstract. Keratins K14 and K5 have long been considered to be biochemical markers of the stratified squamous epithelia, including epidermis (Moll, R., W. Franke, D. Schiller, B. Geiger, and R. Krepler. 1982. *Cell.* 31:11–24; Nelson, W., and T.-T. Sun. 1983. *J. Cell Biol.* 97:244–251). When cells of most stratified squamous epithelia differentiate, they downregulate expression of mRNAs encoding these two keratins and induce expression of new sets of keratins specific for individual programs of epithelial differentiation. Frequently, as in the case of epidermis, the expression of differentiation-specific keratins also leads to a reorganization of the keratin filament network, including denser bundling of the keratin fibers. We report here the use of monospecific antisera and cRNA probes to examine the differential expression of keratin K14 in the complex tissue of human skin. Using in situ hybridizations and immunoelectron microscopy, we find that the patterns of K14 expression and filament organization in the hair follicle are strikingly different from epidermis. Some of the mitotically active outer root sheath (ORS) cells, which give rise to ORS under normal circumstances and to epidermis during wound healing, produce only low levels of K14. These cells have fewer keratin filaments than basal epidermal cells, and the filaments are organized into looser, more delicate bundles than is typical for epidermis. As these cells differentiate, they elevate their expression of K14 and produce denser bundles of keratin filaments more typical of epidermis. In contrast to basal cells of epidermis and ORS, matrix cells, which are relatively undifferentiated and which can give rise to inner root sheath, cuticle and hair shaft, show no evidence of K14, K14 mRNA expression, or keratin filament formation. As matrix cells differentiate, they produce hair-specific keratins and dense bundles of keratin filaments but they do not induce K14 expression. Collectively, the patterns of K14 and K14 mRNA expression and filament organization in mitotically active epithelial cells of the skin correlate with their relative degree of pluripotency, and this suggests a possible basis for the deviation of hair follicle programs of differentiation from those of other stratified squamous epithelia.

KERATINS are especially abundant in stratified squamous epithelial tissues, where they compose the major differentiation-specific products of these cells (for reviews, see Moll et al., 1982*a*; Sun et al., 1984; Fuchs et al., 1987). Type I keratins (K9–K19) are relatively small (40–56.5 kD) and acidic (pKi = 4.5–5.5), whereas type II keratins (K1–K8) are larger (52–67 kD) and more basic (pKi = 5.5–7.5) (Fuchs et al., 1981; Moll et al., 1982*a*). In epithelial tissues, keratins are differentially expressed as specific pairs of type I and type II proteins, both of which are essential for filament formation (Eichner et al., 1984). In epidermis, tongue, cornea and esophagus, K5 and K14 are the pair expressed in the basal, mitotically active layer, and this pair has been considered to be a biochemical marker of stratified squamous epithelia (Moll et al., 1982*a*; Nelson and Sun, 1983). Both in vivo and in vitro, filaments composed of these keratins are loosely bundled (Eichner et al., 1986). Upon stratification, these epithelial cell types diverge, and each expresses a unique set of keratins that are tissue-specific for their particular program of differentiation (Fuchs and Green, 1980; Viac et al., 1980; Woodcock-Mitchell et al., 1982; Skerrow and Skerrow, 1983; Crewther et al., 1983; Sun et al., 1984; van Muijen et al., 1986; Schermer et al., 1986). The expression of these new sets of keratins is frequently associated with an increase in bundling of keratin filaments (Steinert, 1978; Eichner et al., 1986).

In the hair follicle, the programs of differentiation and patterns of keratin expression are far more complex than in other stratified squamous epithelia (Steinert and Rogers, 1973; Steinert, 1978; Crewther et al., 1980; Baden, 1981; Moll et al., 1982*b*, 1988; Lane et al., 1985; French and Hewish, 1986; Ito et al., 1986; Lynch et al., 1986; Heid et al., 1986, 1988*a*; Stark et al., 1987). Surrounding the hair shaft are two sheaths, an outer (ORS)[1] and an inner (IRS) root sheath (Fig. 1). Most of the ORS originates from one to

1. *Abbreviations used in this paper*: IRS, inner root sheath; ORS, outer root sheath.

two layers of cells on the surface of the mid-region of the hair bulb (Birbeck and Mercer, 1957; Pinkus, 1958; Hashimoto and Shibazaki, 1975; Montagna, 1976; Ito et al., 1986). At the beginning of each hair cycle, these progenitor ORS cells divide rapidly and give rise to a population of self-propagating cells that extend along the outer surface of the ORS to approximately half way up the follicle (Bullough and Laurence, 1958; Chapman et al., 1980; Clausen et al., 1982). ORS cells in this lower region seem to migrate both upwards and inwards as they differentiate. In contrast, cells of the upper portion of the ORS migrate predominantly inwards as they differentiate. Moreover, [^{3}H]thymidine labeling of sheep follicles has indicated that cells in the upper portion of the follicle seem to be less active mitotically (Chapman et al., 1980). Despite these differences, both populations of mitotically active ORS cells seem to have the capacity to regenerate epidermis during wound-healing. Conversely, however, epidermis does not seem to be able to regenerate ORS cells (Weiss et al., 1984; Demarchez et al., 1986; Mansbridge and Knapp, 1987; Lenoir et al., 1988).

At the base of the follicle are relatively undifferentiated and mitotically active cells of the matrix, which differentiate upward as concentric cylinders of six distinct cell types, making up both the hair shaft and the inner root sheath (Fig. 1). Seemingly even more pluripotent than ORS cells, matrix cells apparently have the capacity to choose among multiple pathways of differentiation. However, the pathways of differentiation leading to the production of the hair and the IRS are characteristic of other stratified squamous epithelia in that unique sets of keratins are produced by the fully differentiated cells of these structures (Crewther et al., 1980; Moll et al., 1982*b*; Lynch et al., 1986; Heid et al., 1986; Stark et al., 1987).

Despite major differences in the behavior of the mitotically active epithelial cells of the hair follicle, it has been assumed widely that these cells are similar in their biochemistry to

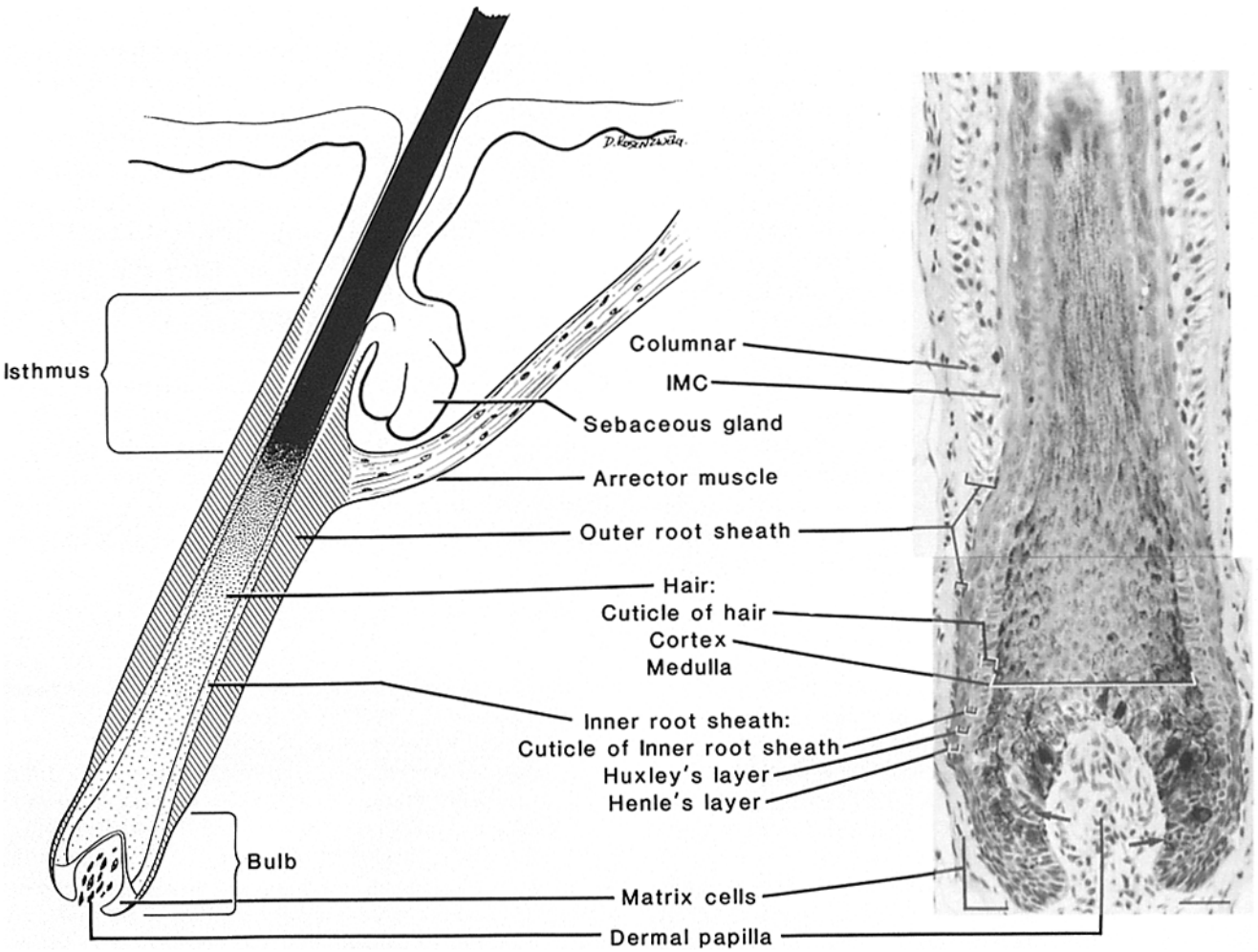


Figure 1. Differentiated cell types within the adult hair follicle. (*Left*) Schematic representation of a human hair follicle. (*Right*) Hematoxylin and eosin stained section (5 μm) of a human hair follicle (bar at lower right, 30 μm). The hair follicle forms as a bulb around the specialized mesenchymal cells, i.e., dermal papillae. The relatively undifferentiated matrix cells are derived from the embryonic basal layer of developing skin, and give rise to concentric rings of differentiated cell types, including the medulla, the cortex, and the cuticle of the hair. The hair is surrounded by two root sheaths: the inner root sheath is composed of three cell types: the IRS cuticle, and Huxley's and Henle's layers; the ORS is composed of multiple layers of a single cell type. The stem cells of the IRS, namely the matrix cells, extend to the dermal papilla, where they are active mitotically (*arrows*) and give rise to cells that migrate upward in single cell layers of distinct differentiative pathways. The ORS stem cells do not come into direct contact with the dermal papilla. They are contiguous with the basal epidermal layer, but they are distinct and self-propagating.

basal epidermal cells. It is known, for example, that ORS cells express K5 and K14 (Moll et al., 1982*b*; Lynch et al., 1986; Stark et al., 1987; Heid et al., 1988*a*, 1988*b*; Moll et al., 1988; Kopan and Fuchs, 1989), and recently it was claimed that matrix cells also express K14 (Heid et al., 1988*a*, 1988*b*; Moll et al., 1988). However, it is not known whether the temporal and spatial expression of K5 and K14 in the ORS is similar to that of epidermis, and moreover, it is controversial whether matrix cells truly contain K14: the only antibody which has detected K14 in matrix is an mAb (KA1) whose antigenic specificity has not yet been fully analyzed (Nagle et al., 1984, 1986). That this antibody has not yet been well characterized becomes an especially important issue in light of findings by other researchers that (*a*) common antigenic epitopes exist between keratins and nonkeratin proteins in hair (Lynch et al., 1986) and (*b*) other K14- and K5-specific probes have not detected these or other keratins in matrix cells (Moll et al., 1982*b*; Heid et al., 1986; Lynch et al., 1986; Kopan and Fuchs, 1989). Hence the extent to which the mitotically active cells of the follicle are truly biochemically similar to epidermal basal cells remains to be determined.

Several lines of recent evidence suggest that levels of K5 and K14 in mitotically active cells may correlate with their relative degree of differentiation: An increase in expression of K14 was found to accompany commitment of embryonic basal cells to differentiate into basal epidermal cells (Kopan and Fuchs, 1989), and a reduction in expression of K14 seemed to be associated with tumorigenesis leading to basal cell carcinomas (Stoler et al., 1988; Roop et al., 1988). Because mitotically active cells of the hair follicle seem to have different capacities to undergo cell division and differentiation, we wanted to explore the possibility that these cells might also have different levels of K14 expression. In addition, we wanted to investigate in more detail the notion that filament organization may provide some clues as to relative differentiation states of stratified squamous epithelial cells. In this study, we focused on the epidermis and hair follicle of human skin and conducted (*a*) immunocytochemistry at the light and electron microscopy levels using a monospecific anti-K14 antiserum, and (*b*) in situ hybridizations with a specific K14 cRNA probe. Our results have uncovered biochemical and morphological differences in the pattern of K14 and K14 mRNA expression and filament organization in the mitotic epithelial cells of the skin. These differences provide valuable insights into the distinct differentiative programs of these cells and the degree to which these cells remain pluripotent in the adult.

Materials and Methods

Tissues

Normal human scalp skin was obtained as material which would otherwise be discarded during the course of surgery.

Immunohistochemistry: Light Microscopy Level

Skin from a patient with white hair was used, and tissue was fixed for 6 h in 4% paraformaldehyde at room temperature. After fixation, samples were embedded in paraffin and sectioned (5 μm). Sections of tissue were treated for ~30 min at room temperature with a solution of 2% BSA in PBS (BSA-PBS). The solution was then replaced with fresh BSA-PBS containing a 1:200 dilution of a monospecific rabbit polyclonal antiserum against a synthetic 15-mer coding for the carboxy terminus of human K14 (Stoler et al., 1988). After a 1-h incubation at room temperature, sections were washed three times in PBS (10 min each) and then treated with a gold-conjugated goat anti-rabbit antiserum (5-nm gold particles; Janssen Life Science Products, Piscataway, NJ). After an overnight incubation at room temperature, sections were washed six times in PBS (10 min each), fixed for 15 min in PBS containing 2% glutaraldehyde, and washed three times in PBS (10 minutes each) followed by three times in glass distilled water (10 min each). Sections were then placed in a single glass jar, along with a negative control (section in which the primary antiserum was omitted) and a positive control (section of previously analyzed human epidermis) (Stoler et al., 1988). Sections were then subjected to silver enhancement (IntenSE; Janssen Life Science Products), according to the manufacturer's instructions. Enhancement was stopped for all sections when the positive control section showed the familiar pattern of basal-preferred K14 staining. Specificity of the cytochemical labeling was assessed by repeating the above procedure with preimmune antiserum.

Immunocytochemistry: Electron Microscopy Level

Skin from a patient with dark hair was fixed and embedded according to Stromer and Bendayan (1988) with some modifications (Coulombe, P. A., M. Bendayan, D. Gingras, and E. Fuchs, manuscript in preparation). All fixatives were prepared in 0.1 M sodium phosphate buffer, pH 7.4, supplemented with 0.5% glucose and 1.2 mM $MgCl_2$. After surgical removal, the scalp tissue was fixed by immersion in freshly prepared 4% paraformaldehyde at 4°C. Within 2 h, single hair follicles were isolated using a dissecting microscope and then further fixed in 4% paraformaldehyde, with or without 0.05% glutaraldehyde. Total fixation time was ~6 h. The best anti-K14 labeling was observed when paraformaldehyde alone was used as a fixative. Addition of 0.05% glutaraldehyde greatly improved the morphology, but altered the overall quality of the cytochemical signal. Thus, only where the quality of the cytochemical signal was of utmost importance, i.e., in the bulbar section of the hair follicle, did we use paraformaldehyde-fixed sections. After fixation, hair follicles were sectioned in three segments (bulb, suprabulbar, and isthmus) along their main axis, and then processed for low-temperature embedding in Lowicryl K4M as previously described (Bendayan, 1984). Before UV polymerization, the follicle segments were oriented such that their longitudinal axis would be parallel to the plane of sectioning. Thin sections (50 nm) were recovered on Parlodion- and carbon-coated nickel grids.

For labeling, all incubations were performed at room temperature. Grids were first floated (sections downward) on a drop of 2% BSA in 0.01 M PBS solution, containing 0.02% sodium azide, pH 7.2, for 6 min. They were then transferred to the same solution containing either (*a*) a rabbit polyclonal anti-K14 antiserum specific for the carboxy-terminal 15-amino acid sequence of K14 (Stoler et al., 1988), used at a 1:300 dilution, or (*b*) a rabbit polyclonal anti-type I keratin antiserum made against gel-purified 50-kD human epidermal keratins (Fuchs and Marchuk, 1983), used at a 1:200 dilution. After antibody incubation, grids were rinsed briefly in PBS, incubated in the BSA-PBS solution as above, for 6 min, and finally incubated in a solution containing 10 nm gold-conjugated goat anti-rabbit IgG antiserum (Janssen Life Science Products) for 1 h. Grids were washed three times in PBS, once in distilled water, dried on filter paper, and then contrasted with 3% uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Prepared grids were then examined using an electron microscope (model 410; Philips Electronic Instruments, Mahwah, NJ). The specificity of the cytochemical labeling was assessed through (*a*) repeating the above procedure using preimmune sera, rather than primary antikeratin antisera, and (*b*) repeating the above procedure, but omitting the primary antikeratin antisera incubation step.

In Situ Hybridizations

Tissue sections were subjected to in situ hybridizations according to the procedures of Cox et al. (1984) as described previously (Tyner and Fuchs, 1986; Stoler et al., 1988). ^{35}S-UTP-labeled cRNA probes were generated from (*a*) plasmid 3sp, a derivative of plasmid pSP65 (Promega Biotec, Madison, WI) containing a 1,080-bp Bste II/Stu I fragment cloned into the Hinc II site of the vector. The K14 cDNA fragment encompassed nucleotide residues 170–1,250 of KB-2, a 1,410-bp cDNA encoding human keratin K14 (Hanukoglu and Fuchs, 1982; Tyner and Fuchs, 1986; Stoler et al., 1988); and (*b*) K14-3'NC, a derivative of plasmid pGEM3 containing a 452-bp Aha II/Eco RV fragment cloned into the Acc I/Eco RI site of the vector after Klenow treatment and blunt-end ligation. The K14 3' noncoding segment was a hybrid, encompassing nucleotide residues 1,267–1,349 of KB-2 (Han-

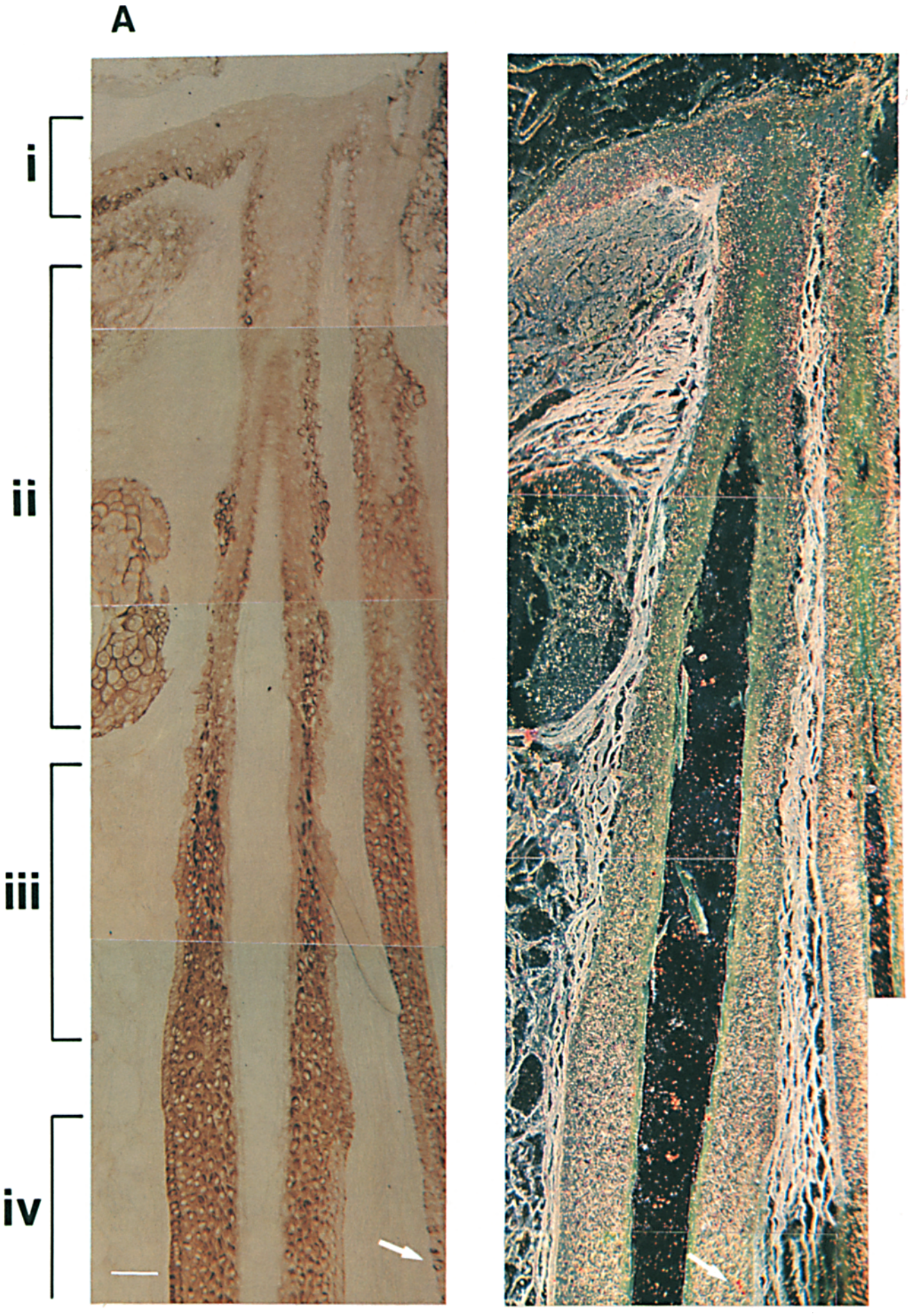


Figure 2.

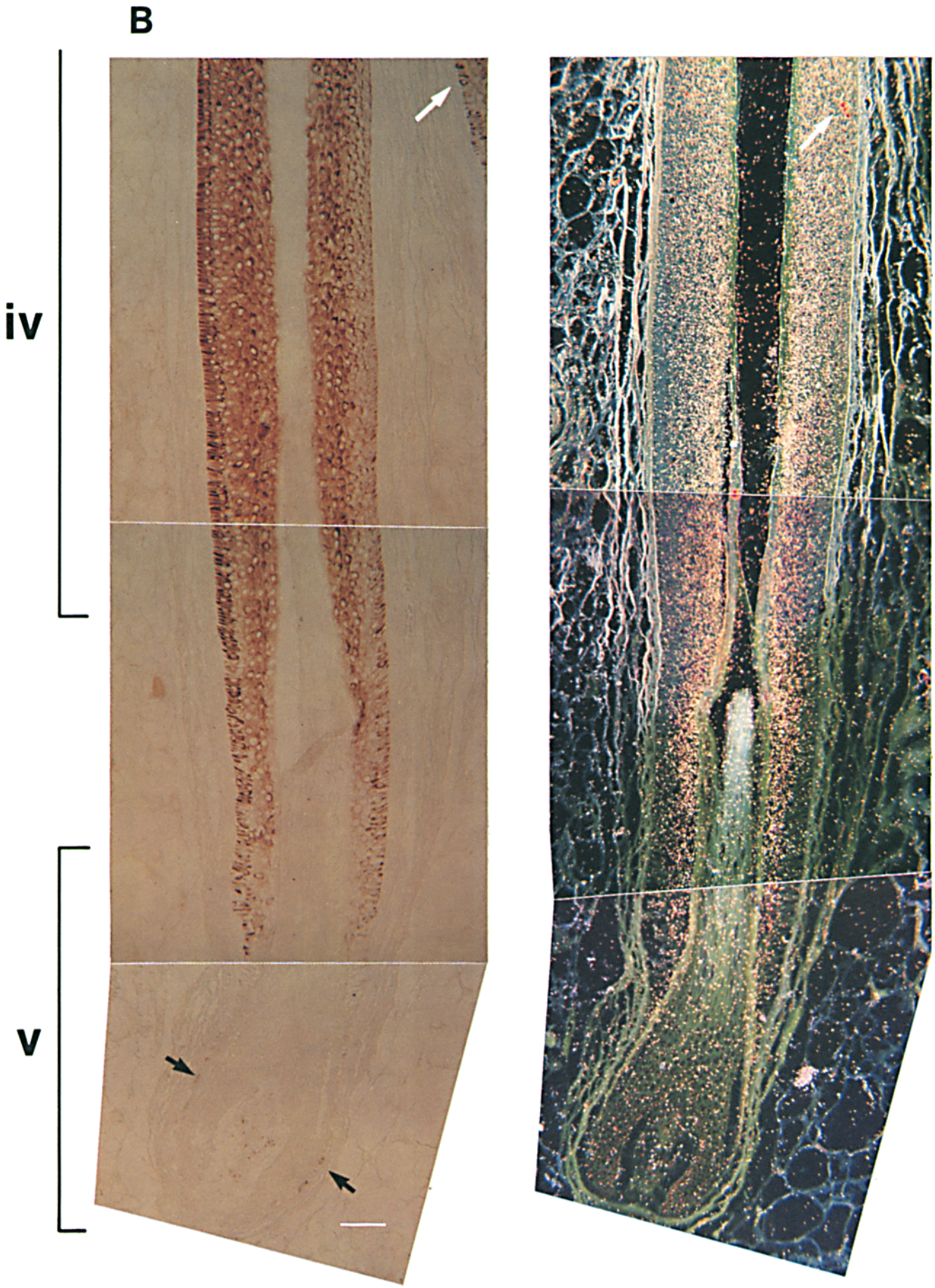


Figure 2.

ukoglu and Fuchs, 1982) coupled to 370 nucleotide residues extending from the BstX I site to the Eco RV site of the GK-1 genomic K14 clone (Marchuk et al., 1984). Antisense cRNAs were generated by linearizing the plasmids with Hind III, and using (*a*) SP6 RNA polymerase and (*b*) T7 polymerase, respectively, in the presence of ^{35}S-UTP and unlabeled ribonucleotides. The specificity of the probes was assessed by repeating the in situ hybridization with sense rather than antisense K14 cRNA probe.

Results

General Approach

To examine the pattern of K14 expression at the level of light microscopy, we stained sections of human scalp skin with an antiserum prepared against the 15 amino acid residues of the carboxy terminus of human K14. This antiserum has been well characterized, and it is monospecific for K14 (Stoler et al., 1988). Immunogold enhancement was used to visualize the bound primary antibody. To investigate K14 mRNA expression, we used in situ hybridization with ^{35}S-labeled cRNA probes complementary to either (*a*) a large portion of the coding region of the K14 mRNA (shown) or (*b*) a portion of the noncoding segment of the K14 mRNA (not shown) (Tyner and Fuchs, 1986). Hybridization conditions were chosen to exclude the possibility of cross-hybridization with other keratin mRNAs. Although the signal-to-noise ratio was always much better with a near full-length K14 probe, both probes consistently showed similar patterns of hybridization. Previous studies also confirmed the specificity of the K14 cRNA probe under the conditions used (Tyner and Fuchs, 1986; Stoler et al., 1988; Kopan and Fuchs, 1989). In addition to carefully testing the specificity of the K14 cRNA probe, we also verified that for all positive signals obtained with this probe, no signal was produced with the corresponding sense strand probe.

Fig. 2 shows adjacent sections of human skin: the section on the left was stained with anti-K14 and the section on the right was subjected to in situ hybridization with the larger K14 cRNA probe. Fig. 2 *A* illustrates the upper portions of the two skin sections, encompassing the epidermis and upper follicle region. Fig. 2 *B* shows the lower portions of the same two skin sections, encompassing the lower portion of the follicle.

Each region of the skin showing different patterns of K14 protein and mRNA expression was further examined at the ultrastructural level to investigate the organization of K14-containing keratin filaments in the cytoplasm of individual epithelial cells.

Expression of K14 and K14 mRNAs and Organization of Keratin Filaments in Epidermis

Previous studies at the light microscope level have indicated that as epidermal cells undergo a commitment to terminally differentiate, they downregulate the expression of K5 and K14 and induce expression of new and larger keratins, K1 and K10 (Fuchs and Green, 1980; Woodcock-Mitchell et al., 1982; Schweizer and Winter, 1983; Moll et al., 1982*a*; Roop et al., 1983, 1988; Tyner and Fuchs, 1986; Stoler et al., 1988; Kopan and Fuchs, 1987, 1989). Our K14 and K14 mRNA expression data at the light microscopy level are in agreement with these earlier studies (see zone *i* of Fig. 2 A, *left* and *right*, respectively).

To examine the epidermal localization of K14 more closely, we subjected a section of scalp skin to immunoelectron microscopy using the anti-K14 antiserum as primary antibody, followed by 10-nm gold particle–conjugated goat anti-rabbit IgG as secondary antibody. Fig. 3 *A* clearly illustrates that the K14 staining observed in basal epidermal cells was localized to the keratin filaments, which were organized into loose bundles. Typical of most intermediate filament networks, keratin bundles extended from the nuclear envelope to the cell periphery. As expected, dermis, melanocytes, and Merkel cells were devoid of labeling, as were most regions of each epidermal cell, including the nucleoplasm and the extracellular matrix.

As cells transited through the spinous layers, they flattened progressively and their axis became parallel to the skin surface. Keratin filaments were still present, and although they

Figure 2. K14 and K14 mRNA expression in the hair follicle. Human scalp skin was fixed in paraformaldehyde and sectioned (5 μm) as described in Materials and Methods. Serial sections were analyzed by immunohistochemistry and in situ hybridization as follows: (*Left*) K14 expression in the hair follicle. This section was stained with a polyclonal antiserum, specific for the 15 carboxy-terminal residues of the human K14 protein (Stoler et al., 1988). Staining was visualized by immunogold enhancement. The figure shown was assembled piecemeal, from serial photographs taken with a Standard 16 microscope (Carl Zeiss, Inc., Thornwood, NY) and a 16X objective. The top of the skin section is shown in *A* and the bottom of the same skin section is shown in *B*. A small portion of the photograph at the bottom of *A* and the top of *B* was duplicated for reference (white arrows denote distinguishing features of this duplicated portion). Note that the background level of staining is most easily seen in the dermal portions surrounding the sebaceous gland. Staining above background levels was only observed in the epidermis, the sebaceous gland and the outer root sheath cells of the follicle. With the exception of some of the highly polarized columnar cells along the left side of the follicle, staining was highest in the inner layers of the outer root sheath at the central portion of the follicle. Black arrowheads indicate anti-K14 staining cells within a single layer of ORS on the bulbar surface. Bar, 75 μm. (*Right*) Expression of K14 mRNA in the hair follicle. A serial section of human scalp was hybridized with ^{35}S-UTP labeled RNA complementary to (*a*) human K14 mRNA (shown) or (*b*) the noncoding portion of human K14 mRNA (not shown). A set of hybridization and wash conditions was chosen such that (*a*) there was no cross-hybridization of the radiolabeled anti-sense RNA with other non-K14 keratin mRNAs in the tissue sections, and (*b*) there was no hybridization of a radiolabeled sense-strand control RNA with any mRNAs in the tissue sections (see Materials and Methods). After hybridization, sections were washed and exposed to photographic emulsion for 3 d before developing. Hybridizing radiolabeled cRNAs were visualized as developed silver grains. Sections were assembled piecemeal from photographs as described above. Sections shown are in darkfield. White arrows are as described above. Note the abundance of K14 mRNA in the basal layer of the epidermis and in the innermost layer of the outer root sheath at the lower portion of the follicle. Further up the hair follicle, K14 mRNAs are more uniform throughout the outer root sheath. At the middle of the isthmus, there is a transition, where above this zone, K14 mRNA becomes localized in the outermost layer. Zones: for purpose of discussion in the text, the follicle was arbitrarily divided into zones *i–v*.

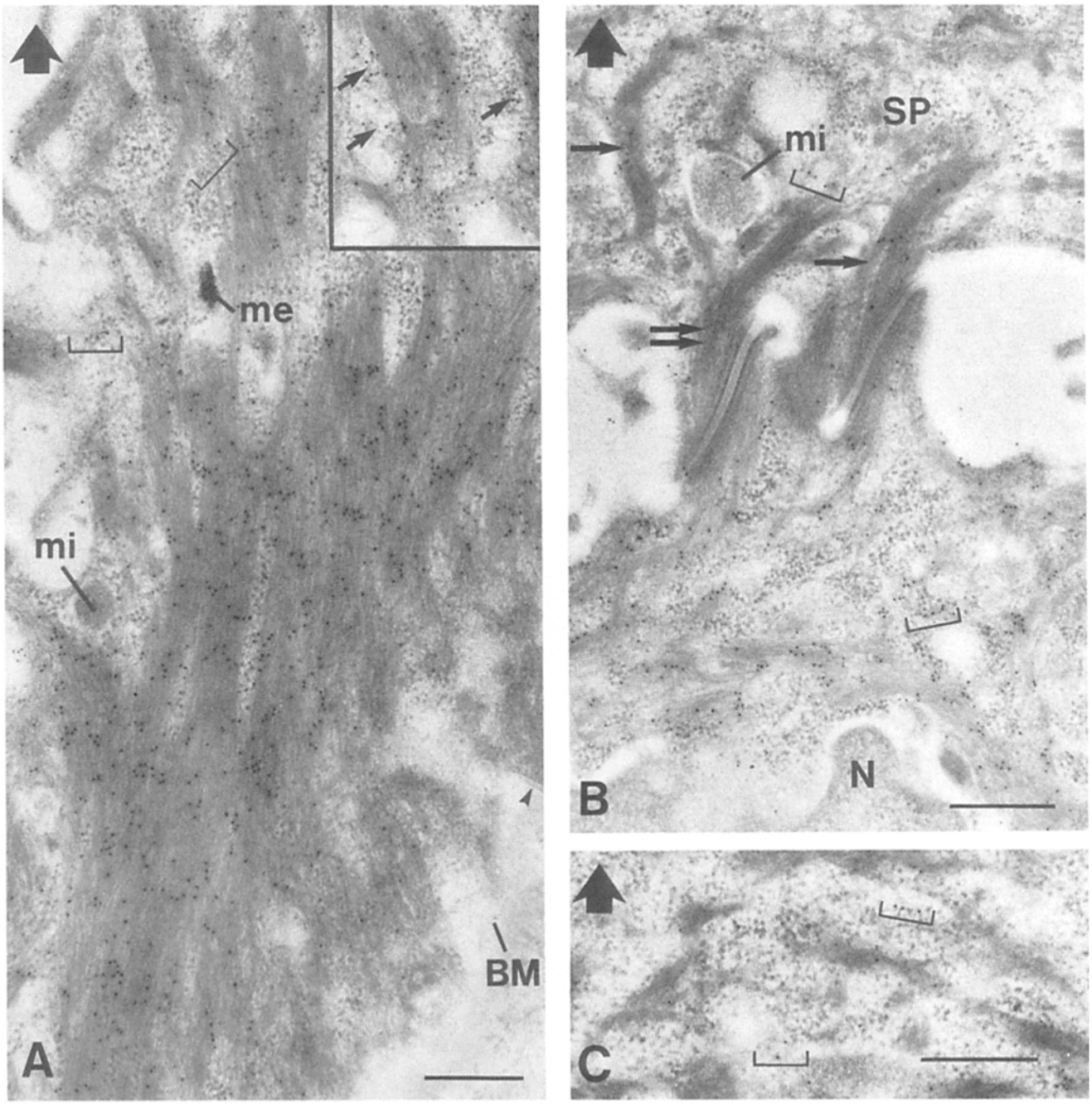


Figure 3. Immunoelectron microscopy of epidermal cells labeled with anti-K14. Human scalp epidermis was fixed (4% paraformaldehyde, 0.05% glutaraldehyde), embedded in Lowicryl K4M, and sectioned perpendicular to the skin surface. Sections were subjected to indirect immunoelectron microscopy, using anti-K14 antiserum to detect all K14 with exposed COOH-terminal ends, and 10 nm gold-conjugated IgG to visualize the bound anti-keratin antibodies. (*a*) Basal cell: brackets denote presence of labeling over polysomes, which are closely associated with the rough endoplasmic reticulum and with keratin filaments (see also the inset for closer inspection: labeling denoted by arrows). Note that the filaments near these labeled ribosomes are somewhat less bundled than elsewhere in the cytoplasm. *mi*, mitochondria; *me*, melanin granules; *BM*, basement membrane. Arrowhead in *a* points to a hemidesmosome, characteristic only of basal cells. (*b*) Transition zone between the basal layer and first spinous layer (*SP*) of the epidermis. Note the dramatic decrease in density with which tonofilaments are labeled between these two layers: double arrow denotes a bundle of keratin filaments which is devoid of labeling: single arrows denote filament bundles which are weakly labeled. Note also that the filament bundles are thicker in the spinous cells than in the basal and first suprabasal layer. Abbreviations are the same as in *b*. (*c*) Detail of the cytoplasm of a spinous cell depicting the persistence of labeling over polysomes (labeling indicated over the brackets). Bars, 0.5 μm. Thick arrow in each frame indicates direction of the skin surface.

did show labeling with a monospecific anti-K1 antiserum (data not shown), they did not label well with anti-K14 (Fig. 3 *B*: double arrows in *B* indicate absence of labeling in filament bundles; single arrows in *B* indicate low-level labeling). A priori, the markedly reduced level of anti-K14 labeling in the spinous layers might have been expected, because the K14 mRNA levels were much lower in spinous than in basal cells (see Fig. 2 *A*, *right*). However, epidermal keratins are very stable, and hence we expected that a significant amount of K14 protein might persist in the spinous layers. Since we detected only low levels of anti-K14 labeling in spinous cells, we suspect that either the K14 was processed or modified, or that the K14 carboxy terminus was masked so as to escape detection by the antiserum.

The difference in antibody labeling of keratin filaments was accompanied by a change in filament organization: the filaments of the spinous layers were more densely bundled than those of the basal layer. Many of these bundles appeared to be anchored to the numerous desmosomes, which interconnected all spinous and granular cells within the epidermal tissue (Fig. 3 *B*). Interestingly, the increase in filament bundling that took place during the transition from the basal to the spinous layer was consistent with a similar difference in filament bundling observed when K1 and K10, rather than K5 and K14, were used in in vitro filament assembly studies (Eichner et al., 1986). Whether other factors might also contribute to the in vivo change in filament organization remains to be determined. However, a strong parallel could be drawn between the relative stage of differentiation and the degree of filament bundling.

Localization of Nascent K14 Polypeptides by Immunoelectron Microscopy

The use of immunoelectron microscopy seemed to enable us to localize nascent K14 polypeptides as their synthesis was completed on polysomes. In the basal layer, although the intensity of labeling over polysomes was weaker than that over the keratin filament, it was nevertheless readily detectable (see arrows in Fig. 3 *A*, *inset*). Anti-K14 labeling over polysomes was even seen in the spinous layers, where overall anti-K14 labeling was markedly reduced (see gold particles over the brackets in *B* and *C*). Labeled polysomes were often associated with the rough endoplasmic reticulum, but no labeling was seen within the endoplasmic reticulum cisternae (see *C*). Labeling was also frequently associated with the presence of delicate (nascent?) bundles of keratin filaments, suggesting that keratins were assembled into filaments shortly after they were synthesized, and that organization of filaments into thicker bundles occurred after filament formation.

Interestingly, anti-K14 labeling of polysomes did not seem to be confined to any one discrete region within the cell. Labeling thus was seen near the nuclear envelope as well as the cell periphery and generally throughout the cytoplasm. If the anti-K14 labeling associated with polysomes reflected the location of nascent K14 synthesis, then the more uniform distribution of keratin synthesis in vivo differs from the apparent nuclear location of vimentin mRNAs in fibroblasts in vitro (Lawrence and Singer, 1986). Moreover, although our data are consistent with in vitro studies suggesting that keratin filament assembly initiates at the nuclear envelope and that filaments grow towards the cell periphery (Albers and Fuchs, 1987, 1989), they argue against the notion that all keratin subunits are added to growing filaments at a single discrete site, e.g., the nuclear envelope. Studies involving in situ localization of K14 mRNAs at the electron microscopy level will be necessary to confirm this notion.

Filament Organization and Expression of K14 in the Upper Portion of the Outer Root Sheath: Parallels with Epidermal Differentiation

Extending from the epidermis-follicle border to the center of the isthmus region between the arrector muscle and the sebaceous gland, the upper portion of the ORS showed a morphology that was similar to epidermis (Fig. 2 *A*, *left*: zone *i*). In this region, anti-K14 staining was only present in the ORS, and it was most prominent in the outermost layer of the outer root sheath (ORS), contiguous with the basal epidermal layer. K14 mRNAs were also most abundant in the outermost layer of the ORS, and the level was comparable to that in the basal epidermal layer (Fig. 2 *A*, *right*: zone *i*).

At the ultrastructural level, the outermost cells of the upper ORS contained loosely organized bundles of keratin filaments, and the bundles showed morphology and anti-K14 labeling similar to that seen in basal epidermal cells (Fig. 4 *A*). In addition, polysomes in the ORS cells showed labeling with anti-K14 antibodies, both near the nuclear envelope and elsewhere in the cytoplasm (see gold particles denoted by brackets in *A*). As expected, the nucleoplasm and other cellular organelles of the cells were negative for anti-K14 labeling.

In this upper region of the follicle, differentiating ORS cells move progressively inward towards the hair shaft, and perpendicular to the movement of terminally differentiating cells of the epidermis. Thus, cells in the middle of the ORS stemmed from the outermost layer, and they had a more elongated and flattened shape than the outermost layer (Fig. 4 *B*). Abundant cytoplasmic keratin filaments were present and were organized into dense bundles that were often associated with desmosomes (see vertical arrow in *B*). The filament bundles were markedly reduced in anti-K14 labeling (horizontal arrows in *B*), and K14 mRNA levels were also downregulated (Fig. 5 *A*). Hence, in many morphological and biochemical respects, these cells were similar to those of the spinous layers of the epidermis. However, whereas spinous epidermal cells expressed abundant levels of the terminal differentiation-specific keratins K1 and K10 (Fuchs and Green, 1980; Moll et al., 1982*a*; Sun et al., 1984; Roop et al., 1983), the keratin filaments in the inner layers of the upper ORS labeled only weakly with an anti-K1 antiserum (Coulombe, P. A., and E. Fuchs, unpublished observations). Moreover, whereas the normal epidermis does not seem to express K6 and K16 (Moll et al., 1982; Weiss et al., 1984; Tyner and Fuchs, 1986), the inner layers of the upper ORS expressed mRNAs encoding K6 (Kopan, R., and E. Fuchs, unpublished observations) and probably K16 (not investigated). These observations extend previous studies reporting the presence of these keratins in the ORS (Moll et al., 1982*b*, 1988; Lynch et al., 1986; Stark et al., 1987; Heid et al., 1988*a*,*b*).

In the upper follicle zone, the innermost cell layer (*IMC*) of the ORS had undergone terminal differentiation, as evidenced by the flattened appearance of the cells, the absence of a nucleus, the presence of keratohyalin granules (*KG*) in the cytoplasm, and the relative sparsity of cytoplasmic organelles (Fig. 4). Filaments were still evident in these cells, but the bundles were short, randomly oriented, and only

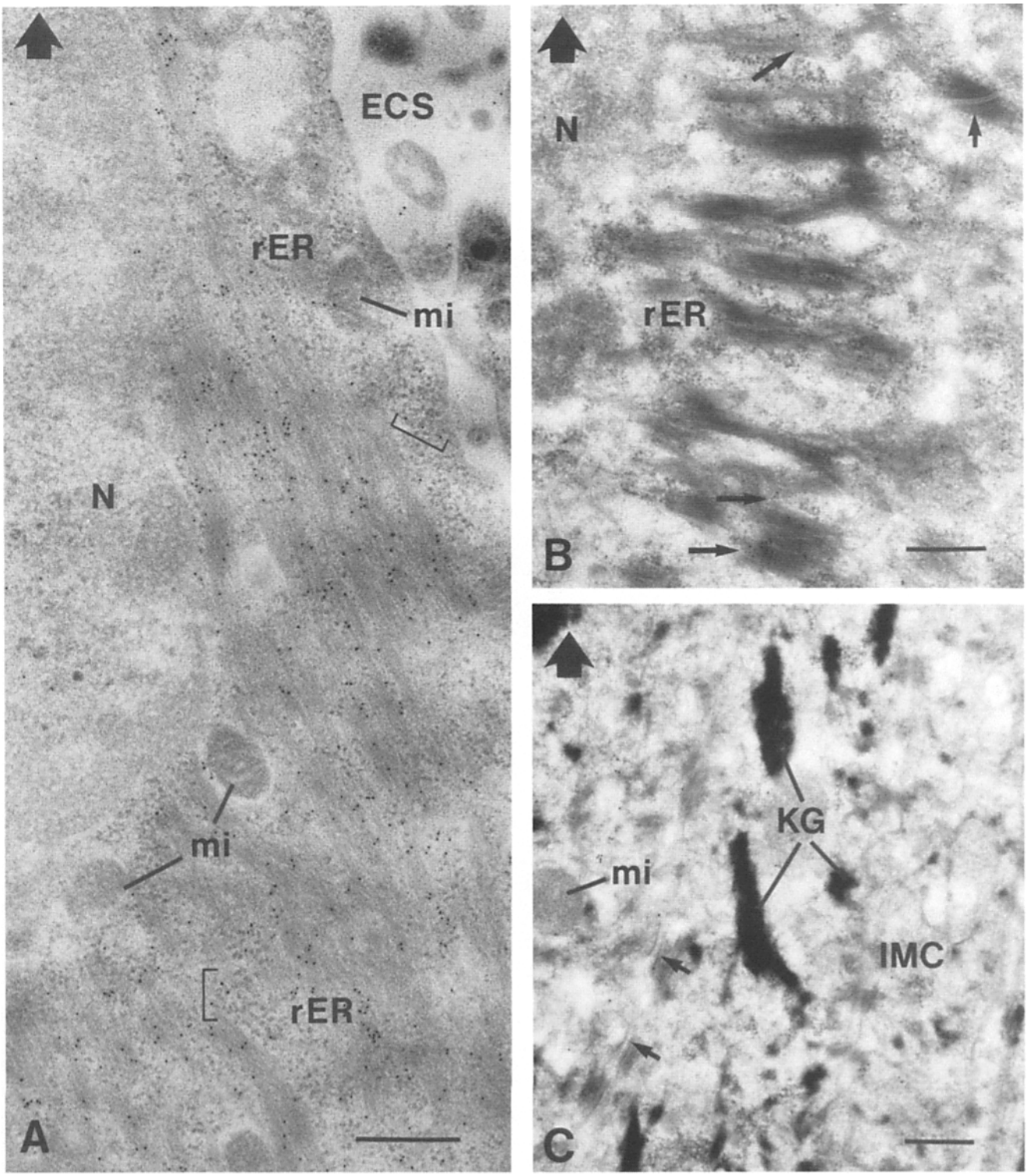


Figure 4. Immunoelectron microscopy of the ORS: upper portion above the isthmus. Human scalp tissue was fixed and embedded as described in the legend to Fig. 3, and then sectioned parallel to the main axis of the hair shaft. Sections were subjected to indirect immunoelectron microscopy, using anti-K14 antiserum to detect all K14 with exposed COOH-terminal ends, and 10 nm gold-conjugated IgG to visualize the bound anti-keratin antibodies. (*a*) Outer cell layer of the ORS: note the presence of abundant anti-K14-labeled keratin filaments in the cytoplasm, similar to what is seen in the basal layer of the epidermis. Note that labeled filaments extend from the cell periphery to the nuclear envelope. *N*, nucleus; *rER*, rough endoplasmic reticulum; *mi*, mitochondria; *ECS*, extracellular space. Brackets denote labeling over polysomes. (*b*) Middle portion of the ORS: vertical arrow points to desmosome; horizontal arrows point to weak anti-K14 labeling of the bundles of keratin filaments. (*c*) Innermost layers of the ORS: note the absence of a nucleus and the presence of keratohyalin granules (*KG*) in the cytoplasm, indicative of terminal differentiation in these cells. *IMC*, innermost layer of the outer root sheath; arrows indicate desmosomes that depict the border of an IMC cell with the rest of the ORS. Bars, 0.5 μm. Thick arrow on each frame points parallel to the main axis of the hair shaft.

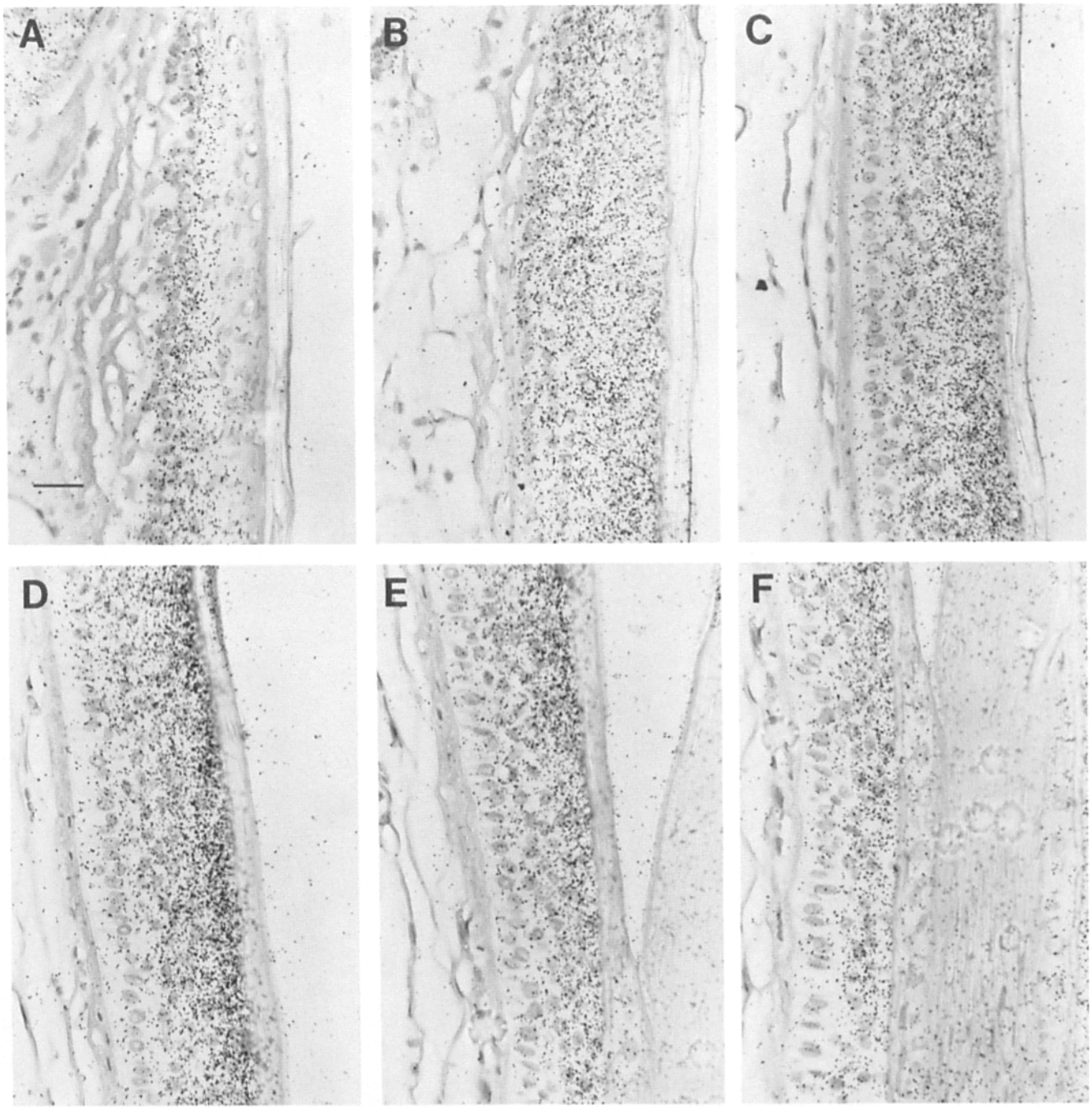


Figure 5. A closer inspection of K14 mRNA expression in the ORS. Serial sections of human scalp were hybridized with ^{35}S-UTP labeled RNA complementary to (shown) or identical to (not shown) human K14 mRNA as described in the legend to Fig. 2, *right*. Sections are of the outer root sheath and are shown in brightfield. Zones are assigned according to Fig. 2. (*A*) section from zone *i*: above the isthmus; (*B*) section from zone *ii*: just below the central portion of the isthmus; (*C*), section from zone *iii*: middle of the hair follicle; (*D*) section from zone *iv*: lower third of the follicle; (*E*) section just below zone *iv*: region where K14 mRNA expression is most specifically localized to the innermost layers of the ORS; (*F*) zone *v*, just above the hair bulb: region K14 mRNA expression first begins to show signs of elevation. Bar in *A*, 30 μm.

weakly labeled by gold particles. Overall, this morphology and biochemistry was similar to that seen in the upper (granular) layers of the epidermis.

Collectively, these data provide biochemical and morphological support for the notion that the upper portion of the outer root sheath, which is contiguous with the epidermis, shares many features characteristic of the epidermis: abundant K14 and K14 mRNA expression were characteristic of the outermost basal-like cells, and increased keratin filament bundling, the presence of keratohyalin granules, and loss of

the cell nucleus were observed in the inner, terminally differentiating cells.

Biochemical and Morphological Differences between the Upper and Lower Portions of the ORS

Although the ORS in the upper follicle region appeared to be similar to that of epidermis in its K14 and K14 mRNA expression patterns, a marked change in K14 expression was observed in the ORS near the center of the isthmus. Above this region, both anti-K14 staining (Fig. 2 *A*, *left*: zone *i*) and K14 cRNA hybridization (Fig. 5 *A*) were largely confined to the outermost layer. In the center of the isthmus, however, anti-K14 staining appeared weak and variable (Fig. 2 *A*, *left*: zone *ii*). This biochemical difference coincided with major morphological changes stemming from cells differentiating upward from the hair bulb: at the isthmus, the innermost layer of the ORS became fully differentiated, the keratinized IRS was shed into the hair canal, and the hair shaft became detached from its sheaths (see also Pinkus, 1958; Hashimoto, 1970; Montagna, 1976; Ito, 1986). Hence, it seemed that the disorganized morphology and variability of keratin expression in the isthmus resulted from a merging of differentiation programs in the upper part of the follicle with those of the lower part of the follicle.

Below the isthmus and extending to the region just above the follicle bulb, the ORS widened (see zones *iii* and *iv* in Figs. 2, *A* and *B*). Resting on the basement membrane surrounding the follicle, the outermost cells of the ORS became larger and their orientation was perpendicular to the axis of the hair shaft. In zone *iii* and in the upper half of zone *iv*, anti-K14 staining and K14 mRNAs seemed to be relatively evenly distributed throughout the ORS layers (Fig. 2 *A*). For K14 cRNA hybridization, this was most clearly seen at higher magnification (Fig. 5, *B* and *C*). Interestingly, the intensity of both anti-K14 staining and K14 cRNA hybridization suggested that K14 expression may be very high in this region and even exceed that which is present in the basal layer of the epidermis.

In the lower half of zone *iv*, K14 mRNA distribution changed markedly: K14 cRNA hybridization became most prominent in the innermost layer of the ORS (Fig. 2 *A* and *B*, *right*; see also Fig. 5, *D* and *E*). In this region, autoradiographic grains were barely above background in the outermost columnar cells. In many cases, the pattern of K14 mRNA expression was paralleled by anti-K14 staining patterns. Hence, many outer layer cells stained only weakly with anti-K14, while inner layer cells showed more prominent staining (see the right side of the follicle, zone *iv*, Fig. 2 *B*, *left*). In some cases, the anti-K14 staining pattern and cell morphology showed a peculiar asymmetry which was not reflected in the K14 mRNA pattern. For example, in the follicle shown in Fig. 2 *B* (zone *iv*), the outermost cells on the left side stained strongly with anti-K14, and they were highly columnar in shape, each with their nucleus eccentrically located at their apex (i.e., at the end opposite to the basement membrane). The functional significance of this asymmetry is unknown.

Overall, the biochemical differences in K14 expression between the upper and lower portions of the ORS suggested that the differentiation programs were markedly distinct. To investigate this in greater detail, we examined the lower region at the immunoelectron microscopy level. Fig. 6, *A–C*, illustrates a montage from the outer to the innermost layer of a single section of ORS in zone *iv* of the follicle. Given the ultrastructural morphology and the pattern of K14 labeling, the cells were from a region analagous to that of the right side of the follicle shown in Fig. 2 *B*, zone *iv*. *D* illustrates a columnar cell from the opposite side of the follicle. The outermost cells were oriented perpendicularly to the hair shaft and were larger than either basal epidermal cells or cells of the outermost layer of the upper ORS (*A* and *D*). Irrespective of whether the keratin filaments were more (*D*) or less (*A*) labeled with anti-K14 antibodies, filament bundles were less ordered and much thinner than in other basal cells. Cells that showed the weakest labeling with anti-K14 (*A*) also appeared to have the fewest filaments. Collectively, many of the outermost cells of this zone seemed to be less differentiated both in their morphology and in their biochemistry than either basal epidermal cells or outermost cells of the upper ORS.

The differentiating cells of the suprabasal layers of zone *iv* ORS shifted their axis to one which was parallel to the hair shaft (Fig. 6 *B*; see also Fig. 2 *B*, *left*). As in the epidermis, this change in orientation of the differentiating cells occurred within one to two cell layers from the basal layer. The orientation of keratin filaments had also shifted, so that they too were parallel to both the cell axis and the hair shaft. However, in this case, the filaments were organized into prominent dense bundles, which were intensely labeled with anti-K14 antibodies (Fig. 6 *B*). These bundles appeared to be similar, although somewhat tighter, than those seen in basal epidermal cells (compare with Fig. 3 *A*). Collectively, these data suggest that as the lower ORS cells differentiate, they pass through a stage where they display morphological and biochemical characteristics that are similar to those of the epidermal basal layer.

The finding that the columnar ORS cells seemed to be less differentiated than basal epidermal cells prompted us to expect that the process of differentiation in the lower portion of the ORS might be more delayed than in epidermis. Indeed, even though the nucleus (*N*) of each innermost cell was somewhat condensed, an early sign of keratinization, the cytoplasm did not contain keratohyalin granules (Fig. 6 *C*). Moreover, although the keratin filaments were organized into thicker bundles in the innermost layers, these bundles were clearly different from those seen in the terminally differentiating cells of the upper ORS and epidermis, and moreover, they were intensely labeled with anti-K14 antibodies (*C*). Hence, both morphologically and biochemically, the innermost layers of the ORS in the lower portion of the hair follicle did not appear to be as keratinized as those in the upper ORS.

Absence of K14 and K14 mRNA Expression in the Matrix

As evidenced by immunohistochemistry at the light microscopy level, anti-K14 staining was extremely low, if not absent, in most regions of the hair bulb (Fig. 2 *B*, *left*, zone *v*). Upon closer inspection of the bulbar region, where the ORS was only one to two layers thick, anti-K14 staining could be seen, but it was only present in a few cells (see black arrows in Fig. 2 *B*, *left*, zone *v*). When radiolabeled K14 cRNA was hybridized with hair follicle sections, similar results were obtained: silver grains were extremely low over most regions of the bulb and did not appear to be significantly greater than

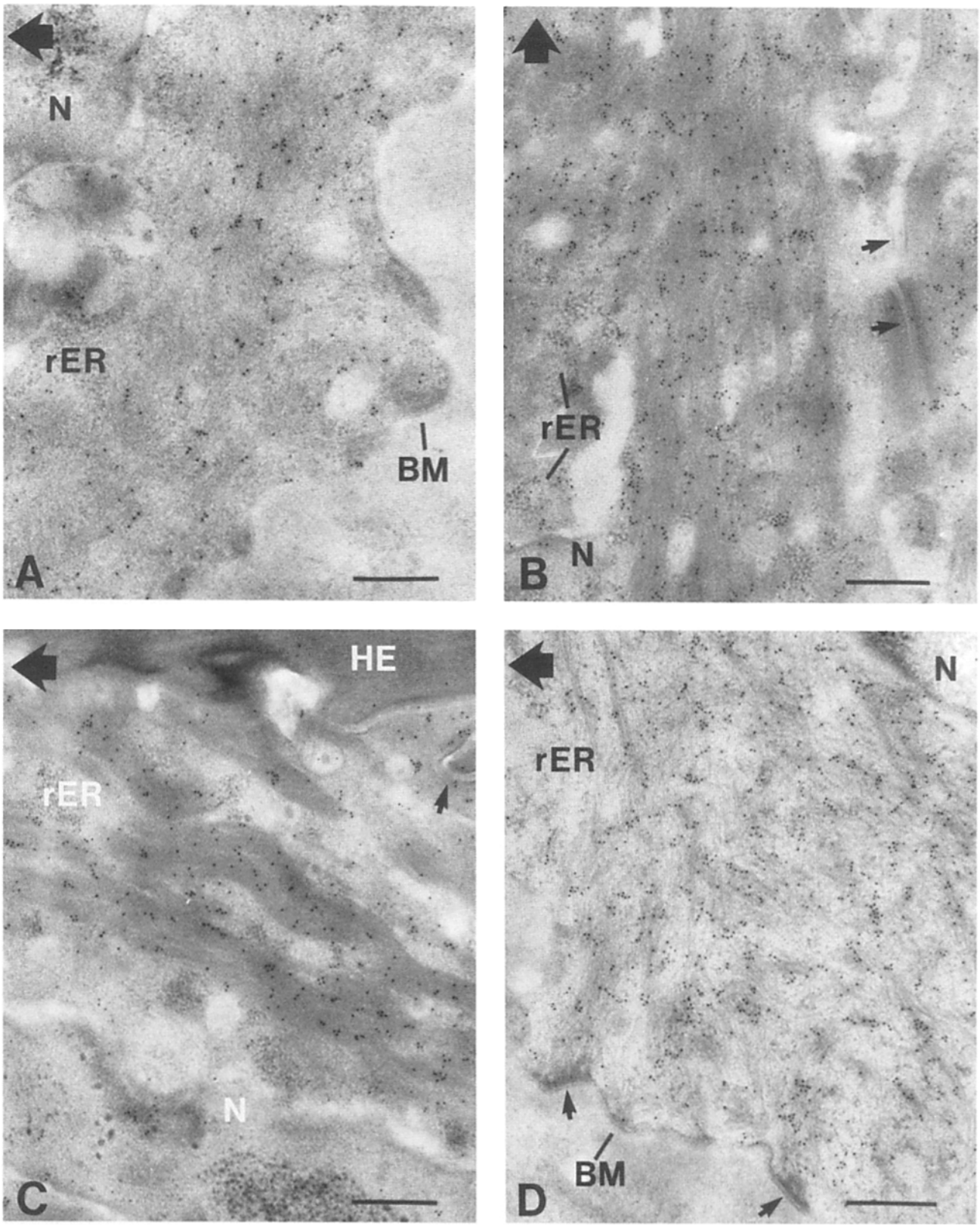


Figure 6. Immunoelectron microscopy of the ORS: lower portion, above the bulb. Human scalp sections (see legend to Fig. 4) were subjected to indirect immunoelectron microscopy, using anti-K14 antiserum to detect all K14 with exposed COOH-terminal ends, and 10 nm gold-conjugated IgG to visualize the bound anti-keratin antibodies. (*a*) outer columnar cell layer of the ORS: note the presence of "delicate" anti-K14 labeled keratin filaments, with only very low levels of bundling. *rER*, rough endoplasmic reticulum; *N*, nucleus; *BM*, basement membrane surrounding the hair follicle. (*b*) Middle portion of the ORS, several cells inward from the cell shown in *a*: note that these cells have a very dense cytoplasm filled with thick tonofilaments that are heavily labeled with gold particles. The cells as well as the filament bundles are parallel to the hair axis. Short arrows point to desmosomes. (*c*) Innermost layer of the ORS, several cells inward from the

background levels (Fig. 2 *B*, *right*, zone *v*). The grain levels in this region did not change when a radiolabeled control RNA corresponding to the sense strand, rather than the complementary strand, of the K14 mRNA was used for hybridization (not shown).

To investigate in more detail whether any low, albeit specific, labeling could be detected in the bulbar cells, we examined antikeratin labeling at the ultrastructural level. For these studies, we used both an anti-K14 antiserum and a general anti-type I keratin antiserum (Fig. 7). From the base of the follicle to approximately one third of the way up the hair bulb, the cells of the bulb appeared relatively undifferentiated, as evidenced by their high nucleocytoplasmic ratio as well as a paucity of cellular organelles in an otherwise very dense cytoplasm. In this lower region of the bulb, all of the cells appeared to be uniform and matrix-like: the IRS and hair shaft structures were not yet discernible, and the ORS progenitor cells on the bulb surface did not extend around the base of the bulb. Throughout this lower portion, no specific labeling by gold particles was detected, and there was no evidence of keratin filaments (Fig. 7 *A*). Even when an antiserum which is broadly crossreacting for many type I epidermal keratins (Fuchs and Marchuk, 1983; Kim et al., 1984), including hair-specific keratins (Kopan and Fuchs, 1989) was used for labeling, matrix cells (*MC*) adjacent to dermal papilla cells remained unlabeled (Fig. 7 *B*). Since our antikeratin labeling technique appeared to be sufficiently sensitive to detect the presence of keratins both in assembled filaments and in polysomes, and since we detected no specific labeling in matrix cells, it seems unlikely that K14 or any other type I epidermal keratins could have escaped our detection. These results confirm our immunohistochemical and in situ hybridization studies, and suggest that if type I epidermal keratins are present in matrix cells, their level of expression must either be extremely low, or their antigenic determinants must be highly masked.

While our studies are consistent with previous morphological studies (Birbeck and Mercer, 1957; Hashimoto and Shibazaki, 1975) and some biochemical studies (Moll et al., 1982*b*; French and Hewish, 1986; Lynch et al., 1986; Heid et al., 1986), they differ from some recent reports (Heid et al., 1988*a*; Moll et al., 1988). However, in reports claiming K14 expression in matrix cells, an mAb (KA1) was used in the identification. This antibody seems to be of questionable specificity (see, for example, Nagle et al., 1984 vs. Nagle et al., 1986). Hence, it seems most likely that the matrix staining pattern which Heid and co-workers observed was due to the presence of some protein other than K14. Whether this protein was a different keratin, or a nonkeratin protein which shared an antigenic determinant with one of the keratins remains unknown.

Keratin Expression in the Bulbar Region of the Follicle: Differentiation of Matrix Cells into IRS

Above the lower third of the hair bulb, definite signs of matrix cell differentiation were apparent (see Fig. 2 *B*, *left*, zone *v*). In the initial phases of differentiation, the two layers of the inner root sheath formed. Cells of the innermost of these layers marked the formation of the Huxley's layer of the IRS, as evidenced at the ultrastructural level by the appearance of small trichohyalin granules in the cytoplasm (not shown). Surrounding this layer was the Henle's layer of the IRS: at this stage, the cells were poorly differentiated, and displayed few distinguishing morphological features. At approximately the midregion of the bulb, both layers of the developing inner root sheath began to differentiate, a process which proceeded much more quickly than that of the outer root sheath (see also Hashimoto, 1970; Montagna, 1976; Pinkus et al., 1981; Ito, 1986).

Above the midregion of the bulb, filamentous structures were readily seen in the cytoplasm of both developing IRS layers. These filaments were negative for anti-K14 staining (not shown), but were stained weakly with a general anti-type I epidermal keratin antiserum (Fig. 7 *C*). Hence, although matrix cells showed no signs of keratin expression, the IRS cells synthesized keratin and made keratin filaments soon after their morphological programs of differentiation were initiated.

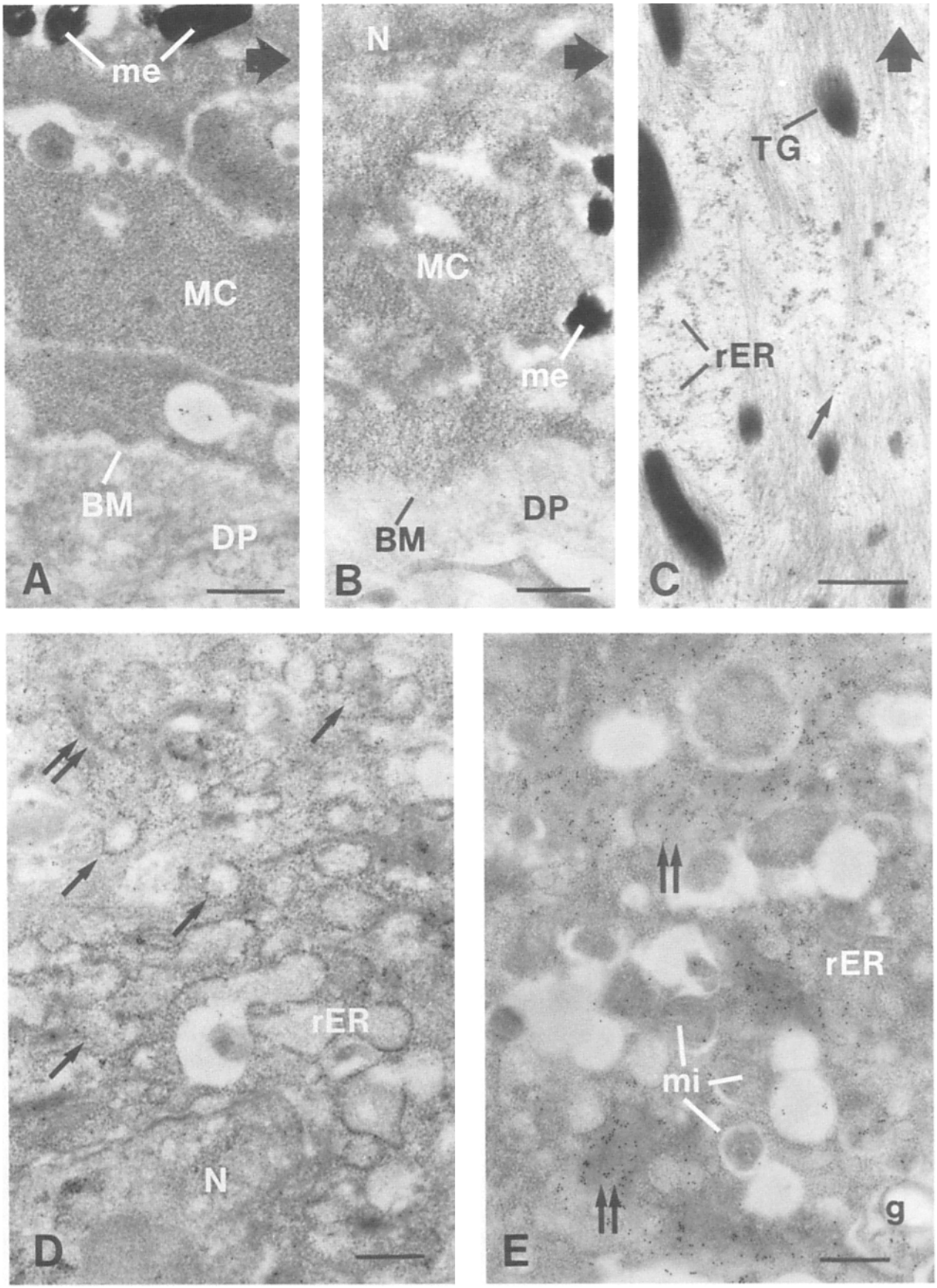
K14 and K14 mRNA Expression in Progenitor ORS Cells

ORS cells differ from those of the IRS in that they do not appear to arise from matrix cells and they maintain their contact with a basement membrane (Hashimoto and Shibazaki, 1975; Montagna, 1976; Pinkus et al., 1981; Ito, 1986). The earliest signs of differentiation in the progenitor cells of the ORS could be seen in a single layer of cells on the bulbar surface between the lower third and midregion of the bulb (see Fig. 2 *B*, *left*, zone *v*). At the light microscopy level, it was evident that these cells stained weakly with anti-K14 antiserum (see cells identified by black arrows). At the ultrastructural level, it was apparent that these cells contained an enlarged cytoplasm, a prominent rough endoplasmic reticulum network, and the presence of weak, but detectable, anti-K14 labeling (Fig. 7 *D*). At this early stage, however, only a few discernable keratin filaments could be detected (*double arrow*), and much of the labeling was found over polysomes (*single arrows*). Slightly higher in the bulb, where the ORS became bilayered, filaments were visible and were labeled with anti-K14 antibodies (*double arrows* in *E*). These keratin filaments had a very delicate appearance somewhat similar to that seen in the more differentiated columnar cells above them (see Fig. 6 *A*). However, there seemed to be fewer keratin filaments in the bulbar ORS cells than in the columnar ORS cells just above the bulb.

Keratin Expression in Cortex and Hair

When cells of the cortex or hair shaft were examined at either the light or immunoelectron microscopy levels for the pres-

cell shown in *b*: this cell layer is more flattened compared with the middle region of the ORS; the nuclei (*N*) show some signs of chromatin condensation, characteristic of terminal differentiation. Note also that the tonofilament bundles are heavily labeled with anti-K14. *HE*, Henle's layer of the inner root sheath (note absence of labeling). (*d*) Outer columnar cell layer of the ORS, on the side of the shaft opposite to that of the cell shown in *a*: note the presence of more abundant and more heavily labeled filaments than those seen in *a*. The filaments are still delicate, and in thin bundles, similar to those in *a*, but unlike those in *b* and *c*. Short arrows point to hemidesmosomes. Bars, 0.5 μm. Thick arrow on each frame points parallel to the direction of the main axis of the hair shaft.


me
MC
BM
DP
A
N
MC
me
BM
DP
B
TG
rER
C
rER
N
D
rER
mi
g
E

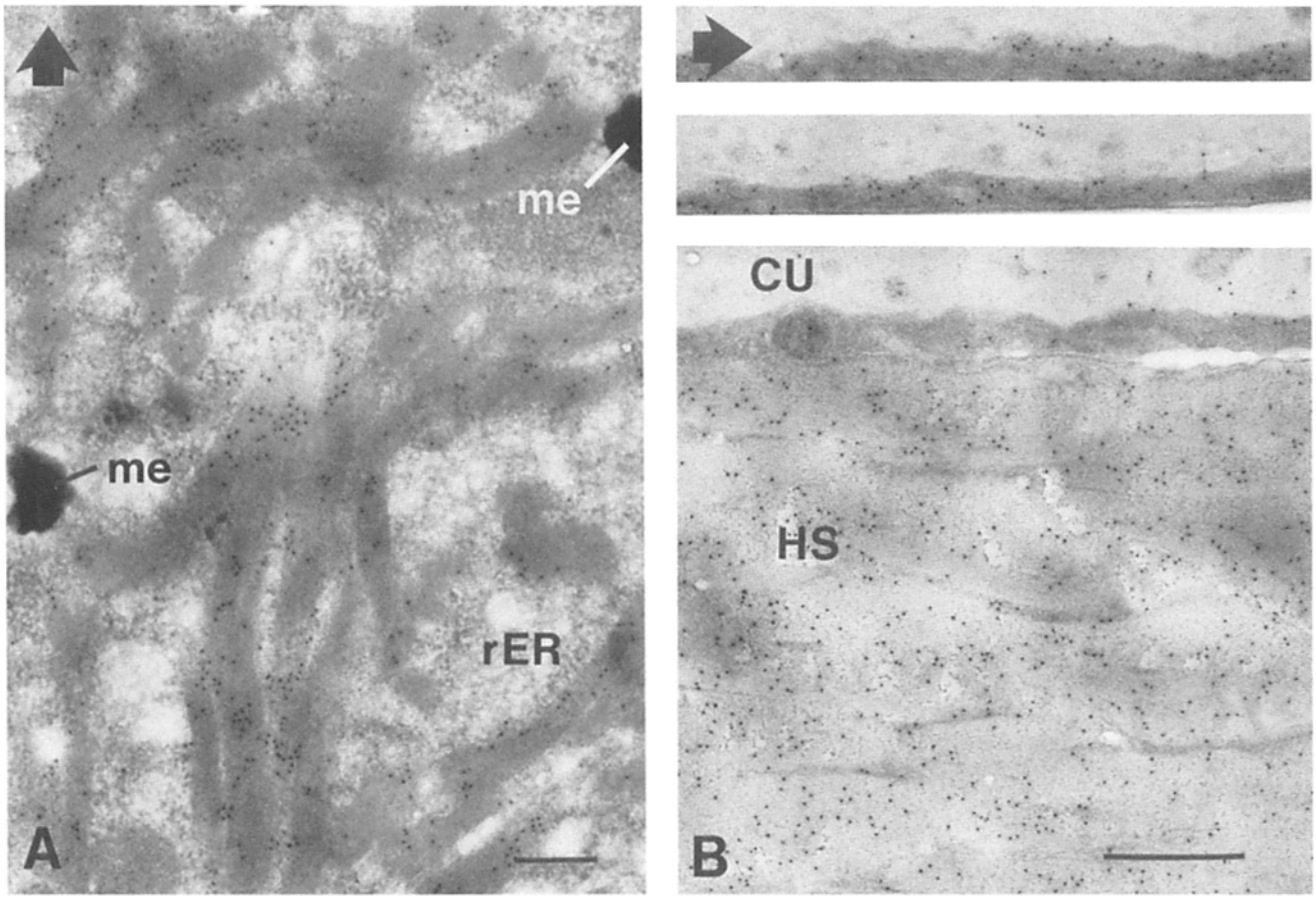


Figure 8. Immunoelectron microscopy of anti-type I keratin labeling of the cortex and hair shaft. Human scalp sections (see legend to Fig. 4) were subjected to indirect immunoelectron microscopy, using a general anti-type I epidermal keratin antiserum to detect keratin and 10 nm gold particle-bound IgG to visualize the bound anti-keratin antibodies. This antiserum has been previously shown to cross-react with hair-specific type I keratins (Kopan and Fuchs, 1989). (*a*) cortex at the upper-level of the hair bulb. Note the dense bundles of keratin filaments, heavily labeled with the anti-type I keratin antibody. *me*, melanin granules; *rER*, rough endoplasmic reticulum. (*b*) hair shaft (*HS*) in the lower portion of the follicle, above the bulb, and hair cuticle (*CU*). Note the polarized labeling over the cuticle cells: only the electron dense halves are labeled. Bars, 0.25 μm in *a* and 0.5 μm in *b*. Thick arrow on each frame indicates direction of the main axis of the hair shaft.

ence of K14, we never detected labeling above background. These observations further strengthened the notion that the basal epidermal keratins are not present in the differentiating cells of the hair (Lynch et al., 1986; Heid et al., 1986, 1988*a*,*b*; Moll et al., 1988). Previous studies using in situ hybridization of a radiolabeled hair-specific cRNA probe with a section of adult rat skin demonstrated that hair-specific keratin mRNAs are first made in the cortex cells, located in the upper bulb region of the follicle (Kopan and Fuchs, 1989). These studies were in agreement with immunofluorescent data involving the use of mAbs to hair-specific keratins (Lynch et al., 1986; Heid et al., 1988*a*,*b*; Moll et al., 1988).

To examine the keratin filaments at the immunoelectron microscopy level, we used our general anti-type I keratin antiserum, which we had previously shown to cross-react with the hair-specific keratins (Kopan and Fuchs, 1989). The earliest stage at which keratin filaments are morphologically visible in the cytoplasm of differentiating cortex cells is in the upper bulbar portion of the hair follicle. Although our

Figure 7. Immunoelectron microscopy of the hair bulb. Human scalp sections (see legend to Fig. 4) were subjected to indirect immunoelectron microscopy, using either anti-K14 antiserum (*A*, *D*, and *E*) or anti-type I keratin antiserum (*B* and *C*) followed by 10 nm gold particle-bound IgG to visualize the bound anti-keratin antibodies. *a*, dermal papilla cell (*DP*) and matrix cell (*MC*) at approximately the midlevel of the hair bulb: note the absence of anti-K14 labeling. *me*, melanin granules; *BM*, basement membrane. (*b*) same region as *a*, but labeled with a general anti-type I epidermal antiserum. *N*, nucleus. All other abbreviations as in *a*. (*c*) Huxley cell, just above the midlevel of the bulb, where the two IRS layers begin to develop: note the presence of 10-nm filaments that show weak labeling (*arrow*) with a general anti-type I keratin antiserum (shown), and no labeling with anti-K14 (not shown). *TG*, trichohyalin granule characteristic of the IRS; *rER*, rough endoplasmic reticulum. (*d*) ORS, single-layer stage, mid-level of the bulb: note that labeling is mostly associated with polysomes (*single arrows*), but the delicate filament bundles (nascent keratin filaments?) are also labeled by gold particles (*double arrows*). (*e*) ORS, two-layer stage, just above the midlevel of the bulb. These cells contain more prominent filament bundles, which while still delicate, are densely labeled by gold particles (*double arrows*). *mi*, mitochondria; *g*, Golgi apparatus. Bars, 0.5 μm. Thick arrow on *A–C* points parallel to the direction of the main axis of the hair shaft.

anti-K14 antiserum showed no evidence of labeling these cells (not shown), the anti-type I keratin antiserum showed prominent labeling of the filament bundles (Fig. 8 *A*). Both the enhanced anti-type I keratin labeling of these filament bundles and the organization of filaments suggested that the filaments in the cortex were much different from those in the early differentiating IRS cells (compare Fig. 8 *A* with Fig. 7 *C*). Labeling extended all the way to the hair shaft, where abundant gold particles were seen over the densely packed keratin arrays (*HS*, Fig. 8 *B*). Interestingly, in each cuticle cell (*CU*) of the hair, the densely packed keratin filaments appeared to be localized in that half of the cell which was closest to the central hair shaft. Collectively, these data indicate that despite the apparent absence of keratin filaments in undifferentiated matrix cells, the differentiating matrix cells (i.e., cortex) leading to hair production showed abundant keratin expression and filament organization that seemed to be distinct from that in differentiating IRS cells.

Discussion

Two Distinct Programs of Differentiation in the ORS

Previous studies have demonstrated similarities between the epidermis and the ORS: both cell types express K5 and K14 (Moll et al., 1982; Lynch et al., 1986; Heid et al., 1986) and both cells behave in a highly similar fashion when cultured in vitro under conditions that permit epidermal differentiation (Lenoir et al., 1988). However, the ORS differs from the epidermis in that it expresses K6, K16, K17, and K19 (Bartek et al., 1986; Stark et al., 1987; Heid et al., 1988*a*; Moll et al., 1988). Moreover, although K1 and K10 expression is high in the differentiating cells of the epidermis, their expression is extremely low in the ORS (Moll et al., 1988). Our data have extended these earlier studies, and demonstrated that even in the upper portion of the ORS, where K14 mRNA and K14 protein expression paralleled that of the epidermis, and where K1-containing keratin filaments were found in the differentiating cells of the ORS, the levels of K1 expression were distinctly lower than that in the epidermis, and this appeared to be compensated for by the presence of K6 in the differentiating cells.

Our data have also uncovered distinct biochemical and morphological differences between the cells in the upper and lower portions of the ORS. Below the transition zone of the isthmus, the pattern of K14 and K14 mRNA expression bore little resemblance to that observed in the epidermis: K14 and K14 mRNA expression was low in all of the progenitor (bulbar) and most of the columnar cells of this part of the ORS, and inward modes of differentiation gave rise to increased levels of these keratins, rather than decreased levels as is typically associated with the epidermis and with other stratified squamous epithelia. We cannot account for these differences on the basis of cross-reactivity between the anti-K14 antiserum or K14 cRNA probes and other type I keratins or keratin mRNAs, respectively, of the ORS. As judged by immunoblot analyses, anti-K14 does not cross-react with any of the other ORS keratins (Stoler et al., 1988) and moreover, the K14 carboxy-terminal sequence used to make this antiserum is not shared by any keratin sequences that have thus far been reported. Similarly, as judged by in situ hybridization, the near full-length K14 cRNA probe does not show cross-hybridization with the closely related K16 mRNA (Stoler et al., 1988). Moreover, the 3′ noncoding K14 cRNA probe shares no significant homology with other ORS keratin mRNAs (Hanukoglu and Fuchs, 1983; Rosenberg et al., 1988; Lersch and Fuchs, 1988; Stasiak et al., 1989) and yet its pattern of expression was analogous to that of the full-length K14 probe. Hence, it seems that the program of K14 and K14 mRNA expression in the lower region of the ORS is truly different from that in the upper ORS.

Our immunoelectron microscopy studies have suggested a possible basis for why a difference in differentiation programs might exist between the lower ORS and the upper ORS and epidermis, even though all mitotically active ORS and epidermal cells seem to give rise to similar structures when placed in the same environment, e.g., in tissue culture (Lenoir et al., 1988). Indeed, the organization of K14-containing filaments in the lower ORS indicated that many of its basal-like cells may be less mature than basal epidermal cells: the lower ORS basal cells contained filaments that were generally more delicate and less bundled in appearance than basal epidermal or upper ORS basal cells. In fact, for all epithelial cells examined in our studies, we consistently found an increase in filament bundling associated with differentiation. Hence, it seems possible that if these lower ORS cells are merely less differentiated than basal epidermal cells, they could become indistinguishable when placed in a common and appropriate environment.

Although this hypothesis seems an attractive one, it does not explain why the inner layer cells of the ORS in this region showed increased K14 mRNA expression concomitantly with nuclear condensation, features that are mutually exclusive in terminally differentiating epidermal cells. At the moment, we can only suggest that if indeed, the progenitors of the lower ORS are merely less differentiated counterparts to the basal epidermal cells, that some component of their environment must be directing these cells through a different morphological and biochemical program of differentiation than that which occurs in the epidermis. Further experiments will be necessary to explore these possibilities.

Does the Level of K14 and K14 mRNA in Dividing Stratified Squamous Epithelial Cells Reflect Their Relative Degree of Differentiation?

In this study, we discovered differences in the level of filament bundling and the expression of K14 and K14 mRNAs between the mitotically active cells of the epidermis and hair follicle. Interestingly, a parallel can be drawn between the patterns of K14/K14 mRNA and differentiation in adult skin and the patterns of K14/K14 mRNA and differentiation during embryonic development of skin. During development, embryonic basal cells are the first cells of the skin to express K14, and they express it at a very low level (Kopan and Fuchs, 1989). At this stage, cells seem to remain pluripotent. Concomitant with determination, these cells give rise to either the basal epidermal layer, with subsequent elevation of K14 expression, or matrix cells, with subsequent downregulation of K14 expression (Kopan and Fuchs, 1989). In some respects, some of the progenitor/columnar ORS cells might be similar to embryonic basal cells. They not only showed few and delicate keratin filaments and expressed low levels of K14 and K14 mRNA, but they also seem to be pluripotent: the progenitor ORS cells must fully regenerate the ORS during hair cycling, and these ORS cells are also recruited to

regenerate the epidermis during wound healing (Weiss et al., 1984; Demarchez et al., 1986; Mansbridge and Knapp, 1987; Lenoir et al., 1988). Hence, at least a subpopulation of ORS cells may be less differentiated than their basal epidermal neighbors, which conversely do not seem to have the capacity to differentiate into either ORS cells, or into any other epidermal appendages.

If the levels of filament bundling and K14 and K14 mRNA expression reflect the degree of differentiation of mitotically active populations of stratified squamous epithelial cells, then this might also explain why we did not detect K14/K14 mRNA or keratin filaments in matrix cells: these cells can undergo periods of extreme mitotic activity, and they can partake in multiple pathways of differentiation, leading to the development of the Henle's and Huxley's layers of the inner root sheath, the IRS cuticle, the hair shaft cuticle, the cortex and the medulla. Hence, matrix cells have even more options than basal epidermal or ORS cells, and in this regard, are less differentiated.

Finally, it is interesting to note that basal cell carcinomas tend to express significantly lower levels of K14 and K14 mRNAs than normal basal epidermal cells (Stoler et al., 1988; Roop et al., 1988). Correspondingly, these cells have also been found to spontaneously undergo programs of differentiation characteristic of other epidermal appendages, including sebaceous gland differentiation and follicular differentiation (Tozawa and Ackerman, 1987; Carter, 1988). Thus, collectively, there seems to be a marked correlation between the level of expression of the so-called basal-specific keratins and the degree to which the mitotically active populations of stratified squamous epithelial cells can proliferate and choose among different programs of differentiation.

We do not yet know the molecular mechanisms that operate to regulate the levels of K14 and K14 mRNA in the epithelial cells of the skin. Previously, we demonstrated that suppression of K14 expression in developing hair follicle cells seems to arise from direct contact of embryonic basal cells with a specialized mesenchymal component of the follicle, the dermal papilla cells (Kopan and Fuchs, 1989). Although interactions with dermal papilla cells seem to play a role, at least in early stages of development, the variety of K14 expression patterns observed in this study indicates that the regulation of K14 is likely to be more complex than this. Recently, we showed that a construct containing ~2.5 kb of 5′ upstream sequence corresponding to a human K14 gene was properly regulated in the epidermis of a transgenic mouse tail (Vassar et al., 1989). As additional molecular and morphological studies are conducted, the sequences involved in the regulation of this keratin gene and the factors that influence its expression should help to uncover some of the mysteries still remaining.

We would like to extend a special thank you to Dr. Moise Bendayan (University of Montreal, Montreal, Canada) for his helpful advice, and to Ms. Diane Gingras (University of Montreal, Montreal, Canada) for help in preparing the thin sections of human skin which were used in our immunoelectron microscopy studies. We thank Dr. Robert Josephs (The University of Chicago) for use of his electron microscope, and Gerry Grofman for his assistance in preparing micrograph prints. We thank Ms. Linda Foster for her valuable assistance in sectioning of tissues used for light microscopy and in situ hybridizations. Finally, we thank Mr. Philip Galiga for his artful presentation of our data.

This research is supported by a grant from the Howard Hughes Medical Institute. P. A. Coulombe is the recipient of a Centennial Research Fellowship funded by the Medical Research Council of Canada. R. Kopan is the recipient of a William Rainey Harper Fellowship for graduate study.

Received for publication 30 May 1989 and in revised form 18 July 1989.

References

Albers, K., and E. Fuchs. 1987. The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. *J. Cell Biol.* 105:791–806.

Albers, K., and E. Fuchs. 1989. Expression of mutant keratin cDNAs in epithelial cells reveals possible mechanisms for initiation and assembly of intermediate filaments. *J. Cell Biol.* 108:1477–1493.

Baden, H. P. Characterization of hair keratins. 1981. *In* Hair Research. C. E. Orfanos, W. Montagna, and G. Stuttgen, editors. Springer-Verlag, Heidelberg, FRG. 73–75.

Bartek, J., J. Barkova, J. Taylor-Papadimitriou, A. Rejthar, J. Kovarik, Z. Lukas, and B. Vojtesek. 1986. Differential expression of keratin 19 in normal human epithelial tissues revealed by monospecific monoclonal antibodies. *Histochem. J.* 18:565–575.

Bendayan, M. 1984. Protein A-gold electron microscopic immunocytochemistry: methods, applications and limitations. *J. Electron Microsc. Tech.* 1:243–270.

Birbeck, M. S. C., and E. H. Mercer. 1957. The electron microscopy of the human hair follicle. I. Introduction and the hair cortex. *J. Biophys. Biochem. Cytol.* 3:203–214.

Bullough, W. S., and E. B. Laurence. 1958. The mitotic activity of the follicle. *In* The Biology of Hair Growth. W. Montagna and R. A. Ellis, editors. Academic Press, New York. 171–186.

Carter, D. M. 1988. Basal Cell Carcinomas. *In* Dermatology in General Medicine. T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg, and K. F. Austen, editors. McGraw-Hill Book Co., New York. 759–765.

Chapman, R. E., A. M. Downes, and P. A. Wilson. 1980. Migration and keratinization of cells in wool follicles. *Austr. J. Biol. Sci.* 33:587–603.

Clausen, O. P. F., E. Thorud, and K. Elgjo. 1982. Epidermal proliferation characteristics are similar in the pilary canal of mouse hair follicles and in interfollicular epidermis. *Virchows Arch. B.* 39:259–266.

Cox, K. H., D. V. DeLeon, L. M. Angerer, and R. C. Angerer. 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev. Biol.* 485–502.

Crewther, W. G., L. M. Dowling, K. H. Gough, R. C. Marshall, and L. G. Sparrow. 1980. The microfibrillar proteins of alpha-keratin. *In* Fibrous Proteins: Scientific, Industrial and Medical Aspects, Volume 2. D. A. D. Parry and L. K. Creamer, editors. Academic Press, New York. 151–159.

Crewther, W. G., L. M. Dowling, D. A. D. Parry, and P. M. Steinert. 1983. The structure of intermediate filaments. *Int. J. Biol. Macromol.* 5:267–282.

Demarchez, M., P. Sengel, and M. Prunieras. 1986. Wound healing of human skin transplanted onto the nude mouse. *Dev. Biol.* 113:90–96.

Eichner, R., P. Bonitz, and T. -T. Sun. 1984. Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression. *J. Cell Biol.* 98:1388–1396.

Eichner, R., T. -T. Sun, and U. Aebi. 1986. The role of keratin subfamilies and keratin pairs in the formation of human epidermal intermediate filaments. *J. Cell Biol.* 102:1767–1777.

French, P. W., and D. R. Hewish. 1986. Localization of low-sulfur keratin proteins in the wool follicle using monoclonal antibodies. *J. Cell Biol.* 102:1412–1418.

Fuchs, E., and H. Green. 1980. Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell.* 19:1033–1042.

Fuchs, E., and D. Marchuk. 1983. Type I and type II keratins have evolved from lower eukaryotes to form the epidermal intermediate filaments in mammalian skin. *Proc. Natl. Acad. Sci. USA.* 80:5857–5861.

Fuchs, E., S. M. Coppock, H. Green, and D. W. Cleveland. 1981. Two distinct classes of keratin genes and their evolutionary significance. *Cell.* 27:75–84.

Fuchs, E., A. L. Tyner, G. J. Giudice, D. Marchuk, A. RayChaudhury, and M. Rosenberg. 1987. The human keratin genes and their differential expression. *Curr. Top. Dev. Biol.* 22:5–34.

Hanukoglu, I., and E. Fuchs. 1982. The cDNA sequence of a human epidermal keratin: divergence of sequence but conservation of structure among intermediate filament proteins. *Cell.* 31:243–252.

Hashimoto, K. 1970. The ultrastructure of the skin of human embryos. IX. Formation of the hair cone and intraepidermal hair canal. *Arch. Klin. Exp. Dermatol.* 238:333–345.

Hashimoto, K., and S. Shibazaki. 1975. Ultrastructural study on differentiation and function of hair. *In* Biology and Disease of the Hair. T. Kobori and W. Montagna, editors. University Park Press, Baltimore, MD/Tokyo. pp. 23–58.

Heid, H. W., I. Moll, and W. W. Franke. 1988*a*. Patterns of expression of trichocytic and epithelial cytokeratins in mammalian tissue. I. Human and bovine hair follicle. *Differentiation.* 37:137–157.

Heid, H. W., I. Moll, and W. W. Franke. 1988*b*. Patterns of expression of trichocytic and epithelial cytokeratins in mammalian tissues. II. Concomitant and mutually exclusive synthesis of trichocytic and epithelial cytokeratins in diverse human and bovine tissues (hair follicle, nail bed and matrix, lingual papilla, thymic reticulum). *Differentiation.* 37:215–230.

Heid, H. W., E. Werner, and W. W. Franke. 1986. The complement of native alpha-keratin polypeptides of hair-forming cells: a subset of eight polypeptides that differ from epithelial cytokeratins. *Differentiation.* 32:101–119.

Ito, M. 1986. The innermost cell layer of the outer root sheath in anagen hair follicle: Light and electron microscopic study. *Arch. Dermatol. Res.* 279:112–119.

Ito, M., T. Tazawa, N. Shimizu, K. Ito, K. Katsuumi, Y. Sato, and K. Hashimoto. 1986. Cell differentiation in human anagen hair and hair follicles studied with anti-hair keratin monoclonal antibodies. 1986. *J. Invest. Dermatol.* 86:563–569.

Kim, K. H., F. Schwartz, and E. Fuchs. 1984. Differences in keratin synthesis between normal epithelial cells and squamous cell carcinomas are mediated by vitamin A. *Proc. Natl. Acad. Sci. USA.* 81:4280–4284.

Kopan, R., and E. Fuchs. 1989. A new look into an old problem: keratins as tools to investigate determination, morphogenesis and differentiation in skin. *Genes & Dev.* 3:1–15.

Kopan, R., G. Traska, and E. Fuchs. 1987. Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. *J. Cell Biol.* 105:427–440.

Lane, E. B., J. Bartek, P. E. Purkis, and I. M. Leigh. 1985. Keratin antigens in differentiating skin. *Annu. NY Acad. Sci.* 455:241–258.

Lawrence, J. B., and R. H. Singer. 1986. Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell.* 45:407–415.

Lenoir, M. -C., B. A. Bernard, G. Pautrat, M. Darmon, and B. Shroot. 1988. Outer root sheath cells of human hair follicle are able to regenerate a fully differentiated epidermis in vitro. *Dev. Biol.* 130:610–620.

Lynch, M. H., W. M. O'Guin, C. Hardy, L. Mak, and T. -T. Sun. 1986. Acidic and basic hair/nail ("hard") keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to "soft" keratins. *J. Cell Biol.* 103:2593–2606.

Mansbridge, J. N., and A. M. Knapp. 1987. Changes in keratinocyte maturation during wound healing. *J. Invest. Dermatol.* 89:253–262.

Marchuk, D., S. McCrohon, and E. Fuchs. 1984. Remarkable conservation among intermediate filament genes. *Cell.* 39:491–498.

Moll, R., W. Franke, D. Schiller, B. Geiger, and R. Krepler. 1982*a*. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell.* 31:11–24.

Moll, R., H. W. Heid, W. W. Franke, and R. Moll. 1988. Patterns of expression of trichocytic and epithelial cytokeratins in mammalian tissues. III. Hair and nail formation during human fetal development. *Differentiation.* 39: 167–184.

Moll, R., I. Moll, and W. Wiest. 1982*b*. Changes in the pattern of cytokeratin polypeptides in epidermis and hair follicles during skin development in human fetuses. *Differentiation.* 23:170–178.

Montagna, W. 1976. General review of the anatomy, growth and development of hair in man. *In* Biology and Disease of Hair. T. Kobori and W. Montagna, editors. University Park Press, Baltimore, MD/Tokyo. pp xxi-xxxi.

Nagle, R. B., W. Bocker, J. R. Davis, H. W. Heid, M. Kaufmann, D. Lucas, and E. D. Jarasch. 1986. Characterization of breast carcinomas by two monoclonal antibodies distinguishing myoepithelial from luminal epithelial cells. *J. Histochem.* 34:869–888.

Nagle, R. B., D. O. Lucas, K. M. McDaniel, V. A. Clark, and G. M. Schmalzel. 1984. New evidence linking mammary and extramammary Paget cells to a common phenotype. *Am. J. Cell. Pathol.* 83:431–438.

Nelson, W., and T. -T. Sun. 1983. The 50- and 58-kdalton keratin classes as molecular markers for stratified squamous epithelia: cell culture studies. *J. Cell Biol.* 97:244–251.

Pinkus, H. Embryology of hair. 1958. *In* The Biology of Hair Growth. W. Montagna and R. A. Ellis, editors. Academic Press, New York. 1–23.

Pinkus, H., T. Iwasaki, and Y. Mishima. 1981. Outer root sheath keratinization in anagen and catagen of the mammalian hair follicle. A seventh distinct type of keratinization in the hair follicle: trichilemmal keratinization. *J. Anat.* 133:19–35.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208–212.

Roop, D. R., P. N. Hawley, C. K. Cheng, and S. H. Yuspa. 1983. Keratin gene expression in mouse epidermis and cultured epidermal cells. *Proc. Natl. Acad. Sci. USA.* 80:716–720.

Roop, D. R., T. M. Krieg, T. Mehrel, C. K. Cheng, and S. H. Yuspa. 1988. Transcriptional control of high molecular weight keratin gene expression in multistage mouse skin carcinogenesis. *Cancer Res.* 48:3245–3252.

Schermer, A., S. Galvin, and T. -T. Sun. 1986. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial cells. *J. Cell Biol.* 103:49–62.

Schweizer, J., and H. Winter. 1983. Keratin biosynthesis in normal mouse epithelia and in squamous cell carcinomas. *J. Biol. Chem.* 258:13268–13272.

Skerrow, D., and C. J. Skerrow. 1983. Tonofilament differentiation in human epidermis: isolation and polypeptide chain composition of keratinocyte subpopulation. *Exp. Cell Res.* 143:27–35.

Stark, H. -J., D. Breitkreutz, A. Limat, P. Bowden, and N. E. Fusenig. 1987. Keratins of the human hair follicle: "hyperproliferative" keratins consistently expressed in outer root sheath cells in vivo and in vitro. *Differentiation.* 35:236–248.

Stasiak, P. C., P. E. Purkis, I. M. Leigh, and E. B. Lane. 1989. Keratin 19: predicted amino acid sequence and broad tissue distribution suggest it evolved from keratinocyte keratins. *J. Invest. Dermatol.* 92:707–716.

Steinert, P. M. 1978. Structural features of the alpha-type filaments of the inner root sheath cells of the guinea pig hair follicle. *Biochemistry.* 17:5045–5052.

Steinert, P. M., and G. E Rogers. 1973. Characterization of the proteins of guinea-pig hair and hair follicle tissue. *Biochem. J.* 135:759–771.

Stoler, A., R. Kopan, M. Duvic, and E. Fuchs. 1989. Use of monospecific antisera and cRNA probes to localize the major changes in keratin expression during normal and abnormal epidermal differentiation. *J. Cell Biol.* 107: 427–494.

Stromer, M., and M. Bendayan. 1988. Arrangement of desmin intermediate filaments in smooth muscle cells as shown by high resolution cytochemistry. *Cell Motil. Cytoskeleton.* 11:117–125.

Sun, T. -T., R. Eichner, A. Schermer, D. Cooper, W. G. Nelson, and R. A. Weiss. 1984. Classification, expression, and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. *In* The Cancer Cell. Vol. 1: The Transformed Phenotype. A. Levine, W. Topp, G. van de Woude, and J. D. Watson, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 169–176.

Tozawa, T., and A. B. Ackerman. 1987. Basal cell carcinoma with follicular differentiation. *Am. J. Dermatopathol.* 9:474–482.

Tyner, A. L., and E. Fuchs. 1986. Evidence for posttranscriptional regulation of the keratins expressed during hyperproliferation and malignant transformation in human epidermis. *J. Cell Biol.* 103:1945–1955.

van Muijen, G. N. P., D. J. Ruiter, W. W. Franke, T. Achtstatter, W. H. B. Haasnoot, M. Ponec, and S. O. Warnaar. 1986. Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins nos. 4 and 13. *Exp. Cell Res.* 162:97–113.

Vassar, R., M. Rosenberg, S. Ross, A. Tyner, and E. Fuchs. 1989. Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 86:1563–1567.

Viac, J., M. J. Staquet, J. Thivolet, and C. Goujon. 1980. Experimental production of antibodies against stratum corneum keratin polypeptides. *Arch. Dermatol. Res.* 267:179–188.

Weiss, R. A., R. Eichner, and T. -T. Sun. 1984. Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J. Cell Biol.* 98:1397–1406.

Woodcock-Mitchell, J., R. Eichner, W. G. Nelson, and T. -T. Sun. 1982. Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. *J. Cell Biol.* 95:580–588.