Keratin 17 Gene Expression during the Murine Hair Cycle

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Keratin 17 (K17) expression is currently considered to be associated with hyperplastic or malignant growth of epithelial cells. The functions of this keratin in normal skin physiology and the regulation of its gene expression, however, are still unclear. As one possible approach to further explore K17 functions, we have studied the differential patterns of mouse K17 (MK17) transcription during the murine hair cycle by means of *in situ* hybridization, using a digoxigenin-labeled riboprobe. Cycling hair follicles in the skin of C57BL/6 mice were found to be the only skin structures expressing MK17 under physiologic conditions. MK17 transcripts were constantly observed throughout all hair cycle stages in the suprainfundibular outer root sheath (ORS). The MK17 expression was also evident in the isthmus part of the ORS, where it was expressed weakly and was spatially restricted during telogen, with an increase in early anagen and stable expression during mid- and late anagen, localizing to the zone of so-called trichilemmal keratinization. In addition, in early anagen, a group of epithelial cells in or next to the bulge region stained weakly for MK17. With progressing anagen development, MK17 expression in this region increased and was consistently localized to keratinocytes at the advancing front of the emerging epithelial hair bulb. In mid- and late anagen, this zone of MK17 expression spread along the proximal ORS, with a maximal level of expression in the innermost cell layer of the ORS. Overall, these findings provide data on the MK17 expression profile of normal murine skin and demonstrate hair-cycle–dependent regulation of MK17 expression. ***Key words: keratin 1/translational regulation/trichilemmal keratinization. J Invest Dermatol 108: 324–329, 1997***

A prominent characteristic of stratifying epithelial cells is their ability to express keratins, a family of intermediate filament proteins consisting initially of 19 members catalogued by Moll and co-workers (Moll *et al*, 1982a) for the human system. In this list, number 17 was assigned to an acidic type-I cytokeratin with a molecular mass of about 46 kDa.

Human keratin 17 (HK17) is apparently not expressed in normal stratified human epidermis (Moll *et al*, 1982a). It is expressed, however, in hyper-plastic epidermis and is found in association with several hyper-proliferative epithelial diseases, including squamous and basal cell carcinoma as well as psoriasis (Moll *et al*, 1982b; Weiss *et al*, 1984; de Jong *et al*, 1991; Leigh *et al*, 1995).[1] HK17 was also been suggested to be a prospective marker of malignant cell transformation (Proby *et al*, 1993; Guelstein *et al*, 1988). In normal human skin, evidence for HK17 expression was shown for tactile epidermal hair discs (Haarscheiben) (Moll *et al*, 1993), for the basal cells of sweat glands (Leigh *et al*, 1995), and for the myoepithelial cells of sebaceous glands (Troyanovsky *et al*, 1989). In addition, HK17 polypeptide was detected in cells of the proximal outer root sheath (ORS) by two-dimensional gel electrophoresis (Moll *et al*, 1982b) and immunohistochemistry (Leigh *et al*, 1995). These data, however, did not sufficiently define the precise follicular location of HK17 protein, and no data regarding hair-cycle–associated changes in HK17 expression are as yet available.

A partial murine cDNA clone (pkSCC50) that selects an mRNA coding for an acidic 50-kDa keratin has been isolated from hyperproliferative murine epidermis. Based on an approximately 98% sequence homology at the nucleotide and amino acid levels, this keratin has been proposed to represent an analog of the human type-I 46-kDa keratin protein HK17 (Schweizer, 1993). By *in situ* hybridization, the 50-kDa murine keratin (MK17) mRNA was found to be expressed in keratinocytes of hyper-proliferative (wounded) tail epidermis and in unidentified cells of the hair follicle, suggesting that MK17 may be a normal hair follicle constituent (Knapp *et al*, 1987). Thus, information on keratin 17 (K17) expression and location in normal mammalian skin is currently limited to the statement that this protein is a normal constituent of "Haarscheiben," some glandular epithelia, the proximal outer root sheath of anagen hair follicles in human skin (Moll

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Abbreviations: K1, keratin 1; MK1, murine keratin 1; K17, keratin 17; HK17, human keratin 17; MK17, murine keratin 17; ORS, outer root sheath; IRS, inner root sheath; cRNA, complementary RNA.

[1] Komine M, Freedberg IM, Sémat A, Blumenberg M: Th-1 and Th-2 lymphocytes differently regulate epidermal keratin gene expression. *J Invest Dermatol* 104:586, 1995, (abstr).

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et al, 1982b, Troyanovsky *et al*, 1989; Moll *et al*, 1993; Leigh *et al*, 1995), and undefined epithelial cells in murine hair follicles, "probably of the ORS" (Knapp *et al*, 1987). The functions of K17 in normal and pathologic skin are even less clear. Recent findings that a mutation in the HK17 gene causes severe epithelial dysfunction (Pachyonychia congenita) associated with hyper-trophic nail dystrophy, multiple epidermal cysts, and some hair abnormalities (McLean *et al*, 1995), however, attest to the potential functional significance of HK17.

To gain further insight into K17 function by the systematic characterization of its *in vivo* transcription in a well-defined developmentally regulated model system, we have analyzed the pattern of MK17 mRNA expression in normal murine hair follicles throughout various stages of their cyclic growth and regression (Hardy, 1992; Paus *et al*, 1994a; Paus, 1996) by using *in situ* hybridization with specific digoxigenin-labeled riboprobes. For comparison and as a positive control, murine keratin 1 (MK1) expression was analyzed as a differentiation-associated cytokeratin (Moll *et al*, 1982a; Weiss *et al*, 1984; Schweizer, 1993; Leigh *et al*, 1994).

MATERIALS AND METHODS

Animals and Skin Samples Seven- to 12-wk-old female C57BL/6J mice with normal fur and normal hair cycling behavior (Charles River, Sulzfeld, Germany) were used throughout. The animals were kept under standard conditions, following all relevant guidelines for laboratory animal care, and were free of cutaneous lesions.

For normal hair cycle induction, 7-wk-old mice with all back skin follicles in telogen were depilated with a mixture of wax and rosin (Paus *et al*, 1990). Dorsal skin was harvested during defined stages (days 0, 3, 5, 12, and 18) of the depilation-induced hair cycle parallel to the paravertebral line to obtain longitudinal hair follicle sections (Paus *et al*, 1994b). Three mice were studied for each hair cycle stage.

Animals were killed by cervical dislocation under general anesthesia. Dorsal skin was fixed in 4% paraformaldehyde and embedded in paraffin (JUNG-Histowax, Reichert-Jung, Heidelberg, Germany) according to standard procedures. Five-micrometer sections were mounted on silane-coated glass slides (six slides with four sections per slide for each mouse).

MK1 cDNA Template Total RNA was isolated according to Chomczynski *et al* (1987) and transcribed into cDNA with an reverse transcription-coupled polymerase chain reaction kit (Stratagene, Heidelberg, Germany). For studies of reverse transcription-coupled polymerase chain reaction of MK1, the primers used contained nucleotides 1664–1681 and 1881–1905 (accession number M10937) at 0.1 μM. The identity of the product was confirmed by restriction enzyme analysis. The MK1 cDNA fragment obtained was ligated into a pGEM-T vector (Promega, Madison, WI), and clones containing the specific cDNA were identified by restriction enzyme analysis.

MK17 cDNA Template A defined 255-bp fragment of the MK17 cDNA clone (Knapp *et al*, 1987) was a kind gift from Dr. J. Schweizer, Deutsches Krebsforschungszentrum, Heidelberg, Germany (Schweizer, 1993). The fragment was ligated into a pSP72 vector (Promega, Madison, WI) and then cloned according to the procedure used with the MK1 fragment.

Preparation of Riboprobes Aliquots of the plasmids enclosing MK1- and MK17-specific cDNA were linearized with *Nco*I or *Not*I (MK1) and *Hin*dIII or *Eco*RI (MK17) restriction enzymes (Boehringer-Mannheim, Mannheim, Germany). Digoxigenin-labeled complementary RNAs (cRNAs) were prepared *in vitro* by using the "DIG RNA Labeling Kit" (Boehringer-Mannheim, Mannheim, Germany). T7 and Sp6 RNA polymerases were used to produce sense and anti-sense probes for MK1 and MK17 according to the kit protocol. The amount of cRNA obtained was estimated by standard paraformaldehyde-containing agarose gel electrophoresis.

***In Situ* Hybridization** *In situ* hybridization was performed as described (Nürnberg *et al*, 1995a; Nürnberg *et al*, 1995b). Briefly, deparaffinized and deproteinized sections were acetylated in acetic anhydride solution (Merck, Darmstadt, Germany) and then dehydrated. Pre-hybridization was performed in humidified chambers at 50°C with a mixture containing 50% deionized formamide (Merck, Darmstadt, Germany). Hybridization with freshly denatured cRNA probes at 50 ng per section was performed at 50°C for 17 h in the same humidified chambers. The MK17 sense probe was used as a negative control and the MK1 anti-sense probe as a positive control. Sections were washed after hybridization at 50°C under highly stringent conditions for 5 h. Prior to immunodetection of the *in situ* hybridization signal, the slides where incubated with normal sheep serum (Sigma, St. Louis, MO) in the presence of levamisol (Sigma, St. Louis, MO) and blocking solution (DIG Nucleic Acid Detection Kit, Boehringer-Mannheim, Mannheim, Germany). Incubation with sheep alkaline phosphatase-labeled anti-digoxigenin antibodies (DIG Nucleic Acid Detection Kit, Boehringer-Mannheim, Mannheim, Germany) was performed for 3 h in humidified chambers at room temperature. The slides were stained by incubation in nitroblue tetrazolium and β-chloroindolyl phosphate solution (Boehringer-Mannheim, Mannheim, Germany) for 16–20 h in complete darkness at room temperature. After a brief wash, the sections were mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany). The pictures were prepared at different magnifications by using an "Axiophot" light microscope (Zeiss, Oberkochen, Germany) and Kodak 160 film. From 25 to 45 appropriately sectioned follicles derived from three mice were analyzed at each stage of hair follicle development.

RESULTS

MK17 mRNA Is Exclusively Expressed in Hair Follicles In the skin of C57BL/6 mice with normal fur and normal patterns of hair follicle cycling, a signal for MK17 mRNA was detected exclusively in the hair follicles, was characterized by a discrete spatial distribution, and was localized to both the permanent and the cycling part of the hair follicle **(Figs 1, 2)**. Strong immunoreactivity denoting MK17 transcripts was consistently found throughout all stages of the murine hair cycle in the suprainfundibular part of the follicle ORS (zone 1), just above the sebaceous duct orifice **(Figs 1, 2, 3)**. This part of the ORS displayed MK17 mRNA immunoreactivity mainly in its inner cell layers **(Fig 1*B,D*; 3*B*)**. The most distal part of the ORS directly adjacent to the interfollicular epidermis as well as the sebaceous gland epithelium did not show MK17 mRNA immunoreactivity **(Figs 1, 3)**.

MK17 mRNA immunoreactivity was also noted in the hair follicle isthmus **(Figs 1*A,B,C,G*; 3)**, as defined by Pinkus, who designated this region as "the stretch between the opening of the sebaceous gland duct and the upper end of the inner root sheath (IRS), where the IRS disintegrates, letting the hair shaft emerge free into pilary canal" (Pinkus, 1969; Pinkus *et al*, 1981). In telogen, MK17 mRNA immunoreactivity in this region (zone 2) was lower than in the suprainfundibular portion of the ORS (zone 1) **(Figs 1*A*, 2)**, was maximally increased in early anagen **(Fig 1*B*)**, and decreased in mid-anagen **(Fig 1*C*)**. In late anagen, this region of MK17 mRNA immunoreactivity coincided with the zone of so-called "trichilemmal keratinization" (Pinkus *et al*, 1981), with maximal expression of the signal localized to the outer cell layers of the ORS at the isthmus region **(Fig 3)**.

The most proximal cycling epithelial part of the hair follicle also displayed MK17 mRNA immunoreactivity (zone 3), although it was subject to prominent hair-cycle–dependent changes in this region. In telogen, signs of staining were absent proximal to hair follicle **(Fig 1*A*)**. In early anagen, a group of epithelial cells in or next to the bulge region and directly above the dermal papilla stained only weakly **(Fig 1*B*)**. With progressing anagen development, the MK17 mRNA immunoreactivity increased in this region and consistently localized to the cells at the advancing front of the emerging epithelial hair bulb **(Fig 1*C,D*)**. By anagen IV and V, these cells produced a new zone of very active MK17 mRNA immunoreactivity in the proximal part of the ORS, where the signal was maximally displayed in the inner cell layer of the ORS **(Fig 1*D,E*)**, with no MK17 mRNA immunoreactivity in the inner root sheath **(Figs 1*E,F*; 3)**. During anagen VI, most of the proximal ORS displayed MK17 mRNA immunoreactivity in its innermost cell layer **(Fig 1*G,H*)**. **Figure 2** shows a schematic summary of the data described so far for the MK17 transcription pattern in defined hair follicle compartments during the normal experimentally induced murine hair cycle.

MK1 mRNA Is Expressed in Suprabasal Cell Layers of the Interfollicular Epidermis *In situ* hybridization with a digoxigenin-labeled riboprobe specific for MK1 was used as positive control. As reported by Schweizer (1993), MK1 mRNA immunoreactivity was present in the suprabasal cell layers of the interfol-

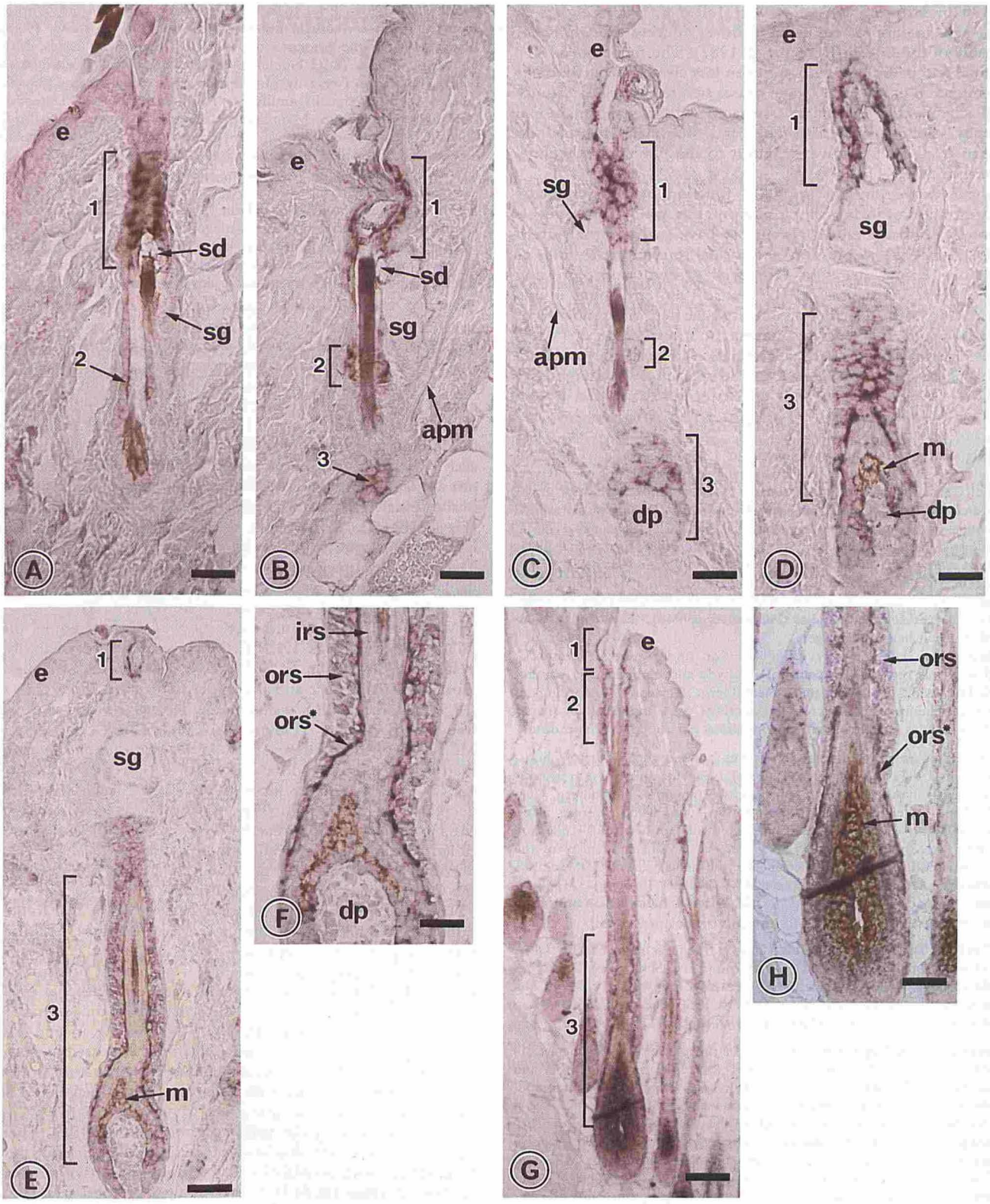

Figure 1. MK17 is expressed only in hair follicles but with different expression patterns during the hair cycle. *In situ* hybridization with a digoxigenin-labeled anti-sense MK17 cRNA probe was performed in paraffin-embedded sections of adolescent C57BL/6 mouse skin. The hair cycle was induced by wax depilation. The following hair cycles are shown: *(A)* Telogen. *(B)* Anagen I. *(C)* Anagen II. *(D)* Anagen IV. *(E* and *F)* Anagen V. *(G* and *H)* Anagen VI. Depending on the hair cycle, MK17 mRNA immunoreactivity was noted in the suprainfundibulum (zone 1), isthmus (zone 2), and the proximal part of the ORS (zone 3). Note that the black-staining sructures in the matrix region of *Panels D–H* represent melanin granules and should not be confused with MK17 mRNA immunoreactivity. apm, Arrector pili muscle; dp, dermal papilla; e, epidermis; irs, IRS; m, matrix; ors, ORS; ors*, innermost cell layer of the ORS; sd, sebaceous gland duct; sg, sebaceous gland. *Scale bars: (A–D,F)* 23 μm; *(E,H)* 45 μm; *(G)* 90 μm.

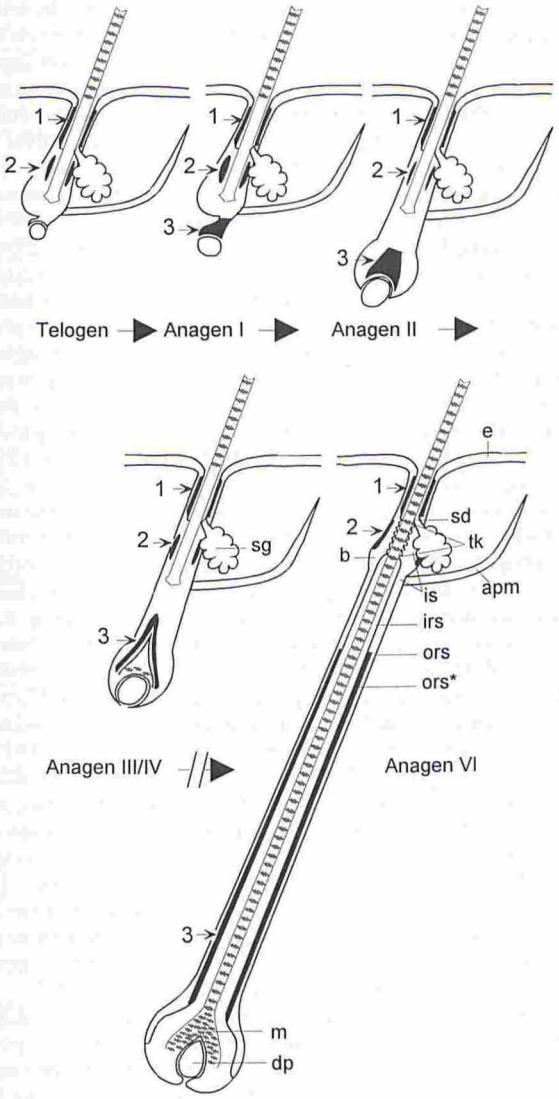

Figure 2. Scheme of MK17 expression during the murine hair cycle. Three different zones of MK17 mRNA immunoreactivity are present: Persistent expression in the suprainfundibulum (zone 1), expression in the isthmus (zone 2), expression in the innermost cell layer of the ORS during anagen stages (zone 3). b, Bulge; is, isthmus; tk, area of trichilemmal keratinization. For further abbreviations, see **Fig 1**.

licular epidermis **(Fig 4*A,B*)**. In addition, MK1 expression was detected in the most distal portion of hair follicle ostium **(Fig 4*B*)**, which has been suggested to represent an epidermal type of keratinization (Kopan and Fuchs, 1989; Leigh *et al*, 1994). The expression patterns of MK1 were stable during all stages of hair follicle cycling **(Fig 4)** and showed no spatial overlap with MK17 expression in the skin of C57BL/6 mice **(Figs 1, 3, 4)** under physiologic conditions. This MK1 expression pattern confirms the specificity and sensitivity of our *in situ* hybridization technique and is in accordance with previously published results (Schweizer, 1993).

DISCUSSION

The current results extend the available information on follicular K17 expression. By using a sensitive and specific *in situ* hybridization methodology, we show that the hair follicle ORS is the only structure in murine skin to express the MK17 gene under physiologic conditions, supporting the previous assumption that MK17 transcription is a common constituent of normal murine hair

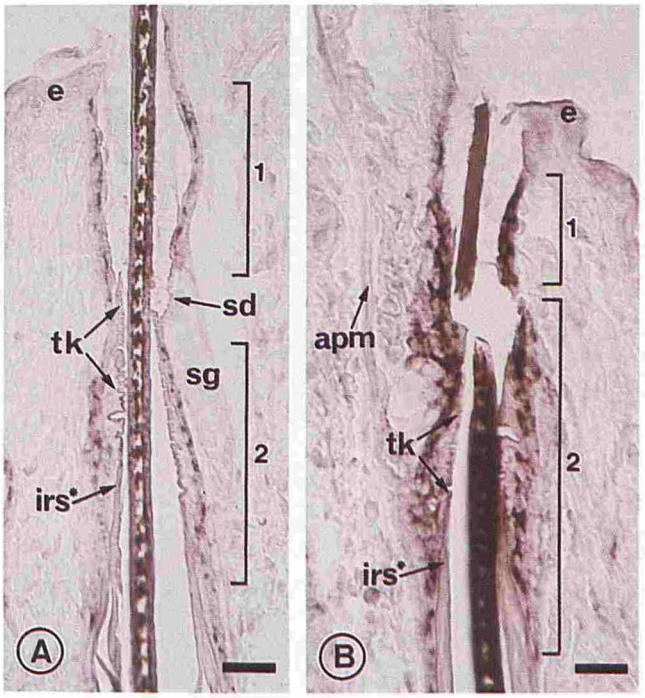

Figure 3. MK17 mRNA immunoreactivity is expressed in the distal part of two hair follicles in anagen VI. irs*, Terminal end of IRS. For further abbreviations, see **Figs 1** and 2. *Scale bars*, 23 μm.

follicles (Knapp *et al*, 1987), including resting (telogen) follicles. This is important in that K17 has previously been detected in human and murine skin only under hyper-proliferative conditions, the only exception being "Haarscheiben" (Moll *et al*, 1993). The exclusive MK17 transcription in distinct regions of the ORS during telogen and anagen VI **(Figs 1*A,G,H*; 3)** indicates that at least in selected keratinocyte populations, MK17 expression is associated with functions other than enhanced proliferation.

The high level of MK17 mRNA immunoreactivity in normal murine skin throughout all hair cycle stages in the suprainfundibular ORS (zone 1, **Figs 1, 3**) contrasts with the report by Moll *et al* (1982b), who noted absence of HK17 protein in the distal portion of human follicle ORS. This may reflect differences between K17 expression patterns in human and murine skin. Alternatively, it could indicate that K17 is actively transcribed in the distal ORS but not translated at this location. K17 mRNA may be continuously synthesized in this follicle region to allow for a rapid synthesis of K17 protein once a translational block is removed. This might occur under conditions of epidermal damage, epidermal stimulation by hyper-proliferative agents, changes in the local keratinocyte environment (e.g., cell culture), or during pathologic hyper-proliferative states such as psoriasis. All these conditions are characterized by very active K17 protein synthesis (Weiss *et al*, 1984; Knapp *et al*, 1987; de Jong *et al*, 1991; Leigh *et al*, 1995; Paladini *et al*, 1996) and by a keratinization pathway related to wound healing and epidermal regeneration (Mansbridge and Knapp, 1987; Leigh *et al*, 1994).

During wound healing, epidermal repair starts from the hair follicle ORS, which provides a rich source of keratinocytes, contributing to the epidermal renewal by migration and proliferation (Chapman and Hall, 1981; Lenoir *et al*, 1988; Lavker *et al*, 1993). The induction of K17 as well as of K6 and K16 in murine and human epidermis occurs rapidly after the application of a hyper-proliferative stimulus (Tyner and Fuchs, 1986; Schweizer, 1993). This has invited the speculation that corresponding mRNAs are permanently present in normal epidermis and are released from a translational block (Curtis *et al*, 1995) in response to skin damage (Tyner and Fuchs, 1986).

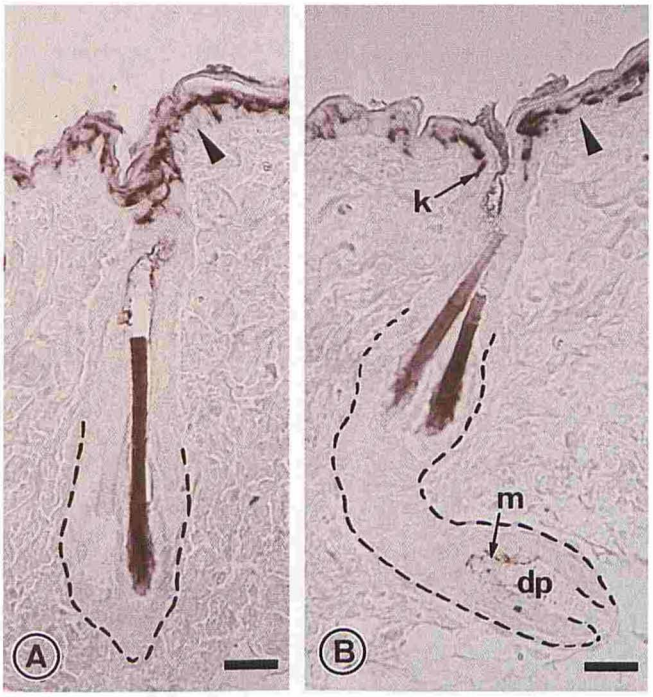

Figure 4. MK1 mRNA is present in suprabasal epidermal keratinocytes and in the most distal part of the ORS. *In situ* hybridization with a digoxigenin-labeled anti-sense MK1 cRNA probe was performed in C57BL/6 mouse skin during the same hair cycle stages as for MK17 studies. Two representative examples are *(A)* Telogen. *(B)* Anagen IV. Arrowheads, epidermal basal cell layer; . . . . , shape of the proximal portion of the hair follicle; k, most distal portion of the ORS. *Scale bars,* 23 μm.

By *in situ* hybridization techniques, however, there are no detectable MK17 transcripts in normal murine epidermis (Knapp *et al*, 1987), as confirmed by our studies **(Figs 1, 3)**. Since the existence of a "repair pool" of keratinocytes derived from hair follicle stem cells in the bulge region has been proposed (Lavker *et al*, 1993; Yang *et al*, 1993), our results may cast new light onto this old controversy and possibly explain why previous attempts to find K17 transcripts in normal interfollicular epidermis have failed: The suprainfundibular ORS of the hair follicle may represent the main reservoir of MK17 transcripts for epidermal repair. At this location, we have found substantial and constant MK17 transcription and, at least in human skin, no apparent translation of this protein under normal conditions (Moll *et al*, 1982b; Leigh *et al*, 1995). In extension of this idea, K17 may be part of a specific keratin filament network used by actively migrating keratinocytes, designed to provide cells with a less rigid cytoskeleton to facilitate rapid changes in cell contacts with neighboring cells (Moll *et al*, 1993; Heyden *et al*, 1994; Paladini *et al*, 1996).

Since no antibodies against MK17 are currently available, this concept remains to be verified on the protein level. The data regarding HK17 expression in human hair follicles (Moll *et al*, 1982b; Leigh *et al*, 1995) may well be relevant for the interpretation of our results, given the high level of homology between the clone 226, encoding HK17 (Kartasova *et al*, 1987), and the murine clone pkSCC50 (Knapp *et al*, 1987) used here. Compared to the human clone, the MK17 clone has only two amino acid changes in the α-helical domain and a proline residue that is absent from the carboxyl terminus of HK17. At the nucleotide level, this high homology extends to the 3′ noncoding region, which is known to be very specific for each keratin (Schweizer, 1993). Therefore, the findings regarding MK17 biology in murine skin may also hold for human skin.

In contrast to MK17 expression in the suprainfundibular ORS, MK17 transcription in the two proximal MK17 expression zones (zones 2 and 3) is shown to be hair-cycle–dependent. In telogen, MK17 expression was present in a spatially confined portion of the ORS isthmus **(Fig 1*A*)**. This region of MK17 expression (zone 2) coincides with the zone of so-called "trichilemmal" keratinization, which is thought to represent the end point of a specific differentiation pathway of isthmus ORS keratinocytes, which differ from other ORS cells (Pinkus, 1969; Pinkus *et al*, 1981). Therefore, with progressing anagen development, the MK17 mRNA immunoreactivity in zone 2 was mainly concentrated at the outermost cell layers of the ORS **(Fig 3)**, in contrast to the suprainfundibular region **(Fig 1*D*)**. The MK17-expressing part of the hair follicle isthmus is recognized to be richly innervated by circular nerve fibers and palisaded nerve endings that have long been considered to provide tactile functions in the hair follicle (Pinkus, 1969; Montagna and Parakkal, 1974). Therefore, it is reasonable to speculate that K17 expression in this follicle region serves to provide a more flexible and more easily deformed epithelial pad, in a manner comparable to the one proposed for human "Haarscheiben" (Moll *et al*, 1993).

The most prominent hair-cycle–dependent changes in MK17 expression were revealed in the most proximal part of the murine hair follicle (zone 3), which undergoes substantial cyclic transformation **(Fig 1)**. MK17 mRNA immunoreactivity in this portion of the ORS started in early anagen, in a small group of epithelial cells between the dermal papilla and the bulge region **(Fig 1*B,C*)**, spreading along the proximal ORS, with maximal expression in its innermost cell layer, directly adjacent to the inner root sheath **(Fig 1*D–H*)**. This immunoreactivity pattern suggests that MK17 expression in this region in mid- and late anagen identifies a specific cell layer, designated as the "companion layer" (Orwin, 1971) or "innermost cells of the ORS" (Ito *et al*, 1986). This keratinocyte layer is distinguished by a unique differentiation pathway and is morphologically and immunologically distinct from other ORS keratinocytes (Orwin, 1971; Ito, 1986). The function and origin of this cell layer are still discussed (Rothnagel and Roop, 1995). According to one suggestion, the innermost cell layer of the ORS provides a "slippery" plane to allow the growing hair shaft to move past the surrounding tissue sheaths (Rogers, 1964). This suggestion is in line with our concept of K17 as a "flexible keratin."

Our studies raise the possibility that there are three different patterns of K17 expression in murine hair follicles: one possibly denoting a putative "repair pool" of keratinocytes in the suprainfundibular portion of the ORS; another at the isthmus of the ORS, probably reflecting trichilemmal keratinization; and a third one involved in hair bulb construction during anagen. The function of K17 expression may be to allow keratinocytes to display greater structural flexibility and to promote easier reorganization of the cytoskeleton (Moll *et al*, 1993). The same suggestion has recently been made for K6 and K16 (Heyden, 1994; Paladini *et al*, 1996), both representing keratins with expression patterns similar to that of MK17 (Stark *et al*, 1987; Schweizer, 1993).

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