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# Structural features and sites of expression of a new murine 65 kD and 48 kD hair-related keratin pair, associated with a special type of parakeratotic epithelial differentiation

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**Abstract.** In the course of studies on local keratin phenotypes in the epidermis of the adult mouse, we have identified a new 65 kD and 48 kD keratin pair. In mouse skin, this keratin pair is only expressed in suprabasal cells of adult mouse tail scale epidermis which is characterized by the complete absence of a granular layer and the formation of a remarkably compact stratum corneum. A second site in which the 65 kD and 48 kD keratin pair is suprabasally expressed and whose morphology corresponds to that of tail scale epidermis is found in the posterior unit of the complex filiform papillae of mouse tongue. The causal relationship of the expression of the 65 kD and 48 kD keratins with this particular type of a non-pathological epithelial parakeratosis is emphasized by the suppression of the mRNA synthesis of the two keratins during retinoic acid mediated orthokeratotic conversion of tail scale epidermis. Apart from tail scale epidermis and the posterior unit of the filiform papillae, the 65 kD and 48 kD keratin pair is, however, also coexpressed with "hard" α keratins in suprabulbar cells of hair follicles and in suprabasal cells of the central core unit of the lingual filiform papillae.

The non α-helical domains of the two new keratins are rich in cysteine and proline residues and lack the typical subdomains into which epithelial keratins of both types can be divided. This structural resemblance of the 65 kD and 48 kD keratins to "hard" α keratins is supported by comparative flexibility predictions for their non α-helical domains. Phylogenetic investigations then show that the 65 kD and 48 kD keratin pair has evolved together with hair keratins, but has diverged from these during evolution to constitute an independent branch of a pair of hair-related keratins. In view of this exceptional position of the 65 kD and 48 kD keratins within the keratin multigene family, their expression has apparently been adopted by rare anatomical sites in which an orthokeratinized stratum corneum would be too soft and a hard keratinized structure would be too rigid to meet the functional requirement of the respective epithelia.

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## Introduction

Keratins are a family of about 30 structurally related proteins that are responsible for the formation of the 10 nm intermediate filaments in almost all cells of epithelial origin. According to the charge properties of the proteins, one distinguishes between basic to neutral, type II keratins and acidic type I keratins (for reviews see [13, 28, 50, 53]). At least one member of each subfamily is required for intermediate filament formation. Detailed studies of epithelial keratin expression patterns have revealed that, as a rule, a distinct keratin of one type tends to be coexpressed with a specific partner of the opposite type and that the resulting keratin pairs constitute useful markers for various states or pathways of epithelial differentiation [13, 28, 44, 50, 53].

In the mammalian epidermis for example, keratins K5 (type II) and K14 (type I) are typical for basal cells, whereas in suprabasal cells the synthesis of this keratin pair is replaced by the expression of a new keratin pair, K1 and K10, that is characteristic for mammalian epidermal or "skin type" differentiation [13, 28, 39, 44, 50, 53].

Occasionally, however, deviations from this basic epidermal keratin pattern which consist of the expression of additional suprabasal members of the keratin family, are observed in distinct skin sites of various species. This is, for example, the case for the callus-forming epidermis of human palmar and plantar skin or its anatomical equivalents in other species i.e., bovine heelpad on the posterior side of the hoof or the footpads of some rodents, in which besides keratins K1 and K10 an additional type I keratin – K9 – is expressed in suprabasal cells [22, 28, 46]. Since K9 is expressed without a new type II keratin, it has to compete with K10 for filament formation with K1. This implies the occurrence of two kinds of differentiation specific keratin pairs, i.e., K1/K10 and K1/K9, respectively which then specify the local epidermal phenotype of callus-forming skin [28, 46].

We have recently described a similar deviation from the basic epidermal keratin pattern in adult mouse skin. In this case a type II 70 kD keratin is expressed besides

K1 and K10 without a defined new type I keratin in suprabasal cells, thus producing a local keratin pair combination K1/K10 and 70 kD keratin/K10 [36]. Unlike the keratin K9 expressing epidermal phenotype, the 70 kD keratin expressing phenotype is, however, not restricted to a single, morphologically distinct skin site. It is found in the thin ear epidermis but also in the thick epidermis of the sole of the foot proper [36]. Moreover it occurs in the morphologically complex tail skin in which a remarkable parakeratotic scale epidermis regularly alternates with an orthokeratotic interscale epidermis [40, 41, 45, 49]. Whereas the latter represents the 70 kD keratin expressing local epidermal phenotype [36], the parakeratinizing scale epidermis even lacks the expression of keratins K1 and K10 [33] and thus appears to be fundamentally different from the above named local epidermal phenotypes of mouse skin. This assumption is supported by the finding that the keratin pattern of adult mouse tail epidermis contains a genuine type II 65 kD keratin which is not only absent from any other skin site [36, 39, 45] but also acquired postnatally [39, 45] concomitant with the onset of paraorthokeratotic morphological patterning of tail epidermis [41]. Moreover the expression of the 65 kD keratin is completely suppressed after orthokeratotic conversion of the scale epidermis under the influence of excess retinoic acid [45]. These properties of the 65 kD keratin strongly suggest that its expression is involved in the formation of parakeratosis in tail scale epidermis. In view of the fact that non-pathological parakeratosis represents an exceptionally rare phenotype in mammalian epidermis we have decided to investigate the nature of the 65 kD keratin in more detail. In the present study we describe the unusual structural properties of the 65 kD keratin. We also report on the identification of a specific, structurally equally remarkable type I 48 kD keratin partner of the 65 kD keratin and show that the suprabasal expression of the 65 kD/48 kD keratin pair is not restricted to tail scale epidermis but is also implicated in parakerotic differentiation of internal stratified squamous epithelia.

## Methods

*Isolation of epithelia.* Newborn and adult mouse epidermis from various skin sites, (back, footsole, tail) and internal stratified epithelia (tongue, palate) were generally obtained by shortly incubating the tissues in 60° C hot water (about 20–30 seconds) and separation of the epithelia from connective tissue with fine forceps. In most cases, freshly isolated epithelia were used for the various purposes, occasionally, however, also lyophilized material was used.

*Isolation of keratins, one and two dimensional gel electrophoresis.* Keratins were isolated from the various epithelia as described previously [55]. One dimensional SDS PAGE was carried out according to Laemmli [24]; two dimensional non equilibrium pH gradient electrophoresis (NEPHGE) was performed as described by O'Farrell et al. [32]. Gels were stained with 0.5% Coommassie blue in 40% methanol, 10% glacial acetic acid and destained in the same solution without the dye.

*cDNA cloning and screening procedures.* PolyA$^+$ RNA of adult mouse tail epidermis was isolated according to the method of Gough [15] and used for the construction of a cDNA library in λgt10 [26]. The library was screened by hybridization with a [$^{32}$P]-labeled 535 bp *ClaI/KpnI* fragment of the previously described cDNA clone pkt57 which encodes the type II keratin K4 of internal stratified epithelia [21]. The fragment spans from position 601 to 1136 of pkt57 and thus contains a portion of the sequence coding for the α-helical domain of K4 [21]. Hybridization was carried out for 18 h at 42° C [35% formamide, 5 × (0.9 M NaCl, 50 mM $NaH_2PO_4$, pH 7.4, 5 mM EDTA, pH 7.4)]; subsequent washing was at 52° C [final wash with 0.1 × standard salina citrate (SSC)]; 0.1% sodium dodecy sulphate (SDS). The resulting positive phage clones were further hybridized with a mixture of [$^{32}$P]-labeled specific 3′ cDNA fragments of type II keratin clones pkt70, coding for the 70 kD keratin [36] and of two clones encoding either the basal keratin K5 or the suprabasal keratin K1 (unpublished sequence data). This hybridization was performed for 18 h at 42° C (50% formamide, 5 × SSPE) and the final wash was at 68 °C (0.1 × SCC, 0.1% SDS).

For the isolation of type I keratin clones from the library, an identical screening procedure was carried out using first a 645 bp *Sac/Sac* α-helical fragment (position 82 to 727) of the previously described cDNA clone pkt47 coding for the type I keratin K13 of internal epithelia [21] and subsequently the specific 3′ cDNA fragments of type I keratin clones pke59 and pkSCC52 coding for keratin K10 and K14, respectively [23].

*RNA slot hybridization.* Inserts of phage clones which did not hybridize with the specific 3′ cDNA fragments of K1, K5 and the 70 kD keratin (type II) or of K10 and K14 (type I) were subjected to slot blot RNA hybridization. Total RNA was isolated as indicated above from the epidermis of various skin sites, 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate(DMBA/TPA) – induced benign and malignant skin tumors and internal stratified epithelia. Slot blots were performed by spotting aliquots of the RNA preparations on Gene screen plus membranes (DuPont, Dreieich, FRG) using a Minifold I system (Schleicher & Schüll, Dassel, FRG). Hybridization was carried out for 18 h at 42° C (50% formamide, 1 *M* NaCl, 1% SDS, 50 m*M* TRIS-Cl, pH 7.6, 104 dextrane sulfate) with the [$^{32}$P]-labeled phage inserts. Membranes were washed stringently (0.2 × SSC, 0.1% SDS at 68° C) and exposed at −80° C to Kodak X-Omat films.

*Subcloning and sequencing.* One type II phage clone, λktII-1 and one type I phage clone, λktI-2, resulting from the RNA slot blot screening procedure were chosen for further characterization. The inserts of λktII-1 and λktI-2, attached to *Eco*RI adaptors were cloned into the transcription vector Bluescript II KS+ (Stratagene, La Jolla, USA). Sequencing of both strands of the resulting plasmid vectors pktII-1 and pktI-2 was performed according to the dideoxy sequencing method of Sanger et al. [38] using first M13 and T3 primers and subsequently 17mer oligonucleotides as walking primers. To prepare 3′-specific cDNA probes, a 696 bp *XhoI/StyI* fragment of pktII-1 and a 396 bp *HindIII/SspI* fragment of pktI-2 was subcloned into Bluescript II KS+. These subclones were designated pktII-1–3′ and pktI-2–3′, respectively.

*In situ hybridization.* The protocol used for in situ hybridization to frozen tissue sections (nominally 6 μm thick) was essentially as described previously [34, 35], however, with some modifications [1, 2]. Upon linearization of subclones pktII-1–3′ and pktI2–3′ with *XhoI* and *HindIII*, riboprobes were obtained that were labeled with [$^{35}$S]-UTP by in vitro transcription with T7 RNA polymerase. A sense probe was used as negative control. Slides were dipped in Kodak NTB2 photoemulsion, developed after 1 to 2 days and stained with H&E.

*Hybrid selection analysis and in vitro translation.* PolyA$^+$ RNA obtained by oligo(dT) cellulose chromatography of total RNA of tail epidermis was hybridized to filter bound DNA of the two 3′ specific subclones ptkII-1–3′ and pktII-2–3′. The selected mRNAs were translated in vitro in the presence of [$^{35}$S]-methio-

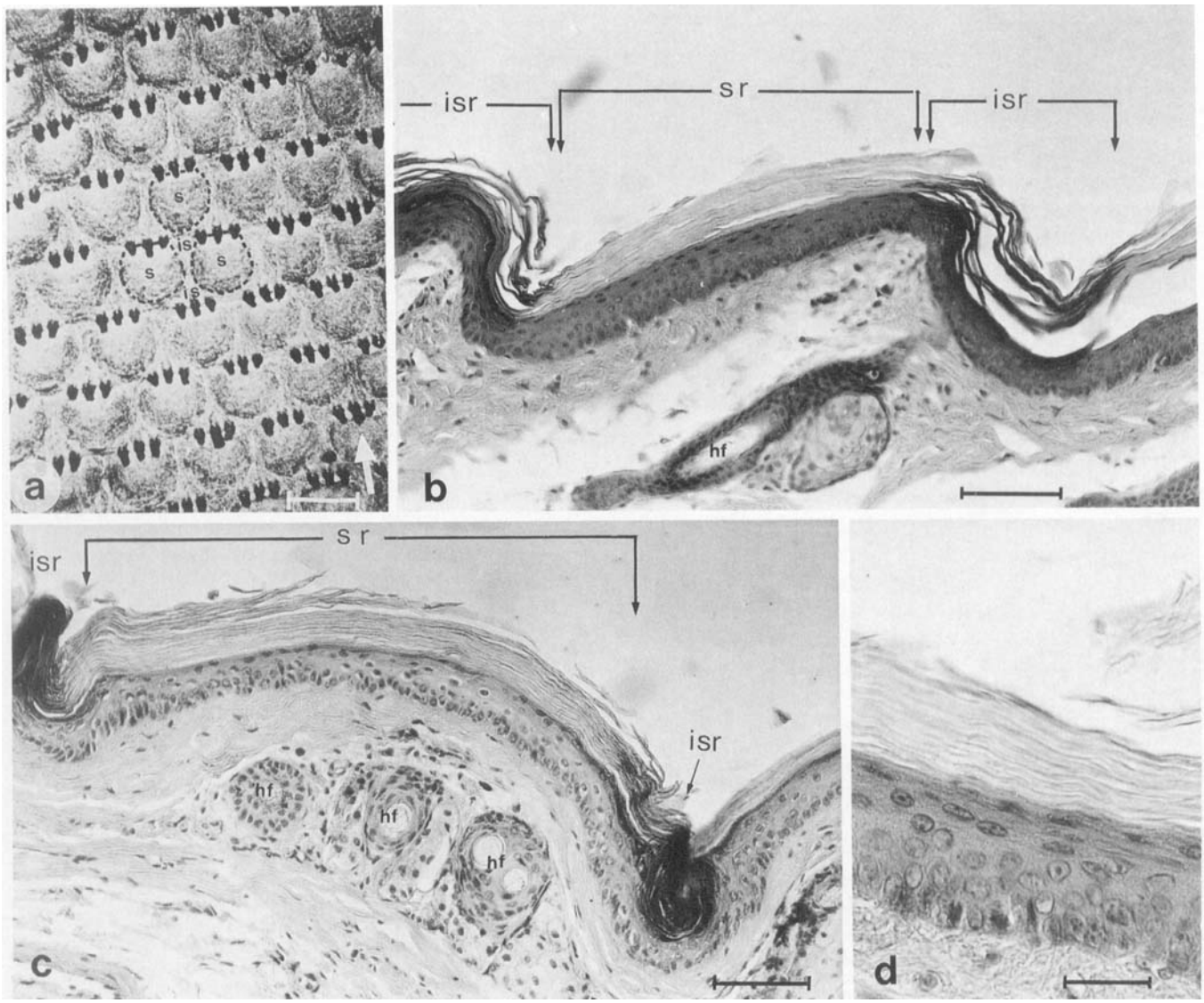


**Fig. 1 a–d.** Morphological appearance of adult mouse tail epidermis. **a** Whole mount of tail epidermis viewed from the dermal side. Note the parallel rows of scales (*s*) which are surrounded by interscale epidermis (*is*). The neck region of three hair follicles, consistently associated with each scale [40, 41] is visible. The *large arrow* points to the tip of the tail. **b** Longitudinal and **c** cross section through tail skin. **d** Higher magnification of scale epidermis. All samples are stained with H&E. *sr*, parakeratotic scale region; *isr*, orthokeratotic interscale region. *Bars*, 100 μm in **b** and **c**, 50 μm in **d**

nine. PolyA$^+$ RNA from both footsole epidermis and tongue epithelium was also translated in vitro. One dimensional SDS PAGE of the in vitro translation products and autoradiography was performed as described [23].

*Treatment of mice with retinoic acid and TPA.* The topical application of both retinoic acid and TPA to adult mouse tail epidermis has previously been described in detail [41, 45]. Briefly, groups of mice received either daily applications of 30 μg retinoic acid in 100 μl acetone or applications of 20 nmol TPA in 100 μl acetone every two days. Maximal application time was 14 days. Beginning from day 0, two mice of each group were sacrificed every two days and RNA was extracted from tail epidermis and used for slot blot hybridization. At later stages of treatment, portions of the epidermis were also used for keratin extraction and frozen sections of skin pieces were prepared for in situ hybridization.

*Phylogenetic tree construction.* The construction of a phylogenetic tree on the basis of the sequences of the α-helical 2B subdomains of various type II and type I keratins including the new keratins described here was performed using program TREE of the HUSAR System (Heidelberg Unix Sequence Analysis Resources) of the German Cancer Research Center. The logic of TREE is essentially described by Feng and Doolittle [11]. TREE produces a multiple alignment for a set of protein sequences by iteratively acting at the sequences. An approximate phylogenetic order of the sequences is first determined by a series pairwise alignments by the Needleman and Wunsch method [29]. Any subclusters that may exist in the set are prealigned before the final alignment. Finally, the phylogenetic tree of the sequences is plotted in the form of a dendrogram.

## Results

### *Morphology of adult mouse tail epidermis*

Macroscopically, mouse tail epidermis is made up of a regular system of slightly elevated scales arranged in rings around the tail's long axis. When isolated sheets

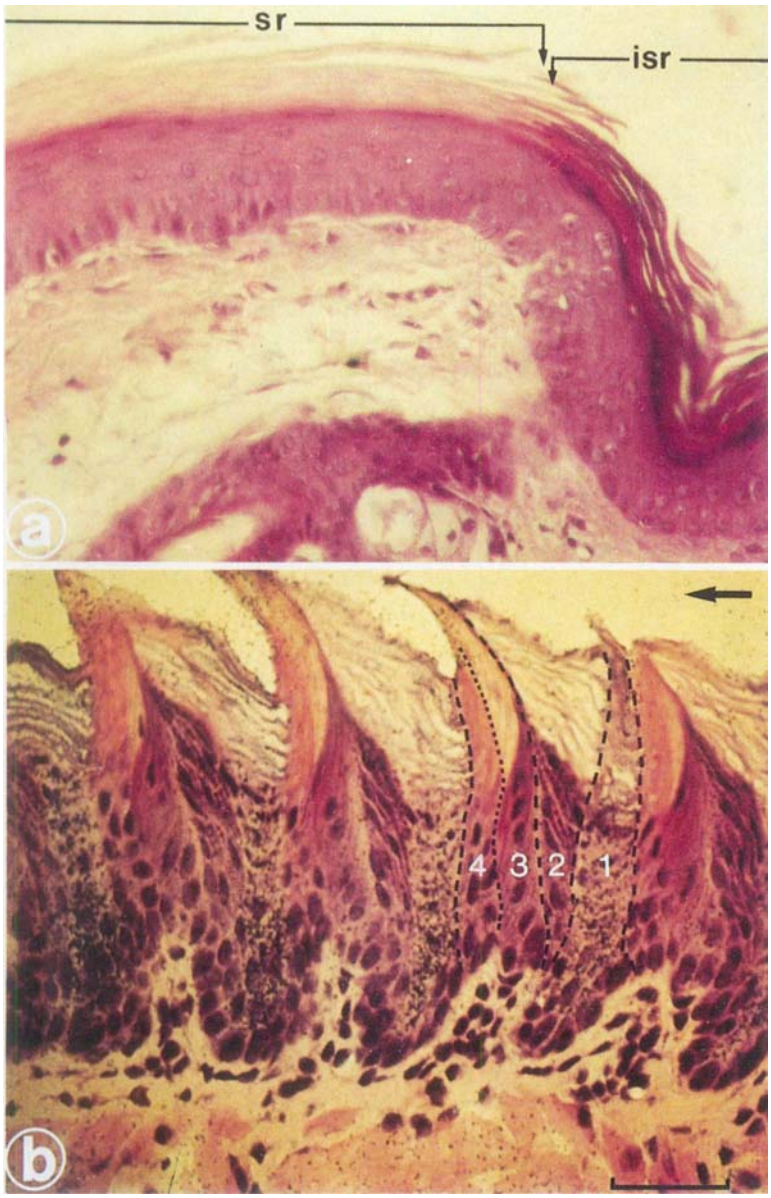


**Fig. 2a, b.** Hematoxilin-eosin staining properties of adult mouse tail epidermis (**a**) and of filiform papillae of mouse tongue epithelium (**b**). Note the red staining of the orthokeratotic stratum corneum of the epidermis of the interscale region (*isr*) and the pink staining of the parakeratotic stratum corneum of the scale epidermis (*sr*) in (**a**). These differences are also visible in black and white microphotographs (see Fig. 1a, b). In (**b**) the different compartments of the dorsal tongue epithelium are indicated by (*1*), interpapillary lingual epithelium; (*2*), anterior unit; (*3*), central core unit and (*4*), posterior unit of the filiform papillae. The three major units of the filiform papillae are equally well visible in the adjacent papillae at left. Note the almost unstained horny spine of the central core unit and the pink staining of the stratum corneum of the posterior unit of the two papillae in the center. The *arrow* points toward the pharynx. (The microphotograph in (**b**) stems from previous work on in situ hybridization with a specific cDNA probe for keratin K4. This explains the presence of silver grains in the interpapillary epithelium [35]. *Bars*, 100 μm

of hematoxilin-eosin stained tail epidermis are viewed from the dermal side, these scales appear as well outlined half circles that are surrounded by an interscale epidermis (Fig. 1a). Under each scale, consistently groups of three hairs are located which emerge in the interscale epidermis between two scale rings [40, 41, 49]. The scale rings are out of phase so that interscale regions between two scales of one ring are opposite to the midscale region of the adjacent rings (Fig. 1a) [30, 41, 49].

Both longitudinal (Fig. 1b) and cross sections (Fig. 1c) of mouse tail skin reveal that the cells of the scale epidermis keratinize without passage through a granular layer (see also Fig. 1d), whereas the interscale epidermis between the scale rings (Fig. 1b), and also lateral to each scale of one ring (Fig. 1c) contains a pronounced stratum granulosum. The cells of the scale epidermis keratinize abruptly into a prominent stratum corneum that consists of numerous densely packed, well discernable lamellar cell layers in which, however, nuclear remnants cannot be demonstrated (Fig. 1b, c, d, and [49]). In contrast the stratum corneum formed in the interscale epidermis appears loose and flaky and exhibits a typical basket-weave structure (Fig. 1b). The transitional zone between the two differently keratinizing epidermal compartments is relatively sharp. It can best be visualized by the different staining properties of the corresponding cornified layers. Whereas the flaky stratum corneum of the interscale epidermis stains deeply red with hematoxilin-eosin, the densely packed stratum corneum of the parakeratotic scale epidermis displays a weak pink (Fig. 2a).

*Keratin pattern of adult mouse tail epidermis*

Two dimensional resolution of keratins of adult mouse tail epidermis results in a body site specific pattern

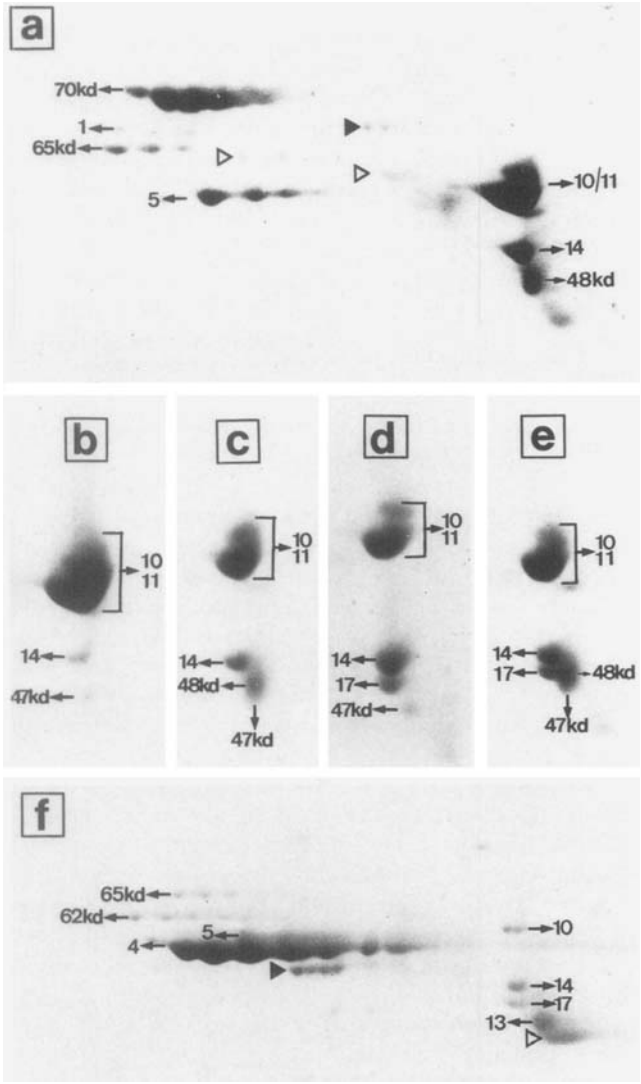


**Fig. 3a–f.** Two dimensional keratin patterns of adult mouse tail epidermis (**a**) and mouse tongue epithelium (**f**). Panels **b** to **e** show the acidic keratins of newborn mouse back epidermis (**b**), normal adult tail epidermis (**c**), tail epidermis treated either daily for two weeks with 30 μg of retinoic acid (**d**) or every two days for two weeks with 20 nmol TPA (**e**). In (**a**) the *solid arrowhead* indicates the 67.5 kD stratum corneum equivalent of the 70 kD keratin. The 64 kD and 62 kD stratum corneum equivalents of K1 are marked by *open arrowheads*. In (**f**) the *solid arrowhead* indicates the 56 kD stratum corneum equivalent of keratin K4, the *open arrowhead* denotes the 46 kD stratum corneum equivalent of keratin K13 [31]. Keratins were resolved by non equilibrium pH gradient gel electrophoresis in the first dimension (NEPHGE) and by 8.5% SDS PAGE in the second dimension (SDS)

which, besides the normal suprabasal keratins K1 and K10/11 (plus their stratum corneum equivalents) and the basal keratins K5 and K14, exhibits additional members of the murine keratin family (Fig. 3a). These members comprise the type II 70 kD keratin, that is expressed in the interscale epidermis [36] and the type II 65 kD keratin whose occurrence in the integument is virtually restricted to adult mouse tail epidermis [36, 45]. A further unusual feature of the keratin pattern of tail epidermis which we have payed little attention

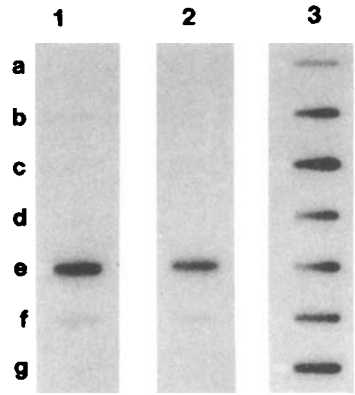


**Fig. 4a–g.** RNA slot blot hybridization. Total RNA from (*a*) neonatal mouse back epidermis, (*b*) a 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA) – induced back skin papilloma, (*c*), a DMBA/TPA induced squamous cell carcinoma, (*d*) mouse footsole epidermis, (*e*) mouse tail epidermis, (*f*) mouse tongue epithelium, (*g*) mouse palate epithelium was spotted onto a nylon membrane in a concentration of 2 μg each and hybridized with the inserts of clones λktIII-1 (*lane 1*) and λktIII-2 (*lane 2*). Lane 3 represents a control lane in which the insert of a clone coding for keratin K5 was used for hybridization

to in previous investigations [36, 42, 45], is encountered in the acidic 47–48 kD region. In this molecular weight range, the keratin patterns of other skin sites usually exhibit a generally faint 47 kD keratin which is slightly more acidic than K14 (Fig. 3b) [39, 44]. Together with K5 and K14, this keratin is expressed in basal epidermal cells [39, 44] and has most probably no counterpart in human epidermis [39]. In contrast, tail epidermis displays a prominent 48 kD keratin that partly obscures the faint 47 kD keratin (Fig. 3a and c).

*Identification of cDNA clones*

The strategy used for the isolation of cDNA clones encoding the 65 kD keratin consisted of an initial selection of all type II keratin cDNAs present in a λgt10 library constructed with polyA$^+$ RNA of adult mouse tail epidermis. To this purpose, the library was screened with a cDNA probe corresponding to a portion of the α-helical domain of the type II keratin K4 of internal stratified epithelia [21]. From the bulk of clones identified by this procedure we subsequently eliminated those clones that hybridized with the specific 3′ ends of the cDNAs of K1 and K5 (unpublished data) and the 70 kD keratin [36]. Considering that the expression of the 65 kD keratin in mouse skin is obviously restricted to adult mouse tail epidermis, each of the remaining radiolabeled clones was subjected to slot blot hybridization with RNA from various skin sites, epidermal tumors and internal stratified epithelia. This screening procedure yielded, among others [54], the phage clone λktIII-1, whose insert strongly reacted with RNA of tail epidermis and which also exhibited a weak but significant signal with RNA of tongue epithelium (Fig. 4, lane 1). At first glance this finding seems to disclose a relationship of clone λktIII-1 with the 65 kD keratin. However, pre-

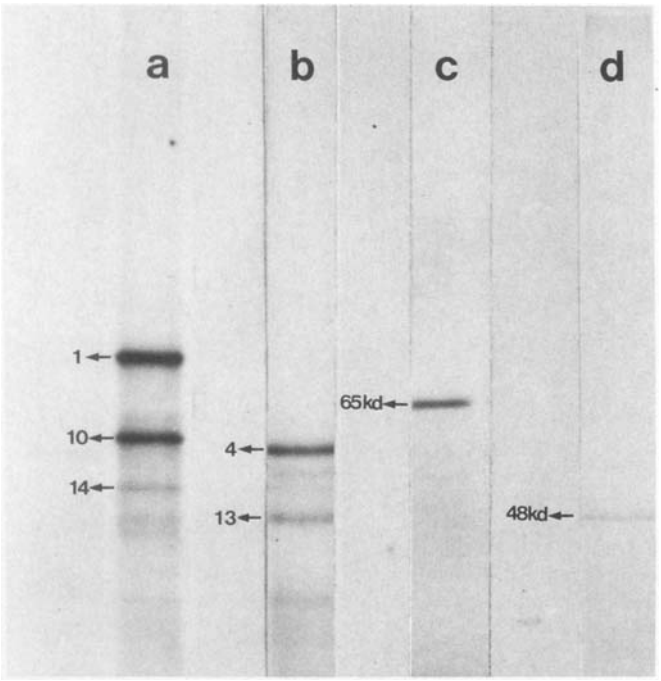


**Fig. 5a–d.** Hybrid selection analysis. The mRNA species selected by hybridization of polyA$^+$ RNA of tail epidermis to the filter-bound subclones pktlII-1–3′ (*lane c*) and pktlI-2–3′ (*lane d*) were translated in vitro in the presence of [$^{35}$S]-methionine. The translation assays were extracted for keratins as described [55] and the translation products were resolved by one dimensional SDS PAGE on 9% gels. Lanes *a* and *b* represent the keratin profiles obtained by in vitro translation of polyA$^+$ RNA of footsole epidermis (*lane a*) and tongue epithelium (*lane b*). The molecular weights of the keratins in *lane c* and *d* were estimated relative to the molecular weights of the reference keratins K1 (67 kD), K10 (60 kD), K14 (52 kD) in *lane a* and K4 (57 kD) and K13 (47 kD) in *lane b*

vious investigations in our laboratory had shown, that among the internal stratified epithelia covering the oral cavity and the upper digestive tract, tongue epithelium is the only one that also expresses a weak 65 kD keratin (Fig. 3f) [35]. Coelectrophoresis of keratins from tail epidermis and tongue epithelium revealed that the respective 65 kD keratins comigrate in two dimensional gels (results not shown), thus indicating that the two keratins are identical. Further evidence that clone λktlII-1 contains sequence information for the 65 kD keratin was gained from hybrid selection analysis of polyA$^+$ RNA of tail epidermis with subclone pktlII-1–3′. As shown in Fig. 5, lane c, upon release and in vitro transition, the selected mRNA yields a 65 kD protein.

In order to investigate whether the 65 kD keratin possesses a distinct type I partner, the λgt10 library was screened for type I keratin cDNAs by means of cDNA fragment encoding a portion of the α-helical domain of the type I keratin K13 [21]. After elimination of clones which hybridized with the specific 3′ ends of the previously isolated cDNAs of keratin K10 and K14 [23], the remaining clones were again subjected to RNA slot blot hybridization as described for type II clones. As shown in Fig. 4, lane 2, one type I clone λktlI-2, virtually exhibited the same hybridization pattern as type II clone λktlII-1, including the difference in the intensity of the hybridization signals between tail and tongue RNAs. Hybrid selection analysis with subclone pktlI-2–3′ specifically selected a mRNA which encodes a 48 kD protein (Fig. 5, lane d). It therefore appears that the 48 kD keratin of adult tail epidermis (Fig. 3a, c) represents the type I partner of the 65 kD keratin. In this context it should be mentioned that due to the strong expression of the differentiation specific type I 47 kD keratin K13 in tongue epithelium, the 48 kD keratin is apparently not detectable in two dimensionally resolved keratins of this epithelium (Fig. 3f).

### *Sequence characteristics of the clones coding for the 65 kD and 48 kD keratins*

The nucleotide sequence of the 1598 bp insert of pktlII-1 (plasmid clone of λktlII-1) and the deduced amino acid sequence of the encoded keratin are shown in Fig. 6. The insert comprises 573 nucleotides coding for the helical domain (179 amino acid residues), 357 nucleotides coding for the carboxyterminus (119 amino acid residues), the stop codon TGA, an extraordinary long 3′ noncoding tract which contains the polyadenylation signal and the poly A tail. The primary structure of the 65 kD keratin is remarkable in that its carboxyterminus is distinctly different from that of all known type II epidermal keratins. First, it does not contain a conserved H2 subdomain adjacent to the α-helical region [50, 52]. Moreover, although the entire carboxyterminus is generally rich in serine residues, it is, however, devoid of the typical accumulation of GGG or GGX motifs (with X being predominantly serine) [50, 52]. Only seven of these motifs are present that are, however, dispersed throughout the sequence and exhibit rather unusual amino acid residues in the third position (Fig. 6). It is therefore not possible to delineate a defined carboxyterminal V2 subdomain of the 65 kD keratin [50, 52]. Instead the carboxyterminus is strikingly rich in cysteine and proline residues (5 cysteines and prolines each) that are normally absent from or, at most, represent rare amino acids of the carboxytermini of epidermal keratins.

The nucleotide sequence of the 829 bp insert of clone pktlI-2 (plasmid clone of λktlI-2) and the deduced amino acid sequence of the encoded protein is shown in Fig. 7. The insert contains 366 nucleotides coding for the α-helical domain (122 amino acid residues) 228 nucleotides encoding the carboxyterminus (76 amino acid residues), the stop codon TAG, the entire 3′-noncoding tract with the polyadenylation signal and the poly A tail. Again the carboxyterminus of the encoded 48 kD keratin is rich in serine residues, however, completely lacks GGG or GGX motifs. Relative to the length of the carboxyterminus of the type II 65 kD keratin, the corresponding region of the type I 48 kD keratin contains even higher amounts of cysteine and proline residues (9 prolines, 2 cysteines) (Fig. 7).

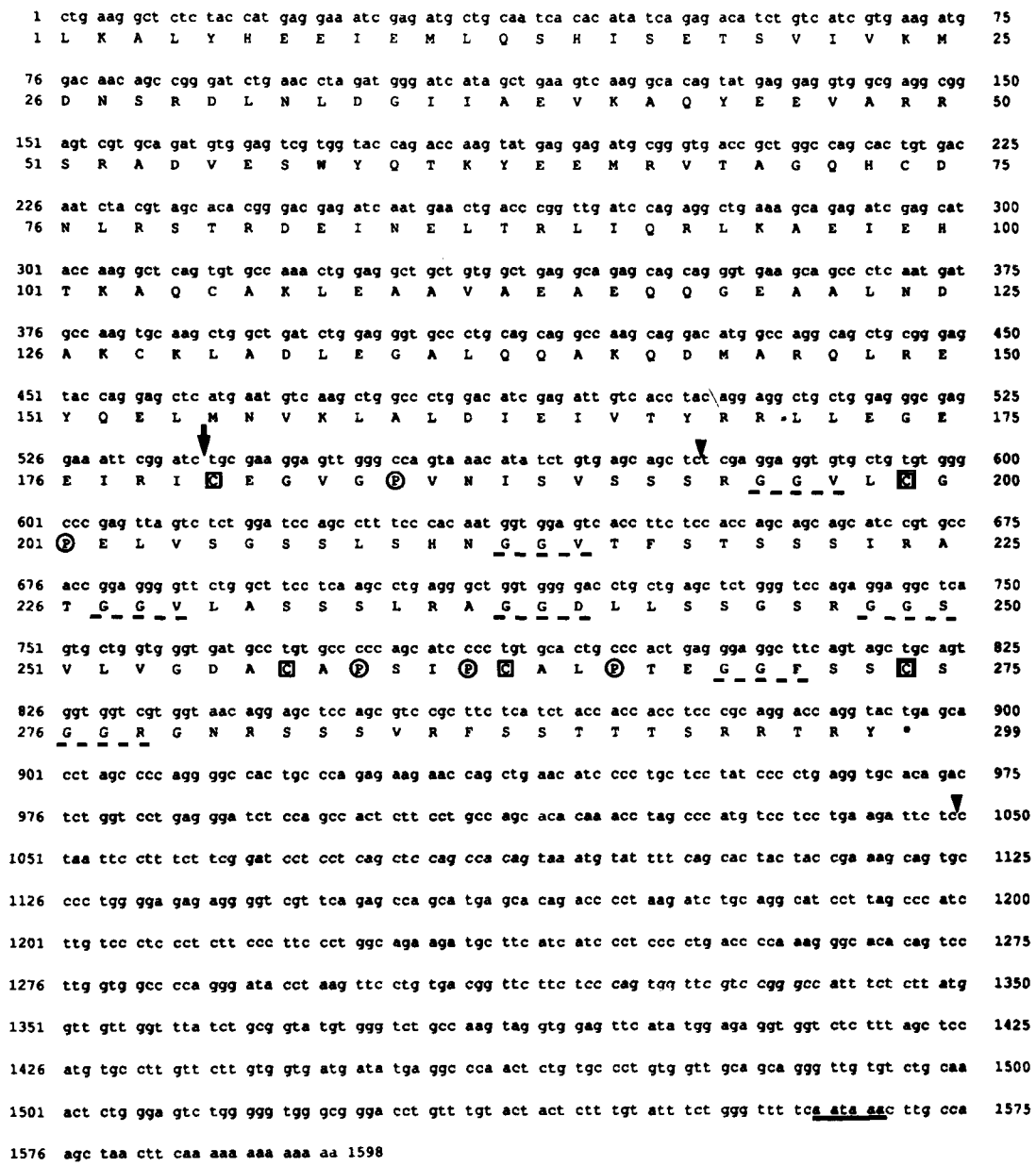

Fig. 6. Nucleotide sequence and deduced amino acid sequence of the insert of clone pktIII-1. The *arrow* indicated the end of the α-helical domain. Proline residues in the carboxyterminal domain are *encircled*, cysteine residues are *boxed*. GGX motifs are *underlined by a broken line*, the polyadenylation signal is marked by a *solid line*. The *small arrowheads* indicate the cDNA fragment inserted in subclone pktIII-1–3′ that was used for hybrid selection, slot blot hybridization and in situ hybridization

*In situ hybridization to tail skin*

To investigate the site of expression of the mRNAs of the 65 kD and 48 kD keratins, radiolabeled riboprobes complementary to the 3′-terminal regions of the corresponding mRNAs were used for in situ hybridization to frozen sections of mouse tail skin.

As shown in Fig. 8a, b and c, the expression of the 65 kD keratin mRNA is restricted to suprabasal cells of the parakeratotic scale epidermis. No hybridization signals occur in the orthokeratotic interscale epidermis. Moreover, control sections of other orthokeratinizing skin sites (i.e., newborn mouse back skin, adult mouse footsole skin) were equally devoid of hybridization signals in the corresponding epidermis (results not shown). Virtually identical results were obtained by in situ hybridization with the 3′-riboprobe of the 48 kD keratin clone pktII-2 (Fig. 8d). There is a clear cut gradient with regard to the intensity of the hybridization signals within the suprabasal compartment of tail scale epidermis (Fig. 8a, c, d). Whereas generally, parabasal and lower suprabasal cells show low grain counts, the highest density of grains clearly occurs in the uppermost living cells and abruptly ceases at the transition from living to dead cell layers (Fig. 8c). It is worth mentioning that in situ hybridization with both probes to skin sections which

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  1  tgg ttc atc gct cag acc gag gag ctg aac cag cag gtg gta tcc agc tca gag cag ctg cag tgt tgc cag aca   75
  1  W   F   I   A   Q   T   E   E   L   N   Q   Q   V   V   S   S   S   E   Q   L   Q   C   C   Q   T    25

 76  gag atc att gag ctg aga cgc act agt caa ctc ctg gag atc gag ctg cag gct cga cag agc atg agg cat tct   150
 26  E   I   I   E   L   R   R   T   S   Q   L   L   E   I   E   L   Q   A   R   Q   S   M   R   H   S    50

151  ctg gaa tct acc ctg gct gag aca gag gcc cgc tac agc tcc cag ctg ggc cag atg cag tgc ctg atc acc aag   225
 51  L   E   S   T   L   A   E   T   E   A   R   Y   S   S   Q   L   G   Q   M   Q   C   L   I   T   K    75

226  gtg gaa tcc cag ctg gct gag att cgt tgt gac ctg gag cgg cag aac cat gag tat cag gtg tta ctg gat gtc   300
 76  V   E   S   Q   L   A   E   I   R   C   D   L   E   R   Q   N   H   E   Y   Q   V   L   L   D   V   100

301  aag gcc cgg ctg gag tca gag att gcc acc tac cgt cgc ctg ctg gat ggg gag gac tgc aag ctt cct gcc cac   375
101  K   A   R   L   E   S   E   I   A   T   Y   R   R   L   L   D   G   E   D   C   K   L   P   A   H   125

376  cct tgt tcc acg gaa tgc aag cct gct gtt aag agt acc tta tat ccc ttc tac aac ctg cac tcc agc tgg gcc   450
126  P   C   S   T   E   C   K   P   A   V   K   S   T   L   Y   P   F   Y   N   L   H   S   S   W   A   150

451  ctg cac ccc agc tgg gcc ctg cac ccc agc tcc tca agt cag cac cca gat ccg gac cat cac cga gga gat cag   525
151  L   H   P   S   W   A   L   H   P   S   S   S   S   Q   H   P   D   P   D   H   H   R   G   D   Q   175

526  gga tgg aag agt cat ctc ctc cag gga gca cgt ggt gcc ccg ggc aat gtg agc agc cac cag agg gcc tag gcc   600
176  G   W   K   S   H   L   L   Q   G   A   R   G   A   P   G   N   V   S   S   H   Q   R   A   *       199

601  aca gga agg agg aca cct tgt ggt tct tgg cag cta aat gat ctt gca ctt ctc tag ggc cct cct tgc tta tca   675

676  ggt ttt tct act caa att cat cta ttg ttt ctg gtc tta act gtg ctt ttt acg cca tgc aaa acc agg ttc ccc   750

751  tgg aaa tat tcc caa taa agt gtt ctc ttg gca tag caa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa   825

826  aa 827
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**Fig. 7.** Nucleotide sequence and deduced amino acid sequence of the insert of clone pktII-2. For the key of signs, see legend to Figure 6

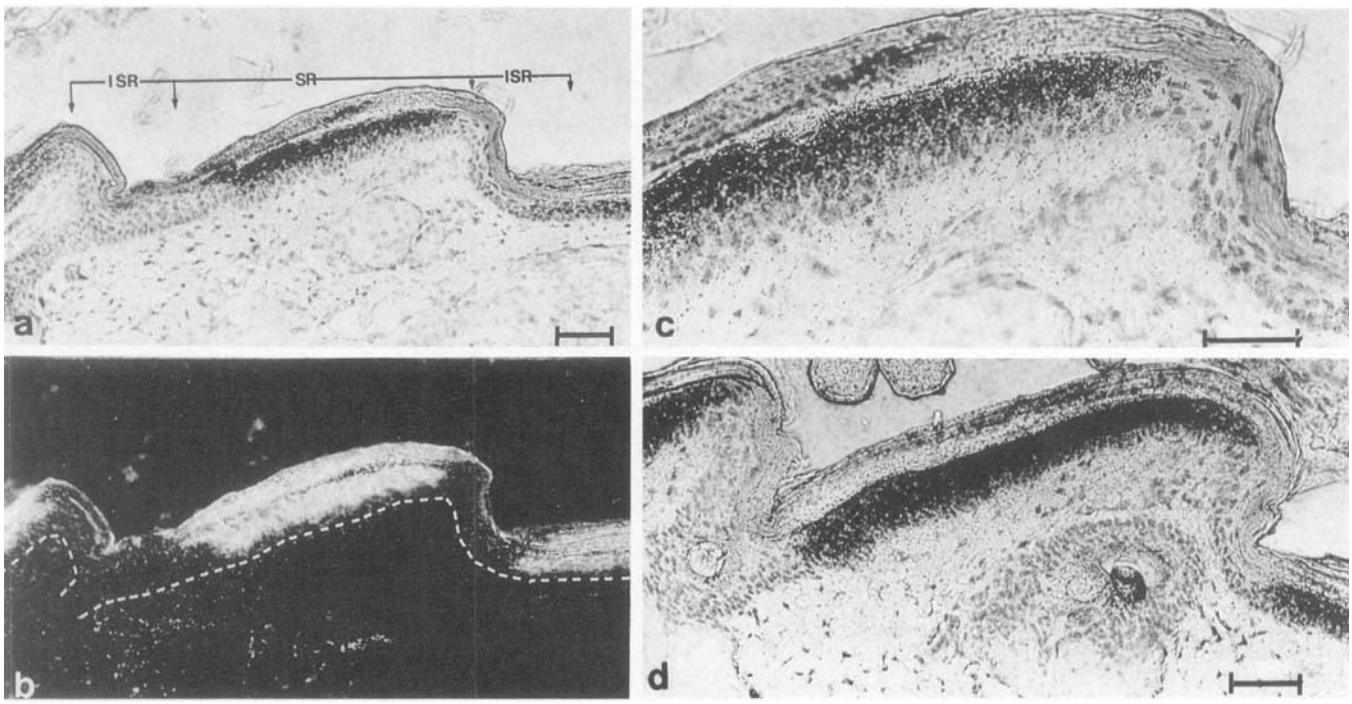


**Fig. 8a–d.** In situ hybridization with the [$^{35}$S]-labeled riboprobes of subclones pktIII-1–3′ (**a–c**) and pktII-2–3′ (**d**) to frozen sections of adult mouse tail skin. **b** is a dark field illumination of **a**; **c** is a higher magnification of **a**. *ISR*, interscale region; *SR*, scale region. *Bars*, 100 μm throughout

contain hair follicles in the dermal part (i.e., newborn mouse back skin, tail skin) also reveals weak hybridization signals that seem to be located over suprabulbar cells of the hair follicles (Fig. 9a, b, c). The localization of this label is reminiscent of that recently observed by in situ hybridization with a specific 3′ cDNA probe of a murine type II hair keratin to hair follicles [54]. On the other hand, the hair keratin specific probe did not label tail scale epidermis [54].

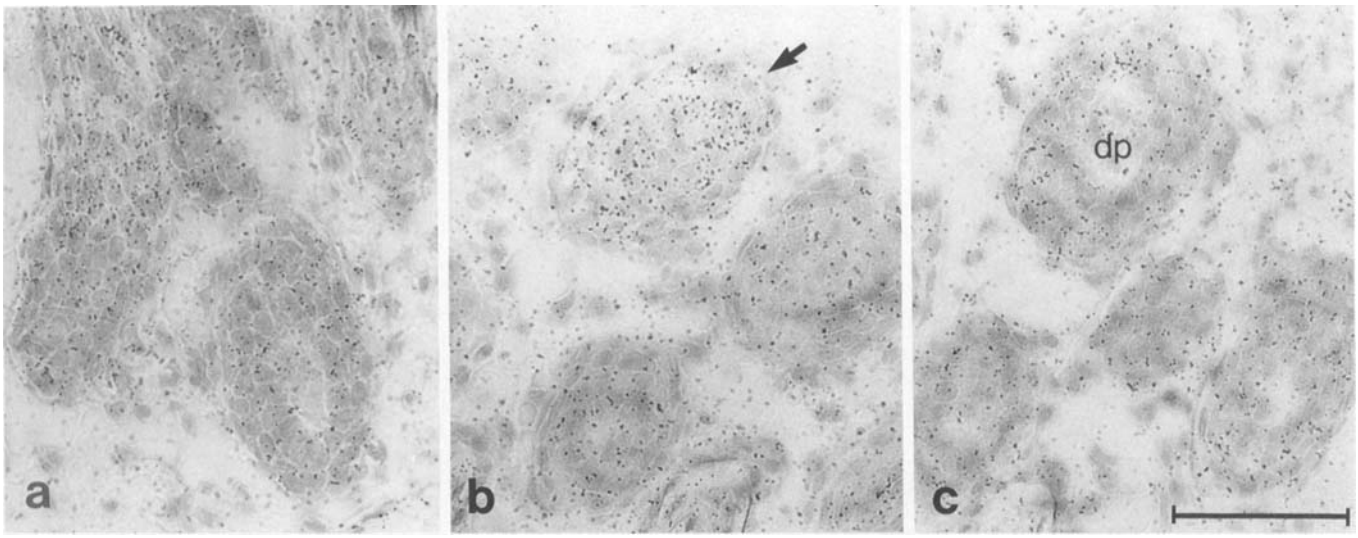


**Fig. 9a–c.** In situ hybridization with the [$^{35}$S]-labeled riboprobes of subclones pktlII-1–3′ **a, b** and pktlI-2–3′ **c** to frozen sections of newborn mouse back skin. The *arrow* in **b** denotes a cross section of a hair follicle in which the suprabulbar localization of hybridization signals is well visible. *dp*, dermal papillae. *Bar*, 100 μm throughout

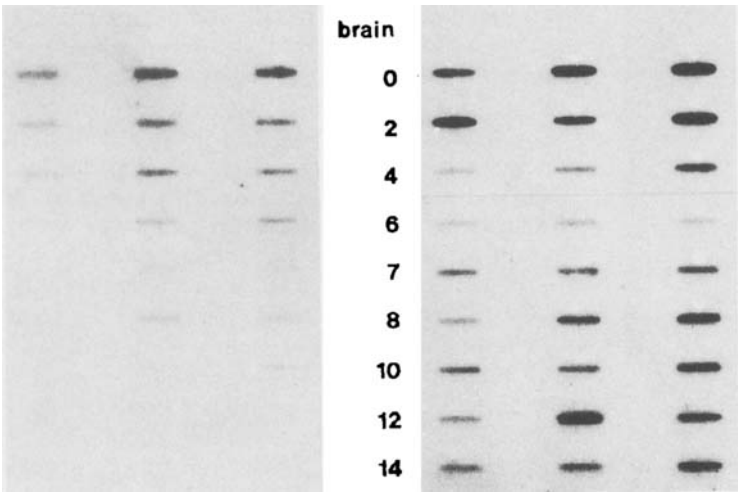


**Fig. 10a, b.** Slot blot hybridization. RNA was isolated from adult mouse tail epidermis after different time intervals of treatment with retinoic acid (RA) (panel **A**) and TPA (panel **B**) and hybridized with the specific cDNA probes for the mRNAs of the 48 kD keratin (panel **A**) or the 65 kD keratin (panel **B**). The time intervals are indicated at the *left* side of each panel. The different RNA concentrations are given on *top* of each panel. RNA isolated from mouse brain was used as control

*Fate of the 65 kD and 48 kD keratins in retinoic acid or TPA treated tail epidermis*

We have previously shown that the continuous treatment of adult mouse tail epidermis with retinoic acid leads to the induction of a generalized epidermal hyperplasia and the complete orthokeratotic conversion of the parakeratotic scale epidermis [41, 45]. Concomitant with the progressive orthokeratinization in the scale epidermis, we observed a gradual disappearance of the 65 kD keratin protein which was no longer detectable electrophoretically after two weeks of regular retinoic acid application [45]. However, in this study, the fate of the 48 kD keratin was not explicitely followed, although we noticed a strong reduction of the keratin proteins in the 47–48 kD molecular weight range [45]. In the present study we have therefore investigated changes in the transcriptional activity of both the 65 kD and 48 kD keratin genes during retinoic acid mediated orthokeratotic conversion of tail scale epidermis. To this purpose we used the specific 3′ cDNA probes of the corresponding keratins for slot blot hybridization to tail epidermis RNA isolated at different time intervals of retinoic acid treatment. We observed an essentially identical time course with regard to the suppression of the synthesis of both mRNAs which is shown here for the 48 kD keratin mRNA in Fig. 10, panel A. It can be seen that already after 10 to 12 days of treatment with the vitamin, the synthesis of the 48 kD keratin mRNA is completely suppressed (Fig. 10, panel A). Two dimensional resolution of keratins obtained 14 days after retinoic acid treatment reveals the complete absence of the 48 kD keratin in the acidic low molecular weight region, in which besides the basal keratin K14 and the hyperproliferation asso-

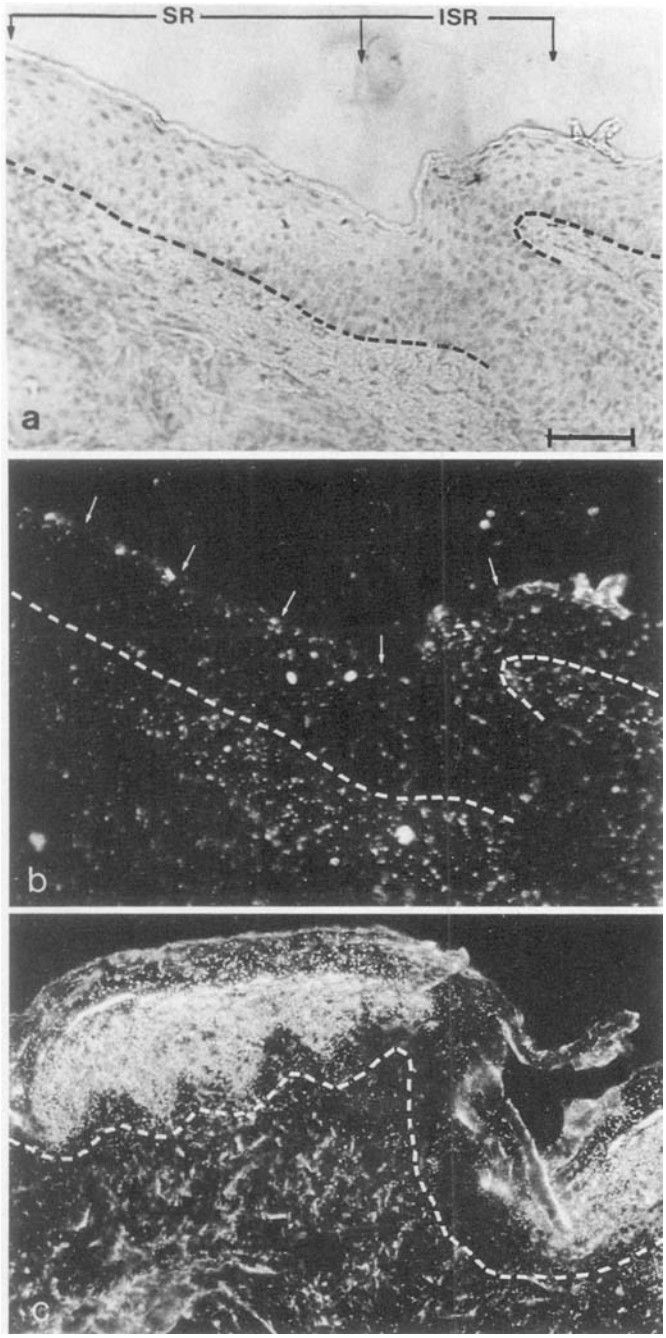


**Fig. 11a–c.** In situ hybridization with the [$^{35}$S]-labeled riboprobes of subclones pktIII-1–3′ **a–b** and pktII-2–3′ **c** to frozen sections of tail skin treated either daily for two weeks with retinoic acid **a, b** or every two days for two weeks with TPA **c**. **b** is a dark field illumination of **a**. The *dashed line* indicates the epidermal-dermal junction. The *small arrows* in **b** denote the stratum corneum. Note the strongly undulated dermal epidermal junction in the scale epidermis of TPA treated tail skin **c**. *Bars*, 100 μm throughout

ciated keratin K17 only the faint 47 kD keratin is visible (Fig. 3d).

In our previous study we were also able to show that a two week treatment of tail epidermis with the hyperplasiogenic phorbol ester TPA did not alter the para-orthokeratotic epidermal patterning and hence did not suppress the synthesis of the 65 kD keratin [45]. Consequently, under these conditions, the expression of its mRNA is not affected (Fig. 10, panel B), although we observed a slight decrease in the expression of the mRNA at days 4 and 6 of TPA treatment which coincides with the onset of epidermal hyperplasia [40, 42, 45]. Essentially the same results were obtained with regard to the expression of the mRNA of the 48 kD keratin (results not shown). Moreover the 48 kD keratin can easily be detected in keratin extracts of TPA treated tail epidermis (Fig. 3e).

The suppression of the synthesis of the 65 kD and 48 kD keratin mRNAs in retinoic acid treated orthokeratotic tail epidermis and the maintainance of their synthesis in TPA treated tail epidermis can also be demonstrated by in situ hybridization to frozen sections of the corresponding skins (Fig. 11a–c).

### *In situ hybridization to tongue epithelium*

The morphologically complex mouse tongue epithelium displays distinct areas in the filiform papillae that exhibit a strikingly similar type of parakeratosis to mouse tail scale epidermis. Three major units with different types of keratinization can be distinguished in the murine filiform papillae [7, 8, 9, 35]: an anterior orthokeratinizing unit whose loose and flaky stratum corneum resembles that of the parapapillary epithelium, a central core unit with a hook-shaped horny spine and a posterior unit (Fig. 2b). Both the central and the posterior units completely lack a granular cell layer (Fig. 2b, and [8]). Whereas the solid spine of the central core unit is almost refractory to hematoxilin-eosin staining (Fig. 2b), the stratum corneum formed in the posterior unit – just as that of parakeratinizing tail scale epidermis (Fig. 2a) – consists of numerous densely packed cell layers without nuclear remnants [49] and stains a weak pink with hematoxilin-eosin (Fig. 2b).

It should be emphasized that a clear cut distinction of the three units of the murine filiform papillae depends essentially on the plane of section. In Fig. 2b, the three papillary units are entirely visible only in the two central papillae, whereas in the two lateral papillae, only the living cells of the central core unit are detectable.

Previous studies have shown that basal cells of the murine filiform papillae express keratins K5 and K14 [35]. In suprabasal cells of the anterior unit, K13 is expressed together with a still unknown type II keratin [9, 35]. Recent immunofluorescence and in situ hybridization studies have revealed that suprabasal cells of the central core unit presumably express the entire set of hair keratins [9, 54].

The results of the in situ hybridization with the specific 3′ riboprobes of the 65 kD and 48 kD keratins to frozen sections of tongue are shown in Fig. 12a–d. It is evident that as in tail epidermis the orthokeratinizing areas of the dorsal tongue epithelium, including fungiform papillae (Fig. 12d) are unlabeled. The hybridization signals clearly occur over suprabasal living cells of the two parakeratinizing papillary units (Fig. 12a–d). In appropriately sectioned papillae, it can readily be seen

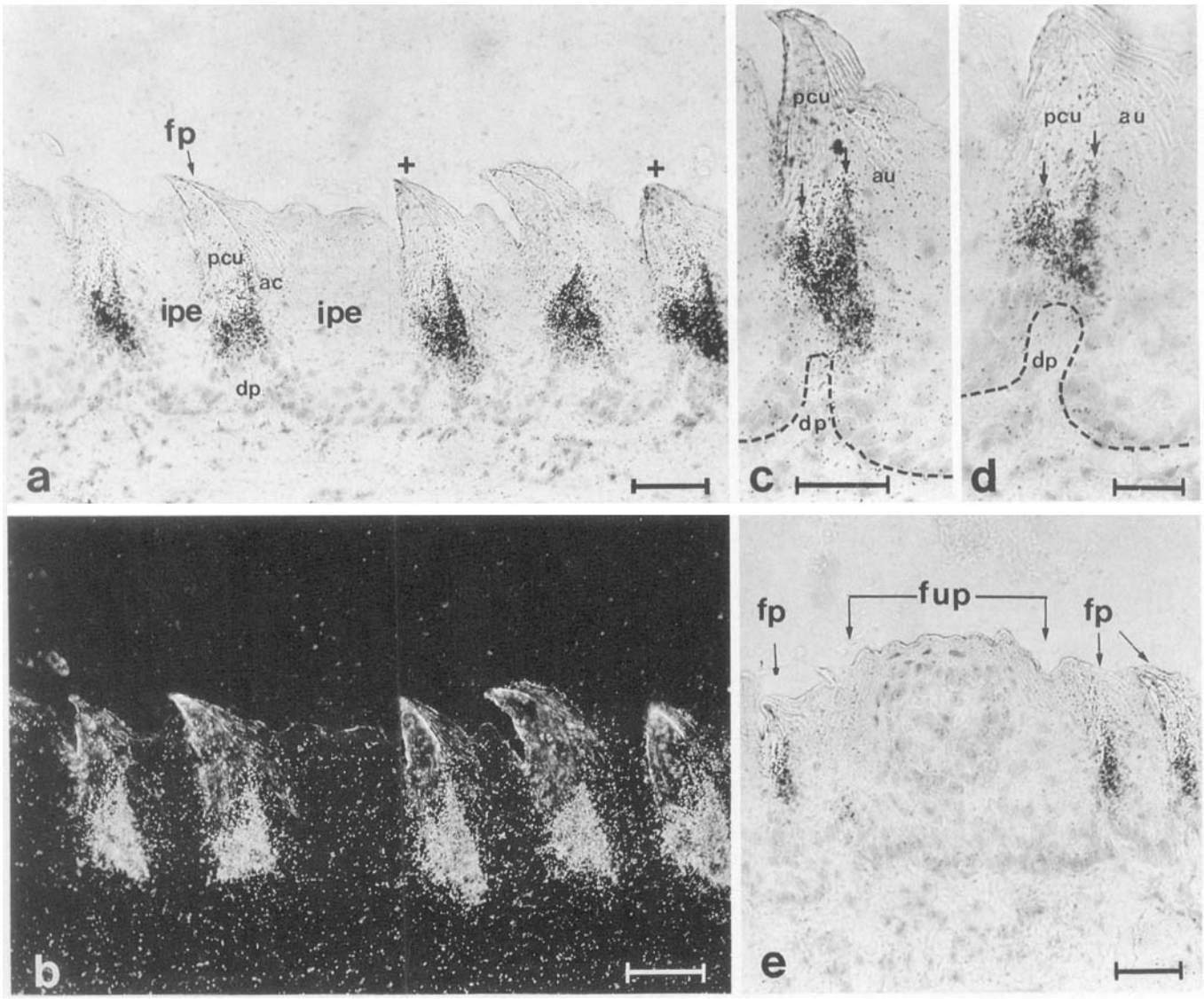


**Fig. 12a–e.** In situ hybridization with the [$^{35}$S]-labeled riboprobes of subclones pktlII-1–3′ **a–d** and pktlI-2–3′ **e** to frozen sections of mouse tongue. **b** is a dark field illumination of **a**; **c** and **d** show two filiform papillae at higher magnification in order to better visualize the hybridization pattern in the posterior and central core unit (*small arrows*). Filiform papillae with a similar pattern are indicated by asterisks in **a**. *fp*, filiform papillae; *ipe*, interpapillary epithelium; *pcu*, posterior and central core unit; *au*, anterior unit; *dp*, dermal papillae; *fup*, fungiform papillae. *Bars*, 100 μm throughout

that living cells of the central core unit generally occupy a higher position than the corresponding cells of the posterior unit (see Fig. 2b). Moreover, the uppermost living cells of the central core unit ascend along the boundary to the anterior papillary unit, whereas the corresponding cells of the posterior unit preferentially ascend along the boundary to the interpapillary epithelium (Fig. 2b). This positional difference is clearly reflected by the hybridization pattern of the two papillae that are marked with an asterisk in Fig. 12a, and the magnified papillae in Fig. 12c and d.

## Discussion

In this study we present a new murine 65 kD and 48 kD keratin pair whose suprabasal expression in stratified squamous epithelia is associated with a particular type of parakeratotic differentiation. This epithelial phenotype is characterized by the complete absence of a granular cell layer and the formation of a multilayered, conspicuously compact stratum corneum without nuclear remnants. In mouse skin, this epithelial phenotype is encountered in tail scale epidermis of the adult animal, however, it also occurs in the posterior unit of the filiform papillae of murine lingual epithelium. The causal relationship of the 65 kD and 48 kD keratins with the formation of this parakeratotic phenotype is emphasized by the retinoic acid-mediated suppression of the synthesis of the two keratins which leads to an orthokeratotic conversion of the scale epidermis concomitant with the re-expression of the normal "skin type" keratin pattern of the orthokeratinizing neonatal mouse tail epidermis [33, 42, 45].

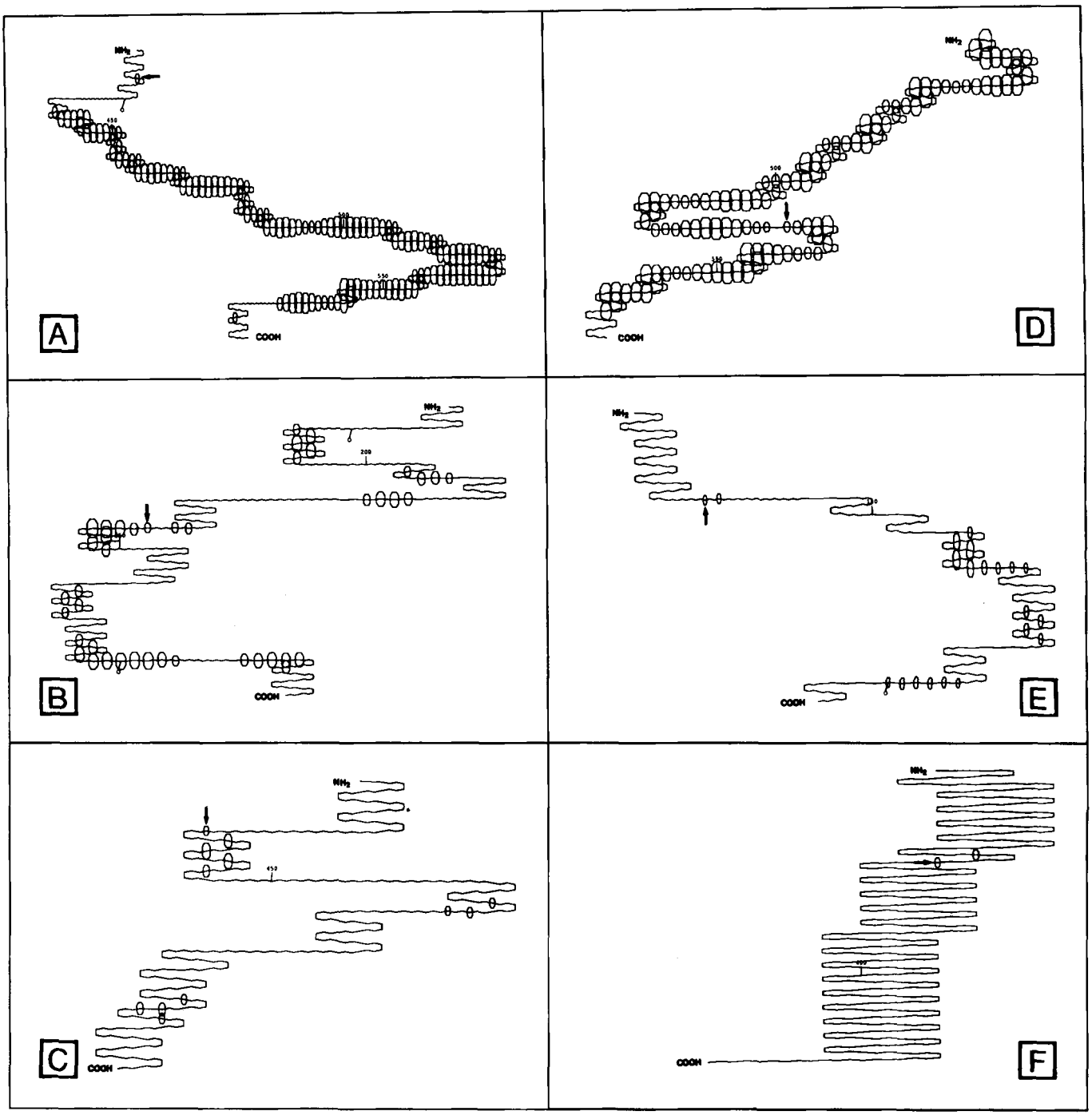


**Fig. 13A–F.** Schematic presentation of the flexibility of the carboxyterminal domains of murine type II and type I "soft" and "hard" α keratins in comparison with the corresponding regions of the 65 kD and 48 kD keratins. **A** epidermal keratin K1 [52]; **B** 65 kD keratin; **C** hair keratin Hb4 [54, 56]; **D** epidermal keratin K10 [51]; **E** 48 kD keratin; **F** hair keratin Ha1 [4]. The secondary structure of the protein sequences was determined according to Garnier et al. [14]. Flexibility predictions were calculated by the method of Karplus and Schulz [20]. Basic flexibility index was set to 1.040. This value is graphically recorded by the oval sign indicated by an *arrow* in each panel. Flexibility indices >1.040 are reflected by the size of the signs. In keratins K1 and K10 (panels **A, D**), sites of highest flexibility occur in the glycine-serine rich V2 subdomain. In the carboxyterminus of the 65 kD keratin (panel **B**) the seven GGX motifs (see Fig. 6) colocalize with or are close to sites of high flexibility

The regulation of expression of the 65 kD and 48 kD keratins appears to be essentially different from that of suprabasal keratins in orthokeratinizing epithelia. In the latter, the highest rate of the expression of the mRNAs of differentiation specific keratins occurs in parabasal and lower spinous cells and gradually decreases toward the uppermost living cell layers [23, 30, 35, 37] in which the genes for loricrin [27, 30] and filaggrin [12] are successively activated. In contrast, the synthesis of the mRNAs of the 65 kD and 48 kD keratin pair in tail scale epider-

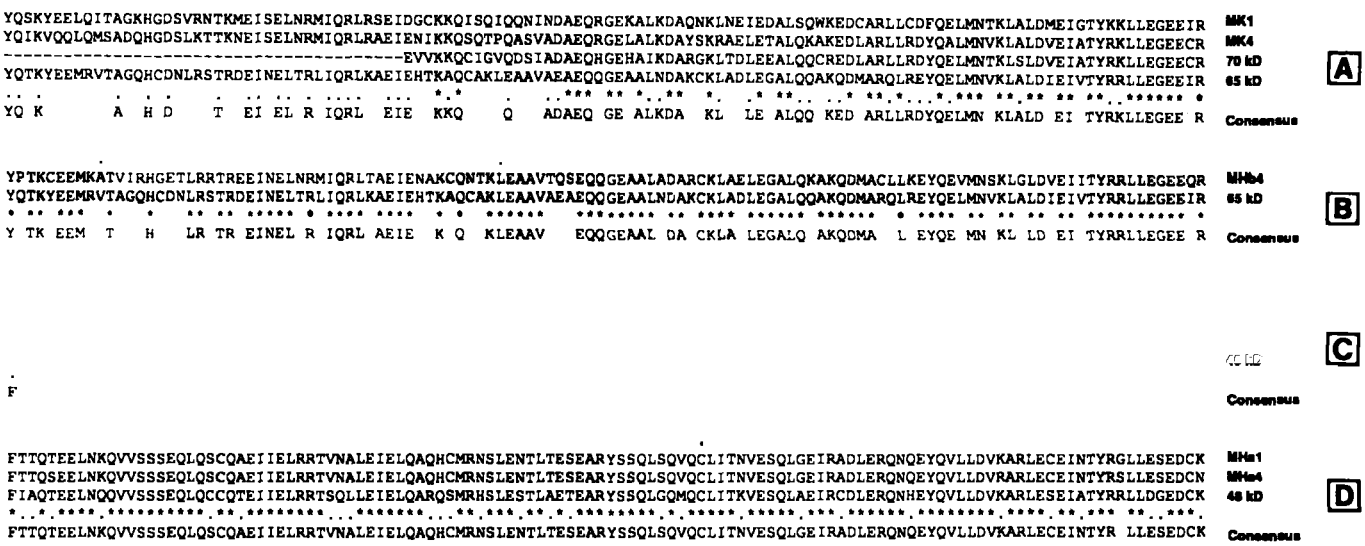


**Fig. 14.** Sequence homology comparisons between the α-helical 2B subdomains of the type II 65 kD keratin and various type II keratins expressed in mouse epithelia [**A**] and the murine type II keratin Hb4 [**B**]. Also shown are the corresponding comparisons between the type I 48 kD keratin and various type I keratins expressed in mouse epithelia [C] and two murine type I hair keratins, Ha1 and Ha4 [**D**]. Under each of the multiple comparisons a consensus sequence is indicated. The sequences are taken from MK1 [52], MK4 [21], 70 kD keratin [36], Hb4 [54, 56], MK10 [51], MK13 [21], MK19 [25], MHa1 [4], Ha4 [5]

mis increases steadily from parabasal to the uppermost living cells in which neither loricrin [30] nor filaggrin (B. Dale, personal communication) are synthesized. The absence of an orthokeratogenous zone with pronounced intracellular autolytic processes in tail scale epidermis may explain why, unlike orthokeratinization-associated keratins, the 65 kD and 48 kD are apparently not subject to posttranslational modifications.

The most striking difference of the new murine 65 kD and 48 kD keratins with those expressed in orthokeratinizing epithelia concerns the amino acid composition and structural organization of their non α-helical domains. It is evident that the carboxyterminal sequences of the 65 kD and 48 kD keratins are devoid of the typical subdomains into which the tail portions of epithelial type II and type I keratins can usually be divided [50, 52]. Interestingly the absence of defined non α-helical subdomains is also a characteristic feature of the "hard" α keratins of wool, hairs and nails [4, 5, 10, 48, 54, 56]. Similarly, the 65 kD and 48 kD keratins share a conspicuously high content of carboxyterminal cysteine and proline residues with "hard" α keratins [4, 5, 10, 48, 54, 56]. However, both the overall content of cysteine and proline residues and, in particular, the occurrence of the two amino acids as multiple proline-cysteine tandems is clearly below that of "hard" α keratins [4, 5, 10, 48, 54, 56]. Moreover, the carboxyterminus of the type II 65 kD keratin has maintained a certain number of dispersed GGX motifs, typical for the non α-helical V2 subdomain of type II epithelial keratins [50, 52] and practically absent from the carboxyterminal tail portion of type II hair keratins [48, 54, 56]. On the other hand, comparative flexibility calculations for the carboxytermini of type II and type I epithelial and hair keratins and the 65 kD and 48 kD keratins suggest a closer relationship of the latter with hair keratins. As can be seen from Fig. 13, with regard to both the number of flexible sites and the flexibility indices of these sites, the 65 kD and 48 kD keratins display a higher resemblance to hair keratins than to epithelial keratins. The apparent structural relationship of the 65 kD and 48 kD keratins with hair keratins is further emphasized by sequence homology comparisons of their terminal α helical 2B subdomains with the corresponding sequences of murine "soft" and "hard" α keratins. Such an analysis reveals a much higher homology of the 65 kD and 48 kD keratins with hair keratins (about 80%) than with epidermal keratins (about 55%) (Fig. 14). If the α-helical 2B subdomains listed in Fig. 14 (supplemented by the α-helical 2B subdomain of sheep wool type II keratin 7c [48]) are used to investigate the evolutionary relationship of the keratins [6], the resulting phylogenetic tree (Fig. 15) clearly shows that the 65 kD and 48 kD keratins have evolved together with hair keratins from a common ancestor, however have diverged during evolution to constitute a separate branch of hair-related keratins.

Considering that the high percentage of cysteine residues in the apparently rather unflexible head and tail portions of "hard" α keratins essentially contributes by extensive disulfide protein bridging to the formation of the hard keratinized structures of hairs and nails, one would expect that stratified epithelia that differentiate via the expression of the hair-related 65 kD and 48 kD keratin pair with a distinctly lower cysteine content in more flexible non α-helical end domains should display less compact keratinized structures. This is clearly the case with regard to the consistence and morphological appearance of the stratum corneum formed in tail scale epidermis and the posterior unit of the murine filiform papillae. Both exhibit well discernable parallel or obliquely staggered arrays of cornified cells whose affinity to hematoxilin-eosin is intermediate between that of

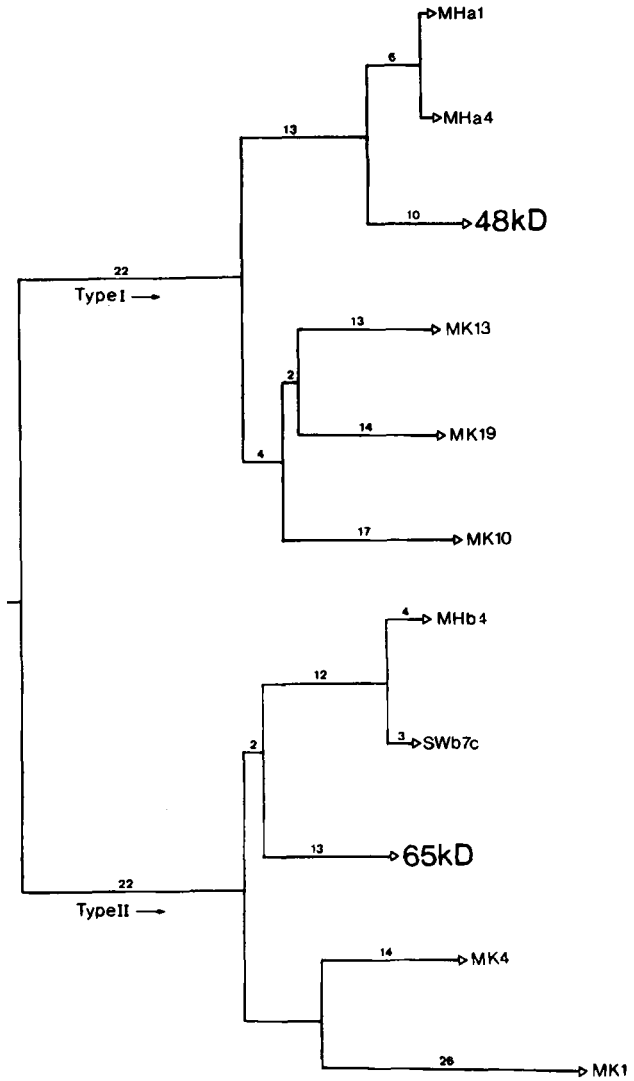


**Fig. 15.** Phylogenetic tree constructed for various murine type II and type I "soft" and "hard" α keratins and the 65 kD and 48 kD keratins on the basis of the α-helical 2B subdomains [6] of the proteins. *Numbers* above the branches indicates branch lengths in amino acid replacements. For the references of the sequences, see legend to Fig. 14; the sequence of the sheep wool type II keratin 7c (SWb7c) was taken from reference [48]

an orthokeratinized stratum corneum and the horny substance formed by "hard" α keratins. These properties are especially well visible in the murine filiform papillae in which the three types of keratinization are adjacent to each other [8, 9, 19, 49].

In this context it should be mentioned that the murine filiform papillae also expresses the skin type keratins K1 and K10 [9, 35]. Both indirect immunofluorescence and in situ hybridization studies suggest that these keratins may be expressed in the parakeratinized parts of the filiform papillae [9, 35]. Recently Dhouailly et al. have tentatively assigned the skin type keratins to the posterior papillary unit [9]. This is, however, highly improbable since the expression of these keratins can hardly be reconciled with parakeratotic differentiation. We do not exclude that the three dimensional structure of the murine filiform papillae is more complex than presently assumed and that it contains small lateral compartments that, similar to the hair surrounding cuticle [16, 17], may express these keratins.

Our investigations have also shown that under certain circumstances i.e., in the central core unit of the murine filiform papillae and in the suprabulbar portion of hair follicles, the hair-related keratin pair is apparently coexpressed with true "hard" α keratins. The demonstration of the mRNAs of the 65 kD and 48 kD keratins in mouse hair follicles is particularly important in view of recent investigations on keratin expression in these skin appendages. Two dimensional resolution of in vivo [$^{35}$S] methionine labeled keratins of murine hair roots, not only yielded the eight members of the murine hair keratin family, but also revealed the presence of a minor acidic protein "of unknown identity" [4, 5]. The protein clearly exhibits a molecular weight of 48 kD [4, 5]. This strongly suggests that it corresponds to our 48 kD hair-related keratin, in as much as the isoelectric points of the two proteins appear identical [4]. Interestingly, recent work by Heid et al. has shown that besides hair keratins, both human and bovine hair follicles also express low amounts of an additional keratin pair that was designated Hbx (type II) and Hax (type I), respectively [16, 17]. SDS PAGE size estimates of the two proteins are strikingly close to the murine 65 kD and 48 kD hair-related keratins. Hbx, both human and bovine, exhibits a molecular weight of 64 kD, whereas the human Hax (about 54 kD) is slightly larger than its bovine 50 kD analogue [17]. Moreover, immunofluorescence studies of human and bovine hair follicles with an antibody that specifically recognized Hax in Western blots, revealed a weak reaction in upper cortex cells [17], thus being reminiscent of the site of expression of the 65 kD and 48 kD mRNAs in murine hair follicles. The obvious conclusion that can be drawn from these data is that Hbx and Hax may represent the human and bovine analogues of the murine 65 kD and 48 kD hair-related keratins. This would imply that keratinized structures of human epithelia that express Hbx and Hax as differentiation specific keratin pair, should be morphologically close to those of mouse tail scale epidermis or the posterior unit of the murine filiform papillae. This assumption is confirmed by investigations of the human filiform papillae [9, 18] which is structurally distinct from its murine analogue [3, 9, 18]. In particular, cells of the core unit differentiate into a tapering projection that virtually lacks the horny and rigid aspect of the stiff spine formed in the murine papillary core unit [3, 9, 18]. Rather its flexible tip displays a tier of flattened, cresent-shaped cornified cells which exhibit a reduced affinity to hematoxilin-eosin in comparison to the adjacent orthokeratinizing epithelium [3, 47]. Recently Heid et al. were able to show that Hbx and Hax are expressed in suprabasal cells of the core unit of the human filiform papillae, however, clearly in the absence of hair keratins synthesis [18], and H.W. Heid, personal communication). These findings were recently confirmed by Dhouailly et al. who reported on the identification of a new "hair-related" suprabasal type I 51 kD keratin

– obviously corresponding to Hax – in the papillary core unit [9]. Also these authors were unable to demonstrate concomitant "hard" α keratin synthesis in the human filiform papillae [9]. We take these biochemical and morphological data as most convincing evidence for an orthologous relationship between the human Hbx/Hax keratin pair and the murine hair-related 65 kD and 48 kD keratins.

In summary this investigation has led to the identification of a new keratin pair that has evolved together with hair keratins. During evolution, however, it has diverged from hair keratins and constitutes an independent pair of hair-related keratins. The highly specialized epithelial phenotype which is associated with the expression of the hair-related keratins in different species is exceptionally rare. Apparently it has been acquired by anatomical sites in which an orthokeratinizing epithelium would be too soft and a hard keratinized structure would be too rigid to optimally meet the functional requirements of the respective epithelium. Thus the function of the mouse tail as a prehensile tail which not only necessitates an acceptable skin flexibility but also requires protection against mechanical wear is fulfilled through the solid parakeratotic scale epidermis in combination with an extensible orthokeratotic hinge region. The murine lingual filiform papillae are important for grooming the hair coat, rasping coarse food, transporting food particles toward the pharynx and for the lapping up of water [9, 49]. For all these functions the exceptionally stiff horny spine of the central core unit plays a pivotal role. It is, however, essentially supported in its manyfold functions by the adhering collar of distinctly more elastic cornified cells formed in the posterior unit which is, not without cause, also called the "buttress unit" [19]. The diversification of the filiform papillae's morphology and keratin expression as a result of adaptation to different functions is best exemplified by the human filiform papillae.

At present the functional significance of the expression of the hair-related keratins in "hard" α keratin expressing epithelial appendages is unknown. Preliminary investigations by Heid et al. on human hair follicles indicate that the hair-related keratins are not obligatory coexpressed with "hard" α keratins [17]. Rather their synthesis seems to occur at later stages of trichocyte differentiation [17]. Further studies with monospecific antibodies against the carboxyterminal end regions of the hair-related keratins are needed to unravel their functional involvement during the formation of hard keratinized structures of hairs, nails and also the central core unit of some filiform papillae.

Finally, since there is obviously no evidence for the existence of more than one pair of hair-related keratins, we propose, by analogy with the nomenclature for hair keratins, to designate the type II member of this keratin pair by HRb-1 (*H*air *R*elated *b*asic) and the type I member by HRa-1 (*H*air *R*elated *a*cidic). Appropriate prefixes may then identify the respective species-specific keratin pair.

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## References

1. Angerer LM, Angerer RC (1989) In situ hybridization with [$^{35}$S]-labeled RNA probes. DuPont Biotech Update 4:3–6
2. Angerer LM, Cox KH, Angerer RC (1987) Demonstration of tissue-specific gene expression by in situ hybridization. Methods Enzymol 152:649–661
3. Bangemann W (1977) Histologie und mikroskopische Anatomie. Thieme Verlag Stuttgart, New York
4. Bertolino AP, Checkla DM, Notterman R, Sklaver I, Schiff TA, Freedberg IM, DiDona GJ (1988) Cloning and characterization of a mouse type I hair keratin cDNA. J Invest Dermatol 91:541–546
5. Bertolino AP, Checkla DM, Heitner S, Freedberg IM, Yu DW (1989) Differential expression of type I hair keratins. J Invest Dermatol 94:297–303
6. Blumenberg M (1989) Evolution of homologous domains of cytoplasmic intermediate filament proteins and lamins. Mol Biol Evol 6:53–65
7. Cameron IL (1966) Cell proliferation, migration and spezialization in the epithelium of the mouse tongue. J Exp Zool 163:271–284
8. Cane AK, Spearman RIC (1968) The keratinized epithelium of the mouse (*mus musculus*) tongue: Its structure and histochemistry. Arch Oral Biol 14:829–841
9. Dhouailly D, Xu C, Manabe M, Schermer A, Sun T-T (1989) Expression of hair-related keratins in a soft epithelium: Subpopulations of human and mouse dorsal tongue keratinocytes express keratin markers for hair-, skin-, and esophageal-types of differentiation. Exp Cell Res 181:141–158
10. Dowling LM, Crewther WG, Inglis AS (1986) The primary structure of component 8c-1, a subunit protein of intermediate filaments in wool keratin. Biochem J 236:695–703
11. Feng DF, Doolittle RF (1987) Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J Mol Evol 25:351–359
12. Fisher C, Haydock PV, Dale BA (1987) Localization of profilaggrin mRNA in newborn rat skin by in situ hybridization. J Invest Dermatol 88:661–664
13. Fuchs E (1988) Keratins as biochemical markers of epithelial differentiation. Trends Genet 4:277–281
14. Garnier J, Osguthorpe DJ, Robson B (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120:97–120
15. Gough NM (1988) Rapid and quantitative preparation of cytoplasmic RNA from small members of cells. Anal Biochem 173:93–95
16. Heid HW, Werner E, Franke WW (1986) The complement of native α-keratin polypeptides of hairforming cells: A subset of eight polypeptides that differ from epithelial cytokeratins. Differentiation 32:101–119
17. Heid HW, Moll I, Franke WW (1988) Patterns of expression of trichocytic and epithelial cytokeratins in mammalian tissues. I Human and bovine hair follicles. Differentiation 37:137–157
18. Heid HW, Moll I, Franke WW (1988) 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 papillae, thymic reticulum). Differentiation 37:215–230
19. Hume WJ, Potten CS (1976) The ordered columnar structure of mouse filiform papillae. J Cell Sci 22:149–160

20. Karplus PA, Schulz GE (1985) Predicting of chain flexibility in proteins. Naturwissenschaften 72:212–213
21. Knapp B, Rentrop M, Schweizer J, Winter H (1986) Nonepidermal members of the keratin multigene family: cDNA sequences and in situ localization of the mRNAs. Nucl Acids Res 14:751–763
22. Knapp AC, Franke WW, Heid H, Hatzfeld M, Jorcano JL, Moll R (1986) Cytokeratin No. 9, an epidermal type I keratin characteristic of a special program of keratinocyte differentiation displaying body site specifity. J Cell Biol 103:657–667
23. Knapp B, Rentrop M, Schweizer J, Winter H (1987) Three cDNA sequences of mouse type I keratins: Cellular localization of the mRNAs in normal and hyperproliferative tissues. J Biol Chem 262:938–945
24. Laemmli UK (1970) Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
25. Lussier M, Quellet T, Lampron C, Lapointe L, Royal A (1989) Mouse keratin 19: complete amino acid sequence and gene expression during development. Gene 85:435–444
26. Maniatis T, Fritsch EF, Sambrook J (1989) In: Molecular Cloning. A Laboratory Mannual, Cold Spring Harbour Laboratory
27. Mehrel T, Hohl D, Rothnagel JA, Longley MA, Bundman D, Cheng C, Lichti U, Bisher ME, Steven AC, Steinert PM, Yuspa SH, Roop DR (1990) Identification of a major keratinocyte cell envelope protein, loricrin. Cell 61:1103–1112
28. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R (1982) The catalog of human cytokeratin polypeptides: pattern of expression of specific cytokeratins in normal epithelia, tumors and cultured cells. Cell 31:11–24
29. Needleman SB, Wunsch CD (1970) A general method applicable to the search for similarities in the aminoacid sequence of two proteins. J Mol Biol 48:443–453
30. Nischt R, Rentrop M, Winter H, Schweizer J (1987) Localization of a novel mRNA in keratinizing epithelia of the mouse: Evidence for the sequential activation of differentiation-specific genes. Epithelia 1:165–177
31. Nischt R, Roop DR, Mehrel T, Yuspa SH, Rentrop M, Winter H, Schweizer J (1988) Aberrant expression during two stage mouse skin carcinogenesis of the type I 47 kDa keratin, K13, normally associated with terminal differentiation of internal stratified epithelia. Mol Carcinog 1:96–108
32. O'Farrell PZ, Goodman HM, O'Farrell PH (1977) High resolution two dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133–1142
33. Rentrop M (1988) Lokalisierung von differenzierungsabhängig exprimierten Keratin mRNAs in stratifizierten Epithelien der Maus. Ph.D. Thesis, University of Kaiserslautern
34. Rentrop M, Knapp B, Winter H, Schweizer J (1986) Aminoalkylsilane-treated glass slides as support for in situ hybridization of keratin cDNAs to frozen tissue sections under varying fixation and pretreatment conditions. Histochem J 18:271–276
35. Rentrop M, Knapp B, Winter H, Schweizer J (1986) Differential localization of distinct keratin mRNA species in mouse tongue epithelium by in situ hybridization with specific cDNA probes. J Cell Biol 103:2583–2591
36. Rentrop M, Nischt R, Knapp B, Winter H, Schweizer J (1987) An unusual type II 70 kilodalton keratin protein of mouse epidermis exhibiting postnatal body site specificity and sensitivity to hyperproliferation. Differentiation 34:189–200
37. Roop DR, Krieg TM, Mehrel T, Cheng CK, Yuspa SH (1988) Transcriptional control of high molecular weight keratin gene expression in multistage mouse skin carcinogenesis. Cancer Res 48:3245–3252
38. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci 75:5463–5467
39. Schweizer J Mouse epidermal keratins. In: Darmon YM, Blumenberg M (eds) Biology of the Skin. Academic Press, submitted for publication
40. Schweizer J, Marks F (1977) Induction of the formation of new hair follicles in mouse tail epidermis by the tumor promoter TPA. Cancer Res 37:4195–4201
41. Schweizer J, Marks F (1977) A developmental study of the distribution and frequency of Langerhans cells in relation to formation of patterning in mouse tail epidermis. J Invest Dermatol 69:198–204
42. Schweizer J, Winter H (1982) Changes in regional keratin polypeptide pattern during phorbol ester mediated reversible and permanently sustained hyperplasia of mouse epidermis. Cancer Res 42:1517–1529
43. Schweizer J, Winter H, Hill MW, Mackenzie IC (1984) The keratin pattern in heterotypically recombined epithelia of skin and mucosa of adult mouse. Differentiation 26:144–153
44. Schweizer J, Kinjo M, Fürstenberger G, Winter H (1984) Sequential expression of RNA encoded keratin sets in neonatal mouse epidermis: Basal cells with properties of terminally differentiating cells. Cell 37:159–177
45. Schweizer J, Fürstenberger G, Winter H (1987) Selective suppression of two postnatally acquired 70 kD and 65 kD keratin proteins during continuous treatment of adult mouse tail epidermis with vitamin A. J Invest Dermatol 89:125–131
46. Schweizer J, Baust J, Winter H (1989) Identification of murine type I keratin 9 (73 kDa) and its immunolocalization in neonatal and adult mouse footsole epidermis. Exp Cell Res 1984:193–206
47. Silverman A (1971) Nonkeratinization and Keratinization: The Extremes of the Human Range. In: Squier CA, Meyer J (eds) Current concepts of the histology of oral mucosa. Charles C. Thomas Publishers, Springfield, Ill., pp 80–113
48. Sparrow LG, Robinson CP, McMahon DTW, Rubira MR (1989) The amino acid sequence of component 7c, a type II intermediate-filament protein from wool. Biochem J 261:1015–1022
49. Spearman RIC (1964) The evolution of mammalian keratinized structures. In: Ebling FJ (ed) Symposia of the Zoological Society of London (No. 12) The mammalian epidermis and its derivatives. Academic Press, London, New York, pp 67–81
50. Steinert RM, Roop DR (1988) Molecular and cellular biology of intermediate filaments. Annu Rev Biochem 57:593–639
51. Steinert PM, Rice RH, Roop DR, Trus BL, Steven AC (1983) Complete amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments. Nature 302:794–800
52. Steinert PM, Parry DAD, Idler WW, Johnson LD, Steven AC, Roop DR (1985) Aminoacid sequences of mouse and human epidermal type II keratins of Mr 67.000 provide a systematic basis for the structural and functional diversity of the end domains of keratin intermediate filaments. J Biol Chem 260:7142–7449
53. Sun T-T, Eichner R, Schermer A, Cooper D, Nelson WG, Weiss RA (1984) Classification expression and possible mechanisms of evolution of mammalian epithelial keratins: A unifying model. In: Levine A, Vande Woude GF, Topp WE, Watson JD (eds) Cancer Cells. Vol. I. Cold Spring Harbor Laboratory, New York, pp 169–176
54. Tobiasch E, Schweizer J, Winter H (1992) Structure and site of expression of a murine type II hair keratin. Mol Biol Rep 16:39–47
55. Winter H, Schweizer J, Goerttler K (1980) Keratins as markers of malignancy in mouse epidermal tumors. Carcinogenesis 1:391–398
56. Yu DW, Pang SYY, Checkla DM, Freedberg IM, Sun T-T, Bertolino AP (1991) Transient expression of mouse hair keratins in transfected Hela cells: Interaction between "hard" and "soft" keratins. J Invest Dermatol 97:345–363