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Sequence and Expression of Murine Type I Hair Keratins mHa2 and mHa3

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A cDNA library was constructed with poly(A)$^+$ RNA from mouse tail epidermis which contained all hair follicles of tail skin. The library was subjected to sequential screening procedures aimed at selecting cDNA clones coding for acidic, type I hair keratins. Two clones, pktI-2 and pktI-3, encoded keratins that could be identified as murine type I hair keratins mHa2 and mHa3, respectively, by positive hybridization selection analysis. Sequence comparisons with the known murine type I hair keratins mHa1 (Bertolino *et al., J. Invest. Dermatol.* 91, 541–546, 1988) and mHa4 (Bertolino *et al., J. Invest. Dermatol.* 94, 297–303, 1990) revealed a structural heterogeneity within the type I hair keratin subfamily. Three keratins, mHa1, mHa3, and mHa4, are highly related, differing mainly in the penultimate part of their amino and carboxy termini. In contrast, mHa2 is structurally distinct from the three other keratins in both the α-helix and, in particular, the non-α-helical domains. These findings are confirmed by evolutionary investigations and flexibility calculations which indicate a more flexible nature of the mHa2 amino terminus when compared to the corresponding region of the three other keratins. *In situ* hybridization experiments with specific 3′ fragments of mHa2 and mHa3 show that mHa3 is expressed in cortex cells, whereas mHa2 transcripts are strictly limited to the cuticle of the hair shaft. mHa3 mRNA expression can also be demonstrated in the central unit of the murine lingual filiform papillae, whereas the cuticular keratin mHa2 is not expressed in this body site. These data indicate that the structural heterogeneity within the type I hair keratin subfamily is functionally relevant in the morphogenesis of hard α-keratin-expressing tissues.

INTRODUCTION

The keratin multigene family consists of about 30 individual but structurally related members which can be grouped into epithelial-type keratins (soft α-keratins) and wool- and hair-type keratins (hard α-keratins). The vast majority of the members of the keratin multigene family belongs to the epithelial-type keratins. Only 8 major and 2 minor hard α-keratins have been identified by two-dimensional gel electrophoresis of keratin extracts of hair and wool follicles of different species [1–4]. Recent findings indicate that a type II 65-kDa keratin and a type I 48-kDa keratin correspond to the minor hard α-keratin components expressed in the murine hair follicle [5]. Since both keratins contain considerably less cysteine residues in their non-α-helical head and tail portions than true hair keratins, they were designated "hair-related" keratins [5]. Four of the major hair keratins appear in a rather narrow molecular weight range of 46–48 kDa and belong to the acidic, type I subfamily of keratins [1, 2, 4]. They were designated Ha1-4 according to a proposal by Heid *et al.* [1]. The remaining 4 keratins with molecular weights between 59 and 63 kDa represent the basic to neutral, type II keratins Hb1-4 [1, 2, 4].

The occurrence of an equal number of type I and type II hair keratins suggests that their expression occurs in the form of four distinct pairs of oppositely charged keratins. Considering that even complex stratified epithelia generally contain only two keratin pairs that define proliferative and differentiating cell compartments [6, 7], the multiplicity of hair keratin pairs expressed within a relatively small area of the hair shaft of the follicle is amazing. Nevertheless, the most reasonable explanation for the multiplicity of hair keratins is that they are also differentially expressed and thus may serve different functions [8].

It is evident that the elucidation of such complex expression pathways is only possible via the knowledge of the DNA and amino acid sequences of the individual hair keratins. This enables the generation of both chain-specific DNA probes and monospecific keratin antibodies in order to analyze the expression pattern of each of the keratins and to deduce the composition of the different keratin pairs.

The first published sequences of hard α-keratins represented a type I keratin, component 8c1, and a type II keratin, component 7c from sheep wool follicles, whose amino acid sequences were determined directly [9, 10].

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With the advent of recombinant DNA techniques, however, further hard α-keratin sequences became available. Thus the current scenario of fully or partially known hard α-keratin sequences comprises component 8c1 [9] and another type I 47.6-kDa keratin [11] as well as the four type II keratins from sheep wool [12], two type I murine keratins, mHa1 and mHa4 [4, 13, 14], the murine type II keratin mHb4 [15, 16],[3] and the recently described human type I keratin hHa3 [8].

In the present study we report on the identification of the murine type I hair keratins mHa2 and mHa3. The corresponding clones were isolated from a cDNA library constructed with poly(A)$^+$ RNA from mouse tail epidermis which also contained the hair follicles of this tissue. Together with the already known type I keratins mHa1 and mHa4 [4, 13], the new keratins complete the sequence information of the acidic subfamily of murine hair keratins. Our investigations indicate that from the structural point of view, the subfamily is heterogeneous, with three of its members being highly related, whereas the fourth member is distinct, exhibiting a different expression pattern in the hair follicle than its highly related partners.

MATERIALS AND METHODS

Isolation of adult mouse tail interfollicular and follicular epidermis. Mouse tail skin was incubated for 30 s in 56°C hot water containing 10 m*M* $ZnCl_2$ [17]. Subsequently both the epidermis and the entire population of hair follicles was collectively removed by scraping the skin with curved forceps. The isolated material was kept at −80°C until further use.

cDNA cloning, screening procedures, and discriminatory RNA slot blot hybridization. Total RNA from tail interfollicular and follicular epidermis was isolated using the guanidinium thiocynate/CsCl method [17]. Poly(A)$^+$ RNA was obtained by oligo(dT) cellulose chromatography as described [17] and used for the construction of a cDNA library in λgt10 [18]. The library was first subjected to a general screening for type I keratin clones under low stringency conditions by means of a 645-bp *Sac*I/*Sac*I α-helical fragment of the previously described clone pkt47 which encodes the type I keratin K13 of internal stratified epithelia [19]. The resulting positive phage clones were further screened for those coding for keratins K14, K17, and K10 using chain-specific 3′ fragments of the corresponding clones [20]. Phage clones which did not hybridize with K14-, K17-, and K10-specific cDNA probes were subjected to a discriminatory RNA slot blot hybridization as described [5]. Briefly, total RNA from adult mouse tail epidermis, the glabrous sole of the foot epidermis, newborn (1 day postnatum) mouse back epidermis, and the internal forestomach epithelium, as well as from carefully excised benign and malignant epidermal tumors, was spotted onto Gene Screen Plus membranes (DuPont, Dreieich, Germany) and hybridized with the remaining phage clones. Phage clones reacting only with RNA from the follicle-containing tail epidermis were selected for further characterization.

Subcloning and sequencing. The inserts of two of the selected phage clones, designated λktI-2 and λktI-3, respectively, were subcloned into the transcription vector Bluescript II KS+ (Stratagene, La Jolla, CA). Sequencing of both strands of the resulting plasmid clones, pktI-2 and pktI-3, was performed according to Sanger *et al.* [21], first using M13 and T3 primers, and subsequently 17mer oligonucleotides as walking primers. To prepare 3′-specific cDNA probes for each clone, a 244-bp *Alu*I/*Alu*I fragment of pktI-2 and a 330-bp *Sty*I/*Sty*I fragment of pktI-3 was subcloned into Bluescript II KS+. These subclones were designated pktI-2′ and pktI-3′, respectively.

Isolation and two-dimensional gel electrophoresis of murine hair keratins. The method described previously by Bertolino *et al.* [22] was used to enriche hair roots from the dorsal skin of 8- to 9-day-old NMRI mice. The material was first homogenized in a high salt buffer [23] to remove soluble proteins and subsequently in a guanidinium thiocyanate/Sarcosyl/β-mercaptoethanol buffer [GTC buffer (17)] for the solubilization of keratins. Simultaneously, clipped hairs from adult mice were mixed with sea sand and reduced to a fine powder in a mortar. The powder was homogenized in GTC buffer. After centrifugation of the homogenate, the supernatant was removed and dialyzed for 2 days against water (four changes). The resulting filamentous aggregate was dissolved in GTC buffer by heating (65°C). Keratin extracts from both enriched hair roots and clipped hairs were mixed (1:1, v/v) and used for two-dimensional gel electrophoresis [IEF/SDS–PAGE (24)]. Isoelectric focusing was performed using a mixture of pH 3.5–10, pH 5–7, and pH 4–6 ampholines (1:2:2; v/v; Pharmacia, Freiburg, Germany). Gels were stained with 0.15% Coomassie blue in 40% methanol, 10% glacial acetic acid and destained in the same solution without the dye.

Positive hybridization selection translation. The specificity of clones pktI-2 and pktI-3 was determined by positive hybridization selection translation as described previously [25]. Briefly, inserts of subclones pktI-2′ and pktI-3′ were denatured and bound to Nylon membranes (Sartorius, Goettingen, Germany) which were hybridized with poly(A)$^+$ RNA from mouse tail interfollicular and follicular epidermis. After washing the membranes, the hybridized RNA was eluted and translated in a reticulocyte lysate system (Serva, Heidelberg, Germany) in the presence of [^{35}S]methionine. Four *in vitro* translation assays of each of the hybrid-selected RNAs were pooled and mixed thoroughly with about 10 μg of isolated hair keratin proteins. After centrifugation, the resulting pellet was extracted once with high salt buffer [23] and dissolved in 10 μl of GTC buffer from which 5-μl aliquots were used for IEF/SDS–PAGE. After staining and destaining, the gels were treated with EN^{3}HANCE (Du Pont, Dreieich, Germany), dried, and exposed to Kodak X-Omat films.

Nonradioactive in situ hybridization. Nonradioactive *in situ* hybridization of paraformaldehyde-fixed paraffin sections of newborn mouse back skin and adult mouse tongue on aminopropyltriethoxysilane-coated glass slides [26] with respective digoxigenin-labeled subclones pktI-2′ and pktI-3′ was performed using a commercially available DIG-labeling and -detection kit (Boehringer, Mannheim, Germany), as described by Fisher *et al.* [27] with minor modifications. These comprised an additional paraformaldehyde fixation step after proteinase K digestion of the sections, followed by treatment with triethanolamine/acetic acid anhydrid to block polar and charged groups. Finally, instead of mounting the stained sections in Glycergel at the end of the *in situ* hybridization, our sections were again taken through ascending ethanol concentrations, rinsed in xylene, and mounted in Eukitt.

Phylogenetic tree construction. The construction of a phylogenetic tree on the basis of the murine type I hair keratins mHa1 [13], mHa4 [4], and the new keratins described here, as well as of the recently described murine hair-related keratin mHRa1 [5], 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 [28]. TREE produces a multiple alignment for a set of protein sequences by iteratively acting at the sequences. An approximate phylogenetic

[3] It should be mentioned that the amino-terminal sequence of mHb4 elucidated in our laboratory [16] is incorrect. The correct sequence has been published by Yu *et al.* [15].

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

Type I Hair Keratin Clones

Figures 1 and 2 show the nucleotide sequences of clones pktI-2 and pktI-3 and the deduced amino acid sequences of the corresponding keratins. Clone pktI-2 represents a full-length clone that encodes a keratin of 407 amino acid residues with a calculated molecular weight of 46390 Da. Clone pktI-3 contains only partial sequence information of the encoded keratin which lacks a few amino acids of the α-helix and the entire amino terminus. The hair keratin nature of the proteins is clearly recognized by the high number of cysteine and proline residues in their non-α-helical domains. Typically, in the carboxy termini, these amino acid residues frequently occur as proline–cysteine dipeptide motifs [4, 8, 13, 15].

Classification of the New Keratins within the Subfamily of Type I Hair Keratins

Since out of the four type I murine hair keratins both the largest (mHa1) and the smallest (mHa4) members have already been elucidated [4, 13], the two new keratin clones described here must encode mHa2 and mHa3, respectively. Fortunately, our present knowledge on the structural features and sequence characteristics of type I hair keratins from various species is so advanced that an attempt at assignment of the clone pktI-2- and pktI-3-encoded keratins can be made on the basis of sequence comparisons. Sequence analyses of mHa1 and mHa4, as well as of two type I sheep wool keratins, component 8cl and a 47.6-kDa keratin, and of the recently identified human keratin hHa3 have shown that independent of the species, all type I hair keratins share a highly homologous amino terminus of 56 amino acid residues and a likewise conserved α-helix of 311 amino acids [8]. This indicates that the chain specificity of type I hair keratins is essentially determined by the sequences of their carboxy terminus. In Fig. 3B we have aligned the carboxy-terminal sequences of hHa3, mHa1, and mHa4 with those of the new murine keratins encoded by clones pktI-2 and pktI-3, respectively. A comparison of the sequences reveals that not only is the length of the hHa3 carboxy terminus (37 amino acid residues) completely identical with that of the keratin encoded by clone pktI-3, but also their protein sequences are almost identical. From this we conclude that clone pktI-3 encodes the murine type I hair keratin mHa3. This assumption is confirmed by positive hybrid

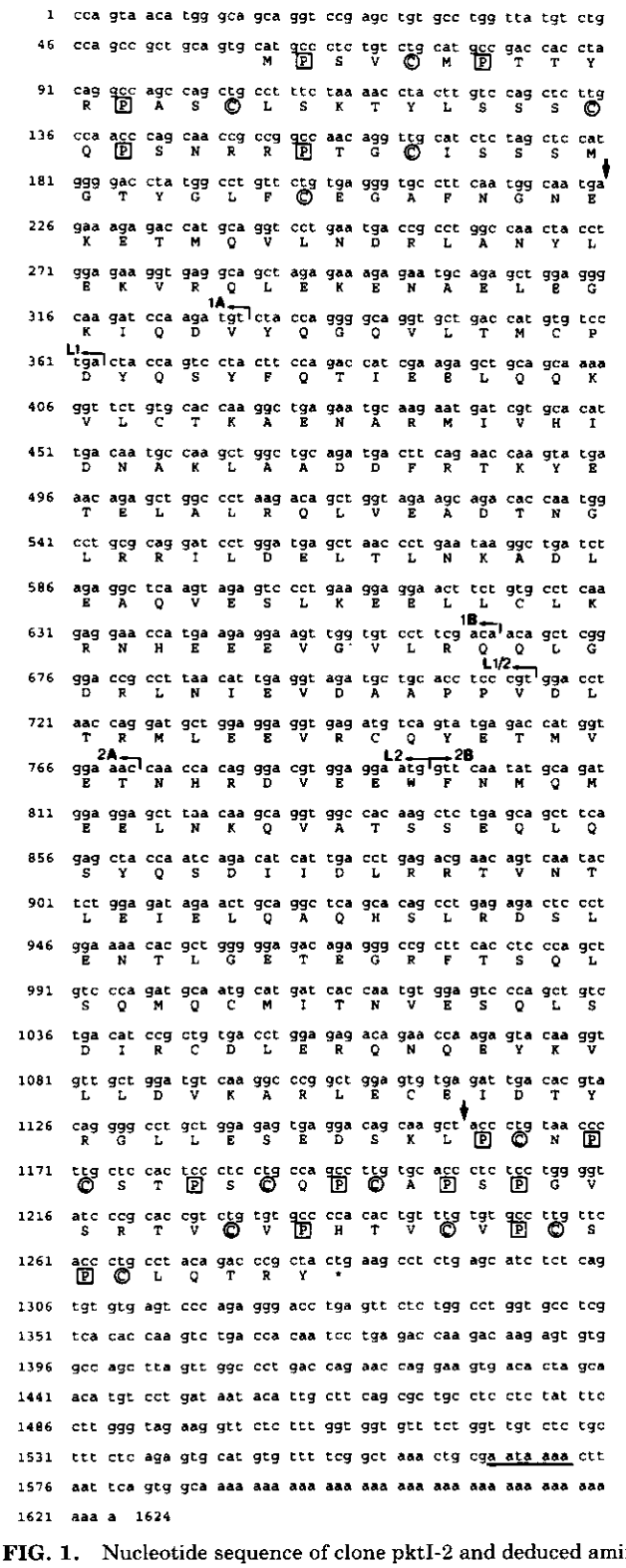

FIG. 1. Nucleotide sequence of clone pktI-2 and deduced amino acid sequence of the encoded keratin. Large arrows demarcate the α-helical domain, whose subdomains are indicated by bent arrows. Proline residues in the amino and carboxy terminus are boxed; cysteine residues are encircled. The polyadenylation signal is underlined. The sequence is available from the EMBL Data Library (Accession No. X 75649).

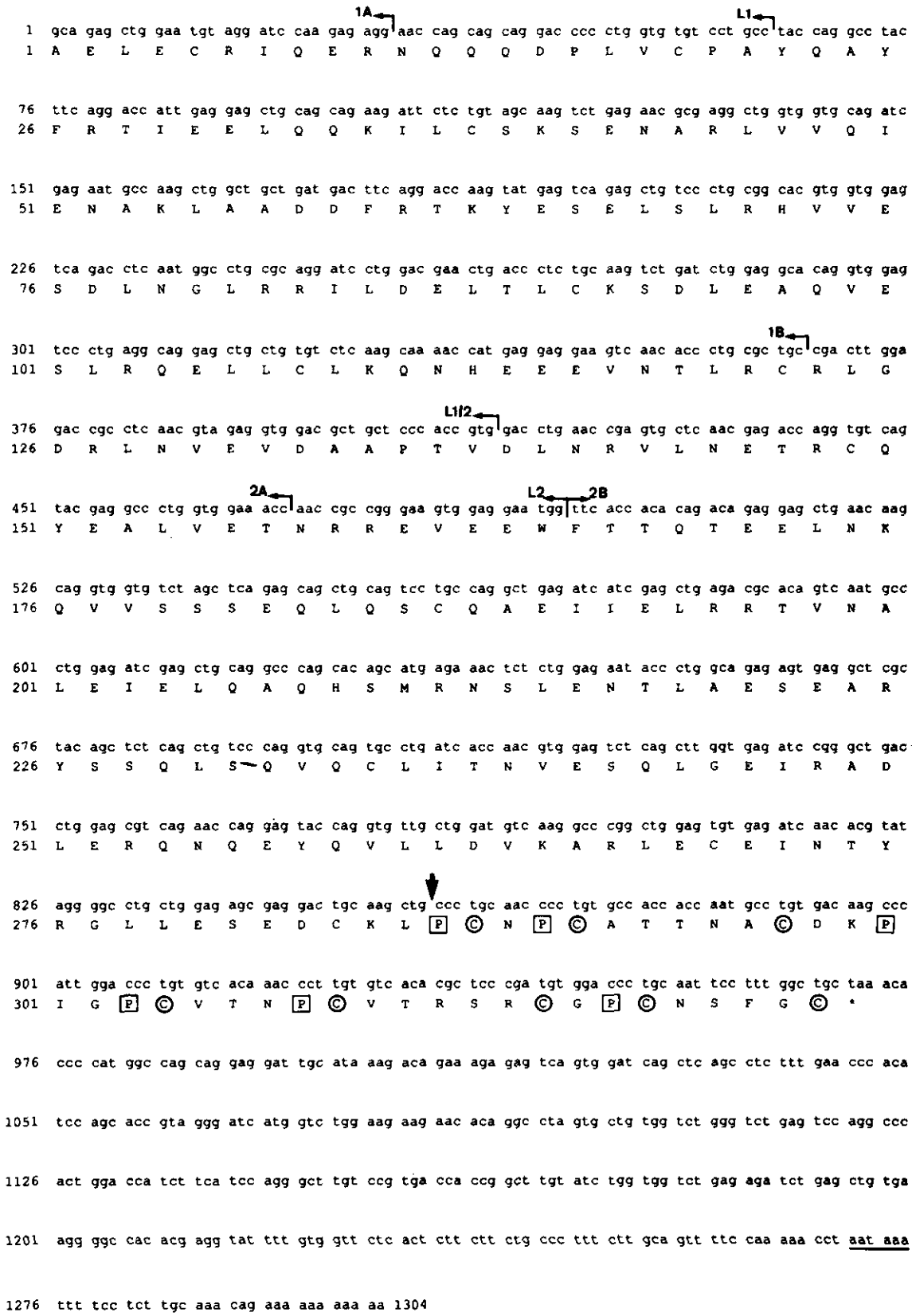

FIG. 2. Nucleotide sequence of clone pktI-3 and deduced amino acid sequence of the encoded keratin. The large arrowhead demarcates the end of the α-helical domain. For further symbols, see legend to Fig. 1. The sequence is available from the EMBL Data Library (Accession No. X 75650).

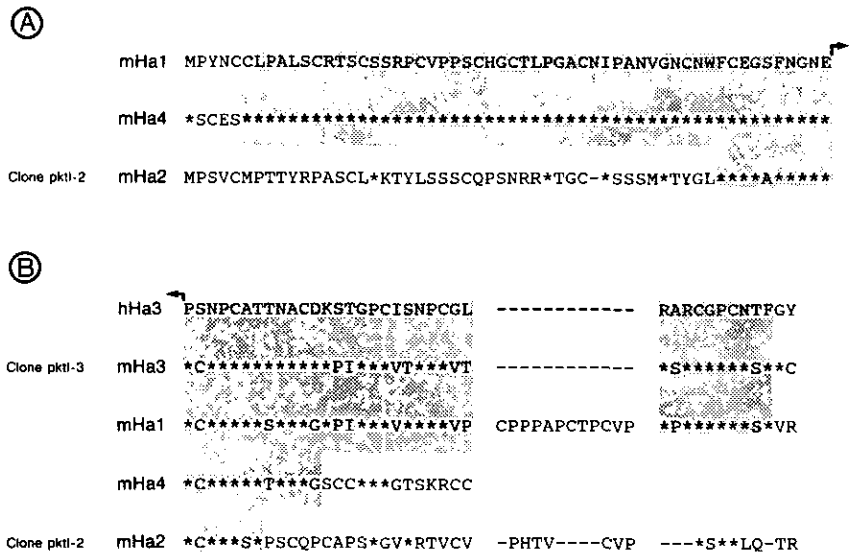

FIG. 3. Sequence comparisons of the amino termini (A) and carboxy termini (B) of various known type I hair keratins from man (hHa3) and mouse (mHa1, mHa4) and of the keratins encoded by clone pktI-2 and pktI-3, respectively. Asterisks indicate amino acid residues that are identically positioned relative to the amino-terminal sequence of mHa1 (A) or to the carboxy-terminal sequence of hHa3 (B). Sequence segments highly conserved among keratins in A and B are shaded. The bent arrows in A and B indicate the beginning (A) and the end (B) of the α-helical domain.

ization selection translation analysis. Figure 4a shows the two-dimensional pattern of the four murine type I hair keratins which are designated according to the nomenclature used by Bertolino *et al.* [4, 13]. Figure 4b clearly demonstrates that the *in vitro* translation product of the mRNA selected by subclone pktI-3′ comigrates with keratin mHa3. As expected, a similar analysis of the mRNA selected by subclone pktI-2′ reveals that the corresponding translation product colocalizes with keratin mHa2 (Fig. 4c).

Sequence Features of mHa2 and mHa3

From its deduced partial structure, mHa3 strikingly conforms to the sequence characteristics of mHa1 and mHa4. Its α-helix shares a 96–98% sequence homology with those of mHa1 and mHa4 (results not shown). Except for a 12 amino acids deletion, the carboxy terminus of mHa3 is highly homologous with that of mHa1 (Fig. 3B). Similarly, the first 12 amino acids of mHa4 are conserved in the corresponding region of mHa3 (Fig. 3B). In contrast, mHa2 exhibits conspicuous deviations from the conserved domains of the three other keratins. First, the α-helices of mHa1, mHa3, and mHa4 have only an average sequence homology of 86% when compared with mHa2 (results not shown). The most striking deviations are, however, found in the amino and carboxy terminus of mHa2 in which only short segments adjacent to the α-helix share homology with mHa1, mHa3, and mHa4 (Figs. 3A and 3B). It should be emphasized, however, that at least for the carboxy terminus, the structural particularity of the non-α-helical domains of mHa2 does not affect the high incidence of proline and cysteine residues as well as the occurrence of proline–cysteine dipeptide motifs (Table 1). In contrast, the frequency of these amino acids in the amino terminus of mHa2 is only about half of that seen in the carboxy termini of mHa1 and mHa4 (Table 1). Moreover, comparative flexibility calculations reveal that the carboxy termini of mHa1 and mHa2 are poorly flexible (Figs. 5C and 5D), whereas the aminoterminus of mHa2 (Fig. 5B) contains essentially more flexible sites than that of mHa1 (Fig. 5A). Finally, phylogenetic investigations on the basis of the terminal 2B subdomain of the four type I murine hair keratin α-helices clearly show that mHa2 is evolutionarily distinct from the three other keratins, among which mHa1 and mHa4 are the most related members of the subfamily (Fig. 6).

In Situ Hybridization

The sites of expression of mHa2 and mHa3 were investigated by nonradioactive *in situ* hybridization using digoxigenin-labeled 3′ cDNA probes of the two corresponding clones. Figures 7a and 7b, which represent almost ideal vertical sections through hair follicles from newborn mouse back skin, show that the mRNA of mHa3 is strongly expressed in cortex cells, whereas both the medulla and the cuticle of the hair shaft are free of mHa3 transcripts. The onset of mHa3 mRNA expression occurs abruptly at the beginning of the "elongation zone" of the follicle [2], i.e., in a two to three cell

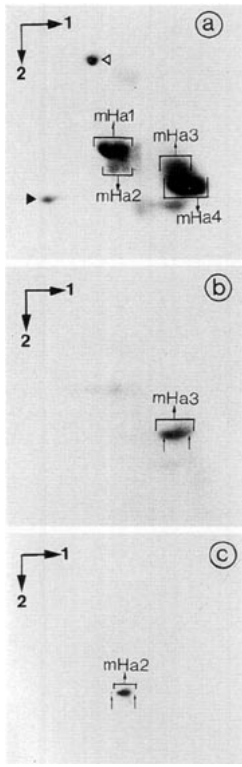

FIG. 4. Identification of murine type I hair keratins by positive hybridization selection translation. (a) Coomassie-stained two-dimensional pattern of the four murine type I hair keratins. 1, Isoelectric focusing; 2, SDS–PAGE. The keratins are designated according to Bertolino *et al.* [4, 13]. The open arrowhead indicates vimentin (57 kDa); the closed arrowhead indicates actin (43 kDa). (b, c) Positive hybridization selection analysis. Two-dimensional gel electrophoresis of the *in vitro* translation product of the mRNA selected by subclones pktI-3′ (b) and pktI-2′ (c) in the presence of authentic hair keratin proteins. Before autoradiography, the X-ray film was attached to the stained and dried gels under IR illumination and a fine needle was used to perforate the film at the position of keratin mHa3 (small arrows in b) and mHa2, respectively (small arrows in c).

layers distance above the basement membrane which separates the follicular bulb from the apical region of the dermal papillae. Its expression terminates at the beginning of the keratogenous zone (Figs. 7a and 7b). The sudden but strong expression of mHa3 mRNA is confirmed by *in situ* hybridization to slightly oblique skin sections in which cross sections through the lower part of the hair follicle exhibited neither weak nor progressive staining for mHa3 transcripts (Fig. 7c). Transcripts of mHa3 could also be detected in the central unit of the filiform papillae of the mouse tongue. Similar to the situation in the hair follicle, the onset of mHa3 mRNA expression occurs in differentiating cells several cell layers above the apex of the dermal papillae (Fig. 7d).

In situ hybridization with the specific 3′ cDNA probe of keratin mHa2 reveals a completely different mRNA expression pattern in hair follicles. Both vertical and cross sections of hair follicles demonstrate that mHa2 transcripts are strictly confined to cuticle cells of the hair shaft (Figs. 8a–8c). The label starts slightly above the critical zone of Auber, i.e., the widest point of the hair bulb [30], and ascends up to the beginning of the keratogenous zone (Figs. 8a–8c). Even after prolonged staining, mHa2 transcripts could not be detected in any compartment of the lingual filiform papillae (results not shown).

DISCUSSION

In the present study we have identified the murine type I hair keratins mHa2 and mHa3. Since both mHa1 and mHa4 have already been characterized previously [4, 13] sequence data are now available for the entire subfamily of murine type I hair keratins. Surprisingly, the comparison of their sequences reveals that from the structural point of view, the subfamily is heterogeneous with three of its members, mHa1, mHa3, and mHa4, being highly related whereas the fourth member, mHa2, is structurally distinct.

The features of the structural relationship between the four type I hair keratins is illustrated schematically in Fig. 9. It is apparent that mHa1, mHa3, and mHa4 share a common building block that comprises almost the entire amino terminus (51 of 56 amino acid residues), the α-helix, as well as the first 12 amino acids of the carboxy terminus (Fig. 9). Considering the apparent

TABLE 1

Number of Proline and Cysteine Residues and Proline–Cysteine Dipeptide Motifs in the Amino and Carboxy Termini of the Four Murine Type I Hair Keratins

Type I hair keratin	Amino terminus			Carboxy terminus		
	Proline	Cysteine	PC dipeptides	Proline	Cysteine	PC dipeptides
mHa1	7	10	1	13	10	7
mHa2	4	5	0	9	8	4
mHa3	nd[a]	nd	nd	6	8	5
mHa4	6	10	1	3	8	3

[a] nd, not determined.

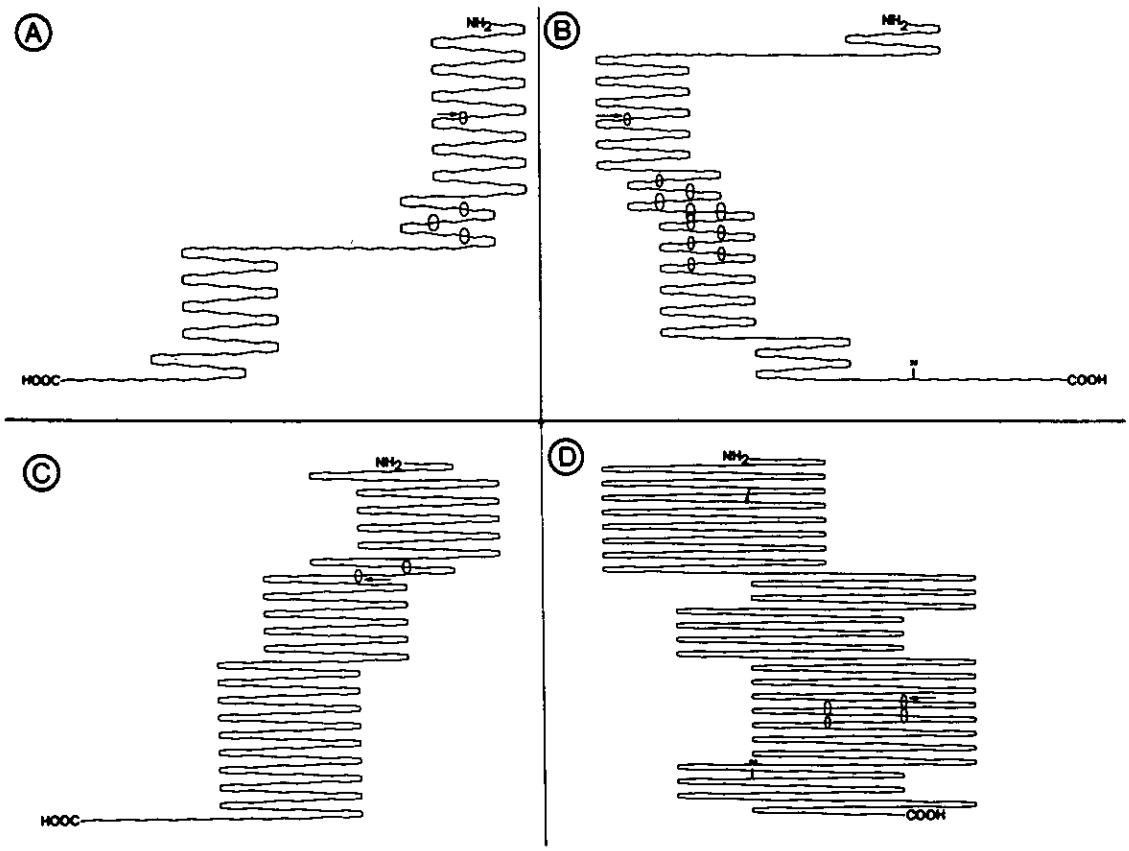

FIG. 5. Schematic presentation of the flexibility of the amino termini of mHa1 (A) and mHa2 (B) and the carboxy termini of mHa1 (C) and mHa2 (D). The secondary structure of the protein sequences was determined according to Garnier *et al.* [39]. Flexibility predictions were calculated by the method of Karplus and Schulz [40]. 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 sign.

length conservation of the amino terminus, this implies that the chain specificity of each of the three keratins resides in a short 5-amino acid sequence motif at the end of the amino terminus and in the penultimate part of the carboxy terminus whose variable length determines also the size of the respective keratin. Thus the smallest keratin, mHa4, exhibits a chain-specific amino-terminal sequence MSCES and a 13-amino-acid-long carboxy terminal end sequence which, however, contains a central tripeptide GPC that is positionally conserved in the corresponding region of mHa1 and mHa3 (see also Fig. 3B). Interestingly, the structural principle that distinguishes the penultimate carboxy terminus of mHa3 from that of mHa1 does not consist, as in mHa4, of a size-dependent elongation of chain-specific amino acid sequences. Instead, both keratins contain, continuous with the 12-amino-acid-long consensus sequence, a further common motif of 23 amino acid residues which in the case of mHa1 is interrupted by the insertion of a 12-amino acid segment that is especially rich in proline residues (Fig. 9 and Fig. 3B). In addition, both keratins carry chain-specific dipeptide motifs at their penultimate carboxy-terminal end (Fig. 9). It is evident that with regard to the structural diversity of mHa1, mHa3, and mHa4, the carboxy-terminal amino acid position 25 plays a pivotal role. Whereas this position demarcates the very end of mHa4, it colocalizes with the site of insertion of the peptide segment that essentially distinguishes mHa1 from mHa3 (Fig. 9).

The structural similarity of the fourth member of the type I murine hair keratins, mHa2, to its highly related partners is confined to the α-helix as well as to relatively short amino-terminal (10 amino acid residues) and carboxy-terminal sequences (7 amino acid residues) adjacent to the rod domain (Fig. 9 and Figs. 3A and 3B). In this context it should be recalled that the α-helical sequence homology between mHa2 and the three other keratins is about 10% lower than that between each of the highly related keratins.

Although the evolutionary principle that determines the particular diversity of the type I hair keratins is now apparent, the question arises whether this diversity is functionally implicated in the formation of hairs. Previous investigations using a mHa1-specific antibody have shown that this keratin is expressed in cortex cells but not in the cuticle of the hair shaft [13]. Here we show

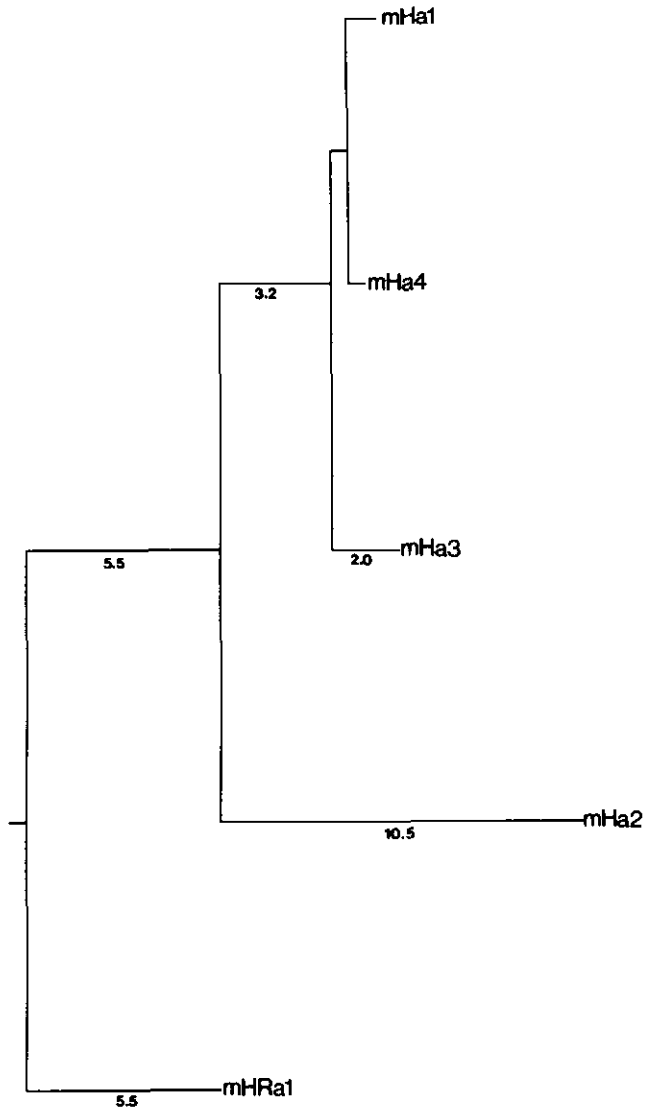

FIG. 6. Phylogenetic tree constructed for the four murine type I hair keratins on the basis of their α-helical 2B subdomain [41]. Also included in the tree is the recently identified murine type I "hair-related" keratin mHRa-1. Numbers above the branches indicate branch lengths in amino acid replacements. Sequences were taken from mHa1 [13], mHa4 [4], and mHRa-1 [5].

that mHa3 exhibits an identical expression pattern in the hair follicle. Although the site of expression of mHa4 has not yet been investigated, it is conceivable that due to its structural homology with mHa1 and mHa3, this keratin is also produced in cortex cells. On the other hand, two independently raised antibodies, AE13 [2] and GP19a [1], both selectively recognizing all four type I hair keratins in Western blots [13, 31], have been shown to decorate both the cortex and the cuticle of the hair shaft [13, 31]. This finding can now be explained by the demonstration that keratin mHa2 represents the cuticular hair keratin.

This type of compartmental expression of the four type I hair keratins can conceptually be related to the different function of cortex cells and cuticle cells in hair formation. It is evident that the expression of multiple, highly inflexible and cysteine-rich keratins in cortex cells is ideally suited for the generation of the tough and hard core of the mature hair. Cuticle cells differentiate into elongated scale-shaped cells that overlap each other at the surface of the hair fiber [3]. Functionally, such an architecture is able to provide flexibility against both stretching and bending of the hair. It is conceivable that these properties of cuticle cells are aided by the expression of the less rigid keratin mHa2 and probably enhanced by the demonstrated coexpression of the cysteine-poor epidermal keratins K1 and K10 [31, 32] whose extraordinary long head and tail portions exhibit high flexibility indices [5]. The expression of ultrahigh sulfur proteins late in cuticular morphogenesis [33] may then be a means to confer the necessary protection to the surface coat of the hair fiber against environmental influences.

Recently preliminary human pedigree studies by Yu *et al.* [8] have shown that within the members of a family, nonexpression of distinct type I hair keratins did not lead to obvious phenotypic alterations of the mature hair. So far, this phenomenon has only concerned keratins hHa1 and hHa4, i.e., those expected to be expressed in the cortex of the hair shaft. In view of the multiplicity of highly related keratins in the cortex, it may be argued that the lack of one of them is tolerated without functional and structural disturbances [8]. On the other hand, one would expect that the nonexpression of the cuticular keratin Ha2, which was not observed in the study by Yu *et al.* [8], should have severe consequences with regard to the proper formation of a functional hair cuticle.

The cuticular association of mHa2 is further emphasized by our investigations in mouse tongue epithelium. Previous studies with antibodies AE13 and GP19a have shown that hair keratins are expressed in the parakeratinizing central unit of the human, bovine, and murine lingual filiform papillae [34, 35]. Using mouse hair keratin-specific cDNA probes for *in situ* hybridization, we have demonstrated that the mRNAs of type II keratin mHb4 [16] and a further type II hair keratin (probably mHb3; Winter *et al.*, manuscript in preparation) as well as of the type I keratin mHa3 (this study) represent differentiation-specific constituents of the core unit of mouse filiform papillae. In agreement with the apparent lack of a cuticular equivalent in the filiform papillae of small rodents [37, 38], mHa2 expression could not be shown in this organ. We do not exclude, however, that orthologs of mHa2 may be present in human and bovine filiform papillae. Except for its outermost horny tip, the central unit of the filiform papillae of small rodents is

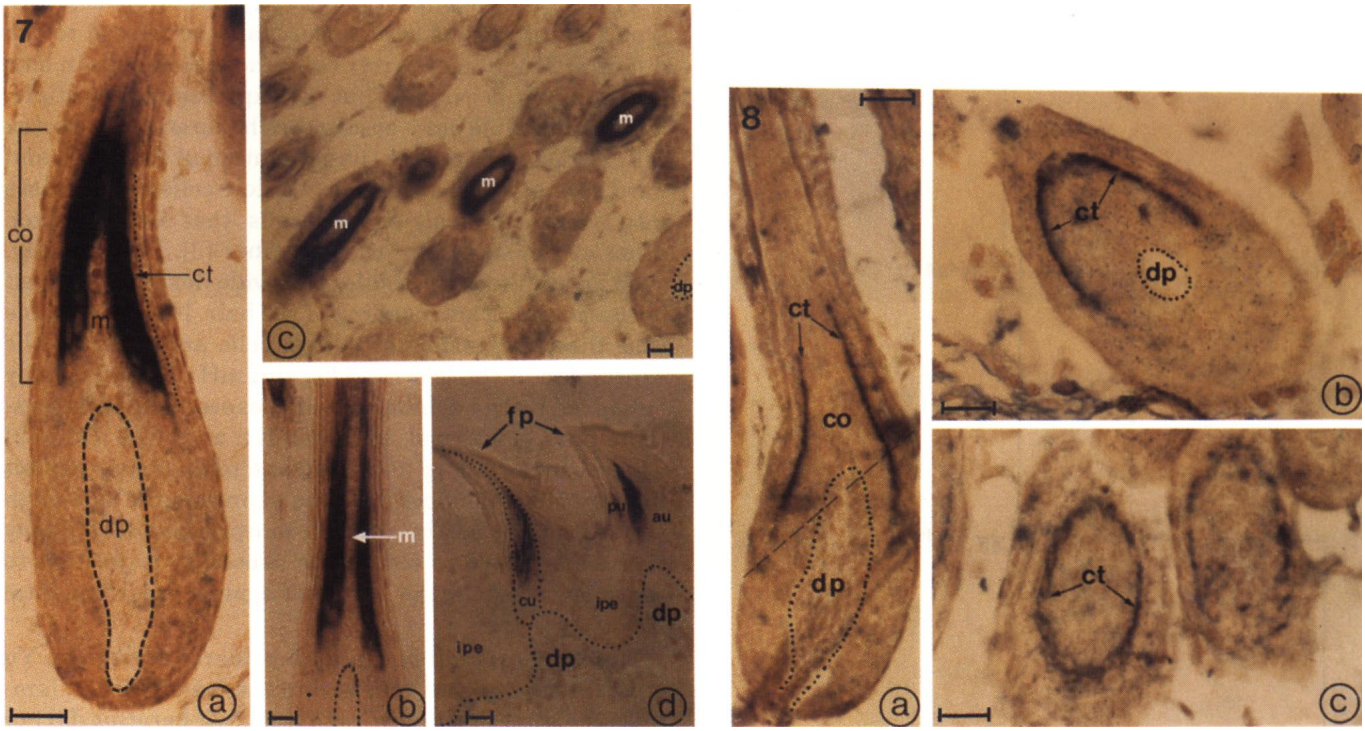

FIG. 7. Nonradioactive *in situ* hybridization with mHa3-specific subclone pktI-3'. (a, b) Vertical sections through hair follicles; (c) oblique section through newborn mouse back skin showing from bottom to top cross sections of hair follicles at different stages of differentiation; (d) vertical section through mouse tongue showing two filiform papillae. co, cortex; ct, cuticle; m, medulla; dp, dermal papillae; fp, filiform papillae; cu, central unit; au, anterior unit; pu, posterior unit; ipe, interpapillary epithelium. Bar, 100 μm throughout.

FIG. 8. Nonradioactive *in situ* hybridization with mHa2-specific subclone pktI-2'. (a) Vertical section and (b, c) cross section through hair follicles. The dashed line in (a) indicates the angle of section through the follicle shown in (b). For symbols, see legend to Fig. 6. Bar, 100 μm throughout.

firmly anchored in and protected by surrounding, morphologically distinct compartments [5, 36, 37]. In contrast, its human and bovine counterparts protrude considerably over the lateral units, forming long and flexible, freely accessible spines [34, 38]. Similar to hairs, these structures may therefore require a peripheral

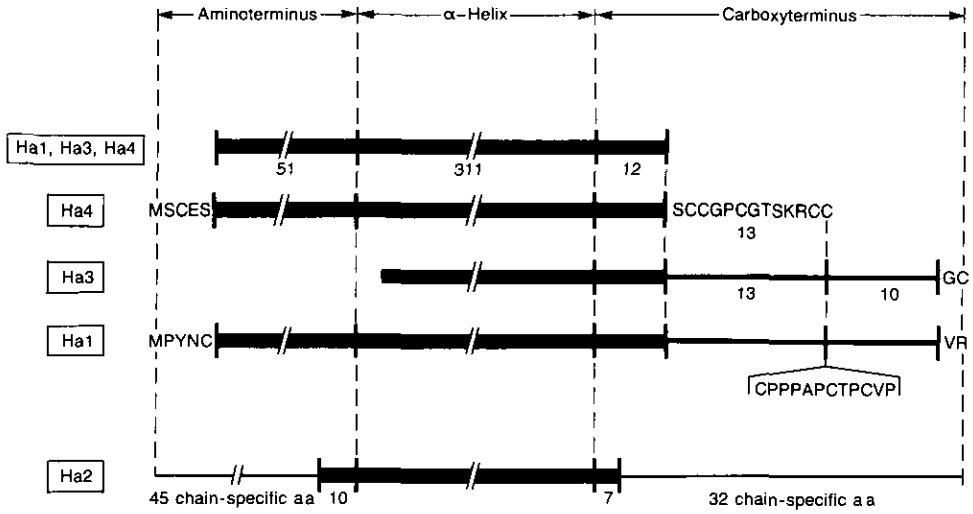

FIG. 9. *Schematic presentation of the structural relationship between the four murine type I hair keratins. Conserved sequences are* indicated by solid bars; chain-specific segments are given by their amino acid sequences. For further details, see text.

sheet of cuticle cell equivalents. In support of this assumption are Western blots with antibody GP19a of two-dimensionally resolved keratins extracted from microdissected bovine filiform papillae which seem to reveal the typical 4 spot pattern of type I hair keratins [36].

It is clear that our demonstration of a distinct structural and functional heterogeneity within the type I murine hair keratin subfamily implies that also the type II hair keratin subfamily is subject to a partitioning into three highly related cortical keratins and a structurally distinct cuticle keratin. Recently, Powell *et al.* have published sequence data for four type II sheep wool keratins [12]. Three of these keratins, designated KII-9, KII-10, and KII-11, proved to be structurally highly related [12]. Interestingly, the fourth keratin, KII-12, from which, however, only 286 amino acid residues of the rod domain are known, differed substantially from KII-9, KII-10, and KII-11 [12]. We have compared the degree of KII-12 α-helical sequence homology with that of the three other keratins and have found a mean value of about 90% versus a mean value of 95–96% among KII-9, KII-10, and KII-11. We therefore interpret these type II wool keratin data as a confirmation of our finding of a structural heterogeneity among murine type I hair keratins.

Unfortunately, the *in situ* hybridization studies by Powell and Rogers with ^{35}S-labeled cDNA probes do not indicate whether the apparent structural heterogeneity among type II wool keratins is also reflected by a differential expression pattern in wool cortex and cuticle [12]. The authors were, however, able to show that KII-9, KII-10, and KII-11 are clearly expressed in the cortex of the wool follicle [12]. Due to the limitation of the known KII-12 sequence to the α-helix, the expression of this keratin could not be studied by *in situ* hybridization. Contrary to the authors' speculation that KII-12 might be a keratin expressed very early in cortex differentiation [12], we would, however, predict that this type II keratin is a cuticular keratin and represents the sheep ortholog of the still unknown type II partner of mouse keratin mHa2.

As the structural entities that distinguish the four type I murine hair keratins are now known, the generation of strictly chain-specific DNA probes as well as monospecific antibodies for each of the murine keratins is feasible. In view of the apparent high sequence conservation of hair keratins among mammals, not only should these tools be helpful for the search and identification of the corresponding orthologous keratins in other species but should also provide the means to answer still open questions on the differential expression of hard α-keratins in hairs, nails, and lingual filiform papillae and thus, in the near future, to gain further insight into the biology of these tissues under both normal and diseased conditions.

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