Krtap16, Characterization of a New Hair Keratin-associated Protein (KAP) Gene Complex on Mouse Chromosome 16 and Evidence for Regulation by Hoxc13*

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Nathanael D. Pruett‡, Tatiana V. Tkatchenko‡, Luis Jave-Suarez§, Donna F. Jacobs‡,
Christopher S. Potter‡, Andrei V. Tkatchenko‡, Jürgen Schweizer§,
and Alexander Awgulewitsch‡¶

From the ‡Department of Medicine, Medical University of South Carolina, Charleston, South Carolina 29425, the §Section of Normal and Neoplastic Epidermal Differentiation, German Cancer Research Center, 69120 Heidelberg, Germany, and the *Department of Dermatology, Medical University of South Carolina, Charleston, South Carolina 29425

Intermediate filament (IF) keratins and keratin-associated proteins (KAPs) are principal structural components of hair and encoded by members of multiple gene families. The severe hair growth defects observed upon aberrant expression of certain keratin and KAP genes in both mouse and man suggest that proper hair growth requires their spatio-temporally coordinated activation. An essential prerequisite for studying these cis-regulatory mechanisms is to define corresponding gene families, their genomic organization, and expression patterns. This work characterizes eight recently identified high glycine/tyrosine (HGT)-type KAP genes collectively designated Krtap16-n. These genes are shown to be integrated into a larger KAP gene domain on mouse chromosome 16 (MMU16) that is orthologous to a recently described HGT- and high sulfur (HS)-type KAP gene complex on human chromosome 21q22.11. All Krtap16 genes exhibit strong expression in a narrowly defined pattern restricted to the lower and middle cortical region of the hair shaft in both developing and cycling hair. During hair follicle regression (catagen), expression levels decrease until expression is no longer detectable in follicles at resting stage (telogen). Since isolation of the Krtap16 genes was based on their differential expression in transgenic mice overexpressing the Hoxc13 transcriptional regulator in hair, we examined whether bona fide Hoxc13 binding sites associated with these genes might be functionally relevant by performing electrophoretic mobility shift assays (EMSA). The data provide evidence for sequence-specific interaction between Hoxc13 and Krtap16 genes, thus supporting the concept of a regulatory relationship between Hoxc13 and these KAP genes.

The distinct functional properties of diverse epithelial cell types are largely determined by their cytoskeletal architecture that includes keratins and keratin-associated proteins (KAPs) as essential components. In vertebrates, there exist two major classes of keratins, acidic and basic, that are commonly known as type I and type II keratins, respectively, and which are encoded by more than 50 genes in mouse and man (1–3). Specific pairs of complementary type I and type II keratins are usually co-expressed and have an intrinsic capacity to form α-helical coiled-coil heterodimers; these heterodimer units are known to self-assemble into 10-nm intermediate filaments (IFs) through both end-to-end association and anti-parallel alignment (4). The cytoplasmic IFs build an intracellular fibrous network that extends from the cell membrane to the nucleus, and its properties are greatly influenced by interactions with keratin-associated proteins, also known as KAPs (1, 5). The keratin and KAP gene expression profiles change with keratinocyte differentiation status, and the progression of hair follicle differentiation is characterized by the sequential activation of distinct sets of hair-specific keratin and KAP genes (5–11). Hair follicle development is initiated prematurely through mesenchymal-epithelial interactions that result in the formation of multilayered cylindrical structures (12). Two major outer layers of functionally distinct keratinocytes known as outer root sheath (ORS) and inner root sheath (IRS) surround the hair shaft which is composed of the cuticle, cortex, and medulla. Differentiation of the cells contributing to the formation of these compartments progresses along the proximal-distal axis of the follicle and originates in the lower bulbous portion that harbors largely proliferating and undifferentiated cells. In terminally differentiated cells of the hair shaft, the bulk of the structural molecules include hair-specific IF keratins and KAPs, which combined are estimated to include up to a 100 different proteins (5). In these cells, IFs are embedded into a dense matrix of KAPs that according to their biochemical composition have originally been classified into 3 major groups including high sulfur (HS), ultra-high sulfur (UHS), and high glycine-tyrosine (HGT). Due to the growing complexity among these KAPs, the three main groups have subsequently been divided into structurally distinct subgroups encoded by at least 23 hair KAP gene families (9–11). The essential role of keratins in the structural organization of hair is underscored by the linkage of severe hair disorders to mutations in human hair keratins as exemplified in monilethrix (13). Furthermore, aberrant expression of keratins and KAPs in transgenic mice has...
been shown to affect hair structure and growth (14–20). Combined, these data suggest that structural integrity and tight regulation of both groups of genes is essential for proper hair growth.

Based on differential gene expression analysis in postnatal skin of mice overexpressing Hoxc13, we have previously identified a novel subset of HGT-type KAP genes that we collectively termed Krtap16-n whose expression was uniformly down-regulated in the skin of this GC13 transgenic mouse; we mapped these genes to a distal region of mouse chromosome 16 (MMU16) that is of conserved linkage with human chromosome (HSA) 21q22.11 (19). Here we demonstrate that these genes are integrated into a larger KAP gene domain of about 0.82 Mb in size and that this region is homologous to a recently described domain of HGT- and HS-type KAP genes in humans (10). We examined the Krtap16 gene expression patterns in both developing and cycling hair follicles by in situ hybridization. Furthermore, we show that a HOXC13 consensic binding motif, 5'-TT(A/T)ATNPuPu-3', implicated in the regulation of human hair keratin genes (21) matches HGT-2, a conserved motif that had previously been speculated to play a role in the transcriptional regulation of certain HGT-type KAP genes (15). Our in vitro DNA binding studies provide evidence for Hoxc13-dependent regulation of Krtap16 genes involving the HGT-2 motif. The Krtap16 gene complex described here might thus serve as a model for examining regulatory interactions of a Hox factor with clustered downstream target genes.

MATERIALS AND METHODS

Genomic Map, Protein Sequence Comparison, and Phylogenetic Tree Analysis—A genomic map of the distal region of MMU16 harboring the Krtap16 genes was derived from the NCBI mouse genome data base (www.ncbi.nlm.nih.gov/genome/guide/mouse). A region of ~0.82 Mb stretching roughly from position 90,094 to 90,912 kb of the MMU16 draft sequence was used for establishing this map. All KAP genes and KAP-related ESTs with continuous open reading frames (ORFs) present in this region were included in the map, and the corresponding protein sequences were derived from conceptual translation of the ORFs starting with an ATG start codon positioned in a sequence context that resembles a common consensus motif for eukaryotic translational initiation (22). Alignment of deduced KAP protein sequences was achieved by using the AlignX program included in the Vector NTI software package. Phylogenetic tree analysis was performed using the same software tool.

Expression Analysis—For determining Krtap16 gene expression patterns in skin of day postnatal FVB mice, the animals were euthanized after substrate reaction and color development.

RESULTS

Genomic and Structural Characterization of Krtap16 Genes—Based on differential gene expression analysis in 5 day skin of normal versus Hoxc13 overexpressing transgenic mice, we have recently identified a subset of new HGT-type KAP genes designated originally Krtap16-1–10, and by using a mouse-hamster radiation hybrid panel we mapped them to the distal portion of MMU16 (Ref. 19; the sequence for Krtap16-10 was subsequently submitted separately to the GenBankTM data base under accession number AF477980). The subsequent publication of a draft sequence of MMU16 and the entire mouse genome (29, 30) facilitated a closer analysis of the corresponding genomic region (see mouse genome data base, www.ncbi.nlm.nih.gov/genome/guide/mouse). This analysis revealed redundancy in the original assignment of these Krtap16 cDNAs to separate genes, thus reducing the actual number of Krtap16 genes isolated to us 8 (see Table I). A map of the region harboring the Krtap16 and neighboring genes is presented in Fig. 1A. Whereas all 8 Krtap16 genes are located on the minus strand within a subregion of about 0.54 Mb of DNA, 6 of them are clustered within less than 100 kb; the seventh (Krtap16-8) and eighth (Krtap16-7) gene are located at a distance of ~90 and
Krtap16 Gene Complex on Mouse Chromosome 16

The first column lists known KAP genes under their originally published name; corresponding GenBank™ accession numbers are listed in the second column. ESTs or cDNAs that have previously not been reported in the literature are only identified by their GenBank™ accession numbers. The third column indicates gene location on the centromeric (+) or telomeric (−) strand of the chromosome. The remaining columns specify characteristics of the conceptual translation product including peptide size, molecular weight, glycine-tyrosine content and cysteine content.

| Gene Designation | GenBank™ accession no. | Strand | Peptide size (amino acid residues) | Molecular mass of protein Da | Gly-Tyr content | Cys content |
|------------------|------------------------|--------|----------------------------------|-----------------------------|----------------|------------|
| Krtap16-1        | AF345291               | (−)    | 141                              | 13,734                      | 59.0           | 5.22       |
| Krtap16-3        | AF345293               | (−)    | 80                               | 8,073                       | 40.7           | 7.65       |
| Krtap16-4        | AF345294               | (−)    | 84                               | 8,624                       | 57.43          | 3.59       |
| Krtap16-5        | AF345295               | (−)    | 87                               | 8,821                       | 48.34          | 7.01       |
| Krtap16-7        | AF345297               | (−)    | 123                              | 12,520                      | 61.92          | 18.0       |
| Krtap16-8        | AF345298               | (−)    | 78                               | 7,942                       | 65.37          | 12.99      |
| Krtap16-9        | AF345299               | (−)    | 87                               | 8,847                       | 52.71          | 8.16       |
| Krtap16-10       | AF477980               | (−)    | 55                               | 5,915                       | 54.89          | 1.76       |
| Krtap13          | AF031485               | (+)    | 197                              | 21,435                      | 13.64          | 12.13      |
| Krtap13-1        | AK009807               | (+)    | 214                              | 23,235                      | 15.02          | 12.53      |
| Krtap14          | AF003601               | (+)    | 200                              | 21,592                      | 16.16          | 12.03      |
| Krtap15          | AF162800               | (+)    | 150                              | 16,240                      | 21.56          | 3.84       |
| Krtap8-2         | D86422                 | (−)    | 62                               | 6,687                       | 59.39          | 3.11       |
| Krtap8-3         | D86423                 | (+)    | 58                               | 6,030                       | 62.99          | 0          |
| Krtap6-1         | D86420                 | (+)    | 78                               | 8,002                       | 66.88          | 11.54      |
| Krtap6-2         | D86421                 | (−)    | 78                               | 8,002                       | 66.88          | 11.61      |
| Krtap6-3         | D86422                 | (−)    | 78                               | 8,002                       | 66.88          | 11.61      |
| Krtap8-2         | D86423                 | (+)    | 57                               | 6,210                       | 65.52          | 13.43      |
| Krtap6-1         | D86420                 | (+)    | 84                               | 5,568                       | 62.23          | 15.38      |
| Krtap6-2         | D86421                 | (−)    | 159                              | 15,258                      | 64.21          | 16.06      |
| Krtap11-1        | AA762330               | (−)    | 183                              | 19,033                      | 8.97           | 11.95      |

450 kb upstream of the 6-member subcluster, respectively. The Krtap16 genes are integrated into a larger domain of 0.82 Mb of genomic DNA harboring predominantly KAP genes (Fig. 1A). Within this domain, the Krtap16 genes are found among 16 annotated KAP genes in addition to 12 ESTs and cDNAs with similarities to known KAP genes. A conceptual translation of the ORFs of these 28 KAP genes and KAP gene-related cDNA sequences revealed considerable heterogeneity with regard to glycine/tyrosine and cysteine content, as well as the presence of conserved amino acid sequence motifs (Fig. 2 and Table I). The glycine/tyrosine content of the predicted Krtap16 proteins ranges from 40.7 mol% (Krtap16-3) to 65.4 mol% (Krtap16-8) (Table I), thus placing them into the HGT-type class (5) that is known to include several structurally distinct subfamilies in both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10). Remarkably, there is a more than 4-fold variation in cysteine content among the Krtap16 proteins ranging from 1.76 mol% for Krtap16-10 to 18 mol% for both mouse and human (5, 10).
FIG. 1. Genomic map of Krtap16-containing KAP gene domain on mouse chromosome 16 (A) and phylogenetic tree analysis of corresponding genes (B). The map shows a 0.82 Mb interval of the distal region of MMU16 (see ideogram of MMU16 to the left) that has been derived from data published by the NCBI Mouse Genome Project (www.ncbi.nlm.nih.gov/genome/guide/mouse). All annotated genes and ESTs assigned to this interval have been included, and their relative positions are shown by rectangular symbols; directions of transcription on the plus and minus strand are indicated by downward and upward pointing arrowheads, respectively. Krtap16 genes are highlighted by red typeface, and ESTs are defined by GenBankTM accession numbers. Color of rectangular symbols reflects assignment of corresponding genes to phylogenetic groups as indicated by the key at the lower right; black symbols mark unknown genes or ESTs without similarities to KAP genes. B, predicted amino acid sequences encoded by KAP genes and KAP-related cDNAs mapped to the MM16 KAP gene domain (A) were subjected to phylogenetic tree analysis by using the AlignX multiple sequence alignment tool from Vector NTI. Graphic representation of this analysis (upper right) reveals distinct clusters of phylogenetically related genes that were assigned to 8 groups and subgroups including I, II, III-A, -B, -C, IV-A and -B, and V. Krtap16 genes are highlighted by red typeface and KAP-related ESTs are denoted by GenBankTM accession numbers. Group V includes two heterogeneous KAP-related ESTs that did not show clustering.
**FIG. 2.** Alignment of putative KAP protein sequences listed in Table I. Optimal alignment was achieved by grouping the sequences according to the clustering observed upon phylogenetic tree analysis (Fig. 1B) and by using the AlignX program included in the Vector NTI DNA analysis software package. Accordingly, sequence alignments are shown as follows: group I (A), group II (B), group III-A (C), group III-B (D), group III-C (E), group IV-A (F), group IV-B (G), group V (H). Blocks of sequence identity and similarity are highlighted by dark and light gray, respectively.
cated just downstream of the III-A distal-most member, Krtap16-3. The group III-C genes encode proteins that share a distinct MSYY(H/Y)GYGG N-terminal domain (Fig. 2E). Members of subgroup III-B form a loose cluster stretched over 100 kb near the middle of this 0.83 Mb domain (Fig. 1A) and contain previously unknown KAP gene sequences. The presumptive proteins encoded by these sequences share a characteristic MCYY(R/G)(G/S)YYGGLG N terminus (Fig. 2D). Interspersed between groups III-A and III-B is a tight cluster of 5 highly conserved Krtap6-1-related KAPs assigned to group IV-A, which includes in addition to Krtap6-1, Krtap16-8, as well as 3 additional KAP genes (GenBank™ accession numbers: D86419, D86421, and AK003924). Conceptual translation of the ORFs of all 5 genes indicates that their protein sequences are nearly identical throughout and start with a distinct MCYYGGYGGYG N terminus (Fig. 2F). The two members of subgroup IV-B, Krtap16-7 and Krtap6-2 are located more than 180 kb distal to the III-B subcluster. Their conceptual protein structures show strong similarities between the N-terminal halves and both have extensive -CGYGSGYG-repeats; however, their C termini are unique (Fig. 2G). Finally, two cDNAs (GenBank™ accession numbers: AK004025 and D86423) were found to encode structurally heterogeneous HGT-type KAPs; since they showed little similarity to members of the other groups, they were assigned to a separate group V (Figs. 1 and 2H). These two genes are located between groups III-A and IV-A (AK004025), as well as group IV-B and the two distal members of group II (D86423).

We have pointed out earlier that the distal region of MMU16 where we originally mapped the Krtap16 genes is of conserved linkage with HSA 21q22.11 (19), and recently a corresponding human KAP gene domain has been defined in this region (10). A comparison between the organization of the genes and subclusters included in the KAP gene domain on MMU16 and those found in the corresponding human KAP gene domain is schematically depicted in Fig. 3 and discussed below. Overall, the results reveal a great degree of similarity with respect to the order and arrangement of subclusters and the orientation of these domains in mouse and human.

Krtap16 Expression Patterns—The circumstance that all Krtap16 genes described here were isolated as differentially expressed cDNAs in 5 day postnatal skin of our GC13 hair mutant (19) implies that they are transcriptionally active in skin at that stage of development. Since the Krtap16 genes bear structural similarities to previously characterized hair-specific HGT-type KAP genes, we focused on examining expression in hair. To define spatial patterns of Krtap16 expression, we performed in situ hybridization analysis in 5-day postnatal skin that is known to harbor fully differentiated hair follicles (35). Furthermore, to determine whether Krtap16 genes might continue to be active also in cycling hair, we examined expression in adult skin containing depilation-induced anagen phase hair follicles.

Expression analyses in 5 day postnatal skin was performed using digoxigenin-labeled RNA probes specific for each of the Krtap16 genes, except for Krtap16-10, whose expression was only examined in cycling hair (see below). Hybridization in longitudinal follicle sections yielded similar hair-specific patterns for all genes examined (Fig. 4). In most cases, expression was restricted to a relatively narrow region of the hair shaft with a proximal boundary near the neck of the bulb, a region known to harbor cells undergoing terminal differentiation (36); for Krtap16-7, however, the proximal expression boundary was shifted slightly more distal compared with the remaining genes (Fig. 4F). Hybridization signals were observed in all differentiated hair follicles found in scapular skin samples, and the patterns suggested expression to be restricted to the cortical region of the hair shaft (Fig. 4, A–D and F–I). Further evidence for the cortically restricted expression was obtained by hybridization to follicular cross sections. In all cases, expression was found to be restricted to a circular layer of uniformly shaped cells surrounding the medulla, which itself lacked detectable hybridization signals (Fig. 5).

For five of the six members of group III, we determined expression in skin at different stages of the hair cycle including the progressive growth phase (anagen), the phase of follicle regression (catagen), and the resting phase (telogen). This was done by either reverse Northern (Krtap16-1, −16-3, −16-5, −16-9) or in situ hybridization (Krtap16-10). The results show a drastic decline in expression levels during follicle regression (catagen), and in skin containing telogen follicles expression was no longer detectable (Fig. 6). In addition, we examined expression of the remaining Krtap16 genes in cycling anagen hair follicles by in situ hybridization with radioactive probes;
this revealed expression patterns that were very similar to those observed upon hybridization with digoxigenin-labeled probes in 5-day postnatal hair (data not shown). Combined, these findings are consistent with the hair cycle-dependent expression of other Krtap16-related HGT-type KAP genes in mouse skin previously reported (37).

Combined, the expression data show that all Krtap16 genes described here are transcriptionally active in both developing and cycling hair follicles and that their expression is restricted to cortical keratinocytes. The reduction and lack of expression demonstrated for a subset of Krtap16 genes during catagen and telogen, respectively, suggests the existence of hair cycle-dependent control mechanisms for the regulation of transcriptional activity. The majority of Krtap16 genes, including Krtap16-1, -16-3, -16-4, -16-5, and -16-9 have, together with the previously isolated Krtap-2 gene (GenBank™ accession no. D86422, Ref. 37), been assigned to group III-A as based on our phylogenetic tree alignment (Fig. 1B), and consequently the great similarities in expression (Fig. 4) to the cortical pattern reported for Krtap-2 (37) are not surprising.

Furthermore, the cortical patterns of group III-A genes seem to be remarkably similar to the spatially restricted patterns of expression of certain members of the KAP19 subfamily of human HGT-type KAP genes in human beard hair, including KAP19.2, -19.3, and -19.7 (10). Group III-A genes are structurally most closely related to KAP19 genes located in a domain of HGT-type KAP genes on human chromosome 21q22.11 (Fig. 3; Ref. 10). However, in contrast to the relative uniformity in Krtap16 expression patterns, some members of the human KAP19 family (i.e. KAP19.1, -19.4, and -19.6) exhibit rather heterogeneous patterns that may either include or be restricted to cuticular cells of beard follicles (10).

DNA Binding Studies—The fact that all Krtap16 genes were found to be down-regulated in the skin of Hoxc13-overexpressing transgenic mice (19) might suggest a regulatory relationship between Hoxc13 and these KAP genes. As a first step toward examining potential direct interactions with Hoxc13, we searched for the presence of presumptive Hoxc13 binding sites in Krtap16 flanking regions. The consensus binding sequence (5′-TT(A/T)ATNPuPu-3′) for human HOXC13 has previously been defined (21), and given the high degree of overall similarity of 98% between mouse Hoxc13 and human HOXC13 proteins, as well as the structural identity of the DNA binding homeodomains in both proteins, it is to be anticipated that murine Hoxc13 will interact specifically with the same consensus sequence. A systematic search for bona fide Hoxc13 binding sites within a genomic interval of 23 kb harboring a subcluster of four tandemly arranged Krtap16 genes, i.e. Krtap16-9, -16-1, -16-5, -16-4 (Figs. 1A and 7A), identified 34 perfect matches for the HOXC13 consensus binding sequence (5′-TT(A/T)ATNPuPu-3′) in this region alone (Fig. 7A). In most cases one or two presumptive binding sites were closely associated with a TATA box (Fig. 7A). To obtain evidence for interaction of HOXC13 with these presumptive binding sites, we selected the two most proximal sites upstream of Krtap16.5 for EMSAs. The experiments were performed with nuclear extracts from HOXC13-transfected and non-transfected PtK2 cells and 32P-labeled double-stranded oligonucleotides containing a bona fide HOXC13 binding sequence and a mutated version of this sequence.

The results show band shifts only for reactions performed with nuclear extracts from HOXC13 transformed cells in the presence of oligonucleotides that contain an intact 5′-TTAATGAG-3′ sequence matching the HOXC13 consensus binding site 5′-TTAATGAG-3′ in both cases, thus indicating the formation of sequence-specific HOXC13/DNA complexes (Fig. 7B and C; compare lanes 1, 2, and 3). In the presence of excess cold competitor oligonucleotide containing either the authentic sequence or an alteration from 5′-TTAATGAG-3′ to 5′-TGCCCGAG-3′, the shifted bands were either abolished or maintained, respectively (Fig. 7, B and C; compare lanes 4 and 5). Super-shift assays performed by adding HOXC13-specific antisera (21) to the reaction resulted in a mobility shift of the band containing the presumptive HOXC13/DNA complex (compare lanes 2 and 6). Specificity of interaction of human anti-HOXC13 antisera with mouse Hoxc13 was examined immunohistochemically in longitudinal sections of hair follicles in 5-day postnatal skin from normal FVB and GC13 transgenic mice. The results show detection of an antigen exhibiting the
Fig. 5. *Krtap16* gene expression patterns in cross sections of postnatal hair follicles. DIG-labeled antisense RNA probes specific for *Krtap16-1,-16-3,-16-4,-16-5,-16-7,-16-8,* and -16-9, 
(A, B and D–H) and a sense (SE) control probe specific for *Krtap16-3* 
(C) were hybridized to 10-μm frozen sections of scapular skin derived from 5-day 
postnatal FVB mice. Hybridization signals were visualized by the NBT/BCIP 
color reaction that resulted in a dark precipitate; no signal was obtained with the 
control probe. A schematic representation of the sections depicting the individual 
follicle layers is shown in the scheme at the lower right (I). Expression of all genes 
is restricted to cortex (Ctx), while no hybridization signal was detected in the 
medulla (M) and surrounding cuticle (Cu). Space bar, 20 μm.

Fig. 6. *Krtap16* gene expression during the hair cycle. A, reverse Northern hybridization to arrayed cDNAs specific for *Krtap16-1,-16-3,-16-5,* and -16-9 as indicated with complex cDNA probes derived from adult mice whose hair growth was at different stages of the hair cycle. The hair cycle had been synchronized by depilation (25) and skin samples were taken at days 9, 17, and 21 post-depilation (p.d.) that corresponded to anagen, catagen, and telogen phases, respectively. Please note the dramatic reduction in signal and loss of signal for all four genes in catagen and telogen, respectively. S, total mouse genomic DNA used as a standard. B′/B′, D′/D′, E′/E′, *in situ* hybridization with 32P-labeled *Krtap16-10*-specific antisense probe to frozen sections of adult skin that was depilated for synchronized entry into anagen, catagen, and telogen phase of the hair cycle as described above; C′/C′, hybridization with *Krtap16-10*-specific sense control probe. Hybridized sections were counterstained with hematoxylin/eosin as shown in brightfield (B–E), and silver grains corresponding to hybridization signal were visualized by light scattering (*bright grains*) using darkfield microscopy (B′–E′). Please note *Krtap16-10* expression in the lower cortex (Ctx) of the growing hair shaft during anagen (B′B′) and the reduced expression in catagen hair (D′D′); no specific hybridization signal was detectable in the shaft of telogen hair (E′E′), and no signal was detectable in anagen follicles upon hybridization with the sense probe (C′C′) used as control. DP, dermal papilla; HS, hair shaft; M, medulla. Space bar, 50 μm.

typical pattern of Hoxc13 expression (Fig. 7D) previously re-
ported in both mouse and human hair follicles at RNA (19, 38), 
as well as both RNA and protein levels (21), respectively. Fur-
thermore, this apparent Hoxc13 expression is shifted toward 
the keratogenous zone of the cortex in GC13 mutant hair 
follicles (Fig. 7E).

**DISCUSSION**

Our data show that the *Krtap16* genes are embedded within 
a larger KAP gene domain that occupies ~0.82 Mb of DNA and 
includes 28 KAP genes (Fig. 1); 20 of these genes, including the 
*Krtap16* genes, have previously been reported in the literature, 
on occasion under alternative names attributed by different 
authors. The remaining 8 KAP genes were listed in the mouse 
genome data base as ESTs and cDNAs with KAP gene similarities ([www.ncbi.nlm.nih.gov/genome/guide/mouse](http://www.ncbi.nlm.nih.gov/genome/guide/mouse)). In addition, 3 ESTs for unknown genes without similarities to KAP genes have been mapped to this region (Fig. 1A). Outside of this 0.82 Mb region, no further KAP genes or KAP-related ESTs were identified, thus suggesting that the map presented in Fig. 1 defines the limits of a murine KAP gene complex in the distal portion of MMU16.

Like all KAP genes reported to date (see Refs. 5, 9–11), the 
*Krtap16* genes have a simple structure consisting of a single 
exon with a short ORF, in this case ranging from 165 to 423 bp. 
Although all *Krtap16* genes may be classified as HGT-type 
KAPs as based on the high glycine/tyrosine content of their 
presumptive products ranging from 40.7 to 65.4%, they do
belong to diverse groups as revealed by phylogenetic tree analysis (Fig. 1B), and they are found scattered throughout much of the 0.82 Mb KAP gene complex. Interestingly, putative proteins encoded by Krtap16-7 and -16-8, the two Krtap16 proteins with the highest glycine/tyrosine content of 61.8 and 65.4%, respectively, have also the highest cysteine content of 18 and 13% (Table I). The same applies also to other members of the Krtap16-8 and -16-7 subgroups IV-A and -B, respectively, as well as members of subgroup III-B (Table I, Fig. 1). This circumstance might illustrate that the traditional dichotomy of KAP classification into HS (and UHS) and HGT-type KAPs is insufficient for adequate categorization, as it has been recognized previously (5).

By and large, the clustering of the described KAP genes into distinct groups and subgroups as demonstrated by phylogenetic tree alignment corresponds well with their spatial clustering in the genome (Fig. 1, A and B). This overall genomic organization is conserved between mouse and human where a corresponding KAP gene domain has recently been defined on HSA 21q22.11 (10). In both cases, the domains start at the centromeric end with a group of Krtap13-related (HS-type) genes that is followed by Pmg-related KAPs, which precede a subcluster of HGT-type KAP genes related to murine Krtap8-2, i.e. groups III-A and KAP19 in mouse and human, respectively (Fig. 3). This is followed by groups IV-A and KAP6 in mouse and human, respectively, which in both cases include genes with similarities to Krtap6-1. Subsequent to this, the density of KAP genes decreases and includes group III-B and IV-B genes in mouse and their respective human KAP20 and KAP21 equivalents. Finally, the complexes terminate distally with heterogeneously Pmg-like genes in both cases. This remarkable conservation in gene order on a relatively small scale reflects on a much larger scale the conserved synteny between distal MMU16 and HSA21 corresponding to segments of 22.37 and 28.42 Mb of the mouse and human chromosomes, respectively (29). The order of 111 genes in this MMU16 segment has been reported to follow exactly the order of corresponding human genes in this region (29), and consequently, the overall conservation of the murine KAP gene domain is of no surprise. Although the mouse and human genomes have been extensively shuffled by chromosomal rearrangements since their phylogenetic separation from a common ancestor ~65–75 million years ago, the gene order in syntenic segments has been found to be largely intact (30). However, this does not exclude isolated local rearrangements and gene family expansions that are likely to have occurred during the evolution of the murine KAP gene domain on chromosome 16 and its human counterpart and resulted in paralogous genes.

The expression patterns of the Krtap16 genes in the keratogenous zone of the hair shaft most closely resemble the patterns of two previously isolated members of their domain including Krtap8-2 (originally isolated under the name HGTtype 1a, see Ref. 37) and Krtap11-1 (originally known as Huc1-1, see Ref. 34). Although these two genes are structurally heterogeneous (Fig. 2) and belong to different groups according to our phylogenetic alignment analysis (Fig. 1B), their reported expression patterns are very similar. This relative uniformity contrasts with a considerable diversity in expression patterns among members of the human equivalent of the MMU16 KAP gene domain. In that case, several genes exhibited expression in both cortical and cuticular cells with remarkable variation in proximal-distal expression domains, which for certain Pmg-like KAP genes, including hKAP13.1 and -15.1, initiated in the matrix of beard follicles (10). Furthermore, several of the human KAP genes showed striking cylindrical asymmetries in expression, a feature that previously had been observed also for Krtap6-1 expression in hair follicles of sheep and rabbit (39). These asymmetries are likely to reflect differential expression
in local subpopulations of cortical keratinocytes and might be a contributing factor in determining the texture and crimp of hair (39). Accordingly, the greater diversity in human KAP gene expression patterns compared with their counterparts on MMU16 is likely part of the molecular signature determining textural differences between human beard and murine coat hair.

The down-regulation of the Krtap16 genes in Hoxc13 overexpressing (GC13) mice led us to speculate about a regulatory relationship between Hoxc13 and these KAP genes. In support of this idea we found multiple putative Hoxc13 binding sites associated with members of the MMU16 KAP gene domain (Fig. 7A); EMSAs carried out for two of these sites located 5′ of Krtap16-5 indicated sequence-specific interaction with human HOXC13, whose DNA binding homeodomain is identical to the murine HOXC13 homeodomain. Furthermore, both HOXC13 and the Krtap16 genes are expressed in the same lineage of cortical keratinocytes, although at different stages of differentiation. While the Krtap16 genes are expressed in the keratogenous zone of the cortex, HOXC13 is expressed in the matrix and the precerical region, which means that there is little overlap between the Krtap16 and HOXC13 expression domains in normal hair follicles. Taking this circumstance into account, one may consider at least two alternative mechanisms for explaining the down-regulation of KAP genes in skin of GC13 mice. The first is based on indirect regulation mediated by another transcription factor, whose expression might directly be controlled by HOXC13. A good candidate for this is HOXC12, whose expression domain in the cortex and precerical region of differentiated hair follicles conspicuously overlaps with both the HOXC13 domain (38) and the Krtap16 expression domains (Fig. 4). During axial patterning, HOX genes frequently restrict the activity of their next downstream neighbor within the same cluster that usually occupies a more anterior expression domain along the longitudinal embryonic axis, a phenomenon known as posterior prevalence (see Refs. 40 and 41). A similar principle might apply to the regulation of HOX gene activities along the longitudinal axis of the differentiating hair follicle (42). In our case, the concept of a regulatory relationship between HOXC13 and Krtap16 is supported by the presence of multiple bona fide HOXC13 binding sites upstream of KRTAP16, (as well as HOXC13; data not shown), which itself might positively regulate Krtap16 expression. In this context it is worth mentioning that HOXC13 might control also its own expression via negative autoregulation as it has been postulated based on the reduced expression of a Hoxc13-lacZ reporter gene construct in Hoxc13 overexpressing mice (19). The presence of putative HOXC13 binding sites found in genomic regions flanking Hoxc13 is consistent with this idea.

The second mechanism for explaining the down-regulation of Krtap16 genes in hair follicles overexpressing HOXC13 is based on an expansion of the HOXC13 expression domain toward the keratogenous zone, thus resulting in HOXC13 expression in cells that normally express Krtap16 genes but not HOXC13. Under the premise that HOXC13 normally acts as a negative regulator of Krtap16 genes to prevent their premature activity, this would result in their reduced expression. Our comparative immunohistochemical analysis of HOXC13 expression in normal and GC13 hair follicles indeed suggests a distal expansion of the Hoxc13 domain into a zone where Hoxc13 and Krtap16 gene expression overlap (compare Figs. 7 and 4). In summary, the phylogenetically conserved KAP gene complex on mouse chromosome 16 might provide a useful paradigm for studying mechanisms that regulate the coordinated expression of clustered hair-specific genes in a cyclical manner.

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