Isolation and Characterization of Mouse High-glycine/Tyrosine Proteins*

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During hair follicle differentiation, several families of keratin proteins are synthesized sequentially. In the present study, cDNA clones encoding six members of mouse high-glycine/tyrosine protein type I and four of type II. Interestingly, one of the four type II proteins had been encoded by two distinct cDNAs. Among the cDNA clones isolated were included the ones encoding a new member of type I and II protein, respectively, which possessed an entire open reading frame. Novel type II protein, termed type II.4, with a molecular mass of 15,130 Da was revealed to have significant direct repeats and a cysteine residue at the carboxyl terminus, which indicates that this protein has characteristics intermediate between high-glycine/tyrosine proteins and cystine-rich proteins. In addition, the new member of type I protein has some features common with type II protein. We propose to term this protein type Ie until it is further characterized. Northern blot analysis demonstrated that gene expression of mouse high-glycine/tyrosine proteins followed the hair cycle growth fundamentally and reached its peak at day 9 in the first hair cycle, while two peaks of their expression were observed at day 33 and day 39 in the second cycle. Their transcripts were expressed in the cortical cells of hair follicles but not in the cells of the outer root sheath, inner root sheath, or medulla. Moreover, their gene expression commenced at different levels in cortical cells. The novel findings that each gene is activated transcriptionally with a distinct expression pattern spatially and temporally suggest that there is a remarkable difference in the distribution of these proteins in hair.

Hair is a keratinized tissue formed within the hair follicle and has a common structure composed of a cuticle sheath, an inner cortex, and a central medulla. The major structural protein of hair is keratin, which forms the rigid hair shaft by formation of a cross-linked network of keratin molecules and associated proteins. Keratin proteins are derived from several multigene families, and have been classified into intermediate filaments and intermediate filament-associated proteins (1). Furthermore, intermediate filament-associated proteins have been classified as high sulfur (16–30% cysteine), ultra-high sulfur (>30% cysteine), and high-glycine/tyrosine proteins (HGTps) according to their amino acid compositions (1).

HGTps are the smallest among the keratin proteins, most having molecular weights below 10,000 (2). They have been demonstrated to contain at least 30 components by two-dimensional electrophoresis (2, 3). However, controversy exists that the apparent heterogeneity may have arisen from the conditions of protein preparation and fractionation (4). On the basis of amino acid content and solubility, these proteins were separated into two distinguishable groups, types I and II. Their amino acid compositions determined primarily from sheep exhibited that the less soluble type I group was comparatively poor in cysteine but rich in phenylalanine, while the reverse was true for type II, and that both types possessed no methionine (2). Sheep type I has been further separated by chromatography on quaternary ammonium ethyl cellulose at pH 10.5 into two major (C and F) and eight minor components. At the present time, amino acid sequences for some sheep HGTps have been identified by the peptide mapping and sequence studies of the purified protein fractions (type I C3 and KAP6.2 corresponding to type II) (4, 5) or cloning their genes (types I C2 and F, and KAP6.1 corresponding to type II) (6, 7). Their sequence information showed that HGTps were enriched in glycine, tyrosine, and serine, accounting for 48–55 mol % of the amino acid content for type I and 74 mol % for type II proteins. Furthermore, the comparisons among their sequence data revealed that types I C2 and C3 were identical, and that there was a high homology between two type II proteins, KAP6.1 and 6.2. In contrast, there was no apparent homology among type I C, I F, and II proteins, except that all of them had a tyrosine residue as the C-terminal amino acid. In addition to these sheep HGTps, a gene for a type II protein has been isolated from rabbit as well. This deduced amino acid sequence showed significant identity with sheep KAP6.1, suggesting that they were evolutionarily conserved in two species.

HGTps have been proposed to form the intermicrofibrillar matrix of cortex together with the high sulfur proteins. Indeed, in situ hybridization experiments using KAP6.1 cDNAs isolated from sheep and rabbit demonstrated that the transcripts of at least one type II were predominantly restricted to the cortical cells of hair follicles (7). However, it is uncertain whether the transcripts of other type II proteins are expressed similarly. On the other hand, the localization of type I transcripts in hair follicles has not yet been described.

HGTps have been estimated to constitute about 19% of mouse hair protein (8). Southern blotting analysis indicated the possibility that unique types I C and F, and about 20 type II genes were present in the mouse genome (7, 9). However, no

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D86419, D86420, D86421, D86422, D86423, D86424, and D89902.

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1 The abbreviation used is: HGTps, high-glycine/tyrosine proteins.
EXPERIMENTAL PROCEDURES

**Materials—**ZAP II and an RNA transcription kit were purchased from Stratagen (La Jolla, CA). A nitrocellulose filter was from Schleicher & Schuell (Dassel, Germany). DNA was from Promega (Madison, WI). [32P]dCTP (3000 Ci/mmol) and 35S-UTP (3000 Ci/mmol) were from Amersham Japan (Tokyo, Japan). Moloney murine leukemia virus Reverse transcriptase and RNA molecular weight markers were obtained from Life Technologies, Inc. Sephadex G-50 was from Pharmacia (Uppsala, Sweden). BcaBEST™ DNA polymerase was from Takara (Kyoto, Japan). Isogen was from Nippongene (Tokyo, Japan). Other chemicals were purchased from Sigma and Wako (Osaka, Japan).

**Animals and Skin Samples—**Syngeneic C57BL/6 female mice (purchased from Charles River, Yokohama, Japan), 1–45 days and 7–8 weeks old, and BALB/c mice, 1–45 days old, were housed under 12-hour light/dark cycles and fed ad libitum. For library construction and screening, hair growth (anagen) was induced as described previously (16). Seven-week-old mice with all skin follicles in telogen were depilated with a mixture of wax and resin. Dorsal skins were harvested at days 0 (telogen) and 6 (anagen) after follicle growth induction. All skin samples were confirmed to be in telogen or anagen (anagen III-IV) phases histologically by a light microscope.

**Library Construction and Screening by Differential Hybridization—**Poly(A)+ RNAs were isolated from the dorsal skins of three C57BL/6 mice in anagen or telogen phases, respectively, by guanidine thiocya-

**Northern Blot Analysis—**Total RNAs were isolated from the dorsal skins of three C57BL/6 mice ranging in age from day 1 to 45, according to manufacturer’s recommendations. Ten μg of total RNA were electro-

**DNA Sequence and Analysis—**The nucleotide sequences of the entire coding region were determined by the dye-deoxy chain terminal method (12) using double-stranded DNA as a template for BcaBEST™ DNA polymerase. The nucleotide sequence of the entire coding region was determined by sequencing both strands. Searching for the known sequences was performed with the GenBank™ and EMBL data bases.

**Northern Blot Analysis—**For Northern blot analysis, total RNAs were isolated using isogen from the skin sections that were excised from three C57BL/6 mice ranging in age from day 1 to 45, according to manufacturer’s recommendations. Ten μg of total RNA were electro-

**Quantitation of mRNA Levels—**Quantitation of the specific mRNA transcripts detected by Northern blot was determined by BioImaging Analyzer BAS 2000 (FUJIX, Tokyo). All values were normalized for the same amount of RNA by determining the relative level of 18 S rRNA in

| Group | cDNA clone | Encoded protein |
|-------|------------|-----------------|
| 1     | 765, 7y1, 7y6 | HGTp type II.1 |
| 2     | 7j8T7    | HGTp type II.1 |
| 3     | 7j3, 7y3, 7z6 | HGTp type II.2 |
| 4     | 7b2    | HGTp type II.3 |
| 5     | 7y9 (2) | HGTp type II.4 |
| 6     | 7y4, 7m1 | HGTp type I F |
| 7     | 7k1, 7j7 | HGTp type I a |

* a The nucleotide sequences of each group were registered in DDBJ, EMBL, and GenBank databases, and their accession numbers are D886420 (group 1), D886421 (group 2), D886419 (group 3), D89901 (group 4), D89902 (group 5), D886423 (group 6), and D886422 (group 7).

Each sample. Further, HGTp transcript levels were normalized to the maximum level of each transcript observed.

In **Stu Hybridization—**Nine-, 33-, and 39-day-old BALB/c mice were sacrificed, and the dorsal skins were taken parallel to the paravertebral line to obtain longitudinal hair follicle sections. The skin samples were fixed in 4% paraformaldehyde and embedded in paraffin according to standard procedures. Five-micrometer sections were mounted on site-

**Characterization of Mouse HGTp Type II—**Comparisons among groups 1, 2, and 3 showed high nucleotide sequence identity of 97.4–98.3% in the coding region. However, there was negligible sequence homology in the 3’-untranslated region. Groups 1 and 2 did not have completely the same nucleotide sequence (98.3% nucleotide homology covering 237 base pairs) in the open reading frame, although they were discussed.
to encode the same protein, HGTp type II.1. These mouse types
II.1 and II.2 proteins had significant identity of 97.4% at the
amino acid level, which revealed one amino acid change and an
II.1 and II.2 proteins had significant identity of 97.4% at the
sequence homology of 53–97.4%. Additionally, these common
features were shown to be a very high content of glycine (40.7–
to 158 residues with a molecular mass of 15,130 Da except for
methionine at the initiation site, because HGTps have been
known to contain no methionine residue (2), and had a high
glycine (43.0%), tyrosine (22.8%), serine (15.2%), and cysteine
(15.2%) content, but lacked 13 kinds of amino acids including a
phenylalanine residue (Fig. 2). Moreover, a remarkable feature
of type II.4 was that it contained 33 direct repeats of a tet-
rapeptide, (C/S)G(Y/S/C)G, ranging from amino acids 9 to 147.

Type II.4 protein, encoded by group 5 possessing a complete
open reading frame of 798 nucleotides, was determined to be a
new member of this subfamily on the basis of its deduced amino
acid sequence information (Fig. 2). This protein was composed of
158 residues with a molecular mass of 15,130 Da except for
methionine at the initiation site, because HGTps have been
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was hybridized with the various 32P-labeled HGTp cDNA probes, followed by washing with 0.1× SSC, 0.1% SDS at 50 °C. The blot was subsequently rehybridized with the 32P-end-labeled oligonucleotide derived from an 18S rRNA probe.

The expression of HGTp mRNA was observed during the first hair growth cycle with their peaks at day 9 and was not detectable during telogen. Again, the levels of their mRNA expression appeared first in the cortical cells possessing the flat nuclei at a distance of about 250 μm above the apex of the dermal papilla (Fig. 6, a–f). The nuclei of cortical cells have been known to change from round through oval to flat and disappear finally, as they differentiate moving upward from the follicle bulb. The expression of type II.1 and II.3 transcripts commenced in the flat nuclei at a distance of about 150 μm above the apex of the dermal papilla (Fig. 6, c–f, h, and i). Their expression appeared first in the cortical cells possessing the flat nuclei at a distance of about 250 μm above the dermal papilla (Fig. 7, a–f). The transcripts of five HGTps were coexpressed in the cortical cells where the transcripts of types II.4 and Ia were detected. The signals of these HGTps were not detected in the inner root sheaths and the medulla cells of the hair shaft. From our in situ hybridization, it was not clear whether their genes were expressed in the hair shaft cuticle cells, since the cuticle was composed of only a single layer of cells. No expression was observed in the epidermis or any other hair follicle cells. Sense probes produce random signals (data not shown). Scale bar, 50 μm.

**DISCUSSION**

Hair contains three major classes of proteins, keratin intermediate filament proteins, HGTps, and cysteine-rich proteins. In the hair follicles of the anagen phase, keratin intermediate cells of hair follicles. However, there were some differences in the starting point of their expression. The transcripts of type I F were first detectable in the cells above the dermal papilla, in general within a three-cell distance above the basement membrane that separates the follicle bulb from the dermal papilla (Fig. 6, a, b, and g). The nuclei of cortical cells have been known to change from round through oval to flat and disappear finally, as they differentiate moving upward from the follicle bulb. The expression of type II.1 and II.3 transcripts commenced in the flat nuclei at a distance of about 150 μm above the apex of the dermal papilla (Fig. 6, c–f, h, and i). Their expression appeared first in the cortical cells possessing the flat nuclei at a distance of about 250 μm above the dermal papilla (Fig. 7, a–f). The transcripts of five HGTps were coexpressed in the cortical cells where the transcripts of types II.4 and Ia were detected. The signals of these HGTps were not detected in the inner root sheaths and the medulla cells of the hair shaft. From our in situ hybridization, it was not clear whether their genes were expressed in the hair shaft cuticle cells, since the cuticle was composed of only a single layer of cells. No expression was observed in the epidermis or any other hair follicle cells. Sense probes produce random signals (data not shown). Scale bar, 50 μm.
filament genes are activated initially, and subsequently expression of HGTps occurs, followed by the expression of cysteine-rich proteins (18). In contrast, the expression of all their transcripts appears to decrease significantly in telogen phase. Mouse HGTps have been determined to constitute about 19% of hair protein. Therefore, it is likely that many HGTp genes are activated, and their transcripts are expressed at high levels in mouse skin of the anagen phase, which implies that many HGTp cDNAs isolated is below 10,000 except for type II.4. First, the predicted molecular mass of type II.4 is 15,130 Da, which is much larger than that of HGTps estimated generally to have molecular weight below 10,000 by chromatography on controlled pore glass in 6 M urea, 0.5% SDS (19) and by ultracentrifugation (20). Indeed, the deduced molecular weight of all HGTp cDNAs isolated is below 10,000 except for that of type II.4. Second, type II.4 is essentially composed of a tetrapeptide repeat. While mouse HGTp types II.1, II.2, II.3, and II.4, and sheep and rabbit KAP6.1 also contain some direct repeats, the frequency of their repeats is much fewer than that of type II.4. Third, type II.4 has a cysteine residue at the carboxyl terminus in which other HGTps have been confirmed to have tyrosine. Cysteine-rich proteins have been exhibited to have a molecular mass range of 10,000 to 30,000 Da (21). The deduced amino acid sequences of their several cDNAs or genes isolated showed that they contained several direct repeats and had cysteine at the C-terminal except for mouse serine-rich ultra-high sulfur protein 1 (22). They are enriched in either glycine or tyrosine, although HGTps have a high content of both amino acids (23). These findings suggest that type II.4 have intermediate characteristics between HGTps and cysteine-rich proteins.

So far, two-dimensional electrophoretic study demonstrated that mouse type II proteins contain approximately 20 components (8). However, there is controversy that the apparent heterogeneity of type II proteins may be due to the conditions of protein preparation and fractionation (4). Instead, Southern blot experiment has showed that about 20 type II genes are likely to be present in the mouse genome (7). In the present study, we isolated five distinct cDNA groups encoding four mouse HGTp type II proteins. This suggests directly that type II proteins are derived from multiple genes, and that their heterogeneity results in part from their discrete genes. However, we still cannot exclude the possibility of the heterogeneity produced by the preparative procedures for HGTps.

Additionally, another novel HGTp was encoded by the iso-

**Fig. 7. In situ hybridization of HGTps in mouse skin.** $^{35}$S-UTP-labeled sense and antisense RNA probes prepared from mouse HGTp type II.4 and IIa cDNAs were hybridized to paraffin sections of BALB/c mouse skin at day 33. a, c, d, and f, and b and e, brightfield and darkfield views, respectively, of longitudinal sections. In order the panels are type II.4 (a–c) and type IIa (d–f). c and f are higher magnifications of a and d, respectively. No hybridization to the epidermis or any other hair follicle cells is seen (data not shown). Sense probes produce random signals (data not shown). Scale bar, 50 μm.
lated cDNAs possessing an entire open reading frame. This protein has a high glycine (37.7%) and tyrosine (26.2%) content and is rich in phenylalanine (6.6%) relative to cysteine (3.3%), which suggests strongly that it belongs to type I. Sheep type I proteins have been separated into 10 components, two major amino acid sequences of which have already been reported, while those of eight minor ones have not as yet. Since the novel HGTp we isolated has no significant homology with sheep types I C and I F at both nucleotide and amino acid levels, it is likely to represent one of the minor components. Until it becomes certain as to which component this protein belongs, we propose to term it HGTp type Ia. Type Ia has some features in common with type II, but types I C and F do not. Indeed, its glycine and tyrosine content is close to that of mouse type II (65.8–71.0%). In addition, it lacks 10 kinds of amino acids in its composition, compared with seven kinds in sheep types I C and F, and 12 to 13 kinds in mouse type II. Type Ia contains some direct repeats, which are found to occur in type II proteins but not in type I. The comparison of it with other HGTps revealed it to share high homology (50–63%) with sheep, rabbit, and mouse type II proteins.

To further characterize mouse HGTps isolated in this study, we examined the expression patterns of their genes both in time and in space. The results from Northern blot experiments showed that the expression patterns of the transcripts of five HGTps essentially followed hair cycle growth, being similar with those of ultra-high sulfur protein and serine-rich ultra-high sulfur protein (22, 24). At the period ranging from day 21 to 27, which covers telogen and early anagen (anagen I–III) phases, the transcripts of HGTps seemed to be either not expressed or expressed at very low levels. In the first hair cycle, the expression levels of the transcripts of five HGTps reached the peak at day 9 corresponding to anagen VI, whereas in the second cycle, two expression patterns were observed. Types Ia, II.3, and II.4 genes were activated at the highest level at day 33, the early stage of anagen VI, while types I F and II.1 genes were at day 39, the late stage of anagen VI. This result indicates that type Ia, II.3, and II.4 proteins in the second cycle are distributed more in the upper portion of hair and decrease toward the hair root, whereas types I F and II.1 are distributed reversely. In contrast, in the first cycle, five HGTps are estimated to be present more in the upper portion.

The distributions of the transcripts of respective HGTps in hair follicles were examined in situ hybridization using 9-, 33-, and 39-day-old mice, which were confirmed to be identical among three age groups. Therefore, their transcripts seem to be expressed in the respectively specific portions of hair follicles during anagen after their appearance. The transcripts of all HGTps were detected exclusively in the cortical cells of hair follicles, and this finding coincides in part with that observed in rabbit and sheep type II protein, KAP6.1, and proposes the possibility that HGTps are expressed in the cortical cells of hair follicles in mammals generally. So far, although sheep and rabbit KAP6.1 transcripts had been shown to be expressed in the cortex in some distance from dermal papilla, type I F transcripts appeared first in the cortical cells in the middle follicle bulb around the dermal papilla. This novel finding suggests that type I F is one of the HGTps transcriptionally activated closest to dermal papilla. Instead, type II.4 and Ia transcripts were detected first in the cortical cells starting to keratinize with flat nuclei, indicating that their genes are likely to be activated in the uppermost region of hair follicles among the HGTp family. It has been reported that the transcripts of rabbit cysteine-rich protein were detected first in the cortical cells about 300 μm above the follicle bulb, compared with about 200 μm measured for rabbit KAP6.1 (18). Our in situ hybridization results demonstrated that the transcripts of type II.4 were expressed first in the higher cortical cells than that of type II.1 corresponding to KAP6.1. This result seems to support our notion that type II.4 protein has intermediate characteristics between HGTp and cysteine-rich protein.

We estimated the distributions of HGTps in mouse hair on the basis of expression patterns of their genes from Northern blot and in situ hybridization observations. As shown in Fig. 8, there was a remarkable distinction of the distributions of HGTps between the hairs in the first and second hair growth cycles. In the second cycle, HGTp transcripts with the earlier peak of expression level were detected in the more distant cortical cells from dermal papilla, while those with the later one were in the closer. Accordingly, HGTps as a whole appear to be present substantially throughout hair from the tip to root. Alternatively, in the first hair cycle, HGTps increase toward the upper portion of hair, because all five HGTp genes are activated at the highest levels at day 9, the early to middle stage of anagen VI. In particular, it is interesting that the transcripts of type II.4, which has been exhibited to have unique characteristics, are expressed at the twice the level of those in the second cycle. The intermicrofibrillar matrix in the cortex has been proposed to be constituted of cysteine-rich proteins and HGTps. Cysteine-rich proteins have been presumed to stabilize the intracellular filament-matrix protein structure of hair cortical cells by forming many interpoly peptide cross-links with their abundant cysteine residues, while the role of HGTps in hair differentiation has remained to be determined. Since the pelage follicles of newborn mice are poorly developed, their first hair cycle actually represents a neogenetic event distinct in some aspects from the subsequent hair cycles (25). During the first cycle, hair passes through hair canals for the first time to emerge from the skin surface. This phenomenon raises the possibility that hair in the first cycle needs to have a more rigid upper portion than that in the subsequent cycles, suggesting that HGTps are likely to relate to strengthening hair.

HGTps have been confirmed to be composed of multiple members in the present study. However, we do not yet know whether there are distinct roles between HGTps expressed with the various patterns spatially and temporally during hair
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follicle differentiation. This study represents the first report on the isolation and the characterization of mouse HGTps. Their nucleotide sequence information will be useful to obtain HGTp genes from the mouse genome. In future, the function of each HGTp in hair follicles will be elucidated further by producing the transgenic or knock out mouse using this gene.

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