Sequential expression of glutathione-S-transferase isoenzymes during hair growth phases in mice and their relationship to caldesmon, phosphotyrosinase and VIP receptor protein

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Summary. The hair follicle is a structure showing a very unique cycling of its epithelial components. It is innervated by an abundance of peptidergic nerves, and neuroendocrine cells occur within the epithelium or in close proximity to it. Recently, it has been suggested that hair growth may be regulated by neuropeptides. We therefore investigated the relationship of VIP receptor expression to intracellular phosphotyrosinase and caldesmon involved in the control of growth and differentiation and metabolizing isoenzymes of the glutathione-S-transferase (GST) family alpha, mu, and pi during induced anagen of C57 BI-6 mice by immunohistochemistry. It was demonstrated that GST isoenzymes were expressed sequentially in the bulge area and the inner hair root sheath as well as in the sebaceous gland epithelium. Caldesmon was present during the early anagen phases within the bulge region, as was phosphotyrosinase. However, phosphotyrosinase expression decreased in late anagen, and recovered again in post-epilation telogen. VIP receptor was expressed within the bulge area during anagen V, but was absent during the other cycle phases. These results suggest a relationship of protein expression to hair cycle phases and in particular a physiological function of VIP/VIP receptor in terminating the extensive hair follicle growth during anagen I to IV.

Key words: Hair follicle, Anagen phase, VIP receptor, Caldesmon, Phosphotyrosinase, Glutathione-S-transferase isoenzymes

Introduction

Mice, in contrast to humans, have a highly synchronized hair cycling pattern (cf. Paus and Czarnetzki,

1992), which makes it possible to investigate the fine control of growth mechanisms involved in hair follicle cycling.

We have employed the C57 BL-6 mouse model, in which melanogenesis is strictly coupled to the growth (anagen) phase of the hair cycle (Slominski et al., 1991). Recent advances in mixed melanogenesis have revealed that the key intermediate of pheomelanin is 5-S-cysteinyl dopa (5SCD) (Prota, 1980). The formation of 5SCD depends upon the availability of the major intracellular thiol compound, reduced glutathione (GSH), and the metabolizing enzymes such as glutathione-S-transferase (GST) and gamma-glutamyl transpeptidase (gamma-GTP) (Chakraborty et al., 1991). Higher levels of GST activity have been found in metastatic versus non-neoplastic cell lines in vitro (Benathan et al., 1992; Chakraborty et al., 1992), suggesting some relationship of GST activity to proliferative activity and differentiation.

Caldesmon is a unique actin-binding protein regulated by calcium and calmodulin. Possible physiological functions of caldesmon in non-muscular cells include granule movement, hormone secretion and cell division and motility (Matsumura and Yamashiro, 1993).

Phosphotyrosine is an immunogenic product of tyrosine phosphorylation by tyrosine kinases. It has been demonstrated that several viral oncogenes like *v-src* and epidermal growth factor receptor (EGF-R) have tyrosine kinase activity. Overexpression of their antagonist protein tyrosine phosphatase has been shown to inhibit cytokinesis in T cells and fibroblasts. It has been suggested that the protein tyrosine phosphatases may act as tumor suppressor genes because they are antagonistic to the growth-promoting and oncogenic potential of the tyrosine kinases. In some models evidence has been gained that neuropeptides like somatostatin, dopamine and angiotensin regulate the activity of protein tyrosine phosphatases (Walton and Dixon, 1993).

The hair follicle is abundantly innervated by

cutaneous nerves forming a distinct and unique network (Halata, 1990; Hashimoto et al., 1990), which consists of free nerve endings, pilo-Ruffini nerve endings, Merkel cells nerve endings, and lamellated nerve endings. The hair growth in C57 BL-6 mice has been shown to be induced by neuropeptides like substance P (Slominski et al., 1991; Paus et al., 1994a,b). But even in human hair follicles, the participation of neuroendocrine factors seems conceivable (Wollina, 1992). Strong evidence has been gained that vasoactive intestinal peptide (VIP), a partial antagonist of substance P, is a growth factor for epidermal keratinocytes (Haegerstrand et al., 1989; Wollina et al., 1992a,b). VIP is present in cutaneous nerve endings, and mast and Merkel cells. VIP exerts specific effects by binding to a specific receptor protein, which can be localized with immunohistochemistry by using a monoclonal antibody.

We therefore investigated the expression of three GST isoenzymes, namely alpha, mu, and pi, calmodulin and calcium binding protein caldesmon and phospho-tyrosinase during induced anagen of C57 Bl-6 mice and their relationship to vasoactive intestinal peptide (VIP) receptor expression in order to better understand the fine control of hair follicle cycling.

Materials and methods

Animals

Telogen C56 BL-6 mice (female, syngenic, 6-8 weeks old) were purchased from Charles River (Kingston, NY, USA), housed with 12-hour light periods and fed ad libitum with water and «rat/mouse chow» (Agway, Syracuse, NY, USA). Telogen mice were anaesthetized with 30 mg sodium pentobarbital/kg b.wt. and hair was stripped with a mixture of beeswax and resin (Paus et al., 1991) to induce anage. Animals were sacrificed by an ethyl ether overdose.

Nine different hair cycle stages were studied (Chase et al., 1951): spontaneous telogen (day 0); anagen I-VI (days 1-18); post-epilation catagen (day 18); and post-epilation telogen (day 25). For each cycle stage five different mice were analysed.

Immunohistochemistry

Back skin samples from mice were snap frozen, cut at 4 μm and fixed in acetone. For immunostaining, we followed our recent protocol for the unlabelled immuno-peroxidase technique (Wollina, 1991) with a minor modification; the primary antibodies were added overnight at -4 °C. In control experiments, primary antibodies were omitted. Visualization of antigen-antibody reaction was performed with 3-amino-9-ethylcarbazole (AEC; EGA-Chemie, Steinheim, FRG). Primary antibodies are summarized in Table 1.

Results

GST isoenzymes showed a sequential expression during the hair cycle. GST alpha was demonstrated at days 0 to 25 with strong immunoreactivity in the bulge area and a weaker expression within the inner hair root sheath around day 18 (Fig. 1). In general, GST mu followed the expression of GST alpha, except for the demonstration of a stronger enzyme activity in the infraglandular portion of the inner hair root sheath. GST pi activity was not seen before day 8 and the activity decreased markedly thereafter. No GST pi was demonstrable from day 12 to 25. GST pi labelled the sebaceous epithelium and the bulge region.

Though major GST immunoreactivity was found at the bulge, some cells near the infundibulum were also labelled with anti-GST pi. With GST alpha, pi and mu immature sebocytes could be identified. During the whole hair follicle cycle, the bulb remained negative for all three GST isoenzymes.

Caldesmon could be detected by immunohistochemistry in the early anagen phases I-IV within the bulge region. The epidermis was stained with anti-caldesmon antibodies during days 0 to 3 and the dermal hair follicle sheath was also stained around day 7.

Phosphotyrosinase was expressed during days 0 to 8 but declined down to zero at day 12. It was weakly expressed during days 18 to 25. Phosphotyrosinase activity was localized in the bulge region only (Fig. 2).

VIP receptor was identified with antibody 109.11. It was not demonstrable before day 12 and was absent from day 17 to 25. Again, immunoreactivity was seen in the bulge

Table 1. Relationship of hair cycle stages to GST, phosphotyrosine (PT), caldesmon (C) and VIP-receptor expression (VIP-R) in the bulge area.

	GST			C	PT	VIP-R
	@	mu	pi			
Telogen	+	±	-	-	+	-
Anagen I	+	+	-	+	+	-
Anagen II	+	+	+	+	+	-
Anagen III	+	+	+	+	+	-
Anagen IV	+	+	+	⊢	±	-
Anagen V	+	+	-	-	-	+
Anagen VI	+	-	-	-	-	-
Post-epilation catagen	-	-	-	-	-	-
Post-epilation telogen	+	-	-	-	+	-

Fig. 1. Immunohistochemistry of GST isoenzymes in the hair follicle of mice. GST alpha: **(a)** day 0; **(b)** day 25; **(c)** day 18. Note the staining in the bulge area in (a) and (b) and the moderate staining of the inner hair root sheath in (c). GST pi **(d)** day 5 (x 150); **(e)** day 8 (x 200). Staining is lacking in (d) but present in the sebaceous gland (e) and the bulge region around day 8. GST mu: **(f)** day 1; **(g)** day 18; **(h)** day 25. Note the staining of the bulge area in (f) and (h) and within the inner hair root sheath in (g). x 320 except otherwise indicated.
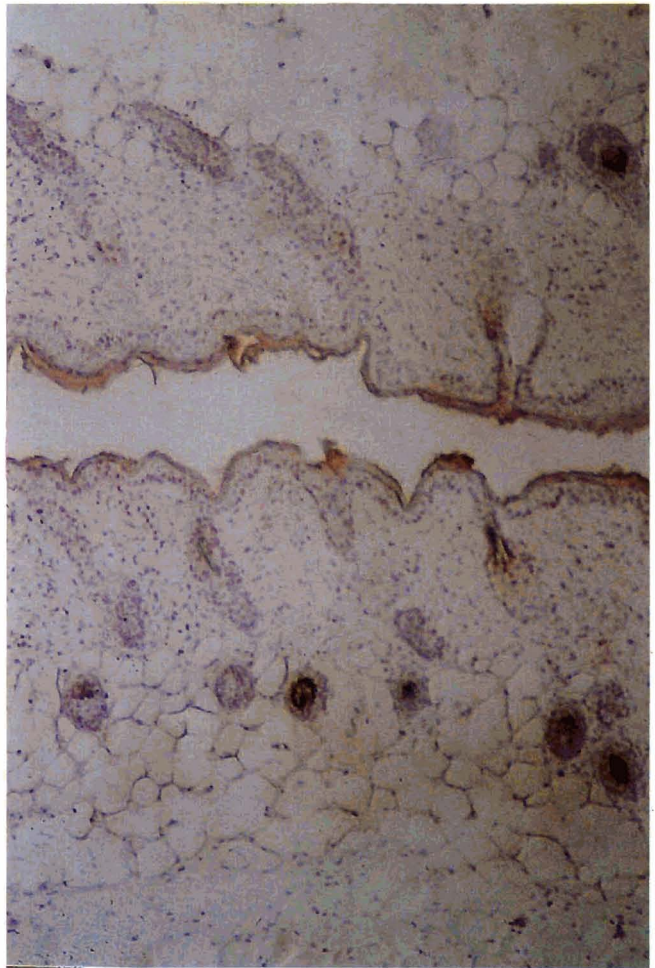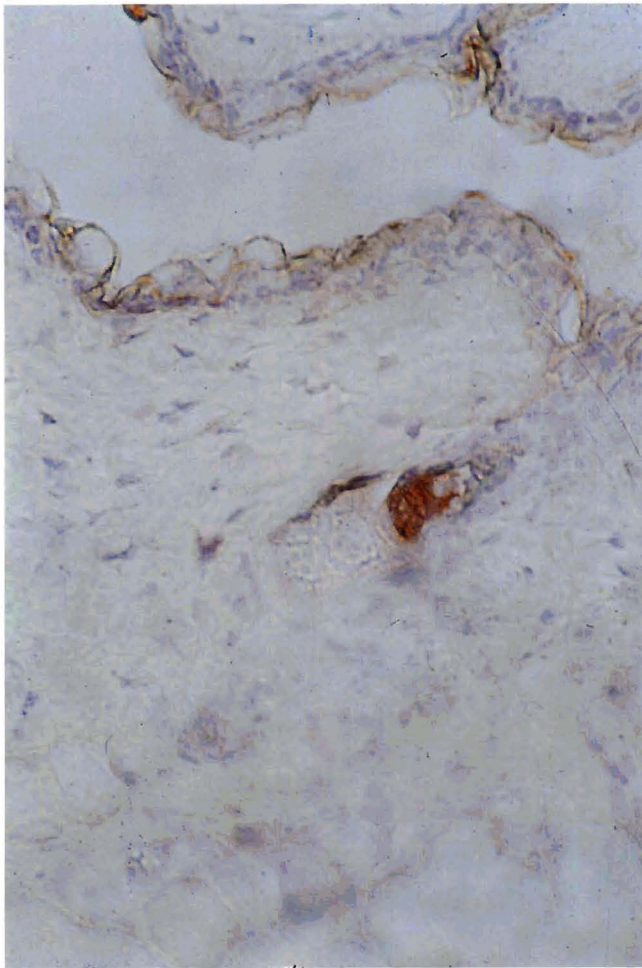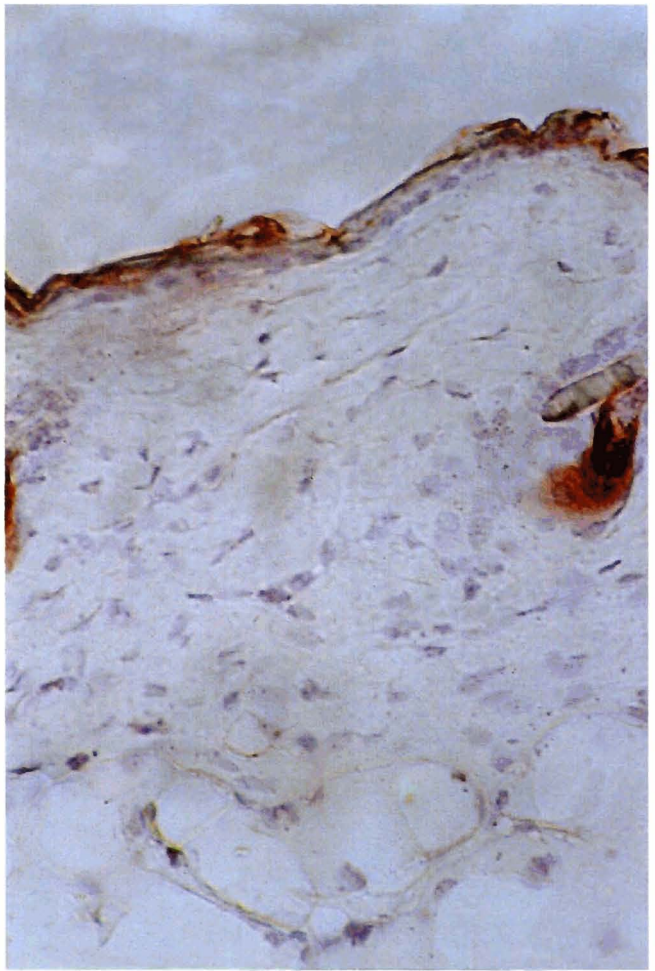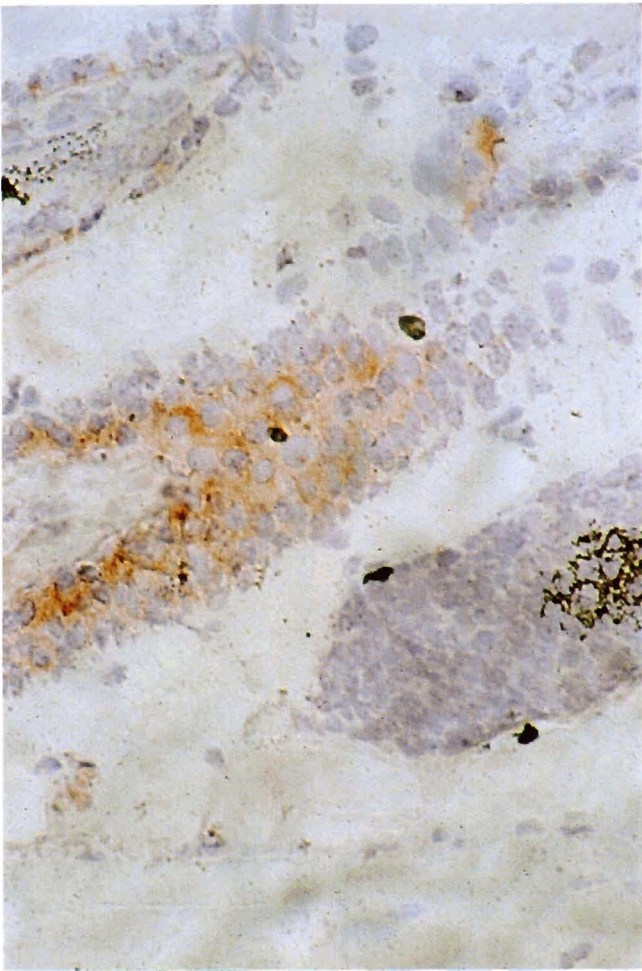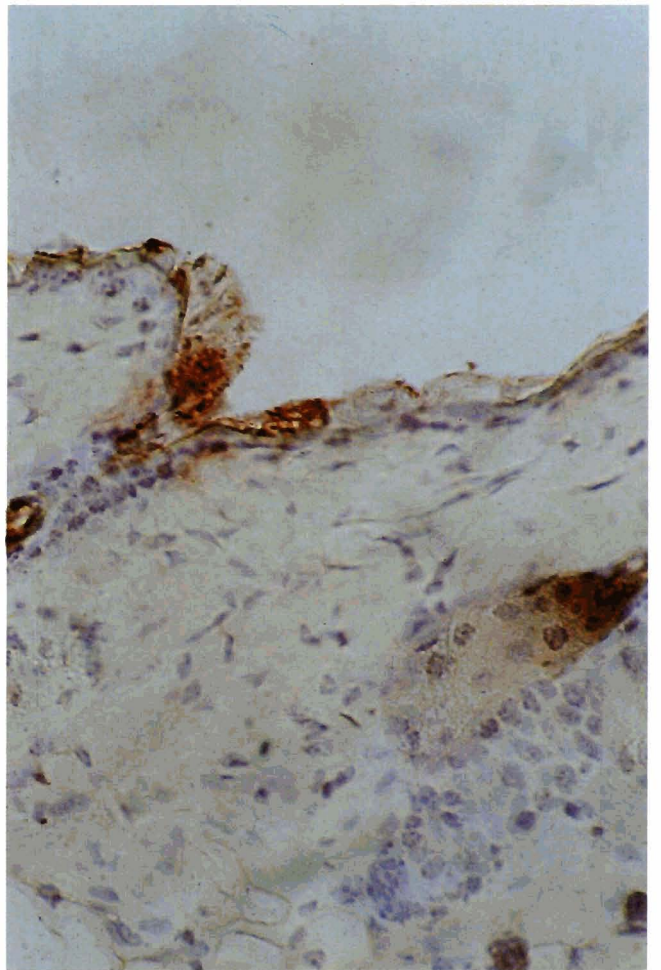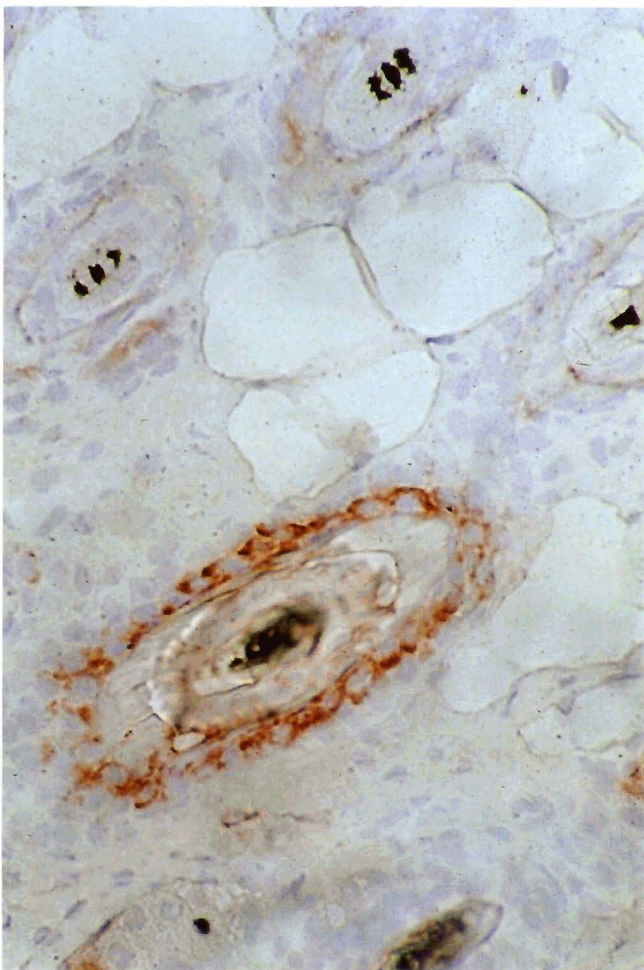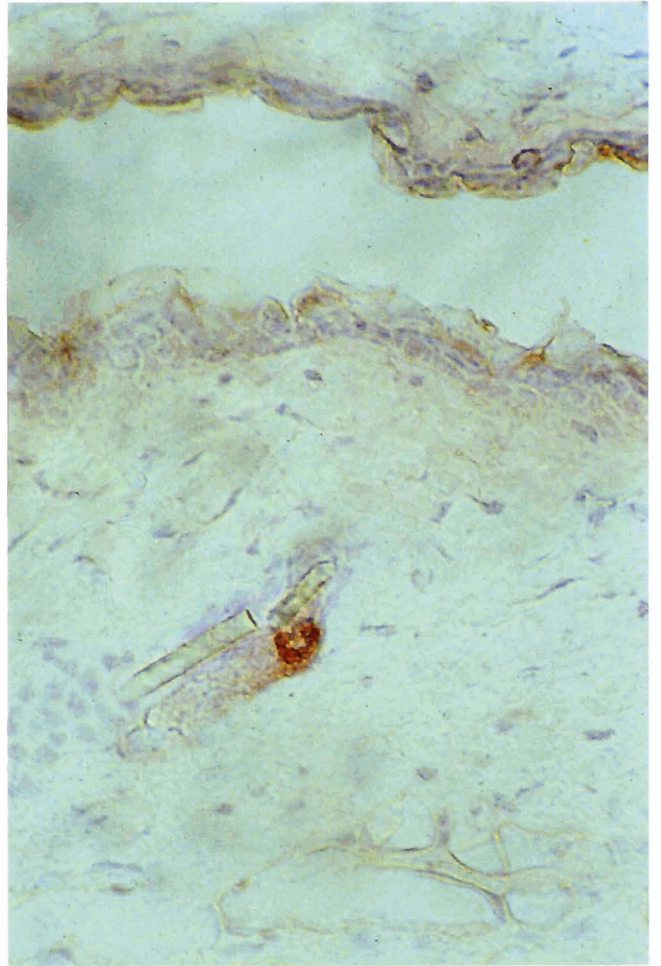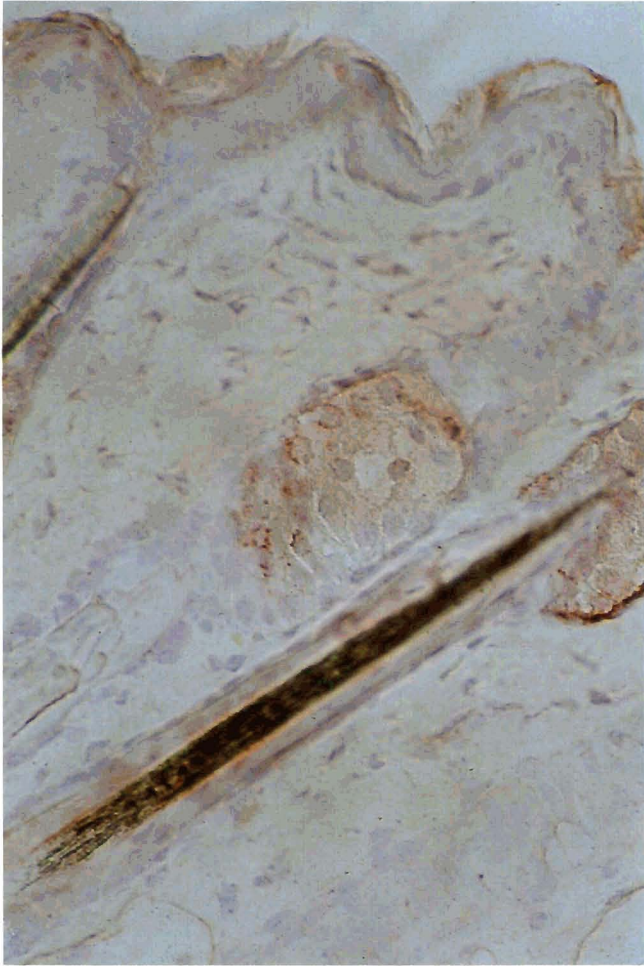
area. Endothelial cells of the vascular sheath were additionally stained. There was no labelling of the bulb (Fig. 2).

The staining patterns of the bulge region have been summarized in Table 1.

Discussion

The hair cycle is a unique example of oscillating growth in skin. It needs a particularly fine regulation involving epithelial, mesenchymal, immunological and neuronal mechanisms. During recent years, neuropeptides have become more interesting for the study of growth regulation of skin cells, including keratinocytes, fibroblasts, etc (Haegerstrand et al., 1989; Paus and Czarnetzki, 1992; Wollina, 1992; Wollina et al., 1992a,b).

The hair follicle is characterized by highly intensive innervation and specialized nerve endings as well as Merkel cell neurite complexes. Within the bulbus, one can find numerous melanocytes. In the outer hair root

sheath Merkel cells are demonstrable. In close proximity to the dermal sheath cutaneous mast cells have been identified (Halata, 1990; Hashimoto et al., 1990; Wollina, 1992). These findings raise the question wether neuronal factors, e.g. neuropeptides, are involved in the biological control of hair cycle movement.

In the present paper, immunohistochemical investigations on GST isoenzyme, caldesmon, phosphotyrosinase and VIP receptor expression have been performed to obtain some insight into this topic.

It could be demonstrated that GST isoenzymes alpha, mu and pi are expressed sequentially. GST alpha and mu are present during the whole cycle, with strong immunoreactivity at the bulge. Minor activity was also seen in immature sebocytes and (for GST mu only) in the infraseboglandular portion of the outer hair root sheath. In contrast, GST pi was expressed only during a short time window around day 8, suggesting a possible function in the anagen IV to V quench.

Caldesmon, one of the important calcium and calmodulin binding proteins, has been demonstrated
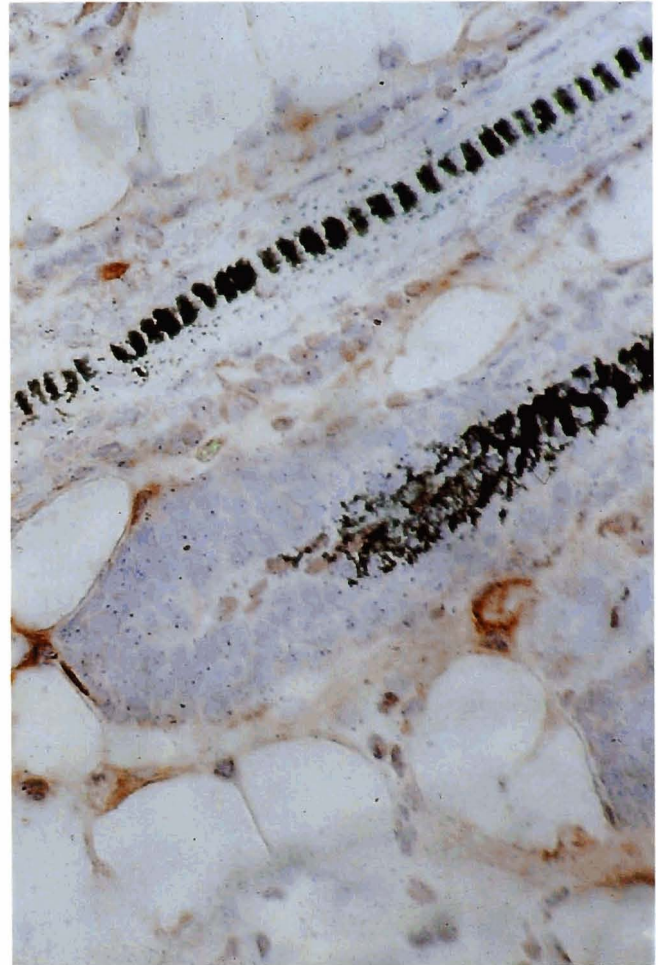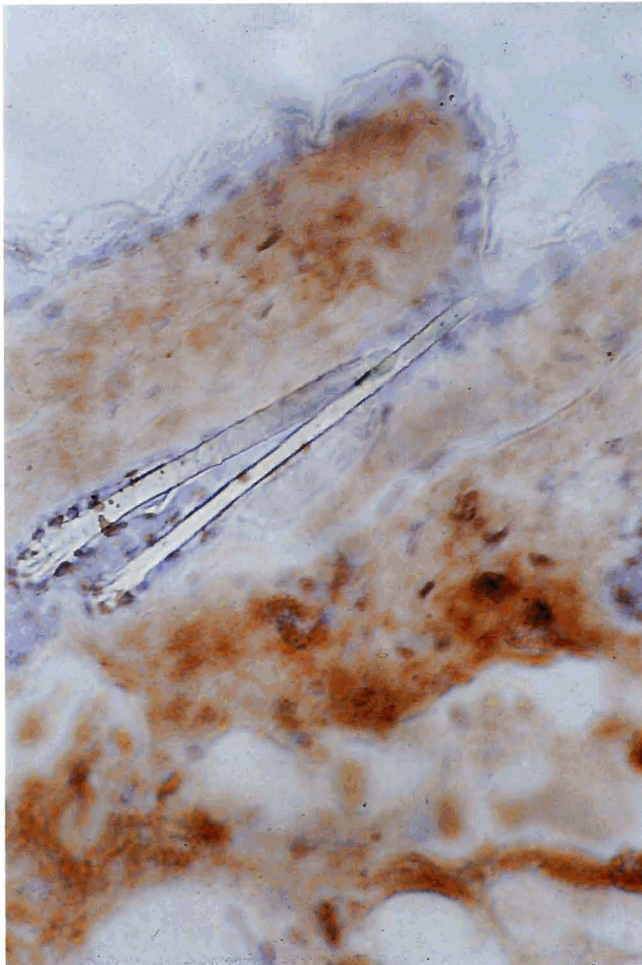
Fig. 2. Immunohistochemistry of caldesmon (a, b), phosphotyrosinase (c, d) and VIP receptor (e, f) in hair follicles of mice. Caldesmon: **(a)** day 1; **(b)** day 8. Note the lack of hair follicle staining in (a) when a diffuse dermal reactivity can be observed. At day 8 (b) some perifollicular dermal cells are labelled. Phosphotyrosinase: **(c)** day 1; **(d)** day 8. A strong immunoreactivity is seen in the bulge region at day 1 (c) but is absent at day 8 (d). VIP receptor: **(e)** day 12; **(f)** day 18. A strong immunoreaction is seen in the bulge region (e) which disappears later on (f). x 320
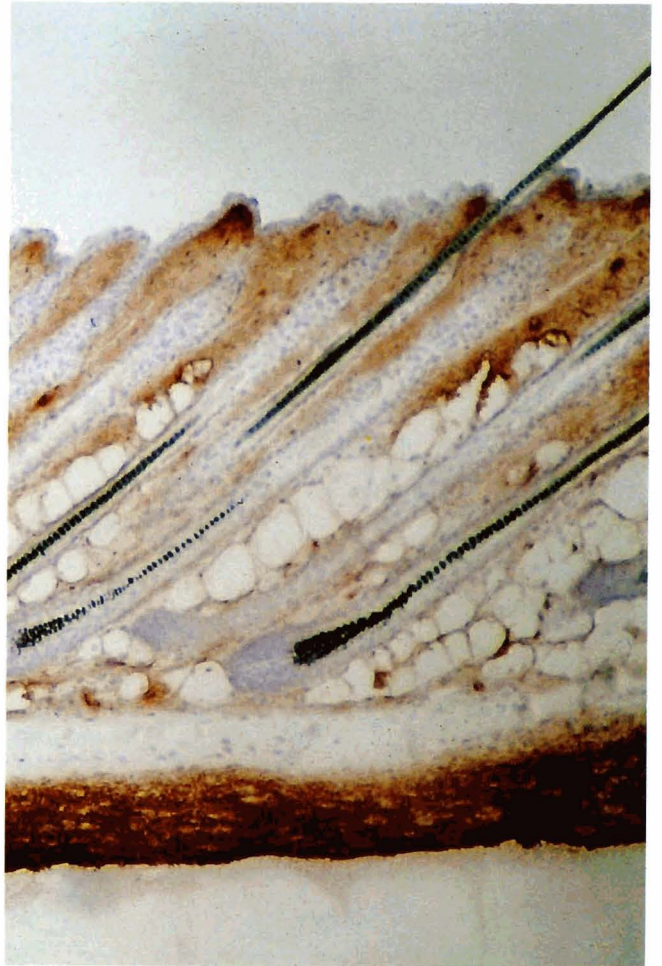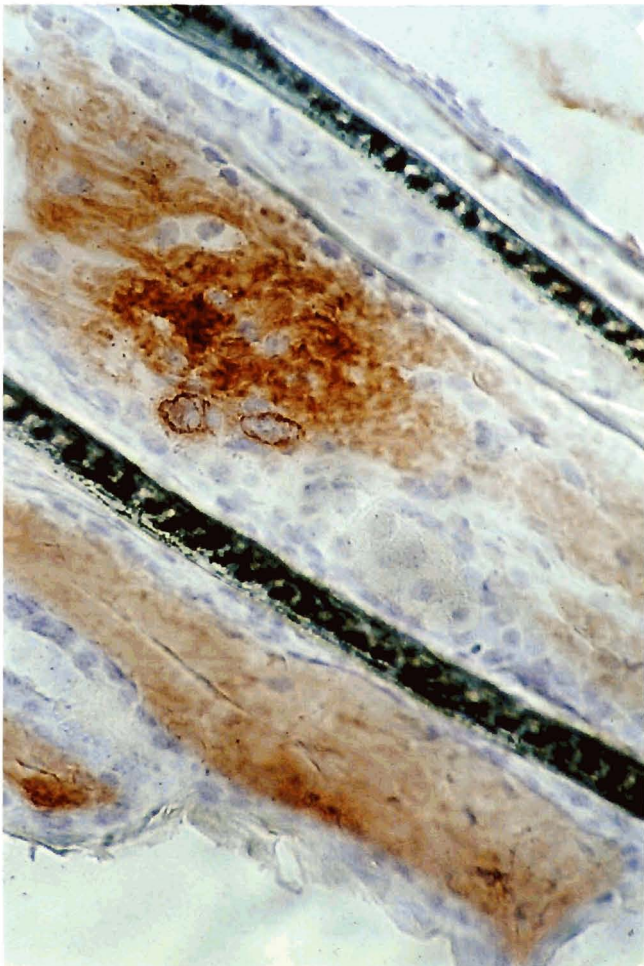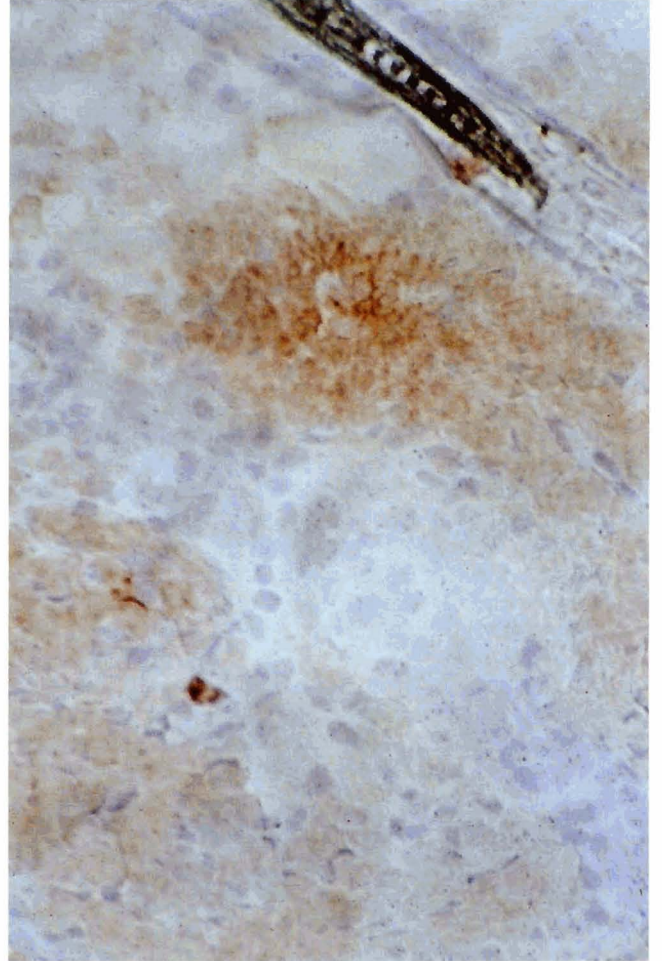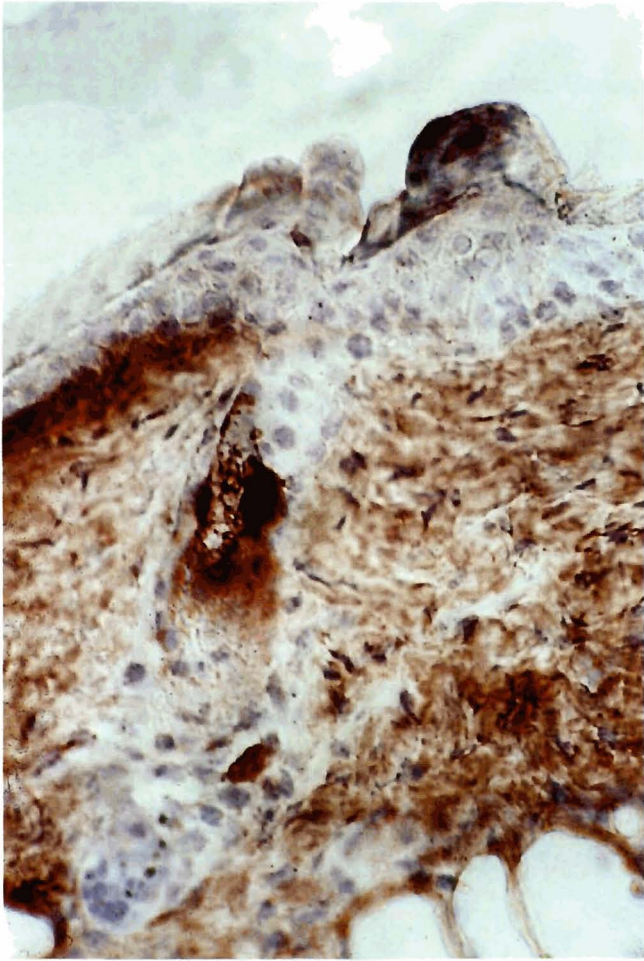
from anagen I to IV. During these cycle phases, the follicle grows extensively downwards at the undersurface of the epidermis. Later on the follicle starts to mature and to produce its specific endproduct - the hair. Caldesmon expression is remarkable when cell proliferation and migration is increasing. Comparing its distribution with another major calcium binding protein, calmodulin, we find a considerably limited caldesmon expression in the hair follicle epithelium (cf. Wollina et al., 1990, 1992c). Whereas caldesmon was detected in the bulge area of mice hair, calmodulin was found within the supraglandular portion of the outer hair rooth sheath, the inner hair root sheath, the shaft and matrix cells, the immature sebocytes and the sebaceous duct cells in humans and (with the exception of shaft and matrix cells) pigs (Wollina et al., 1990, 1992c). This suggests different roles for the various calcium-binding proteins during the hair cycle.

Phosphotyrosinase, a key enzyme of growth and differentiation regulation, was demonstrable during early anagen and telogen, but absent around day 12. On the other hand, VIP receptor could be identified at the bulge around day 12, though it was absent during the remaining cycle (cf. Table 1).

VIP receptor expression seems to be a late event in anagen V when three other antigens have been lost, i.e. caldesmon, GST pi and phosphotyrosine. Considering the time course, VIP itself seems to act as a suppressor of the epithelial follicle enlargement, thereby supporting maturation of the hair. This is in contrast to another neuropeptide affecting hair growth in mice, substance P, which seems to promote anagen hair growth in vivo (Paus et al., 1994a,b).

When we consider protein expression profiles during the hair follicle cycle, it is remarkable that major staining was seen in the bulge area. The bulge is thought to be the reservoir of stem cells (Cotsarelis et al., 1990). Our results do not only demonstrate the sequential expression of selected proteins involved in growth and differentiation, but argue for an involvement of neuropeptides as growth modulators in mice hair.

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