tying[1,6]. Elevated plasma PYY levels are observed in patients with diarrhea due to tropical sprue, pancreatic insufficiency, inflammatory bowel disease or acute infection[18]. It is probable that in such cases PYY cells may function fully to compensate for the loss of electrolytes and water and to maintain homeostasis.

VIP is not only an important neuropeptide in the enteric nervous system but is also thought to be responsible for watery diarrhea in patients with VIP-producing tumors. Since it has been reported that PYY infusion in man was well tolerated[19], and was effective for inhibiting VIP-stimulated ileal secretion[20], PYY or its analogues may possibly be useful as a drug for palliative therapy for patients with VIP-producing tumors.

In conclusion, PYY enhances absorption of sodium chloride with water and antagonizes the secretory effect of VIP in the rat colon.

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Proopiomelanocortin expression in the skin during induced hair growth in mice

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Abstract. We demonstrate for the first time a hair cycle-dependent gene and protein expression of proopiomelanocortin in mouse skin in vivo. Northern blot detected POMC mRNA with an apparent size of 0.9 kb in anagen but not telogen skin. Western blot emphasized a specific protein of 30–33 kDa recognized by anti β-endorphin in late but not early anagen or telogen skin. By immunocytochemistry, β-endorphin antigen was localized in the sebaceous gland in a hair cycle dependent manner.

Key words. Proopiomelanocortin; hair growth; skin; C57B1/6 mice; β-endorphin.

The proopiomelanocortin (POMC) gene is predominantly expressed in the pituitary gland and encodes a single large protein precursor for neuropeptides with multiple regulatory functions, including adrenocorticotropin (ACTH), endorphins (EP), melanotropins (MSH), and lipotrophins (LPH)[1–3]. The POMC gene is also expressed in the brain and many peripheral tissues such as testes, ovaries, placenta, lymphoid cells, adrenals, kidney, lung, liver and gastrointestinal tract[1–4].

Skin, the largest organ of the body, is considered to be a target organ for regulatory functions of some POMC-derived peptides such as MSH or ACTH[3,5–7]. Recent reports showing POMC gene expression and translation in murine and human epidermal keratinocytes in vitro[8,9] suggest that the skin may be among those peripheral tissues that are capable of expressing the POMC gene. This raises the possibility that locally generated POMC products such as β-endorphin, ACTH, LPH and the melanotropins play important roles in the physiology of the skin and its exquisitely hormone sensitive appendages. Utilizing the previously described C57BI/6 mouse model for hair growth and pigment biology stud-

ies[10,11], we examined whether there is POMC gene expression and translation in murine skin and whether it is dependent on the stage of the hair cycle.

Materials and methods

Animals and skin preparation. Telogen C57BI/6 mice (female, syngeneic, 6–8 weeks old) were purchased from Charles River, Kingston, NY, housed with 12-h light periods and fed ad libitum with water and 'rat/mouse chow 3000' (Agway, Syracuse, NY). To induce anagen, telogen mice were anesthetized (30 mg sodium pentobarbital/kg b.wt) and stripped of hair using a warm beeswax/rosin mixture[10,11]. Telogen mice (day 0), and anagen mice (days 1, 3, 4, 5, 6, 7 and 8) were killed by cervical dislocation under ether narcosis, and telogen mice were shaven with an animal clipper. All back skin was dissected at the level of the subcutis, immediately frozen in liquid nitrogen and stored at − 80 °C until used.

Western blots. Frozen skin was pulverized in liquid nitrogen and further homogenized in lysis buffer (10 mM Tris, pH 7.4, 1 mM PMSF and 0.01% aprotinin) at a ratio of 0.5 g tissue/4 ml of buffer using a polytron at maximum speed. Tissue extracts were centrifuged at 15,000 × g for 30 min at 4 °C, supernatants were aliquoted and frozen at − 80 °C. All above manipulations were done on ice. The protein content in the samples was estimated with the aid of a Bio-Rad protein assay kit, using serum albumin (Sigma) as a standard. The supernatants (70 µg of protein) were separated under nonreducing conditions according to a nondenaturing modification of the method of Laemmli by 0.1% SDS/20% polyacrylamide gel electrophoresis as described previously[11]. The proteins were blotted from the polyacrylamide gels to Zeta Probe blotting membranes (Bio-Rad)[11]. After blocking with 10% nonfat dry milk in TBS (20 mM Tris, 0.5 M NaCl, pH 7.5), the membranes were washed in TTBS (TBS plus 0.05% Tween 20), and probed with polyclonal anti-β endorphin antibody (1:200 dilution, gift of Dr. W. Allen[12]). Parallel membranes were incubated with rabbit nonimmune serum. After washing with TTBS, the membranes were incubated with goat anti-rabbit IgG conjugated to horse radish peroxidase (BRL) (1:500 dilution). The immune complexes were visualized by staining for peroxidase activity with diaminobenzidine (DAB, Sigma) and H_2O_2 (Sigma) as substrates.

Northern blots. A heat-denatured random prime-labeled POMC cDNA was prepared from 0.923 kb mouse POMC clone (gift of Dr J. Roberts[13]), with the aid of a Boehringer-Mannheim kit and α-^{32}P-dCTP (DuPont, USA). The skin tissues were pulverized in liquid nitrogen, and the poly $(A)^+$ mRNA was extracted using 'FAST track' mRNA isolation kit according to the manufacturer's instructions (Invitrogen). Fifteen µg of poly $(A)^+$ mRNA and 10 µg of 0.26–9.1 kb RNA ladder (BRL) were separated electrophoretically through formaldehyde/1% agarose gels by standard procedures[11,14] and transferred to Zeta Probe blotting membranes by capillary transfer overnight. To aid in the determination of the size of the transcripts, prior to transfer, the portion of the agarose gel in which the RNA ladder was run was cut, stained with ethidium bromide and photographed under UV illumination. The membranes were dried for 2 h at 80 °C under vacuum, and then soaked in hybridization buffer (50% deionized formamide, 7% SDS, 5 × Denhardt's solution, 5 × SSC, 20 mM NaH_2PO_4, pH 7.0, 10% dextran sulfate and 100 µg/ml denatured salmon sperm DNA) at 42 °C for 12 h. The heat denatured random prime-labeled cDNA probe was added to fresh hybridization buffer at 42 °C and hybridization allowed to proceed for 24 h. The membranes were then washed for 5 min with 2 × SSC plus 0.5% SDS and 15 min with 2 × SSC plus 0.1% SDS at room temperature, then with 0.2 × SSC plus 0.1% SDS at 65 °C for 2 h. After the final 5-min rinse in 0.2 × SSC plus 0.1% SDS, the hybridized blots were exposed on Kodak XAR film at − 70 °C. The size of the RNA transcript was estimated in relation to the RNA ladder.

Immunocytochemistry. The skin from telogen (day 0) and anagen mice (days 1, 3, 5, 8 after anagen induction) were fixed in 10% formalin, embedded in paraffin, and sectioned. For immunostaining, the deparaffinized sections were hydrated in PBS and incubated with a rabbit polyclonal anti β-endorphin antibody (1:1000 dilution, gift of Dr W. Allen[12]) in PBS plus 0.1% Triton X-100 at room temperature for 1 h. In control experiments sections were incubated with rabbit nonimmune serum (1:1000 dilution) or anti β-endorphin antibody (1:1000) preadsorbed overnight with 5×10^{-5} M β-endorphin (Sigma) plus 0.1% Triton X-100. After washing with PBS (3 times for 5 min) the cells were incubated with biotin conjugated goat anti rabbit IgG (1:100 dilution) for 1 h at room temperature. After washing with PBS tissue sections were processed further using a biotin-avidin immunostain kit (Vectastain kit from Vector Laboratories). The immune complexes were visualized by staining for peroxidase activity with diaminobenzidine and H_2O_2 as substrates.

Results and discussion

Northern blot analysis of poly $(A)^+$ mRNA showed detectable POMC mRNA with an apparent size of 0.9 kb in days 4, 6 and 7 after anagen induction but not in telogen skin (fig. 1). The size of the transcript (0.9 kb) was shorter than that in pituitary and hypothalamus (1.1 kb)[1] but corresponded to those detected in several extracranial tissues (0.8–0.9 kb)[1,3,4]. In a repeat experiment POMC transcripts again were not detected in telogen, but by day 1 of anagen induction POMC mRNA was just at the level of dectectability, and on days 3 through 8, Northern blot analysis showed a strong signal for POMC RNA (fig. 2).

On immunoblots with anti β-endorphin antibodies, a protein of apparent molecular weight 30–33 kDa (arrow) was present in day 5 and 8 of anagen skin (fig. 3).

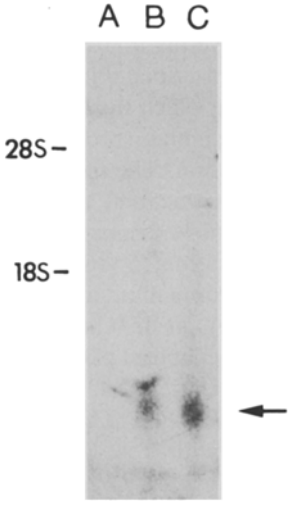

Figure 1. Northern blot analysis of POMC-hybridizable RNA in mouse skin. Fifteen μg of poly $(A)^+$ mRNA were separated on a formaldehyde agarose gel, blotted to nylon membrane, hybridized to ^{32}P-labeled mouse POMC cDNA, washed and placed on Kodak X-Omat AR film for autoradiography. Telogen: *A*; day 4 of anagen skin *B*; days 6 and 7 of anagen skin, combined *C*. Arrow, 0.9 kb transcript hybridized to POMC cDNA.

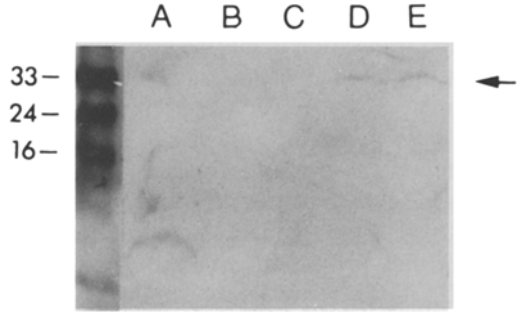

Figure 3. Western blot analysis of POMC antigen in mouse skin. Skin extracts were separated by SDS-PAGE under nonreducing and nondenaturing conditions, blotted to nylon membranes and probed with anti β-endorphin antibody. Telogen (*A*); days 1 (*B*), 3 (*C*), 5 (*D*) and 8 (*E*) after anagen induction. Arrow, 30–33 kDa protein recognized by anti β-endorphin antibody. Left panel, molecular weight markers (kDa).

Since β-endorphin is a part of the POMC protein and the size of the detected protein (30–33 kDa) is similar in molecular weight to POMC protein synthesized in the pituitary[2, 3] we suggest that it represents newly synthesized POMC protein. This protein was not detectable on immunoblots in telogen and days 1 and 3 of anagen skin or in control blots stained with nonimmune rabbit serum. In telogen skin, a protein of low molecular weight was also detected with the β-endorphin antibody, but it was absent in anagen skin and in control blots stained with nonimmune rabbit serum (fig. 3, lane A). Because this low molecular weight, immunoreactive protein was detected only in telogen, it may represent peptide left after the processing of POMC protein during the preceding hair cycle or alternatively it could be a product of an active concentration of plasma β-endorphin peptide in the skin.

Immunoreactivity for anti-β-endorphin antisera was limited to sebaceous glands (figs 4–6). In telogen skin pilosebaceous gland was immunostained, and this reactivity was greatly diminished one day after induction of anagen (figs 4 and 5). The immunoreactivity steadily increased in pilosebaceous glands with increasing time after anagen induction (fig. 4a–e). The immunoreactivity in individual cells of the glands slowly increased over time until the majority of cells were very densely stained. This gradient in staining intensity with time is clearly illustrated in figure 5a–d. Note that the background level of staining of sweat gland remained constant over the 8 days of the induction period (figs 5a–d and 6a and b). The changes in abundance of β-endorphin throughout the hair cycle stages, and the detection of POMC tran-

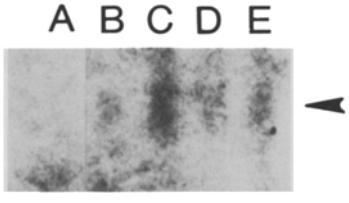

Figure 2. Northern blot analysis of POMC-hybridizable RNA in mouse skin. Seven μg of poly $(A)^+$ mRNA were analyzed for autoradiography (see legend to fig. 1 for method). Telogen (*A*); days 1 (*B*), 3 (*C*), 5 (*D*) and 8 (*E*) after anagen induction. Arrow, 0.9 kb transcript hybridized to POMC cDNA.

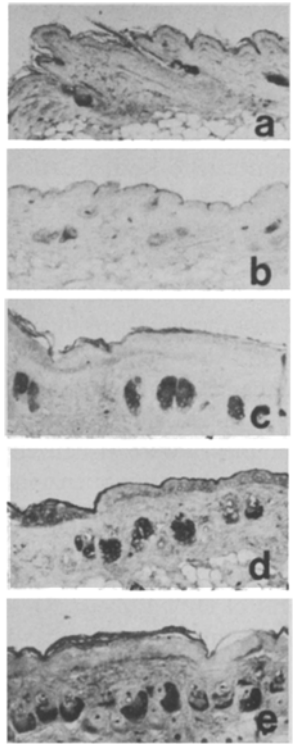

Figure 4. Immunocytochemical localization of β-endorphin in the skin during hair cycle induction. (*a*) Telogen skin; (*b*) day 1, (*c*) day 3, (*d*) day 5 and (*e*) day 8 after anagen induction. Note restriction of dense immunoreactivity to pilosebaceous glands. Magnification 100 ×.

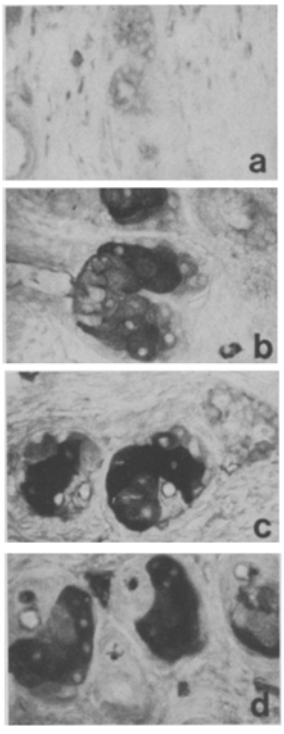

Figure 5. Higher magnification illustrating representative examples of the immunoreactivity for β-endorphin in individual sebaceous glands from (*a*) day 1, (*b*) day 3, (*c*) day 5 and (*d*) day 8 after anagen induction. Note absence of stain on day 1, and the increase in density of staining in sebaceous glands with time after induction. Sweat gland staining is at background levels. Magnification 560 ×.

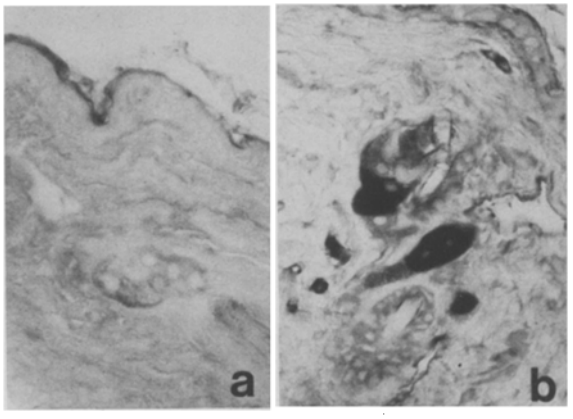

Figure 6. Specificity control for β-endorphin immunocytochemistry. Tissue sections from day 5 anagen skin were reacted with (*a*) Control: 1:1000 dilution β-endorphin antisera pre-adsorbed with 5×10^{-5} M β-endorphin prior to incubation with tissue. (*b*) Experimental: 1:1000 dilution of β-endorphin antisera. Magnification 560 ×.

script only in middle and late anagen suggests that POMC transcription, translation and apparently protein processing are regulated in a differential, hair cycle dependent fashion. Since in rodents, the cyclic activity of hair follicles is associated with dramatic morphological changes of the entire skin [10, 11, 15], we speculate that the peripheral POMC expression and activity observed in the C57BI/6 mouse may be an integral part of the biological clock regulating the cyclic activity of hair follicles, and may serve to modulate skin physiology, including melanocyte activity [11] and the skin immune system [9].

The restricted localization of the β-endorphin antigen to the sebaceous gland suggests that the POMC product β-endorphin may be generated, or alternatively, concentrated by the sebaceous gland, since it is a target for MSH and β-lipoprotein action [16]. Since the sebaceous gland is an integral part of the pilosebaceous apparatus [15], which in turn may communicate with the epidermis [10, 17], this raises the intriguing possibility that sebaceous gland derived POMC peptides are involved in the regulation of follicular and epidermal functions. In support of this we have found that the POMC products γ1-, γ2- and β-MSH can stimulate keratinocyte proliferation in organ culture of mouse telogen skin [18].

During fetal development the sebaceous gland is formed by invagination of the germinative basal epidermal cells into the dermis [19]. Of similar origin is another secretory gland, the mammary gland, that has been reported to accumulate the POMC products: β-endorphin and α-MSH [20]. In this context it is intriguing to note that Köck et al. [9] have reported that undifferentiated epidermal cells cultured in vitro can produce and secrete POMC peptides. Thus further careful studies on the expression of the POMC gene and protein in the fetal germinative basal epidermal cells and glands that are derived from them including sebaceous, mammary and sweat glands, are warranted.

In summary (table) we report that POMC mRNA and protein are expressed in the skin of C57BI/6 mouse during anagen, but not in telogen (the resting phase) and that the immunolocalization of the POMC product, β-endorphin, is restricted to the sebaceous gland. This mouse model may serve to elucidate the physiological role of local POMC expression in the biology of mammalian skin, its pigmentary and immune system as well as in the biology of its appendages.

Differential expression of POMC during induced hair growth

Day after anagen induction	Proopiomelanocortin mRNA [a]	Protein Western blots [b]	Immunocytochemistry [c]
Telogen	−	−	(+)
1	(±)	−	(±)
3 and 4	+	−	+
5–8	+	+	+++

[a] 0.9 kb transcript; [b] 30–33 kDa protein detected by anti-β-endorphin antisera. [c] immunocytochemistry positive for β-endorphin antigen.

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Oxidative stress in the moderately halophilic bacterium *Deleya halophila*: Effect of NaCl concentration

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Abstract. The sensitivity of *Deleya halophila* to oxidative stress caused by hydrogen peroxide (H_2O_2) was found to vary, depending on the NaCl concentration of the growth medium. Pretreatment of the bacteria at a low concentration of H_2O_2 (50 μM) protected the cells against the lethal effects of higher levels (1–2 mM) of H_2O_2. Exposure of *D. halophila* cells to 50 μM H_2O_2 resulted in the induction of several proteins (hydrogen peroxide-inducible proteins, hips). However, the kinetics of induction, the extent of induction and the number of hips appear to be influenced by the salt concentration of the growth medium. Five of the hips exhibited apparent molecular masses identical to those of five heat shock proteins (hsps).
Key words. Deleya halophila; oxidative stress; viability; protein synthesis; heat shock.

In bacteria, in response to non-lethal doses of oxidative agents, there is a stimulation of the production of enzymes that scavenge superoxide radicals, hydroxyl radicals and hydrogen peroxide, as well as of other proteins that alleviate the toxic effect. These induced proteins represent adaptive responses to oxidative stress since they prepare cells against subsequent challenge with otherwise lethal levels of these oxidants[1–3, 6, 18].

In the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*, an H_2O_2-adaptive response stimulates the synthesis of about thirty proteins[2, 13, 17]. Synthesis of several of these polypeptides, including the enzymes catalase and alkylhydroperoxide reductase, is positively controlled by the product of the *oxy R* gene[2, 9, 18]. It has also been shown that *oxy R* mutants are hypersensitive to oxidative stresses and highly mutable under aerobic growth conditions[2, 7, 18]. In the gram-positive bacterium *Bacillus subtilis*, eight proteins were induced by treatment with a low concentration of H_2O_2[14]. In general, many of the proteins induced by H_2O_2 are also induced by a variety of other stresses, including heat shock[2, 13, 17].

Recently, we have demonstrated that the kinetics of induction of the heat shock proteins (hsps) in the moderately halophilic bacterium *Deleya halophila* are influenced by the NaCl concentration of the growth medium. Moreover, it has been shown that growth in high salt (2.5 M NaCl) conditions conferred on these bacteria the ability to maintain a high growth rate at elevated temperatures[10]. In addition, the effects of sudden changes in external NaCl concentration on the patterns of protein synthesis of *D. halophila* have also been investigated[4].

In this study, we report the effect of oxidative stress caused by H_2O_2 on the survival of *D. halophila*. Furthermore, the alteration of protein patterns during the adaptive response to non-lethal levels of H_2O_2 is also investi-