Identification and Sequencing of a Putative Variant of Proopiomelanocortin in Human Epidermis and Epidermal Cells in Culture

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Proopiomelanocortin (POMC) is a precursor polypeptide for various bioactive peptides, including adrenocorticotropic hormone, α-, β-, and γ-melanotropin, β-endorphin, and β-lipotropin. Although the classical source of POMC is the pituitary, various studies indicate the expression of POMC in several nonpituitary tissues. In this study, *in situ* hybridization with anti-sense cRNA riboprobe was used to show expression of POMC mRNA in human epidermis and cultured human epidermal cells (melanocytes and keratinocytes). POMC mRNA was amplified by reverse transcriptase-polymerase chain reaction using anti-sense and sense primers designed from Exons 2 and 3 of POMC gene. A ≈300 bp product was present in normal human skin, grafted human skin, and cultured normal human melanocytes and keratinocytes. By Southern analysis this product was hybridized specifically to the POMC cDNA. Sequence analysis of the reverse transcriptase polymerase chain reaction product from tissues or cells showed 85% homology to POMC cDNA from human, bovine, pig, and monkey sources. This suggests the existence of a putative isoform or variant of POMC mRNA in human epidermis.
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Proopiomelanocortin (POMC) is an ≈30,000 dalton precursor protein for a variety of bioactive peptides (Smith and Funder, 1988), adrenocorticotropic hormone (ACTH), α-, β-, and γ-melanotropin (MSH), β-endorphin, and β-lipotropin. All of these are derived by tissue-specific post-translational proteolytic cleavage from POMC (Evans *et al*, 1983). In the anterior lobe of the pituitary the major secretory products are ACTH and β-lipotropin, whereas in the intermediate lobe the predominant products are α-MSH and corticotropin-like intermediate product (Mains *et al*, 1977; Crine *et al*, 1978). This proteolytic processing is mediated by prohormone convertase 1 and 2 (Bloomquist *et al*, 1991; Lindberg, 1991). Prohormone convertase 1 cleaves POMC to ACTH and β-lipotropin, whereas prohormone convertase 2 cleaves POMC into β-endorphin from β-lipotropin, generating α-MSH and/or des-acetyl-α-MSH by further cleavage of ACTH (Bloomquist *et al*, 1991; Lindberg, 1991).

The POMC gene encodes the precursor to various POMC peptides and has three Exons and two introns. Exon 1 contains a noncoding region, Exon 2 encodes a signal peptide essential to protein processing and secretion (Autelitano *et al*, 1989). Exon 3 encodes various bioactive peptides, such as ACTH, α-, β-, and γ-MSH, β-endorphin, and β-lipotropin. Splicing of the primary POMC mRNA transcript results in ≈1100 nucleotides POMC mRNA (Autelitano *et al*, 1989). It has been shown that the mouse genome contains two POMC related gene sequences, α-POMC and β-POMC (Uhler *et al*, 1983). The functional α-POMC in mouse is very similar to the single gene expressed in human, bovine, and rat genome (Uhler *et al*, 1983). α- and β-POMC genes map to mouse chromosomes 12 and 19, respectively. The β-POMC locus may contain a pseudogene (Uhler *et al*, 1983).

The POMC is expressed in the pituitary gland, its traditional source, and in the hypothalamus, amygdala, and cerebral cortex. POMC expression also occurs in the ovary and testis, adrenal medulla, spleen, duodenum, and stomach (Pintar *et al*, 1984). POMC mRNA and its derived peptides have also been detected in cells of the immune system (Blalock, 1985). Our recent results showed the presence of POMC and its derivative peptides in murine epidermal cells and Thy-1+ dendritic cells (Farooqui *et al*, 1995). The latter cells are bone marrow-derived T lymphocytes found exclusively in murine epidermis. In human skin, α-MSH was the first POMC-derived peptide to be identified (Thody *et al*, 1983). Subsequently, POMC peptides were found in keratinocytes (Slominski *et al*, 1993a; Schauer *et al*, 1994; Chakraborty *et al*, 1995, 1996; Kippenberger *et al*, 1995; Wintzen *et al*, 1996), melanocytes (Farooqui *et al*, 1993; Kippenberger *et al*, 1995; Chakraborty *et al*, 1996), and Langerhans cells (Morhenn, 1991). It remains to be determined whether these molecules were synthesized *in situ* or transported from other sites.

In this study we show the presence of mRNA for POMC in human epidermal cells, including melanocytes and keratinocytes, using *in situ* hybridization. We have also characterized the POMC-like mRNA transcripts in epidermal tissue and isolated cells using reverse transcriptase-polymerase chain reaction (RT-PCR). These data prove that POMC is synthesized in the epidermis. Partial sequence of isolated POMC mRNA transcript from human skin, melanocytes, and keratinocytes indicates that the POMC-like mRNA is an isoform or variant of pituitary POMC.

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Abbreviations: BPE, bovine pituitary extract; MSH, melanotropins; POMC, proopiomelanocortin.

The significance of these results lies in the important pleiotropic effects of POMC-derived peptides on the skin. The POMC-derived peptides play key roles in the communication between the immune system and the neuroendocrine system (Blalock, 1989; McCubbin *et al*, 1991). α-Melanocyte stimulating hormone has potent anti-inflammatory effects that include blockage of chemotaxis of neutrophils, activation of B and T lymphocytes, release of acute-phase reactants from the liver, fever induction by interleukin-1, inflammation induced by chemicals (crude oil), ultraviolet (UV) radiation and psoralen-UVA, or allergic contact dermatitis. It has been suggested that α-MSH also plays a role in the pathogenesis of cutaneous contact hypersensitivity (Rheins *et al*, 1989). Furthermore, α-MSH and interleukin-1 have antagonistic interaction and interleukin-1 antagonizes the melanogenic effect of α-MSH on melanocytes (Daynes *et al*, 1987; Swope *et al*, 1989).

α-MSH plays a major role in stimulating integumental pigmentation (Sawyer *et al*, 1983; Sherbrooke *et al*, 1988). It causes rapid color change in lower vertebrates and coat color darkening in mammals. α-MSH and ACTH directly stimulate proliferation and melanogenesis of cultured human melanocytes and α-MSH augments the melanogenic effects of UV light on these cells (Abdel-Malek *et al*, 1995; Suzuki *et al*, 1996; Im *et al*, 1998). The biologic effects of α-MSH suggest that it might be a transducer of the UV-induced effects on human melanocytes (Bolognia *et al*, 1989; Chakraborty *et al*, 1991; Im *et al*, 1998).

MATERIALS AND METHODS

Cell cultures and human skin Cultures of normal human melanocytes from neonatal foreskins obtained from Caucasian (skin types I–III) and/or African American (skin type V) newborns were established and maintained according to the procedure described previously (Abdel-Malek *et al*, 1995). Cultures of normal human keratinocytes were obtained as previously reported (Boyce and Ham, 1985). The melanocyte and keratinocyte growth media contain bovine pituitary extract (BPE) that contains micromolar concentrations of α-MSH and possibly ACTH (Abdel-Malek *et al*, 1995). Both melanocytes and keratinocytes require BPE for their growth and proliferation in culture. For that, stock cultures were grown in BPE containing medium. Cells were deprived of BPE for 48–72 h prior to experiments, and were maintained in BPE-free medium for the experimental duration. AtT20 cells were obtained from American Type Culture Collection and were maintained in suspension according to the supplier's instructions. Biopsies (4 mm) of normal human skin were obtained from consenting Caucasian (skin types I–III), Chinese American (skin type IV), and/or African American (skin type V) volunteers in our department using an IRB approved protocol (IRB #88–09–09–01).

Riboprobe preparation A human POMC cDNA containing plasmid pH-OX3, a gift from Dr. James L. Roberts (Mount Sinai Medical Center, NY), was used for cRNA riboprobe preparation. Restriction digestion of pH-OX3 with Hind III gave an ≈1146 bp fragment that included full Exon 3 containing coding region (833 bp) and 3′ noncoding sequence (313 bp) (Takahashi *et al*, 1983), which was subcloned into PGEM-3Z expression vector (Promega, Madison, WI). Vector DNA was digested with Hind III followed by dephosphorylation with calf intestinal alkaline phosphatase (Promega). Standard ligation reaction for vector DNA and insert DNA was carried out (Promega). The ligation mixture was transformed into JM 109, competent cells (Promega) and plated on agar plates containing X-gal, IPTG, and ampicillin. We selected two clones under SP6 promoter direction: clone 1 (sense), which contains a DNA fragment insert in the 5′ → 3′, and clone 2 (anti-sense), in which the orientation was from 3′ → 5′ (under SP6 promoter direction).

Sense and anti-sense plasmids were linearized by ECoR1. The linearized templates were treated with proteinase K (0.1 μg per ml) and sodium dodecyl sulfate (0.1%) at 37°C for 30 min and then purified by phenol and chloroform extraction. The radiolabeled sense and anti-sense cRNA probes were synthesized using 1 μg of linearized template and 50 units of SP6 polymerase (Stratagene, La Jolla, CA) in the presence of 1 × reaction buffer, 0.4 mM adenosine-triphosphate, guanosine triphosphate, and thymidine triphosphate, 0.01 mM uridine triphosphate, 30 mM dithiothreitol, and 40 units RNasin (Promega), and 200 μci ^{35}S-UTP (Amersham, Arlington Heights, IL; 800 Ci per mmol, 40 mci per ml) was added in each reaction. The *in vitro* transcription mixture was allowed to react at 37°C for 3 h. Then the mixture was treated with 1 unit RNase free DNase (Stratagene) for 10 min at 37°C. The reaction product was extracted once with phenol, isopropyl alcohol, and chloroform in the presence of 0.1 μg yeast tRNA per ml (BRL, Gaithersburg, MD) and purified by passing through G-50 sephadex Quick Spin Column (Boehringer, Indianapolis, IN).

***In situ* hybridization** Skin biopsies (4 mm) were obtained from the forearm of six consenting normal human subjects and fixed in 4% paraformaldehyde in phosphate-buffered saline for 2 h, cryoprotected in 30% sucrose overnight, embedded in OCT (Tissue Tek, Elkhart, IN), and quickly snap frozen at −80°C. The embedded tissue blocks were stored at −80°C until used. Normal human melanocytes and keratinocytes were either cytocentrifuged on slides or grown in eight well chamber slides (Tissue Tek). The frozen skin sections (7 μm) and cells on Tespa (3-amino propyltriethoxysilane) coated slides were air dried and postfixed in 4% paraformaldehyde for 1 h. The slides were treated with proteinase K (20 μg per ml) for 5 min followed by treatment with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, washed, and air-dried (Witte *et al*, 1991). Prehybridization was carried out at 42°C for 15 min in hybridization cocktail containing 2 × sodium citrate/chloride buffer, 50% formamide, 10% Dextran sulfate, 0.75 mg yeast tRNA per ml, 0.75 mg herring sperm DNA per ml, 5 mM dithiothreitol, 0.1 mg bovine serum albumin per ml, 1 × Denhardts' solution, and 0.1 mM thio-UMP. Following prehybridization, the sections were hybridized by using 1 × 10^6 cpm per slide corresponding anti-sense or sense probes in the hybridization cocktail without thio-UMP at 42°C for 14–16 h with coverslip sealed by Cement (Kindler, Trenton, NJ). Coverslips were removed and the sections were washed in 1 × sodium citrate/chloride buffer and 1 mM dithiothreitol at 50°C for 30 min and then incubated in RNase T1 at 37°C for 30 min. A series of high stringency wash was followed. The washing steps started with 50% formamide in 2 × sodium citrate/chloride buffer at 50°C, 2 × 30 min, 0.5 × sodium citrate/chloride buffer, and finally in 0.1 × sodium citrate/chloride buffer at 55°C, and dithiothreitol was added to a final concentration of 1 mM. After dehydration and drying, slides were dropped in Kodak NTB2 emulsion, stored at 4°C, and developed after 5–14 d.

As a positive control, mouse anterior pituitary tumor cell line (AtT20) obtained from American Type Culture Collection was included throughout *in situ* hybridization experiments.

Combination of immunohistochemistry and *in situ* hybridization Biopsies from normal human skin were processed as described above. The tissue sections were first subjected to immunohistochemical staining to identify epidermal melanocytes and keratinocytes. A melanocyte specific antibody (Mel-5) (Signet Labs, Dedham, MA) that recognizes TRP-1 epitope was used to identify melanocyte, whereas cytokeratin antibody (Zymed Labs, San Francisco, CA) that specifically stains low molecular weight acidic keratins was employed for keratinocyte identification in the tissue sections. The sections were stained using avidin-biotinylated horseradish peroxidase Vector kit (Vector Laboratory, Burlingame, CA) except that all the solutions were prepared in DEPC-treated water. Following immunohistochemical staining, the sections were subjected to *in situ* hybridization as described above.

Total RNA extraction Total RNA used for RT-PCR was isolated using TRI REAGENT method (Molecular Research Center, Ohio). Briefly, normal human skin and grafted human skin were homogenized in TRI REAGENT (50–100 mg tissue per ml) using Polytron homogenizer. Cultures of human melanocytes and keratinocytes were lyzed directly in the culture flask using TRI REAGENT and collected in microfuge tubes. After phenol-chloroform extraction, the RNA was precipitated with isopropanol and the pellet was rehydrated in DEPC-H_20. RNA samples extracted from normal human skin, grafted human skin, cultured human melanocytes, and keratinocytes were purified using Message Clean kit (GeneHunter, Nashville, TN). RNA was treated with 10 units RNase-free DNase 1 for 30 min at 37°C. After phenol-chloroform extraction RNA was precipitated by adding 3 M sodium acetate and 100% EtOH for 1 h at −70°C. The RNA pellet was washed once with 70% EtOH and briefly air-dried. RNA was re-dissolved in DEPC-treated water and quantitated.

Reverse transcription reaction Partial sequence representing Exon 2 (Primer 1) and Exon 3 (Primer 2) was used in the synthesis of the primers: Primer 1, 5′-AGG ACC TCA CCA CGG AAA GCA ACCT-3′ (sense); Primer 2, 5′-AGT GCT CCA TGG AGT AGG AGC GCT T-3′ (anti-sense). Reverse transcription was performed using oligod(T)16 as primer. The reverse transcriptase mixtures contained 0.8 μg of cleaned RNA, 2.5 unit Murine Leukemia Virus Reverse Transcriptase, 1 unit RNase inhibitor, 2.5 mM oligod(T)16, 1 mM of dGTP, 1 mM dATP, 1 mM dCTP, 1 mM dTTP, 5 mM $MgCl_2$, and 10 × PCR buffer II (Perkin Elmer). The reaction was carried out at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min on Perkin-Elmer Gene Amp PCR system 9600.

Polymerase chain reaction (PCR) PCR was performed in 100 μl containing the reverse transcriptase mix (from above) and 80 μl of PCR master mix including 2 mM $MgCl_2$, 1 × PCR buffer II, 0.15 μM Primer 1, 0.15 μM Primer 2, and 2.5 unit of Ampli-Taq DNA Polymerase. The reaction was

carried out using the following conditions: initial denaturization at 75°C for 105 s, 35 cycles at 95°C for 15 s, 60°C for 30 s, and finally 72°C for 7 min. The products were electrophoresed on a 1.5% agarose gel. For negative control the following conditions were used: 1, RNA was not added in reverse transcriptase mix; 2, amplified RNA without reverse transcriptase; 3, PCR with no cDNA as template. None of the negative control lanes showed any bands.

Southern blotting The PCR products were subjected to Southern blot analysis following standard procedure (Sambrook *et al*, 1989). The PCR products were run on 1.5% agarose gel, denatured in 0.5 M NaOH, 1.5 M NaCl, and transferred to nylon membrane. After UV cross-linking, the membrane was prehybridized in solution containing 1% sodium dodecyl sulfate and Formamide at 42° for 4 h. [α-^{32}p]dCTP-labeled human POMC cDNA was added to fresh hybridization buffer and incubation was performed at 42°C overnight. The membrane was washed three times according to the protocol. After final rinse, the membrane was exposed to a X-ray film (Kodak, Rochester, NY) for 2 h at –70°C.

Sequence analysis RT-PCR products were ligated into pCR 2.1C vector (TA cloning kit, Invitrogen, CA) and 2 μl of ligation mixture was transformed into One Shot competent cells and plated on LB-ampicillin agar plate. After incubation of the plates at 37°C overnight, white colonies were picked and placed in LB ampicillin medium overnight at 37°C, 225 r.p.m. The digestion of plasmid DNA with ECoR1 gave a ≈300 bp product. The sequence analysis of the DNA was performed using M13 (Forward/Reverse) primer at the DNA core facility at University of Cincinnati. The sequencing of DNA from each sample was carried out at least three times to ensure the sequence reproducibility.

RESULTS

Tissue localization of POMC mRNA in human epidermis **Figure 1** shows the result of *in situ* hybridization of a representative human skin specimen. The signal for POMC mRNA was specifically confined in the upper layer of epidermis with anti-sense RNA probe (**Fig 1*a***, *arrowheads*). Occasionally, a weak POMC signal was also observed in the dermis. The autoradiography signal in the dermis was confined to individual cells, suggesting that endothelial cells or perivascular cells may also express POMC mRNA. Epidermis labeled with sense probe was used as a measure of nonspecific labeling (**Fig 1*b***). Skin biopsies obtained from Caucasian (skin types I–III), Chinese American (skin type IV), and/or African American (skin type V) volunteers showed no significant variation in the intensity or pattern of POMC mRNA signal by *in situ* hybridization, suggesting similar expression of POMC in different skin types. These experiments were performed in triplicate with identical results.

To evaluate the specificity of the *in situ* hybridization, the cultures of AtT20 cells were used as a positive control. Our results show a strong positive signal with the POMC mRNA anti-sense riboprobe (data not shown), whereas there was only background binding of POMC mRNA with sense probe. These results indicated that our anti-sense probe specifically localized to POMC mRNA.

POMC mRNA expression in cultures of normal human melanocytes and keratinocytes Prior to *in situ* hybridization, the BPE was eliminated from cultures of melanocytes and keratinocytes to eliminate exogenous concentrations of α-MSH or ACTH present in BPE. A strong positive labeling with anti-sense probe was observed in cultures of melanocytes (data not shown). Labeling with sense probe was used as an indication of nonspecific labeling. Although a number of melanocytes were present in the culture, no specific labeling to the cells was noticed with sense probe. Cultures of keratinocytes also showed labeling with anti-sense probe, whereas no specific signal for POMC mRNA was demonstrated with sense probe (data not shown). These results indicate that human epidermal melanocytes and keratinocytes in culture are capable of expressing POMC mRNA. Both cultures of melanocytes and keratinocytes established from Caucasian and/or African American donors did not reveal any variation in the intensity or pattern of POMC mRNA signal by *in situ* hybridization. These experiments were carried out in triplicate to confirm the intensity and pattern of the POMC mRNA signal.

In vivo* identification of the cell types expressing POMC mRNA in human epidermis** A combination of immunohistochemical staining and *in situ* hybridization was employed to identify the cell type (melanocyte and/or keratinocyte) expressing POMC mRNA in human epidermis. A positive staining with mel-5 antibody that recognizes TRP-1 epitope, an enzyme found only in melanocytes, was observed in human epidermis (**Fig 2*a). When the same sections were subjected to *in situ* hybridization a strong positive signal for POMC mRNA was

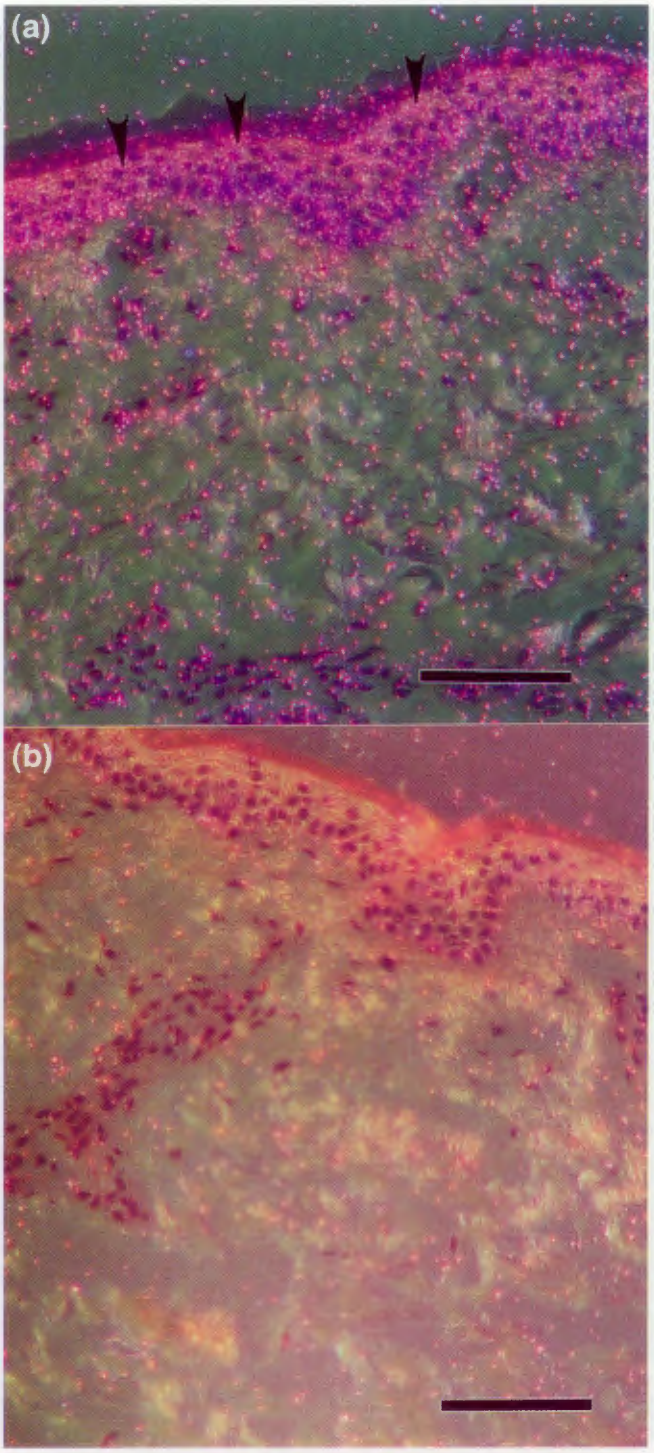

Figure 1. Expression of POMC mRNA in human epidermis by *in situ* hybridization. Skin biopsies (4 mm) from normal human subjects were removed and fixed in paraformaldehyde for 2 h followed by treatment with sucrose overnight. Biopsies were embedded in OCT (Tissue Tek) and frozen at –80°C. Serial sections (7 μm) of skin were subjected to *in situ* hybridization using ^{35}S-labeled anti-sense cRNA probe. Photomicrograph of skin showing positive signal in epidermis by anti-sense probe (*a*: *arrowhead*). *In situ* hybridization with sense probe did not show any specific signal (*b*). *Scale bars*: 100 μm.

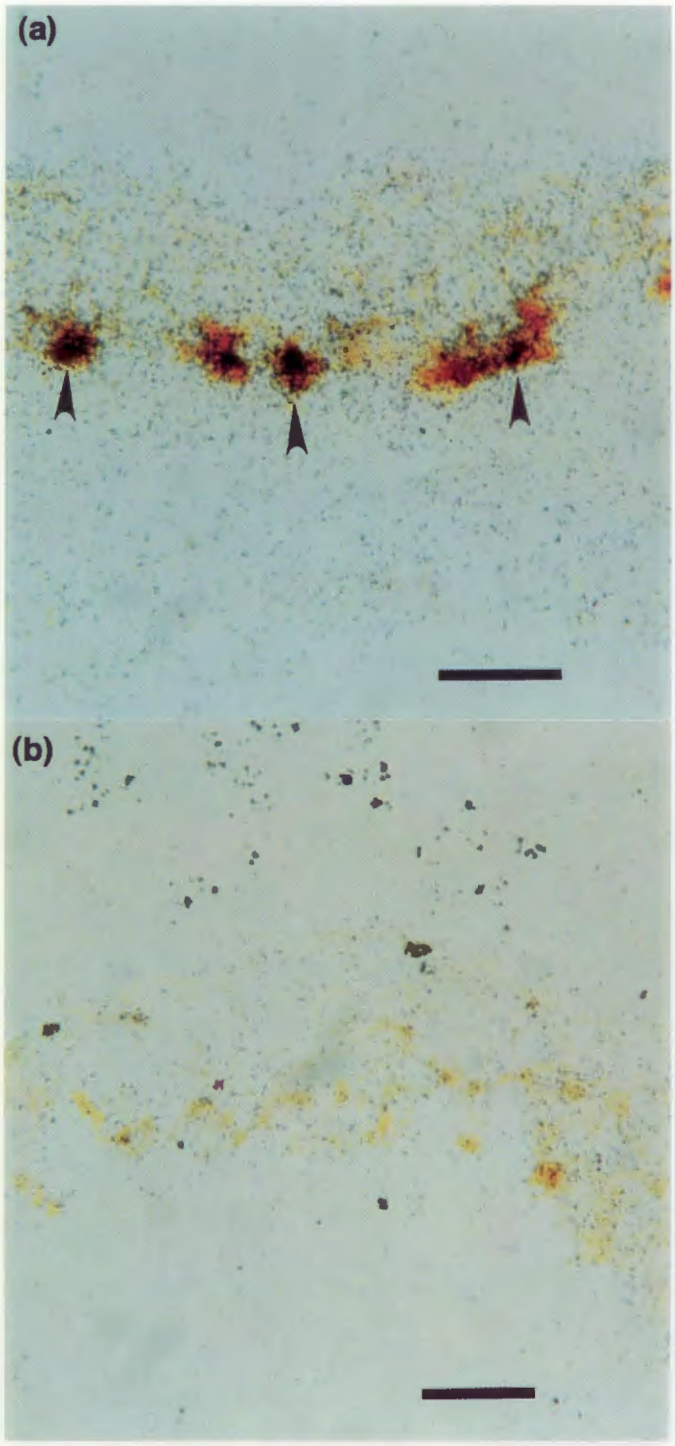

Figure 2. Immunocytochemical staining of epidermal melanocytes followed by *in situ* hybridization to localize POMC mRNA. Sections of normal human skin were stained with DEPC-treated Mel-5 antibody to identify epidermal melanocytes in skin. The same sections were then subjected to *in situ* hybridization using anti-sense and sense riboprobe. Skin sections exhibited positive staining for Mel-5 (*arrowhead*) and POMC mRNA was detected (*a*) with anti-sense POMC riboprobe, whereas no specific signal for POMC mRNA was detected with sense probe (*b*), although the melanocytes were still stained with Mel-5 (*b*). *Scale bar.* 50 μm.

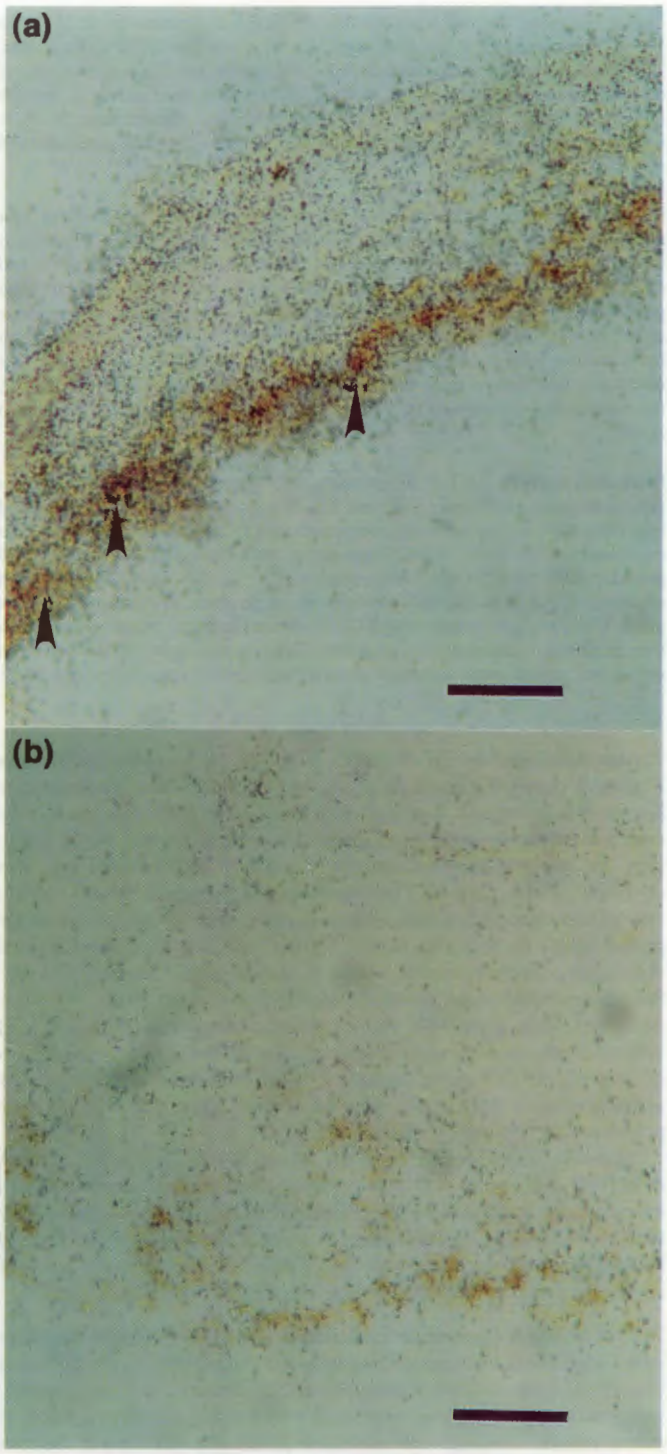

Figure 3. Immunocytochemical staining of epidermal keratinocytes followed by *in situ* hybridization. Immunocytochemical staining of normal human skin was performed using cytokeratin antibody to identify epidermal keratinocytes. The sections were then hybridized with POMC mRNA probe to localize POMC mRNA. A positive staining for keratinocyte was observed (*arrowhead*) for cytokeratin and a positive signal for POMC mRNA was noticed with anti-sense riboprobe (*a*), whereas no signal was observed with sense riboprobe (*b*). *Scale bar.* 50 μm.

detected with anti-sense probe (**Fig 2*a***, *arrowheads*) and only background was noticed with sense probe (**Fig 2*b***). In addition, in control where mel-5 antibody was omitted, only POMC mRNA signal was observed with anti-sense probe (data not shown). **Figure 3(*a*)** shows staining of human epidermis with cytokeratin antibody that specifically stains low molecular weight acidic keratins that are exclusively expressed by keratinocytes. The sections were positively stained for POMC mRNA with the anti-sense cRNA probe (*arrowheads*). Background signal was seen with sense riboprobe (**Fig 3*b***). In control where no primary antibody was added, only the signal for POMC mRNA was observed by anti-sense probe (data not shown). These data indicate that *in*

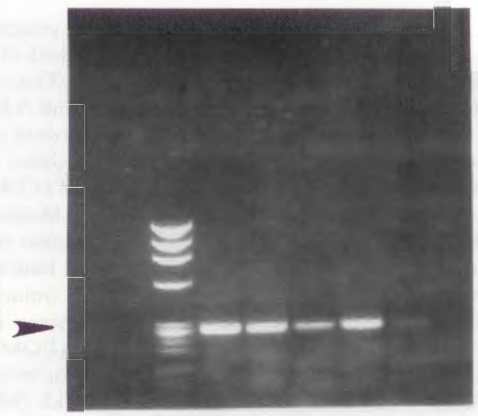

Figure 4. Expression of POMC mRNA by RT-PCR. Total RNA from normal human skin, grafted human skin, and normal human melanocytes and keratinocytes was isolated using TRI-REAGENT following the manufacturer's instructions. The isolated RNA was cleaned by message clean kit from GeneHunter. For RT-PCR, the following 25-mer primers 1 and 2 representing partial sequence of Exon 2 and 3 of POMC gene were used: Primer 1, 5′-AGG ACC TCA CCA CGG AAA GCA ACCT-3′ (sense); Primer 2, 5′-AGT GCT CCA TGG AGT AGG AGC GCTT-3′ (anti-sense). RT-PCR was performed (35 cycles) according to Perkin-Elmer instructions kit. A ≈300 bp product specific to Exon 3 was amplified in all the samples tested (*arrowheads*). **Figure 4** depicts DNA size marker obtained from Gibco/BRL, containing DNA/Hae III fragment ranging from 72 to 1353 bp (*lane 1*), RT-PCR product in AtT20 cells (*lane 2*), normal human keratinocyte (*lane 3*), grafted human skin (*lane 4*), normal human skin (*lane 5*), normal human melanocyte (*lane 6*), and no band in negative control without RNA (*lane 7*).

situ, human epidermal melanocytes and keratinocytes express mRNA for POMC.

Identification of RT-PCR product in human skin RT-PCR was utilized to detect POMC transcripts in human skin and epidermal cells. Using sense and anti-sense primers designed from Exons 2 and 3 of human POMC, respectively, we performed RT-PCR on RNA purified from normal and grafted human skin and cultures of melanocytes and keratinocytes. As shown in **Fig 4**, predicted ≈300 bp RT-PCR product specific to Exons 2 and 3 of the POMC gene was obtained in all the above samples analyzed, except in control where no RNA was added (*lane 7*). The variation in the intensity of the bands specifically in normal human melanocytes could not be due to the variation in sample loading as we used the same concentration of RNA in RT-PCR experiments. The lower intensity of RT-PCR product in melanocytes could possibly be due to the presence of low abundance message of POMC in melanocytes as compared with normal skin and/or keratinocytes. In addition, no RT-PCR product was found in samples that were amplified without cDNA and/or amplified without reverse transcriptase (data not shown). Further identification of the observed RT-PCR products from skin and epidermal cell cultures was carried out by Southern blot analysis using ^{32}P labeled POMC cDNA. Southern blot analysis revealed the presence of ≈300 bp product, which hybridized with cDNA probe (**Fig 5**). Sequence analysis of RT-PCR products from normal human skin, grafted human skin, and cultures of melanocytes and keratinocyte showed identical sequences. Only a representative sequence obtained from human skin or cultured cells is given (**Fig 6**) for comparison with the sequence in human pituitary. The appearance of a reproducible sequence of RT-PCR product eliminates any possibility of RT-PCR artifacts, because the experiment was performed three times independently. Comparison of nucleic acid sequence by Genebank showed an ≈85% homology with human POMC cDNA (**Fig 6**). There are 28 differences between the human pituitary POMC nucleotide sequence data and ours, as depicted by asterisks in **Fig 6**. Owing to some of these differences our deduced amino acid sequence shows the substitution of ala for glu and that of Leu for pro, leu for ala, pro for arg, and ala for ser. The

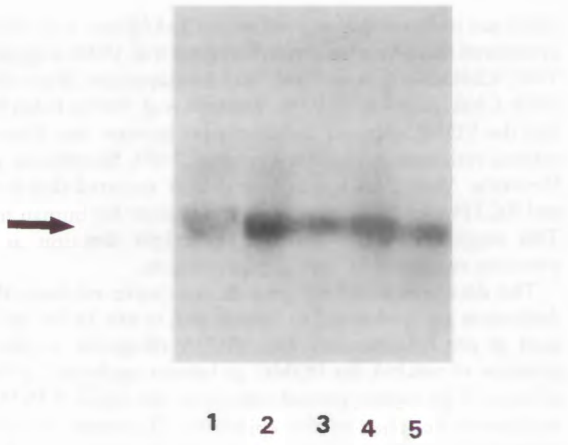

Figure 5. Expression of POMC mRNA by RT-PCR Southern blot analysis. The PCR products obtained under experimental conditions of **Fig 4** were subjected to Southern blot analysis to confirm the identity of the product. After reaction, the bands were transferred onto Nytran membrane and hybridized using ^{32}P-labeled POMC cDNA probe. **Figure 5** shows the presence of ≈300 bp product hybridized to POMC cDNA probe in AtT20 cells (*lane 1, arrow*), normal human keratinocytes (*lane 2*), grafted human skin (*lane 3*), normal human skin (*lane 4*), and normal human melanocyte (*lane 5*).

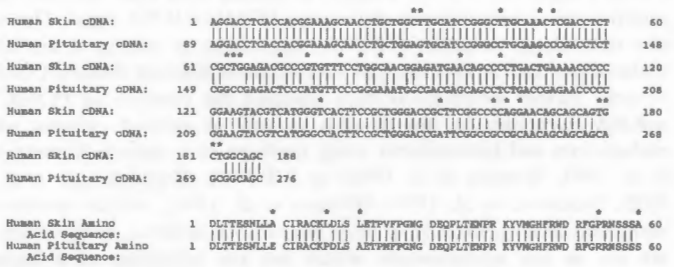

Figure 6. A representative sequence of RT-PCR product of POMC gene obtained from normal human skin, grafted human skin, and cultures of melanocyte and keratinocytes. RT-PCR products obtained from human skin and epidermal cells were subcloned into pCR 2.1 vector using TA cloning kit (Invitrogen, CA) following the manufacturer's instructions. Fresh RT-PCR products were ligated into pCR 2.1 in 10 μl ligation mixture containing 1 μl PCR product, 1 μl 10 × ligation buffer, 2 μl pCR 2.1 vector, 1 μl T_4 DNA Ligase, and 5 μl sterile water. The reaction was carried out by incubating at 14°C for 6 h. Two microliters of ligation mixture were transformed into One Shot competent cells and plated on LB agar plate. After incubation on LB agar plate at 37°C overnight, positive clones were picked up and grown in a larger volume (100 ml) of LB medium overnight at 37°C. The plasmid DNA was isolated and incubated with EcoR1 at 37°C for 1 h to confirm the presence of cloned insert. A ≈300 bp insert band was observed on 1.5% agarose gel. The sequence analysis of the plasmid DNA was performed at the University of Cincinnati DNA Core Facility. Comparison of the RT-PCR product sequence with human cDNA (*upper panel*) indicates an ≈85% homology, suggesting an isoform of POMC mRNA in human skin. Comparison of amino acid sequence (*lower panel*) between pituitary and human skin is also depicted. The differences in nucleotide and amino acid sequences between human skin and human pituitary are represented by asterisks.

sequence data suggest the presence of an isoform or variant of POMC gene in human skin.

DISCUSSION

Previous studies from various investigators revealed the presence of POMC-derived peptides in skin. For example, earlier work by Thody *et al* (1983) demonstrated the occurrence of immunoreactive α-MSH in the skin of humans and other mammals. Subsequent studies documented the presence of α-MSH and other POMC-derived peptides such as ACTH and β-lipotropin in epidermis (Farooqui *et al*, 1993; Slominski *et al*, 1993b). Both melanocytes and keratinocytes are capable of expressing POMC-derived peptides (Farooqui *et al*, 1993; Kippenberger *et al*, 1995; Chakraborty *et al*, 1996; Wintzen *et al*, 1996). POMC mRNA transcripts were also detected in human (Vieau *et al*,

1989) and rodent melanoma cell lines (Chakraborty *et al*, 1995) as well as in cultured human melanocytes (Farooqui *et al*, 1993; Kippenberger *et al*, 1995; Chakraborty *et al*, 1996) and keratinocytes (Kippenberger *et al*, 1995; Chakraborty *et al*, 1996; Wintzen *et al*, 1996). It has been shown that the POMC-derived melanocortins increase skin pigmentation in various vertebrate species (Sawyer *et al*, 1983; Sherbrooke *et al*, 1988). Recently, Abdel-Malek *et al* (1995) have reported that both α-MSH and ACTH were mitogenic and melanogenic for human melanocytes. This suggests α-MSH and ACTH might function as autocrine/paracrine regulators of human pigmentation.

The data presented here provide conclusive evidence that POMC derivatives are synthesized in human skin *in situ*. In this study we have used *in situ* hybridization with cRNA riboprobe to document the presence of mRNA for POMC in human epidermis. In human skin obtained from various normal volunteers, the signal of POMC mRNA was mostly confined to the epidermis. Occasional staining was also observed in the dermis, however, possibly localized to endothelial cells or perivascular cells. The relatively higher signal for POMC mRNA in human epidermis could partly be due to the fact that both epidermal keratinocytes and melanocytes express a strong signal for POMC mRNA as observed by *in situ* hybridization in this study. Together these cells constitute substantial cell population in the epidermis; thus they may contribute to the strong signal. In addition, the extremely sensitive technique of cRNA *in situ* hybridization enabled us to detect a higher signal for POMC mRNA in human skin. Pure cultures of melanocytes or keratinocytes also express POMC mRNA signal. There was no striking variation in POMC expression in various strains of melanocytes and/or keratinocytes derived from different donors. Until recently various investigators have reported the presence of POMC mRNA transcripts in human skin as well as isolated cultures of melanocytes and keratinocytes using northern blot analysis (Farooqui *et al*, 1993; Wintzen *et al*, 1996) or RT-PCR (Kippenberger *et al*, 1995; Slominski *et al*, 1995; Wintzen *et al*, 1996), which involves extraction/homogenization of tissue for mRNA isolation. In this study we use *in situ* hybridization, which has the advantage of a high resolution localization of POMC mRNA in its original anatomical context. It also offers the option of single cell resolution for measuring a biochemical moiety.

In order to identify the cell type expressing POMC mRNA in normal human skin, a combination of immunohistochemistry and *in situ* hybridization was employed. Using a melanocyte specific antibody (which recognizes TRP-1 epitope) the signal for POMC mRNA was localized to melanocytes. Similarly a monoclonal antibody that recognizes various low molecular weight acidic keratins was used for identification of keratinocytes in normal human skin. Again, a strong signal for POMC mRNA colocalized with staining of cytokeratin in keratinocytes, indicating that under normal conditions both melanocytes and keratinocytes *in situ* express POMC mRNA.

Further identification of POMC mRNA in human epidermis as well as cultures of melanocytes and keratinocytes was performed by RT-PCR on RNA isolated from these tissues or cells. Using RT-PCR we were able to show that the POMC gene is expressed in normal skin, grafted human skin, and cultures of melanocytes or keratinocytes as determined by the presence of ≈300 bp RT-PCR product in these tissues or cells. The specificity of the RT-PCR product was confirmed by Southern blot hybridization with ^{32}P-labeled POMC cDNA probe. The RT-PCR product was successfully hybridized with POMC cDNA, indicating that the authentic POMC gene product is expressed by human skin as well as cultured epidermal cells. Using RT-PCR technique, Slominiski *et al* (1995) and Kippenberger *et al* (1995) have recently shown the presence of POMC mRNA transcripts in normal human skin, and in melanocytes and keratinocytes, respectively. Consistent with these results and with our previous findings regarding the increased expression of POMC-related peptides (ACTH and α-MSH) in grafted human skin, in this study we also detected the presence of POMC mRNA by RT-PCR.

Additional confirmation of RT-PCR product was carried out by sequence analysis. RT-PCR products obtained from normal human skin, grafted human skin, and cultures of melanocytes and keratinocytes gave an identical sequence (n = 3). Comparison of sequence by Genebank revealed a homology of ≈85% with human pituitary POMC cDNA. A similar homology (85%) was also observed with cDNA from various species such as bovine, porcine, and monkey. This variation in sequence homology (15%) suggests that the POMC mRNA transcript in human skin as well as in epidermal cells may represent an isoform or variant of POMC mRNA. This observation is consistent with other reports where investigators have shown the presence of POMC mRNA variants in different tissues (Civelli *et al*, 1982; Lacaze-Masmonteil *et al*, 1987; DeBold *et al*, 1988b; Oates *et al*, 1988; Slominski *et al*, 1992; Farooqui *et al*, 1995). Slominski (1991) for the first time has shown the presence of 3.5 kb, 1.5 kb (major), and 1.0 kb (minor) POMC mRNA transcripts in murine and hamster melanoma cells, thus providing strong evidence for both larger and shorter POMC mRNA transcripts under *in vivo* and *in vitro* conditions. Also, trout pituitary expresses larger POMC variants of 1.4 kb and 1.8 kb (Salbert *et al*, 1992). Similarly, longer variants of POMC mRNA have been reported in nonpituitary ACTH-producing tumors, thymic carcinoid, and pancreatic tumor. The processing of the larger mRNA variants in these tumors quite often differs from processing that takes place in the pituitary (de Keyzer *et al*, 1985). On the other hand, using blot hybridization analysis of POMC RNA, investigators have reported the presence of small RNA species ≈200–400 nucleotides shorter than the one expressed in the pituitary. The shorter POMC mRNA transcripts have been reported in normal adrenal glands, ovary, testis, and thymus in animals (Jeannotte *et al*, 1987) and in human tissues (DeBold *et al*, 1988a; Gallinelli *et al*, 1995). Similar differences in transcript size have also been demonstrated in ovaries of rat, mouse, monkey, and sheep (Chen *et al*, 1986). Recently, we have reported the presence of a shorter or truncated POMC mRNA (0.9 kb) in Thy1+ dendritic cells, an epidermal cell of T cell lineage (Farooqui *et al*, 1995). Slominski *et al* (1996) have also demonstrated a shorter 0.9 kb transcript expressed in mouse skin throughout the anagen phase, whereas 1.1 kb POMC transcript is present exclusively during anagen V phase of hair growth and is not detected at early phases of hair growth. It has been postulated that these short POMC mRNA transcripts may arise as a consequence of alternative splicing, variation in the length of the poly+ (A) tail, or use of alternative transcription initiation site (Oates and Herbert, 1984; Jeannotte *et al*, 1987; Lacaze-Masmonteil *et al*, 1987). Lack of Exon 1 and 2 in these transcripts suggests that the products generated are neither processed nor secreted (Lacaze-Masmonteil *et al*, 1987). Using nuclease protection and primer extension assay, Lacaze-Masmonteil *et al* (1987) have shown that the shorter transcripts produced in human testes do not contain 5′ coding sequences and thus may be the products of transcripts initiated from the 3′ end of the second intron rather than alternative processing of the RNA transcripts initiated at the pituitary promoter. The heterogeneity of POMC mRNA due to RNA splicing, however, has been demonstrated in rat pituitary and in AtT20 tumor cell POMC mRNA (Lacaze-Masmonteil *et al*, 1987). Although the functional significance of the heterogeneous/variant POMC mRNA is not clear, they do appear to vary in a tissue-specific manner. Because in this study we have compared only ≈300 bp of the coding region in Exon 3, which encodes for ACTH, α-MSH, and β-lipotropin, it does not necessarily conclude whether human skin expresses normal POMC mRNA like in the pituitary or a variant form reported in peripheral tissues. In the future therefore it will be crucial to look for any other variation in the POMC expression in normal and pathologic skin.

The significance of the occurrence of POMC mRNA variant in normal human skin is unclear at present. One can only speculate that the variant form of POMC mRNA may contribute to the stability or half life of the POMC molecule. Because we have detected immunoreactive POMC peptides such as ACTH, α-MSH, and β-lipotropin in normal human skin as well as in cultured melanocytes and keratinocytes,[1] suggesting the normal translational products of POMC by POMC mRNA variants. It will be interesting to identify

[1]Farooqui J, Gong C, Boyce S, Nordlund J: Expression of pro-opiomelanocortin and its peptides in human epidermis and epidermal cells. *J Invest Dermatol* 106:883, 1996 (abstr.)

these peptides in detail in order to rule out any possible abnormality/variation in their structure/sequence. These studies are beyond the scope of the present investigations; however, experiments are underway to isolate and identify POMC peptides in normal human skin as well as in epidermal melanocytes and keratinocytes.

In summary, our data indicate the expression of POMC mRNA in normal human skin as well as in melanocytes and keratinocytes by *in situ* hybridization. A ≈300 bp RT-PCR product was found in normal human skin, grafted human skin, and cultured melanocytes and keratinocytes. Comparison of the sequence of RT-PCR product by Genebank/Blast revealed 85% homology with human pituitary POMC mRNA, suggesting the presence of an isoform/variant of POMC in human skin.

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REFERENCES

Abdel-Malek Z, Swope VB, Suzuki I, *et al*: Mitogenic and melanogenic stimulation of normal human melanocytes by melanotropic peptides. *Proc Natl Acad Sci USA* 92:1789–1793, 1995

Autelitano DJ, Lundlad JR, Bloom M, Roberts JL: Hormonal regulation of POMC gene. *Ann Rev Physiol* 51:715–726, 1989

Blalock JE: POMC-derived peptides in the immune system. *Clin Endocrinol* 22:823–827, 1985

Blalock JE: A molecular basis for the bidirectional communication between the immune and neuroendocrine systems. *Physiol Rev* 69:1–69, 1989

Bloomquist BT, Eipper BA, Mains RE: Prohormone-converting enzymes: regulation and evaluation of function using anti-sense RNA. *Mol Endocrinol* 5:2014–2024, 1991

Bolognia J, Murray M, Pawelek J: UVB-induced melanogenesis may be mediated through the MSH-receptor system. *J Invest Dermatol* 92:651–656, 1989

Boyce ST, Ham RG: Cultivation, frozen storage and clonal growth of normal human epidermal keratinocytes in serum free media. *J Tiss Cult Meth* 9:83–93, 1985

Chakraborty AK, Orlow SJ, Bolognia JL, Pawelek JM: Structural/functional relationships between internal and external MSH receptors: Modulation of expression in Cloudman melanoma cells by UVB radiation. *J Cell Physiol* 147:1–6, 1991

Chakraborty A, Slominski A, Erinak G, Hwang J, Pawelek J: Ultraviolet B and melanocyte stimulating hormone (MSH) stimulate mRNA production for α-MSH receptors and proopiomelanocortin-derived peptides in mouse melanoma cells and transformed keratinocytes. *J Invest Dermatol* 105:655–659, 1995

Chakraborty AK, Funasaka Y, Slominski A, Ermak G, Hwang J, Pawelek JM, Ichihashi M: Production and release of proopiomelanocortin (POMC) derived peptides by human melanocytes and keratinocytes in culture: regulation by ultraviolet B. *Biochim Biophys Acta* 1313:130–138, 1996

Chen CLC, Chang CC, Krieger DT, Bardein CW: Expression and regulation of proopiomelanocortin-like gene in the ovary and placenta: comparison with the testis. *Endocrinology* 118:2382–2387, 1986

Civelli O, Birnberg N, Herbert E: Detection and quantitation of pro-opiomelanocortin mRNA in pituitary and brain tissues from different species. *J Biol Chem* 257:6783–6787, 1982

Crine P, Gianoulakis C, Seidah NG, Grossand F, Pezalla PD, Lis M, Chretien M: Biosynthesis of β endorphin from β lipotropin and a larger molecular weight precursor in rat pars intermedia. *Proc Natl Acad Sci USA* 75:4719–4722, 1978

Daynes RA, Robertson BA, Cho B-H, Burnham DK, Newton R: α-Melanocyte-stimulating hormone exhibits target cell selectivity in its capacity to affect interleukin 1-inducible responses in vivo and in vitro. *J Immunol* 139:103–109, 1987

DeBold CR, Menefee JK, Nicholson WE, Orth DN: Pro-opiomelanocortin gene is expressed in many normal human tissues and in tumors not associated with ectopic adrenocorticotropin syndrome. *Mol Endocrinol* 2:862–870, 1988a

DeBold CR, Nicholson WE, Orth DN: Immunoreactive proopiomelanocortin (POMC) peptides and POMC-like messenger ribonucleic acid are present in many rat nonpituitary tissues. *Endocrinology* 122:2648–2657, 1988b

Evans CJ, Erdelyi E, Weber E, Barchas JD: Identification of pro-opiomelanocortin derived peptides in the human adrenal medulla. *Science* 221:957–960, 1983

Farooqui J, Medrano EE, Abdel-Malek Z, Nordlund J: The expression of proopiomelanocortin (POMC) and various POMC-derived peptides in mouse and human skin. *Ann NY Acad Sci* 680:508–510, 1993

Farooqui JZ, Medrano EE, Boissy RE, Tigelaar RE, Nordlund JJ: Thy-1+ dendritic cells express truncated form of POMC mRNA. *Exp Dermatol* 4:297–301, 1995

Gallinelli A, Garuti G, Matteo ML, Genazzani AR, Facchinetti F: Expression of pro-opiomelanocortin gene in human ovarian tissue. *Human Reprod* 100:1085–1089, 1995

Im S, Moro O, Peng F, *et al*: Activation of the cAMP pathway by α-melanotropin mediates the response of human melanocytes to UVB light. *Cancer Res* 58:47–54, 1998

Jeannotte L, Burbach JPH, Drouin J: Unusual proopiomelanocortin ribonucleic acids in extrapituitary tissues: Intronless transcripts in testes and long poly (A) tails in hypothalamus. *Mol Endocrinol* 1:749–757, 1987

de Keyzer Y, Bertagna X, Lenne F, Girard F, Luton JP, Kahn A: Altered proopiomelanocortin gene expression in adrenocorticotropin producing non-pituitary tumors. Comparative studies with corticotropic adenomas and normal pituitaries. *J Clin Invest* 76:1892–1898, 1985

Kippenberger S, Bernd A, Loitsch S, Ramirez-Bosca A, Bereiter-Hahn J, Holzmann H: α-MSH is expressed in cultured human melanocytes and keratinocytes. *Eur J Dermatol* 5:395–397, 1995

Lacaze-Masmonteil T, Keyzer YD, Luton JP, Kahn A, Bertagna X: Characterization of proopiomelanocortin transcripts in human nonpituitary tissues. *Proc Natl Acad Sci USA* 84:7261–7265, 1987

Lindberg I: The new eukaryotic precursor processing proteinases. *Mol Endocrinol* 5: 1361–1365, 1991

Mains RE, Eipper BA, Ling N: Common precursor to corticotropins and endorphins. *Proc Natl Acad Sci USA* 74:3014–3018, 1977

McCubbin JA, Kauffmann PG, Nemeroff CB, Strey S: *Neuropeptides and Systemic Disease.* San Diego: Academic Press, 1991

Morhenn VB: The physiology of scratching: involvement of proopiomelanocortin gene-coded proteins-Langerhans cells. *Prog Neuro Endo Immunol* 4:265–267, 1991

Oates E, Herbert E: Sequence of porcine and rat proopiomelanocortin mRNA. *J Biol Chem* 259:7421–7425, 1984

Oates EL, Allaway GP, Armstrong GR, Boyajian RA, Kehr JH, Prabhakar BS: Human lymphocytes produce proopiomelanocortin gene related transcripts. *J Biol Chem* 763:10041–10044, 1988

Pintar JE, Schachter BS, Herman AB, Dugerian S, Krieger DT: Characterization and localization of POMC messenger RNA in the adult rat tissues. *Science* 225: 632–634, 1984

Rheins LA, Cotleur AL, Kleier RS, Hoppenjans WB, Sauder DN, Nordlund JJ: Alpha-melanocyte stimulating hormone modulates contact hypersensitivity responsiveness in C57BL/6 mice. *J Invest Dermatol* 93:511–517, 1989

Salbert G, Chauveau I, Georgette B, Valotaise Y, Jego P: One of the trout proopiomelanocortin messenger RNAs potentially encodes new peptides. *Mol Endocrinol* 6:1605–1613, 1992

Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989

Sawyer TK, Hruby VJ, Hadley ME, Engel MH: α-Melanocyte stimulating hormone: Chemical nature and mechanism of action. *Am Zool* 23:529–540, 1983

Schauer E, Trautinger F, Kock A, *et al*: Proopiomelanocortin-derived peptides are synthesized and released by human keratinocytes. *J Clin Invest* 93:2258–2262, 1994

Sherbrooke WC, Hadley ME, Castrucci AML: Melanotropic peptides and receptors: an evolutionary perspective in vertebrate physiologic color change. In: Hadley ME (ed.). *Melanotropic Peptides*, Vol. II. Washington: CRC Press, 1988, pp. 175–190

Slominski A: POMC gene expression in mouse and hamster melanoma cells. *Febs Lett* 291:165–8, 1991

Slominski A, Paus R, Mazurkiewicz J: Proopiomelanocortin expression in the skin during induced hair growth in mice. *Experientia* 48:50–54, 1992

Slominski A, Paus R, Wortsman J: On the potential role of proopiomelanocortin in skin physiology and pathology. *Mol Cell Endocrinol* 93:C1–C6, 1993a

Slominski A, Wortsman J, Mazurkiewicz JE, *et al*: Detection of proopiomelanocortin-derived antigens in normal and pathologic human skin. *J Lab Clin Med* 122: 658–666, 1993b

Slominski A, Ermak G, Hwang J, Chakraborty A, Mazurkiewicz JE, Mihm M: Proopiomelanocortin, corticotropin releasing hormone and corticotropin releasing hormone receptor genes are expressed in human skin. *Febs Lett* 374:113–116, 1995

Slominski A, Ermak G, Hwang J, Mazurkiewicz JE, Corliss D, Eastman A: The expression of proopiomelanocortin (POMC) and of corticotropin releasing hormone receptor (CRH-R) genes in mouse skin. *Biochim Biophys Acta* 1289:247–281, 1996

Smith AI, Funder JE: Proopiomelanocortin processing in the pituitary, central nervous system and peripheral tissues. *Endocr Rev* 9:159–179, 1988

Suzuki I, Cone R, Im S, Nordlund J, Abdel-Malek Z: Binding capacity and activation of the MC1 receptors by melanotropic hormones correlate directly with their mitogenic and melanogenic effects on human melanocytes. *Endocrinology* 137:1627–1633, 1996

Swope VB, Abdel-Malek ZA, Sauder DN, Nordlund JJ: A new role for epidermal cell-derived thymocyte activating factor (ETAF) /IL-1 as an antagonist for distinct epidermal cell function. *J Immunol* 142:1943–1949, 1989

Takahashi H, Hakamata Y, Watanabe Y, Kikuno R, Miyata T, Numa S: Complete nucleotide sequence of the human corticotropin-β-lipotropin precursor gene. *Nucl Acids Res* 11:6847–6858, 1983

Thody AJ, Ridley K, Penny RJ, Chalmers R, Fisher C, Shuster S: MSH peptides are present in mammalian skin. *Peptides* 4:813–816, 1983

Uhler M, Herbert E, D'Eustachio P, Ruddle FD: The mouse genome contains two nonallelic proopiomelanocortin genes. *J Biol Chem* 258:9444–9453, 1983

Vieau D, Massias JF, Girard F, Luton JP, Bertagna X: Corticotropin-like intermediatry lobe peptide is a marker of alternate proopiomelanocortin processing in ACHT-producing non-pituitary tumors. *Clin Endocrinol* 31:691–700, 1989

Wintzen M, Yaar M, Barbach JPH, Gilchrest BA: Proopiomelanocortin gene product regulation in keratinocytes. *J Invest Dermatol* 106:673–678, 1996

Witte DP, Welch TR, Beischel LS: Detection and cellular localization of human C4 gene expression in the renal tubular epithelial cells and other extrahepatic epithelial sources. *Am J Pathol* 139:717–724, 1991