*Proc. Natl. Acad. Sci. USA*
Vol. 83, pp. 9719–9723, December 1986
Medical Sciences

# Human β-melanocyte-stimulating hormone revisited

(pituitary/hypothalamus/ectopic corticotropin/radioimmunoassay/HPLC)

XAVIER BERTAGNA*, FREDERIC LENNE*, DELPHINE COMAR*, JEAN-FRANCIS MASSIAS*, HENRI WAJCMAN†, VERONIQUE BAUDIN†, JEAN-PIERRE LUTON*, AND FRANÇOIS GIRARD‡

*Laboratoire de la Clinique des Maladies Endocriniennes, and †Institut National de la Santé et de la Recherche Médicale U15, Hôpital Cochin, 27, rue du Faubourg St. Jacques, Paris 75014, France; and ‡Laboratoire d'Explorations Fonctionnelles, Hôpital Trousseau, Paris 75012, France

*Communicated by Elwood V. Jensen, August 18, 1986*

ABSTRACT **It is generally accepted that human β-melanocyte-stimulating hormone (hβMSH) does not normally exist in humans but was merely an artifactually generated 22-amino acid peptide corresponding to a lipotropin (LPH) fragment (residues 35–56). We examined whether the shorter 18-amino acid peptide hβMSH-(5–22) could be detected in some human tissues. Normal human pituitaries and hypothalami as well as corticotropin-secreting pituitary and nonpituitary tumors were extracted and chromatographed on Sephadex G-50, and the fractions were measured with two radioimmunoassays using either a COOH-terminal human γLPH (hγLPH) antiserum that recognized equally hγLPH, hβMSH, and hβMSH-(5–22) or a mid-portion hγLPH antiserum that recognized hγLPH and hβMSH but not hβMSH-(5–22). Normal pituitaries and pituitary tumors contained a single immunoreactive material coeluting with hγLPH. The hypothalami and the nonpituitary tumors all contained hγLPH and a smaller molecular weight material that was only detected in the COOH-terminal hγLPH radioimmunoassay; its elution volume ($V_e/V$, 0.75) was identical to that of hβMSH-(5–22) but different from that of hβMSH ($V_e/V$, 0.60); on reversed-phase HPLC, it coeluted with synthetic hβMSH-(5–22) with a retention time different from that of hβMSH. It is concluded that hβMSH-(5–22) that corresponds to the 18-amino acid peptide hβLPH-(39–56), flanked by two pairs of basic amino acids within the hβLPH molecule, is a normal maturation product of proopiomelanocortin in human nonpituitary tissues.**

A 22-amino acid peptide was isolated from human pituitary extracts that was called human β-melanocyte-stimulating hormone (hβMSH); this molecule had a structure closely related to that of other mammalian βMSHs; it differed, however, by a 4-amino acid extension (Ala-Glu-Lys-Lys) on the $NH_2$-terminal part of the molecule (1, 2).

Scott and Lowry (3) and Bloomfield *et al.* (4) showed that hβMSH did not normally exist in the human pituitary or in human blood. They showed that the peptide isolated by Dixon (1) and Harris (2) had been artifactually generated by a nonspecific proteolysis of human β-lipotropin (hβLPH) under the mild acidic conditions of the extraction procedure used at that time.

Occasionally reports have suggested the presence of a "hβMSH-like" material in extracts of nonpituitary tumors responsible for the ectopic corticotropin (ACTH) syndrome (5–8). In most of these studies it was further noticed that this material behaved somewhat differently from hβMSH on gel chromatography, indicating an apparently smaller molecular weight. Similar results were also obtained in extracts of human hypothalamus where the proopiomelanocortin (POMC) gene is normally expressed (9). These observations again raised the question of the possible existence of an hβMSH-like molecule in humans, at least in nonpituitary tissues.

The primary structures of ovine lipotropins (β- and γLPH) revealed sequence homologies with βMSH (10) that led Chretien and Li (11) to first suggest a precursor-hormone model for βMSH. Steiner and Oyer (12) discovered proinsulin, fully established its precursor function for insulin, and eventually unraveled a maturation process through an enzymatic cleavage at pairs of basic amino acids, a mechanism that has since been applied to most polypeptide hormone precursors, including ovine βMSH (13). Along with this theory, we hypothesized that a βMSH-like molecule could actually exist in humans but correspond instead to the shorter 18-amino acid peptide hβMSH-(5–22), which is identical to hLPH-(39–56), a peptide fragment that, unlike hβMSH, is flanked by two pairs of basic amino acids within the hβLPH molecule (14, 15). We used a combination of gel filtration chromatography, reversed-phase HPLC, and two different radioimmunoassays (RIAs) to detect and discriminate between these two molecules [hβMSH and hβMSH-(5–22)] in extracts of normal human pituitaries and hypothalami, corticotropic adenomas, and nonpituitary tumors responsible for the ectopic ACTH syndrome.

## MATERIALS AND METHODS

**Tissue Collection.** The following tumors were surgically obtained from five patients with ectopic ACTH syndrome: tumors E1–E4 were bronchial carcinoid tumors; tumor E5 was a thymic carcinoid tumor. Fragments of pituitary corticotropic adenomas from two patients with Nelson syndrome (N1, N2) were surgically obtained by the transsphenoidal approach. In all cases, macroscopically homogeneous tumor fragments with no evidence of local necrosis were quickly selected by the surgeon, immediately frozen in liquid nitrogen, and subsequently stored at −85°C. Two normal human pituitaries (P1, P2) and hypothalami (HT1, HT2) were obtained at autopsy 2- and 6-hr post-mortem from patients with no evidence of endocrine disease and no history of prior hormonal treatment. They were frozen and stored as indicated. Patients with ACTH-producing tumors had not been subjected to either radiotherapy or chemotherapy.

**Tissue Extraction.** Tissue fragments were homogenized in ice-cold 5 M acetic acid (10 ml/g of tissue) containing 1 mM phenylmethylsulfonyl fluoride, by three to five 15-sec bursts in a PCU2 Polytron (Polytron, Elkhart, IN). The homogenates were centrifuged at 5000 × *g* for 30 min at 4°C, and the supernatants were lyophilized in multiple aliquots that were subsequently used for RIA and/or chromatographic studies.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: βMSH, β-melanocyte-stimulating hormone; p, porcine; h, human; γLPH, γ-lipotropin; IR, immunoreactive; POMC, proopiomelanocortin; C-hγLPH, COOH-terminal human γLPH; mid-hγLPH, mid-portion human γLPH; ACTH, corticotropin; $V_e/V$, fractional elution volume.

**RIAs.** Two different hγLPH RIAs were used. The COOH-terminal hγLPH (C-hγLPH) RIA was performed as described (16) using antiserum 1547 directed against the COOH-terminal end of hγLPH.

The mid-portion hγLPH (mid-hγLPH) RIA used antibody 3345 obtained from a guinea pig immunized against a "purified" porcine (p) ACTH preparation (17).

Both RIAs used highly purified hγLPH as standard and $^{125}$I-labeled tracer.

The lyophilized tissue extracts were dissolved in 0.1 M HCl, centrifuged at 5000 × *g* for 30 min at 4°C, and the supernatant was serially diluted in the appropriate buffer for RIA measurement and/or for chromatography.

**Sephadex G-50 Gel Chromatography.** A 0.9 × 60-cm column was packed with Sephadex G-50 Fine (Pharmacia), which was equilibrated and developed at 4°C with the RIA buffer (0.06 M $Na_2HPO_4$/0.012 M EDTA/0.1% Triton X-100/0.05% $NaN_3$, pH 7.3). Samples of 0.8 ml were applied and eluted at a flow rate of 20 ml/hr (descending flow, 50 cm of hydrostatic pressure); 1-ml fractions were collected. The column was calibrated with bovine serum albumin (as a void volume marker), hγLPH, hβMSH, hβMSH-(5–22) (each measured by RIA), and dinitrophenyl-alanine (as a total volume marker). Bovine serum albumin and dinitrophenyl-alanine were added to each sample to determine the fractional elution volume ($V_e/V$) of the immunoreactive (IR) materials for each experiment. Fractions eluted from the column were directly analyzed by RIA.

**Reversed-Phase HPLC.** A 0.46 × 25-cm $C_8$ column (Aquapore RP-300; Brownlee Laboratory, Santa Clara, CA) was used with a LKB system consisting of a 2150 pump, a 2152 controller, and a low-pressure mixing chamber. Absorbance was monitored at 226 nm using a 2158 Uvicord-SD spectrophotometer. Solvent A was 15% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid in water, and solvent B was 50% (vol/vol) acetonitrile in water. Using a flow rate of 1.5 ml/min, the elution was obtained as follows: isocratic at 0% solvent B for 8 min, developing a linear gradient from 0% to 13% in 2 min, and from 13% to 18% in 12 min. Fractions (0.5 ml) were collected every 20 sec with an LKB Helirac 2212. For RIA determinations, the fractions were dried and reconstituted in 0.5 ml of the RIA buffer; 10-μl aliquots were used for the RIA. At this dilution there was no solvent interference in the RIA.

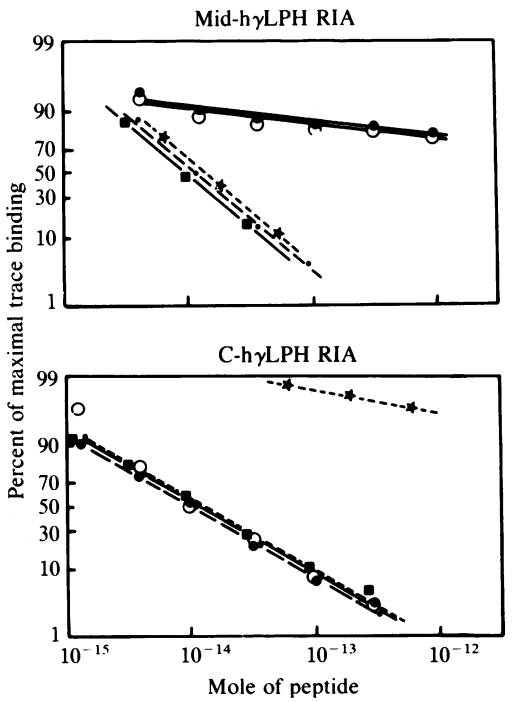


FIG. 1. Mid- and C-hγLPH RIAs. The competitive binding curves generated by highly purified hγLPH (■—■), hβLPH (★—★), hβMSH (●---●), hβMSH-(5–22) (○—○), and pβMSH (●—●) are shown. Data are plotted as percent of maximal trace binding vs. mole of peptide added per tube on a logit-logarithmic scale.

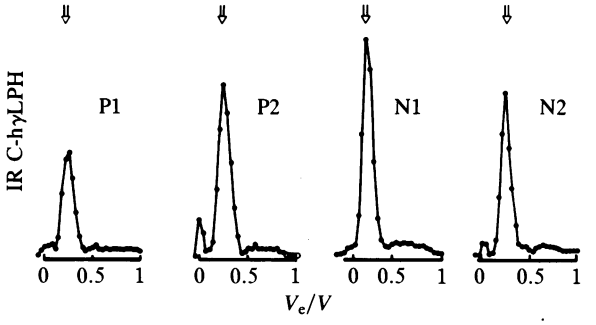


FIG. 2. Sephadex G-50 gel chromatography of tissue extracts from two normal pituitaries (P1, P2) and two corticotropic adenomas (N1, N2). Each fraction was analyzed with the C-hγLPH RIA. The relative distributions of the IR materials are shown on a $V_e/V$ scale; the variable IR C-hγLPH scales are purposely omitted. The position of hγLPH (⇓) is indicated.

**Peptides.** Highly purified hγLPH and hβLPH were provided by D. N. Orth (Vanderbilt University, Nashville, TN); highly purified pβMSH was provided by M. Chretien (Clinical Research Institute, Montreal); hβMSH (Sigma) and monkey βMSH, identical to hβMSH-(5–22), (Peninsula Laboratories, Belmont, CA) were synthetic molecules.

## RESULTS

The specificities of both antisera are shown in Fig. 1. In the mid-hγLPH RIA, antiserum 3345 reacts similarly with hβLPH, hγLPH, and hβMSH. It does not react with the shorter, 18-amino acid βMSHs, pβMSH and hβMSH-(5–22). In the C-hγLPH RIA, antiserum 1547 reacts equally with all βMSHs, pβMSH, hβMSH, and hβMSH-(5–22), as well as with hγLPH. It does not react with hβLPH.

Since the C-hγLPH RIA recognized all types of βMSH-like molecules, it was used as the first screening test to analyze the various tissue extracts.

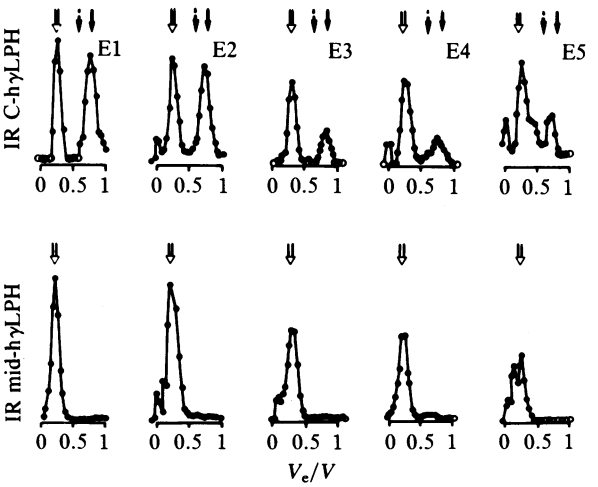


FIG. 3. Sephadex G-50 gel chromatography of tissue extracts from five nonpituitary tumors (E1–E5). Each fraction was analyzed with the C-hγLPH (*Upper*) and the mid-hγLPH (*Lower*) RIAs. The relative distributions of the IR materials are shown on a $V_e/V$ scale; the variable IR-hγLPH scales are purposely omitted. The positions of hγLPH (⇓), hβMSH (⇓), and hβMSH-(5–22) (⇓) are indicated.

As shown in Fig. 2, no hβMSH-like material could be detected in extracts of two normal human pituitaries and two corticotropic adenomas where the C-hγLPH RIA revealed a single peak of hγLPH.

In contrast, extracts of five nonpituitary tumors (Fig. 3, *Upper*) all contained, in addition to hγLPH, a smaller molecular weight material that was detected by the C-hγLPH RIA at a $V_e/V$ of 0.75, identical to that of hβMSH-(5–22). As shown in Fig. 3, *Lower*, this material was not detected by the mid-hγLPH RIA.

Further characterization of the hβMSH-like material from nonpituitary tumors was obtained from reversed-phase HPLC (Fig. 4). The system used could separate hβMSH-(5–22) and hβMSH (Fig. 4*A*). The hβMSH-like material of the nonpituitary tumor E1 had a retention time different from that of hβMSH, but identical to that of hβMSH-(5–22); a minor peak was also detected with a shorter retention time (15 min) corresponding to an oxidized form of hβMSH-(5–22).

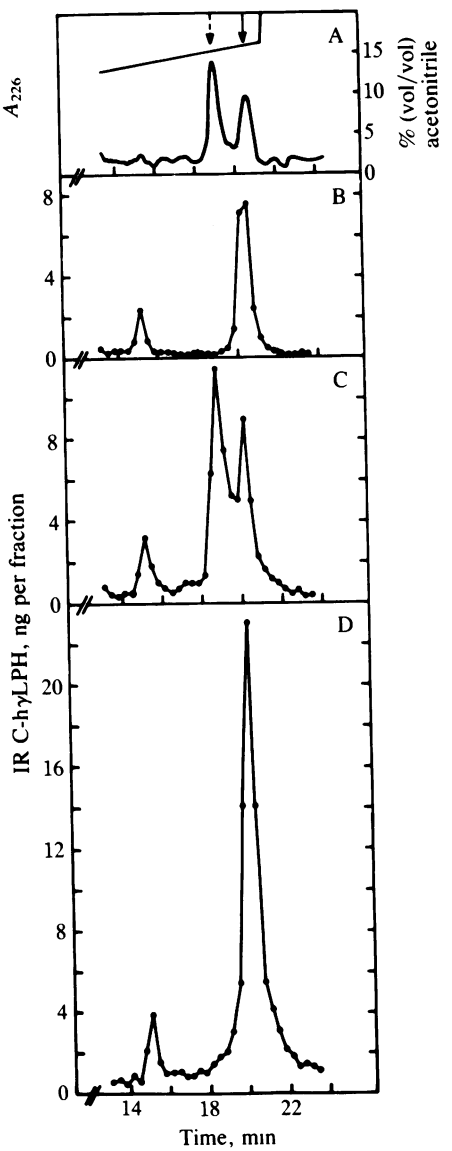


FIG. 4. Reversed-phase HPLC studies. (*A*) Absorbance profile generated by the chromatography of a mixture of hβMSH (↓) and hβMSH-(5–22) (↓). A large amount of nonpituitary tumor E1 extract was gel fractionated on Sephadex G-50 in 0.1 M acetic acid. Fractions corresponding to the hβMSH-like peak as detected by the C-hγLPH RIA were lyophilized and reconstituted. Three identical aliquots of ≈41.4 ng were applied under conditions identical to *A*, either alone (*B*), or with the addition of 100 ng of hβMSH (*C*), or 66.6 ng of hβMSH-(5–22) (*D*); the collected fractions were subsequently assayed with the C-hγLPH RIA.

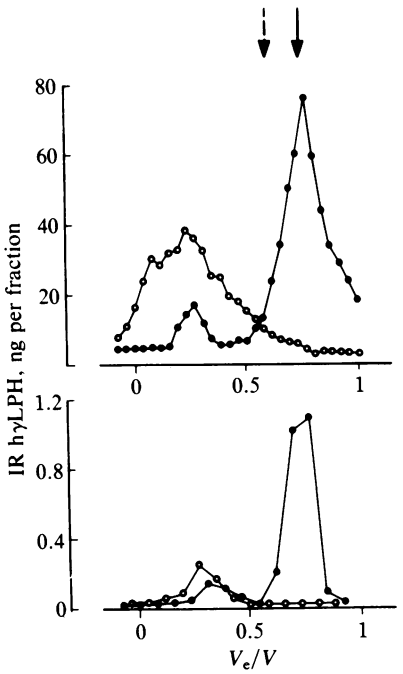


FIG. 5. Sephadex G-50 gel chromatography of human hypothalamic extracts. Each collected fraction was assayed with the mid-hγLPH (○) and the C-hγLPH (●) RIAs. The $V_e/V$ of hβMSH (↓) and hβMSH-(5–22) (↓) are indicated.

As shown in Fig. 5, normal human hypothalamic extracts also contained a βMSH-like material that had chromatographic and IR characteristics identical to those of hβMSH-(5–22).

## DISCUSSION

It had been assumed that an hβMSH did not normally exist in humans (3, 4). However, several authors have reported an hβMSH-like material in extracts from nonpituitary tumors responsible for the ectopic ACTH syndrome (5–8). The following evidence suggested that this molecule was different from the artifactually isolated hβMSH peptide: (*i*) Its elution characteristic on Sephadex G-50 was slightly different from that of hβMSH, indicating an apparently smaller molecular weight (6–8). (*ii*) Its pI value was different from that of hβMSH, but similar to that of pβMSH, an 18-amino acid βMSH molecule closely related to hβMSH-(5–22), except for one amino acid substitution (5). (*iii*) The four amino acids Ala-Glu-Lys-Lys make up an unusual $NH_2$-terminal extension in the hβMSH molecule; if such a molecule was normally generated by the processing of hPOMC, it would be the only exception among all other fragments generated by proteolysis occurring at pairs of basic amino acids (18). This is very unlikely because Barat *et al.* (19) showed that hβMSH can be artifactually produced from hβLPH by cathepsin D.

Thus, it seemed highly reasonable to assume that, if an hβMSH-like molecule could be detected in human tissues, it should correspond to the 18-amino acid peptide hβMSH-(5–22), which is an hβMSH molecule lacking the 4-amino acid $NH_2$-terminal extension, and to the hLPH-(39–56) fragment that is flanked by two pairs of basic amino acids within the hβLPH molecule.

The specificity of antiserum 1547 has been well established by Wilson *et al.* (7), who showed that it is precisely directed against the COOH-terminal residue aspartic acid-56 of hγLPH. The C-hγLPH RIA, therefore, will not react with hβLPH, but will recognize all the βMSH molecules.

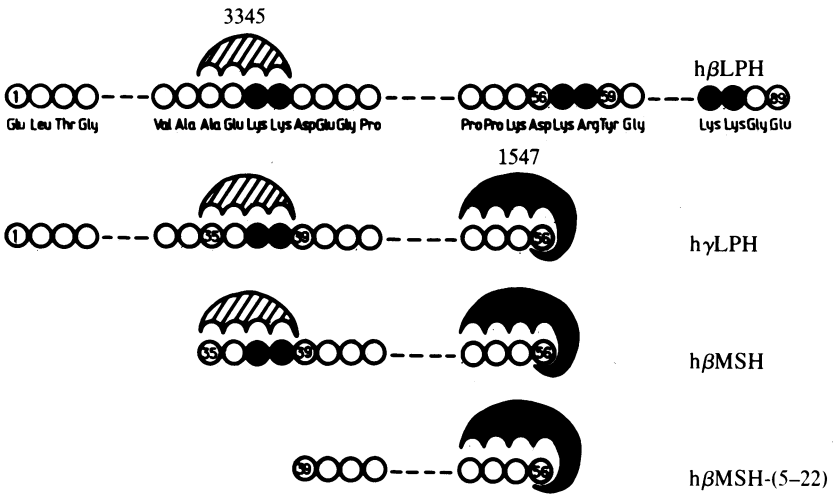


FIG. 6. Schematic presentation of the C- and mid-hγLPH RIA specificities.

Antiserum 3345 had been shown to react with hβMSH but not with bovine βMSH (17). Our results further show that it does not recognize pβMSH or hβMSH-(5–22). Since none of the 18-amino acid βMSHs is recognized by this mid-hγLPH RIA, it indicates that this antiserum is specifically directed against the four amino acids that make up the $NH_2$-terminal extension of hβMSH (Fig. 6).

Another means to separate hβMSH and hβMSH-(5–22) was gel filtration chromatography since the two peptides had different $V_e/V$ on Sephadex G-50 of 0.60 and 0.75, respectively.

Our strategy was to screen for any hβMSH-like molecule in tissue extracts using first the C-hγLPH RIA that recognizes all types of βMSHs. As expected, no such material was detected in extracts of normal human pituitaries. These data confirmed previous results (3–5) and showed that hPOMC processing is not complete in the human pituitary.

It was of interest to examine corticotropic adenomas responsible for Nelson syndrome because it has been suggested that some of these tumors might arise from intermediary lobe remnants (20) where POMC processing would be expected to generate small fragments such as βMSH (21). Nevertheless, we could not detect any such material in these pituitary-derived tumors, which would argue against this hypothesis. It would rather support the concept that hPOMC processing is not qualitatively altered in pituitary tumors.

In contrast, nonpituitary tumors all contained an hβMSH-like material. Since it was not recognized by the mid-hγLPH RIA, it was necessarily different from hβMSH. Its elution volume on Sephadex G-50 was similar to that of hβMSH-(5–22). However, a more discriminative procedure was needed to characterize further the exact molecular nature of this hβMSH-like material: a reversed-phase HPLC system that separates hβMSH and hβMSH-(5–22) could establish that it was indistinguishable from the latter molecule.

That this material might have been generated by nonspecific degradation during the extraction procedure appears highly unlikely for the following reasons. (*i*) Control experiments showed that added $^{125}$I-labeled hγLPH was not degraded during the extraction procedure for pituitary or nonpituitary tissue whether the 5 M acetic acid or 1 M HCl solutions were used; both extraction procedures generated a similar hγLPH/hβMSH-like molar ratio in nonpituitary tumors (data not shown). (*ii*) The 5 M acetic acid extraction did not generate detectable hβMSH-like material in pituitary tissue, either normal or tumoral. (*iii*) Reports have shown hβMSH-like molecules in blood of patients with the ectopic ACTH syndrome, independently of any tissue extraction procedure (22, 23).

The POMC gene can also be expressed in normal nonpituitary tissues especially in the brain (24). It has been shown that POMC processing in the brain is similar to processing in the intermediary lobe of the pituitary where shorter peptides are produced (9). Thus it was logical to look for an hβMSH-like material in this tissue, and we detected a molecule similar, if not identical, to hβMSH-(5–22) in the human hypothalamus. A peptide with an amino acid composition identical to that of hβMSH-(5–22) has also been isolated from human hypothalami extracts (Michel Chretien, personal communication). Whether it has a physiological role as a neurotransmitter remains to be established.

These data show that hβMSH-(5–22) is a normal maturation product of hPOMC in nonpituitary tissues. That this peptide is not present in the human pituitary raises the general question of the cell-specific mechanisms that control the maturation process of polypeptide hormone precursors.

We thank Dr. D. N. Orth for the generous gift of highly purified hγLPH and hβLPH, and antiserum 1547; Dr. M. Chretien for providing us with highly purified pβMSH; and Mrs. M. Le Scouarnec for her excellent secretarial assistance. The study was supported by a Contrat de Recherche Externe de l'Institut National de la Santé et de la Recherche Médicale 834002.

1. Dixon, H. B. F. (1960) *Biochim. Biophys. Acta* **37**, 38–42.
2. Harris, J. I. (1959) *Nature (London)* **184**, 167–169.
3. Scott, A. P. & Lowry, P. J. (1974) *Biochem. J.* **139**, 593–602.
4. Bloomfield, G. A., Scott, A. P., Lowry, P. J., Gilkes, J. J. H. & Rees, L. H. (1974) *Nature (London)* **252**, 492–493.
5. Ueda, M., Toshiyuki, T., Miyakawa, K. A. S., Ohnan, S. & Yanaihara, N. (1980) *J. Clin. Endocrinol. Metab.* **50**, 550–556.
6. Tanaka, K., Nicholson, W. E. & Orth, D. N. (1978) *J. Clin. Invest.* **62**, 94–104.
7. Wilson, R. E., Orth, D. N., Nicholson, W. E., Mount, C. D. & Bertagna, X. Y. (1981) *J. Clin. Endocrinol. Metab.* **53**, 1–9.
8. De Keyzer, Y., Bertagna, X., Lenne, F., Girard, F., Luton, J. P. & Kahn, A. (1985) *J. Clin. Invest.* **76**, 1892–1898.
9. Pique, L., Jegou, S., Bertagna, X., Javoy-Agid, F., Seurin, D., Proeschel, M. F., Girard, F., Agid, Y., Vaudry, H. & Luton, J. P. (1985) *Neurosci. Lett.* **54**, 141–146.
10. Li, C. H., Bonnafi, L., Chretien, M. & Chung, D. (1965) *Nature (London)* **208**, 1093–1094.
11. Chretien, M. & Li, C. H. (1967) *Can. J. Biochem.* **45**, 1163–1174.
12. Steiner, D. F. & Oyer, P. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 473–481.
13. Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J. & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci.* **343**, 1–16.
14. Hsi, K. L., Seidah, N. G., Lu, C. L. & Chretien, M. (1981) *Biochem. Biophys. Res. Commun.* **103**, 1329–1335.
15. Spiess, J., Mount, C. D., Nicholson, W. E. & Orth, D. N. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5071–5075.

16. Bertagna, X. Y., Nicholson, W. E., Sorenson, G. D., Pettengill, O. S., Mount, C. D. & Orth, D. N. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5160–5164.
17. Donnadieu, M. & Sevaux, D. (1971) *Biomedicine* **19,** 272–274.
18. Whitfeld, P., Seeburg, P. & Shine, J. (1982) *DNA* **1,** 133–143.
19. Barat, E., Patthy, A. & Graf, L. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6120–6123.
20. Lamberts, S. W. J., De Lange, S. A. & Stefanko, S. Z. (1982) *J. Clin. Endocrinol. Metab.* **54,** 286–291.
21. Eipper, B. A. & Mains, R. E. (1980) *Endocrinol. Rev.* **1,** 1–27.
22. Bertagna, X., Pique, L., Kuhn, J. M., Seurin, D., Girard, F. & Luton, J. P. (1984) *Riv. Ital. Biol. Med.* **4,** 83–87.
23. McLoughlin, L., Lowry, P. J., Ratter, S., Besser, G. M. & Rees, L. H. (1980) *Clin. Endocrinol.* **12,** 287–292.
24. Liotta, A. S., Gildersleeve, D., Brownstein, M. J. & Krieger, D. T. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 14498–14502.