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Distribution of Lamprey Adrenocorticotropin and Melanotropins in the Pituitary of the Adult Sea Lamprey, *Petromyzon marinus*

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Three peptides containing the melanotropin (MSH)-core sequence, YXMXHFRWG, have been isolated recently from the pituitary glands of adult sea lampreys (*Petromyzon marinus*) and were tentatively assigned as lamprey adrenocorticotropin (ACTH), MSH-A, and MSH-B, respectively. Both MSHs differed significantly from gnathostome MSHs and cannot be assigned as α-MSH, β-MSH, or γ-MSH. The aim of the present study was to localize these peptides in the lamprey pituitary using antisera generated against synthetic lamprey ACTH$^{1-16}$, MSH-A, and MSH-B. ACTH-like immunoreactivity was found in most cells of the rostral pars distalis (RPD) and in a few scattered cells of the proximal pars distalis (PPD). MSH-A-like immunoreactivity was found in most cells of the RPD, a few scattered cells of the PPD, and almost all cells of the pars intermedia (PI). MSH-B-like immunoreactivity was found only in the PI, where almost all cells were stained. Thus, the topographic distributions of ACTH and MSHs in the lamprey pituitary were similar to those in gnathostome vertebrates.

In contrast to recent rapid advances in molecular characterization of teleost pituitary hormones, arginine vasotocin of neurohypophysial origin is the only pituitary hormone that has been identified chemically in the lamprey (Lane *et al.*, 1988). Among several possible tropic functions of the lamprey adenohypophysis, gonadotropic and melanotropic activities appear to be incontrovertible (Sower *et al.*, 1987, 1993; Knox *et al.*, 1994; Baker and Buckingham, 1983), whereas weak adrenocorticotropin (ACTH) activity has been reported in the lamprey pituitary (Eastman and Portanova, 1982; Baker and Buckingham, 1983). In support of the presence of ACTH-like and melanotropin (MSH)-like activities in the lamprey pituitary, Dores *et al.* (1984) demonstrated ACTH-like and MSH-like immunoreactivities in the rostral pars distalis (RPD) and pars intermedia (PI), respectively, of *Lampetra lamolenii* pituitary using immunocytochemical techniques with antisera against mammalian proopiomelanocortin (POMC)-related peptides. Using similar immunocytochemical techniques, however, Nozaki and Gorbman (1984) observed no ACTH-immunoreactivity in the RPD, but observed inconsistent and questionable immunoreactions corresponding to ACTH and α-MSH in some PI cells of *Petromyzon marinus* pituitary.

It has been well established that in the pituitary of gnathostome vertebrates, ACTH and MSHs are produced from a common precursor protein, POMC, by post-translational proteolytic cleavage (Nakanishi *et al.*, 1979; Dores *et al.*, 1993). Dogfish and tetrapod POMC contains three different forms of MSHs, α-, β, and γ-MSH, while salmon POMC only contains

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two forms, α, and β-MSH, and is characterized by the absence of γ-MSH (Kawauchi *et al.*, 1981; Kitahara *et al.*, 1988; Salbert *et al.*, 1992).

Recently, Kawauchi and his colleagues (Takahashi *et al.*, submitted) isolated two MSHs and one ACTH from the sea lamprey (Fig. 1). One MSH (MSH-A) is a 19 amino acid peptide and the other MSH (MSH-B) is a 20 amino acid peptide. Both MSHs differ significantly from gnathostome MSHs and cannot be assigned as α-MSH, β-MSH, or γ-MSH. Lamprey ACTH exhibited an apparent molecular weight of 15 kDa on SDS–PAGE. The N-terminal region of lamprey ACTH, which corresponds with α-MSH of gnathostome vertebrates, is different from dogfish α-MSH by a N-terminal extension of five amino acids and five additional substitutions, followed by three additional residues, four basic residues, and corticotropin-like intermediate lobe peptide (Fig. 1). Recently, Heinig *et al.* (1994) cloned and sequenced a lamprey POMC cDNA which contains the sequence of ACTH and β-endorphin, but which lacks the sequence of either MSH-A or MSH-B. Subsequently, Takahashi *et al.* (unpublished) have isolated another POMC cDNA which does contain the sequence of MSH-A and MSH-B. These results suggest a unique molecular evolution of POMC in lampreys.

The aim of this study was to localize newly identified lamprey ACTH and MSHs in the lamprey pituitary using antisera generated against these peptides.

MATERIALS AND METHODS

Animals. Eight adult sea lampreys (*P. marinus*) of both sexes were collected in May and June 1993 during their upstream spawning migration from the sea, in traps located at the upstream end of a salmon ladder on the Cocheco River in Dover, New Hampshire. The animals were transported to the Fish Hatchery at the University of New Hampshire and were kept in a fresh water flow-through system supplied with ambient reservoir water ranging in temperature between 13° and 19° under a natural photoperiod. During this time, the lampreys were undergoing final reproductive maturation for a period of approximately 6 to 8 weeks until they spawned. They were approximately 65 cm in total length and weighed approximately 900 g at the time of sampling.

Tissue preparations. The animals were killed by de-

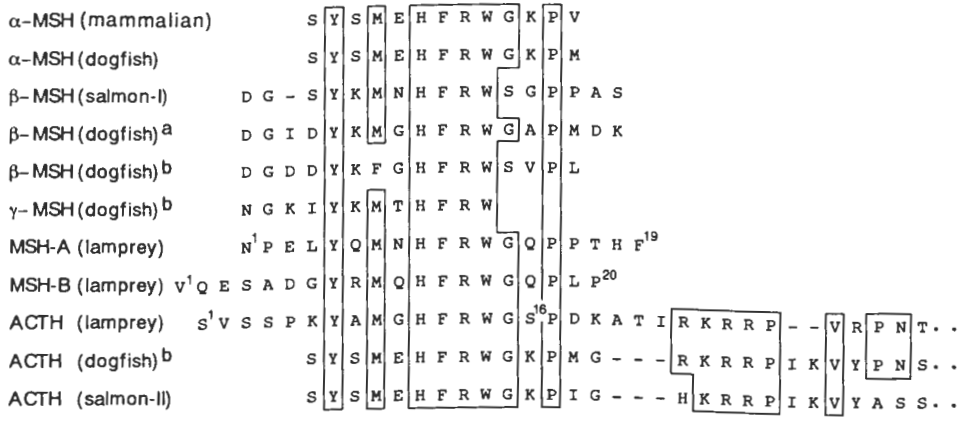


FIG. 1. Comparison of amino acid sequences of lamprey MSHs and ACTHs. Amino acid sequences are taken from mammalian α-MSH (Ney *et al.*, 1965); dogfish α-MSH (Bennett *et al.*, 1974); salmon β-MSH I (Kawauchi and Muramoto, 1979); dogfish, *Scyliorhinus canicula*, β-MSH (Love and Pickering, 1974); dogfish, *Squalus acanthias*, β-MSH (Bennett *et al.*, 1974), dogfish γ-MSH (McLean and Lowry, 1981); lamprey MSH-A, MSH-B, and ACTH (Takahashi *et al.*, 1995); dogfish ACTH (Lowry *et al.*, 1974); and salmon ACTH-II (Kitahara *et al.*, 1988). a, *Scyliorhinus canicula;* b, *Squalus acanthias.*

capitation after being anesthetized by immersion in ethyl *m*-aminobenzoate methane-sulfonate (MS222). After rapid removal of the dorsal fibrocranium and exposure of the dorsal surface of the brain, the dissected brain and the attached pituitary were immersed in Bouin–Hollande sublimate solution (Romeis, 1948) for about 24 hr. The fixed tissues were dehydrated through a series of increasing concentrations of ethanol. Deposited mercuric chloride was removed by treatment with iodine-potassium iodide in 90% ethanol for 1–2 days. Tissues were embedded in Paraplast, and serial sagittal sections of 6 μm were mounted on glass slides.

Preparation of antisera. Lamprey ACTH$^{1-16}$, MSH-A, and MSH-B were synthesized as described previously (Takahashi *et al.*, submitted; Fig. 1). Each peptide sample (2 mg each) was conjugated with bovine serum albumin (Sigma; 6 mg) in water by the carbodiimide method according to Goodfriend *et al.* (1964) and were stored at −30° after dialyzing in water. Each of the conjugated peptides (less than 100 μg) was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected subdermally on the back of the rabbit at intervals of three weeks. Six female rabbits (weighed about 2 kg) were used, two for each of the three conjugated peptides. Blood was first collected 2 weeks after the third injection.

Immunocytochemistry. Immunocytochemical staining was performed by use of a Vectastain avidin–biotin peroxidase complex (ABC Elite) kit. The staining procedures have been described elsewhere (Saga *et al.*, 1993). The lot numbers and optimal working dilutions of the above described antisera used for immunocytochemistry were: (1) antisynthetic lamprey ACTH$^{1-16}$ (Lot No. 9308, ×1500); (2) antisynthetic lamprey MSH-A (Lot No. 9309, ×8000); and (3) antisynthetic lamprey MSH-B (Lot No. 9311, ×8000).

To test the specificity of the immunostaining, the following control stains were done: (1) replacement of primary antisera with normal rabbit serum, and (2) absorption of primary antisera with synthetic lamprey ACTH$^{1-16}$ (50 μg/ml antibody at working dilutions), synthetic lamprey MSH-A (50 μg/ml antibody at working dilutions), or lamprey MSH-B (50 μg/ml antibody at working dilutions). Furthermore, since anti-lamprey ACTH$^{1-16}$ and anti-lamprey MSH-A stained intensely most cells of the RPD (see Results), where Met-enkephalin-like and nasohypophysial factor (NHF)-like immunoreactivities have been reported previously (Nozaki and Gorbman, 1984; Sower *et al.*, 1995), preabsorptions with synthetic Met-enkephalin (Sigma, 100 μg/ml antibody at working dilution) and purified NHF (Sower *et al.*, 1995; 100 μg/ml antibody at working dilution) were also performed.

RESULTS

The results of the immunostaining and preabsorption tests are summarized in Table 1 and Figs. 2 and 3.

ACTH

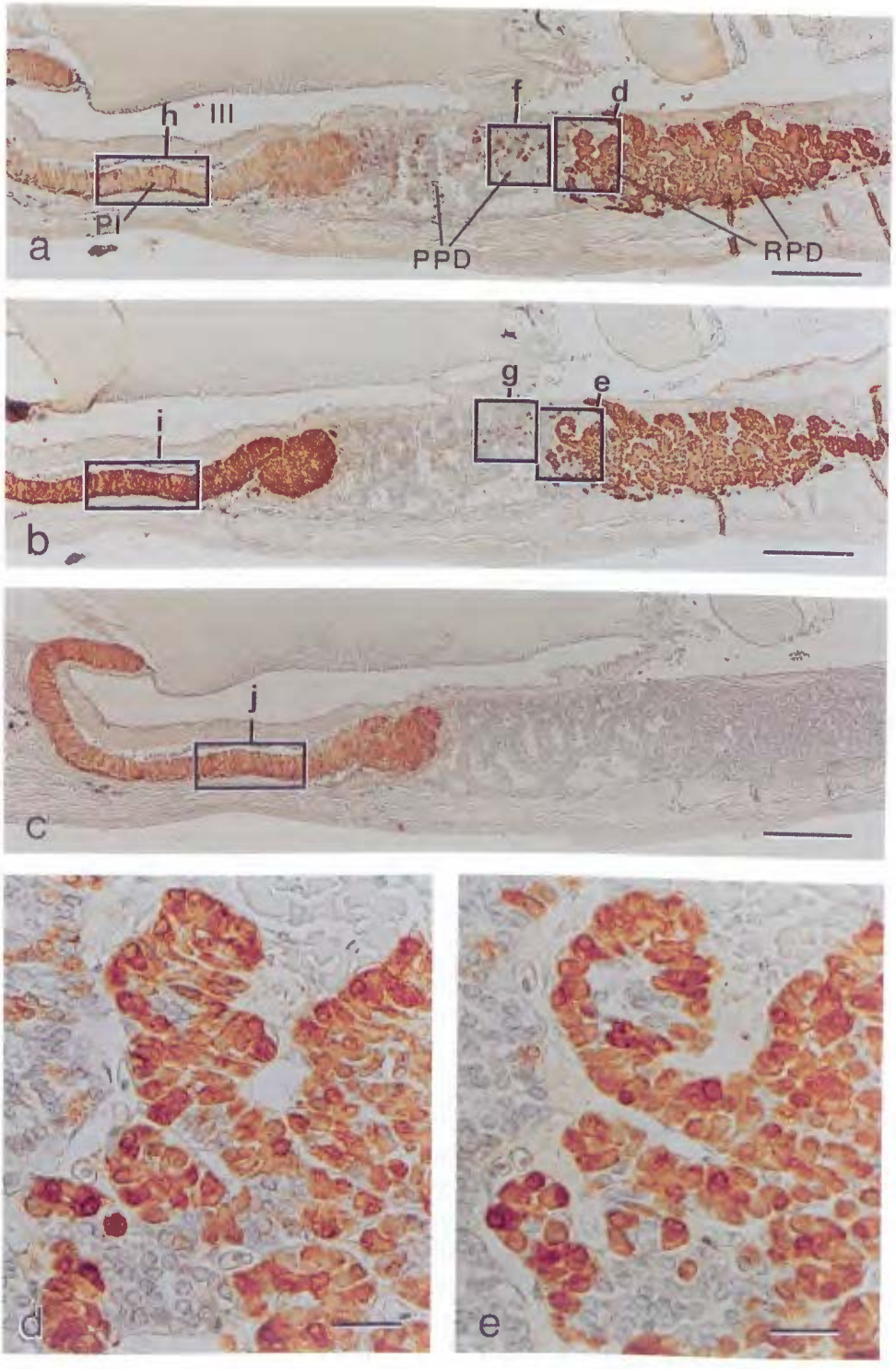
An intense immunoreaction to anti-ACTH was found in most cells of the RPD (Table 1, Figs. 2a and 3a). Moderate and weak immunoreactions to anti-ACTH were observed in a few scattered cells of the proximal pars distalis (PPD) and in most cells of the PI, respectively (Table 1, Figs 2a and 3a). Preabsorption of this antibody with lamprey ACTH$^{1-16}$ resulted in elimination of all the positive reaction in the ade-

TABLE 1

EFFECTS ON IMMUNOSTAINING OF PREABSORPTION OF ANTISERA TO LAMPREY (*l*) ACTH$^{1-16}$, MSH-A, AND MSH-B WITH AUTHENTIC ANTIGENS OR RELATED PEPTIDES IN LAMPREY ADENOHYPOPHYSIS

Antibodies to	Regions	Immunoreactivity			
		Without preabsorption	Preabsorptin with		
			l-ACTH$^{1-16}$	*l*-MSH-A	*l*-MSH-B
l-ACTH$^{1-16}$	RPD[a]	+++[b]	−(Abolished)	++(Reduced)	++(Reduced)
	PPD	++	−(Abolished)	+(Reduced)	+(Reduced)
	PI	+	−(Abolished)	−(Abolished)	−(Abolished)
l-MSH-A	RPD	+++	+++(Not changed)	−(Abolished)	−(Abolished)
	PPD	+	+(Not changed)	−(Abolished)	−(Abolished)
	PI	+++	+++(Not changed)	−(Abolished)	+(Reduced)
l-MSH-B	RPD	−			
	PPD	−			
	PI	+++	+++(Not changed)	+++(Not changed)	−(Abolished)

[a] RPD, rostral pars distalis; PPD, proximal pars distalis; PI, pars intermedia.
[b] +++, Intense immunoreaction; ++, moderate immunoreaction; +, faint immunoreaction; −, no immunoreaction.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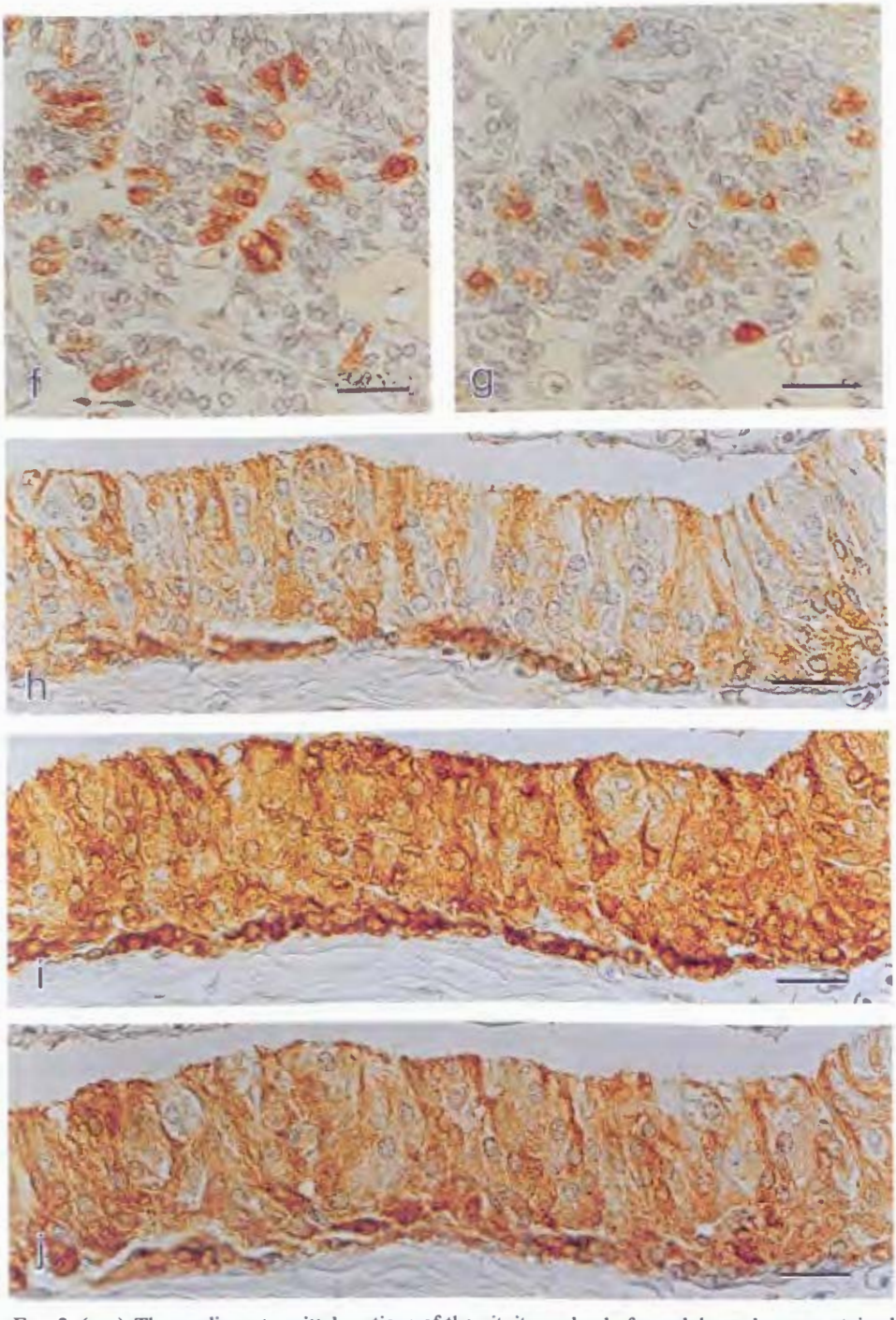


FIG. 2. (a–c) Three adjacent sagittal sections of the pituitary gland of an adult sea lamprey stained with anti-lamprey $ACTH^{1-16}$ (a), anti-lamprey MSH-A (b), and anti-lamprey MSH-B (c), respectively. The anterior end is to the right. The areas outlined by rectangles are enlarged and shown in d–j (d,e, part of rostral pars distalis (RPD); f,g, part of proximal pars distalis (PPD); h,i, part of pars intermedia (PI). III, third ventricle. Scale bars: a–c, 250 μm; d–j, 50 μm.

nohypophysial cells including both the pars distalis (PD) and PI (Table 1). Preabsorption of this antibody with MSH-A or MSH-B resulted in a reduction of ACTH staining intensities in the cells of the RPD and PPD, but abolished the positive reaction in the PI cells (Table 1 and Fig. 3b). Preabsorption of this antibody with Met-enkephalin or NHF had no effect on staining intensities in the cells of either the RPD or PI.

MSH-A

An intense immunoreaction to anti-MSH-A was found in most cells of the RPD and almost all cells of the PI (Table 1 and Fig. 2b). A weak immunoreaction to anti-MSH-A was observed in a few scattered cells of the PPD (Table 1 and Fig. 2b). Preabsorption of this antibody with MSH-A resulted in elimination of all the positive reaction in the adenohypophysial cells including both the PD and PI (Table 1). Preabsorption of this antibody with $ACTH^{1-16}$ had no effect on staining intensities in either the PD or PI, whereas preabsorption with synthetic MSH-B resulted in elimination of the positive reaction in the PD (Table 1). The positive reaction in the PI was not abolished by the latter procedure, but it did result in a major reduction of staining intensity (Table 1). Preabsorption of this antibody with Met-enkephalin or NHF had no effect on staining intensities of the cells in either the PD or PI.

MSH-B

An immunoreaction to anti-MSH-B was found only in the PI, where almost all cells were intensely stained (Table 1 and Fig. 2c). Preabsorption of this antibody with synthetic MSH-B resulted in elimination of the positive reaction in the PI (Table 1). Unlike the other two antibodies, preabsorption of anti-MSH-B with $ACTH^{1-16}$ or MSH-A had no effect on staining intensities in the PI (Table 1).

DISCUSSION

Regional tissue distributions of ACTH and MSHs in the lamprey pituitary were found to be similar to those in pituitaries of gnathostome vertebrates. ACTH-like immunoreactivity was found in most cells of the RPD and in a few scattered cells of the PPD. MSH-A-like immunoreactivity was found in most cells of the RPD, in a few scattered cells of the PPD, and in almost all cells of the PI. MSH-B-like immunoreactivity was limited to the PI, where almost all cells were stained.

The ACTH/MSH-A-positive cells of the lamprey pituitary appeared to correspond to basophils characterized by use of classical tinctorial stains (Hardisty and Baker, 1982). Since basophils in the pars distalis characteristically are observed during the period of sexual maturation, these authors suggested that these cells were possibly gonadotrophs (Ball, 1981; Hardisty and Baker, 1982). In more recent immunocytochemical studies, basophilic cells in lamprey pituitaries were found to be immunostained by antimammalian Met-enkephalin serum (Nozaki and Gorbman, 1984; Dores *et al.*, 1984; Nozaki, 1985) and an antimammalian ACTH serum which was directed to the middle portion of the ACTH molecule (Dores *et al.*, 1984). However, ACTH-immunoreaction was not observed in the lamprey pituitary after the use of other mammalian POMC-related peptide antisera, either a N-terminal (α-MSH) antiserum or a C-terminal (CLIP) antiserum (Dores *et al.*, 1984; Nozaki and Gorbman, 1984). Dores *et al.* (1984) suggested that an antiserum specific to the middle region of the ACTH molecule could account for the ACTH biological activity detected in whole pituitary extracts of lampreys (Eastman and Portanova, 1982; Baker and Buckingham, 1983). In other studies, Nozaki and Gorbman (1985) observed that anti-Met-enkephalin reactive cells stained intensely in the RPD of larval lampreys, as well as in

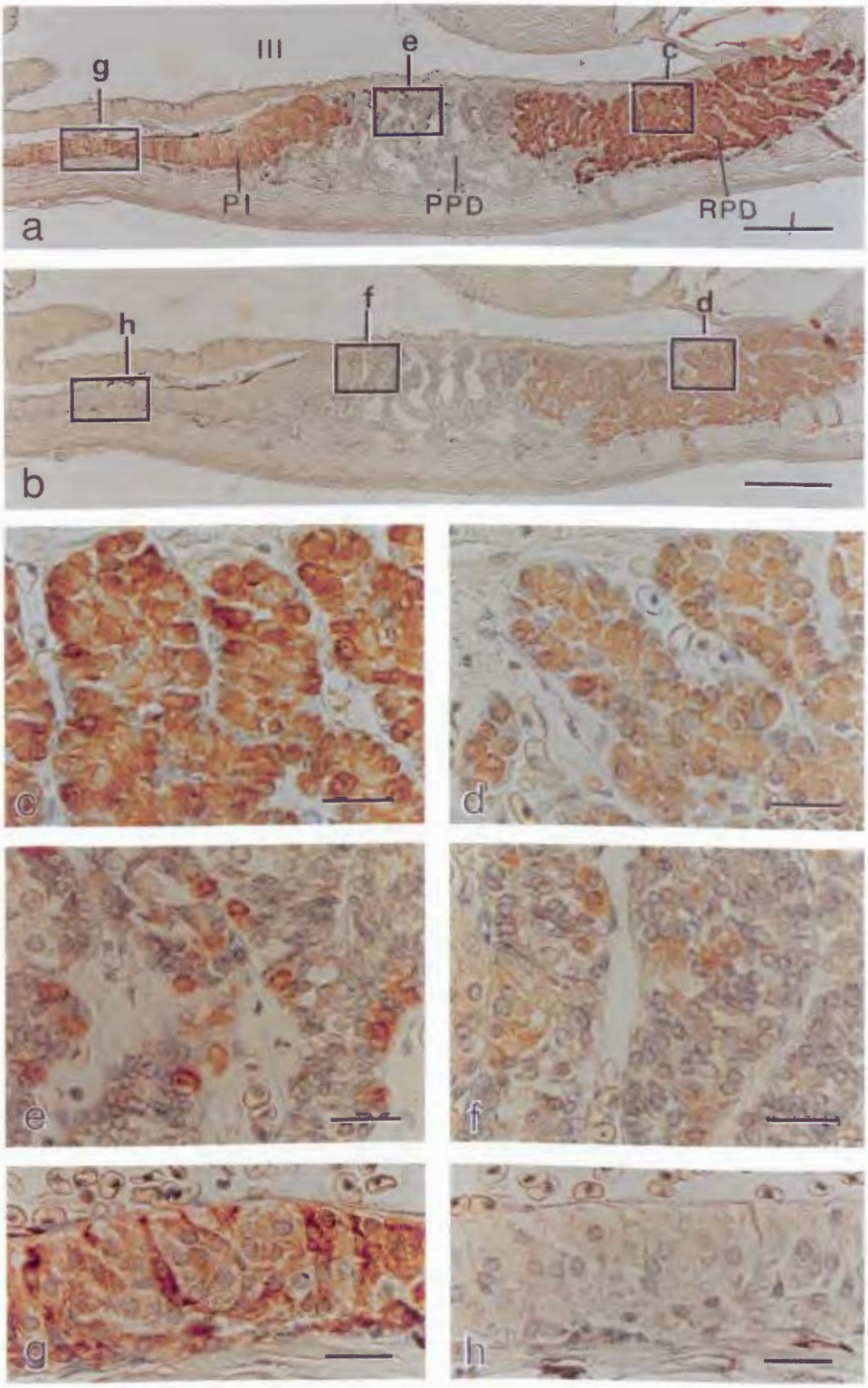
the adult lampreys. These authors suggested that, if there are ACTH cells in the lamprey pituitary, they are the cells reactive to Met-enkephalin/ACTH antisera. The present study confirms the previous immunocytochemical studies, and it is the first to demonstrate the presence of ACTH cells in the lamprey pituitary by use of a homologous immunostaining system.

As shown in Fig. 1, lamprey ACTH, MSH-A, and MSH-B share the same eight amino acid composition/sequences as part of their primary structures. It is likely that the results of the preabsorption tests which showed some crossreactivity of anti-ACTH$^{1-16}$ and anti-MSH-A to related peptides was due to the shared protein structures. The ACTH-immunoreaction in the RPD and PPD was not abolished by the preabsorption with MSH-A or MSH-B, which suggested that an ACTH is present in the RPD and PPD cells, even though anti-ACTH$^{1-16}$ exhibited some crossreactivity to MSH-A. On the other hand, the ACTH-positive reaction in the PI was abolished by preabsorption with MSH-A and MSH-B, which suggests the possibility that the ACTH-positive reaction in the PI may be due to cross reaction of MSH-A and/or MSH-B. It is possible, also, that there is ACTH in the PI. However, at this time, available information does not allow a decision as to whether ACTH occurs in the PI.

MSH-A-like immunoreactivity was found in most cells of the RPD, in a few scattered cells of the PPD, and in almost all cells of the PI. The MSH-A-positive reaction in the RPD and PPD was not abolished by the preabsorption with ACTH$^{1-16}$, but it was abolished by preabsorption with MSH-B. Since MSH-B is not demonstrable in the RPD or PPD, a MSH-A immunoreaction in these regions seems to be specific to MSH-A. The MSH-A reaction in the PI was not abolished by the preabsorption with MSH-B, although this procedure resulted in a major general reduction of staining intensities of the PI cells. This suggests that anti-MSH-A stained both MSH-A and MSH-B in the PI and that MSH-A is clearly present in the PI. Incidentally, our data also showed that preabsorption of anti-MSH-A with MSH-B abolished the positive reaction in the PD, but not in the PI. This indicates that MSH-positive material in the PD has a lower antigenic specificity than that in the PI and, thus, that its immunocytochemical determinants may be different between the PD and PI. Further study is needed to clarify the nature of the difference in MSH-A positive material between the PD and PI. The MSH-B-positive reaction in the PI seems to be specific to MSH-B, since anti-MSH-B showed no crossreactivity to ACTH or MSH-A.

In a previous study, Baker and Buckingham (1983) have reported that extracts of the neurointermediate lobe of *Lampetra fluviatilis* had MSH activity in an *Anolis* skin bioassay, but they were not reactive in an immunoassay using an α-MSH antibody. The found also that the electrophoretic R_f value of lamprey MSH activity was distinct from that of α-MSH. The presence of a MSH-like substance, which is distinct from α-MSH in immunocytochemical determinants, was also reported in the lamprey PI after use of antibodies against mammalian POMC-related peptides (Nozaki and Gorbman, 1984; Dores *et al.*, 1984). The present findings that lamprey MSH-A and MSH-B are distinct from α-MSH, and that they oc-

FIG. 3. (a,b). Two adjacent sagittal sections of the pituitary gland of an adult sea lamprey stained with anti-lamprey ACTH$^{1-16}$ without preabsorption (a) and preabsorbed with synthetic lamprey MSH-A (b), respectively. The anterior end is to the right. The areas outlined by rectangles are enlarged and shown in c–h (c,d, part of rostral pars distalis (RPD); e,f, part of proximal pars distalis (PPD); g,h, part of pars intermedia (PI). (h) An elimination of ACTH-positive immunoreaction following the preabsorption with lamprey MSH-B. III, third ventricle. Scale bars: a,b, 250 μm; c–h, 50 μm.


III
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cur in the lamprey PI, well supports these previous studies.

Recently, Heinig *et al.* (1994) cloned and sequenced a lamprey POMC cDNA which contains the sequence of ACTH, α-MSH, and β-endorphin, but which lacks the sequence of either MSH-A or MSH-B. Subsequently, Takahashi *et al.* (unpublished) have isolated another POMC cDNA which does contain the sequence of MSH-A and MSH-B. From these studies, Takahashi *et al.* (unpublished) cloned two distinct POMC-cDNAs from a lamprey pituitary cDNA library, in which the antisera against lamprey $ACTH^{1-16}$ and MSH-B were used. One POMC encoded ACTH, "nasohypophysial factor" (NHF; Sower *et al.*, 1995) and endorphin and the other POMC encoded MSH-A, MSH-B, and another endorphin. Sower *et al.* (1995) have reported that in adult sea lampreys a newly isolated pituitary protein, NHF, is demonstrable in most cells of the RPD and in a few scattered cells of the PPD, but not in the PI cells. Thus, there are similar distributional patterns between ACTH and NHF in the pituitary of adult sea lampreys. This suggests that the gene encoding ACTH is expressed in the PD, but not in the PI in adult sea lampreys. However, in developing lamprey larvae, the ACTH-gene seems to be expressed in the PI and in the olfactory system as well as the PD, since NHF was found in these regions in the lamprey larvae (Sower *et al.*, 1995). In contrast, the gene encoding MSH-A and MSH-B seems to be expressed in both the PD and PI in adult sea lampreys. Thus, the post-translational proteolytic processing of the precursor protein seems to be different between the two pituitary lobes. One possible explanation based on the presence of MSH-A and the absence of MSH-B in the PD cells is that both MSH-A and MSH-B coexist within the PI cells. Clearly, further studies are needed to delineate the expression of the two POMC genes in the lamprey pituitary and to determine to what extent the POMC peptides may have changed during vertebrate evolution.

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