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MELANOTROPIN AND CORTICOTROPIN ARE ENCODED ON TWO DISTINCT GENES IN THE LAMPREY, THE EARLIEST EVOLVED EXTANT VERTEBRATE*

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SUMMARY: In the lamprey, which is a member of the oldest extant class of vertebrates, the agnathans, melanotropins (MSH) and corticotropin (ACTH) were found to be encoded on two distinct genes. In all other vertebrates, a single precursor gene, proopiomelanocortin (POMC), encodes MSH and ACTH, as well as β-endorphin (END). Two different cDNAs were cloned from a lamprey pituitary λgt11 cDNA library using antisera against lamprey MSH-B and ACTH(1-16). One cDNA encoded MSH-B, MSH-A and β-END, while the other cDNA encoded nasohypophysial factor (NHF), ACTH and a different β-END, but not MSH-A and MSH-B. Northern blot analysis demonstrated that in the adult lamprey pituitary, genes for MSH and ACTH are expressed in the pars intermedia (PI) and pars distalis (PD), respectively.

It has been established that a single POMC gene, encoding ACTH, MSH and β-END, is expressed in two discrete areas in the pituitary gland of vertebrates. ACTH and MSHs are produced by corticotrophs in the PD and melanotrophs in the PI, respectively, through tissue-specific post-translational proteolytic processing (1-5). The MSH sequence repeats three times (α-MSH, β-MSH and γ-MSH) within the POMC gene of tetrapods. α-MSH, corresponding to the N-terminal 13 residues of ACTH, is highly

*The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers: D55628 for sea lamprey proopiocortin cDNA and D55629 for sea lamprey proopiomelanotropin cDNA.

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conserved among species. These data suggest that the gene evolved by intragenic duplication of a primordial MSH gene during the course of early vertebrate evolution (1).

Recently, we identified MSH-A, MSH-B and ACTH from a modern representative of the oldest lineage of vertebrates, the sea lamprey, *Petromyzon marinus*, an agnathan (6). MSH-A, a nonadecapeptide, is free on both ends. MSH-B, an eicosapeptide, is free on the N-terminus and amidated on the C-terminus. The putative MSH sequence of lamprey ACTH is different from α-MSH as well as from lamprey MSH-A and MSH-B (6). The primary structures of lamprey MSH-A and MSH-B exhibit no similarities to any other vertebrate β-MSH and γ-MSH, and thus are not designated as either β-MSH or γ-MSH.

Nozaki *et al.* demonstrated ACTH-like immunoreactivity in cells of the PD and MSH-B-like immunoreactivity in PI cells, which is consistent with the distribution of ACTH and MSHs in all vertebrates (7). These results suggest the existence of two kinds of POMC genes in the lamprey pituitary. Heinig *et al.* cloned cDNA which was amplified from sea lamprey pituitary using polymerase chain reaction (PCR) with primers corresponding to the region conserved among vertebrate growth hormones (8). Sequencing revealed that the cDNA contained the ACTH and β-END sequences, but lacked both the MSH-A and MSH-B sequences (8). In this paper, we report the cloning of a novel cDNA from the adult lamprey pituitary that encodes MSH-A, MSH-B, and a different β-END.

MATERIALS AND METHODS

cDNA cloning. Pituitaries were collected from the mature sea lamprey as described previously (9). Total RNA from the pituitaries was prepared using Isogen (Nippon Gene) and from which poly(A)$^+$ RNA was purified using Oligotex dT30 Super (Takara). cDNA of the poly(A)$^+$ RNA was synthesized using cDNA Synthesis System Plus (Amersham). A cDNA library was constructed using the Complete Rapid Cloning System-λgt11 (Amersham). From 0.05 μg of cDNA and 1 μg of λgt11 arm DNA, a library containing 3.4×10^6 plaques was obtained. Following plaque transfer, Hybond C membrane (Amersham) was immersed in antisera against synthesized lamprey ACTH (1-16) (1:1000) or MSH-B (1:2000) diluted with Tris-buffered saline after blocking with 2% gelatin dissolved in the buffer. The antigen-antibody complex on the membrane was visualized with a Vectastain ABC kit (Vector Laboratories). The cDNA 5'-terminal region was synthesized using a 5'-AmpliFINDER RACE kit (Clontech) or rTth DNA polymerase (Perkin Elmer). The rTth DNA polymerase was used for reverse trascription in the presence of Mn^{2+} and for PCR in the presence of Mg^{2+}.

DNA sequence analysis. The cDNA nucleotide sequence was determined by sequencing both strands according to the dideoxy chain termination method with a DNA sequencer (Applied Biosystems, Model 373S-18) using cDNA subcloned into pUC118/119 plasmid vectors or PCR-amplified cDNA extracted from agarose gel. HIBIO DNASIS, a DNA sequence analysis system (Hitachi),

and HIBIO PROSIS, a protein analysis system (Hitachi), were used for translation and for calculation of sequence identity, respectively.

Northern blot analysis. Total RNA was electrophoresed on a denaturing formamide gel. The RNA was transferred to a nylon membrane (Imobilon-S, Millipore) and the membrane was irradiated with 33,000 $\mu J/cm^2$ at 254 nm (Spectrolinker, Spectronics Co.) to fix the RNA. Denatured double-strand cDNA was labeled using a NEBlot Phototope kit (New England BioLabs). After prehybridization in a sealed plastic bag for one hr at 68°C in a hybridization buffer (Amersham), a cDNA probe was added to the solution and hybridization was performed at 68°C for 12 hr. The membrane was washed twice with 2 × SSC/1% SDS for 5 min at room temperature and then washed again twice with 0.3 × SSC/1% SDS for 15 min at 68°C, and finally brought into contact with X-ray film.

RESULTS

Two kinds of cDNAs encoding either MSH-A and MSH-B or ACTH were cloned from a λgt11 cDNA library of lamprey pituitary using antisera against lamprey MSH-B and ACTH (1-16). Five clones, each MSH-B-positive and ACTH-negative, were obtained from the library and each had a nucleotide sequence of 1802 bp corresponding to base numbers 244-2045 (Fig. 1). However, there was no initiation codon or cluster of hydrophobic amino acids characteristic of a signal sequence. The entire length of the sequence was determined as follows; an AmpliFINDER anchor was connected to the 3' end of a 1st-strand cDNA synthesized with primer A. The primary PCR product was produced using the AmpliFINDER anchor primer and primer B on the connected DNA as a template. The second PCR was performed on the primary PCR product using the AmpliFINDER anchor primer and primer C. After insertion into pUC118 plasmid, a single clone that contained cDNA derived from the 5' region corresponding to base numbers 1-604 was obtained (Fig. 1). This nucleotide sequence was confirmed using PCR-DNA, which was first reverse-transcribed on poly(A)$^+$ RNA using rTth DNA polymerase and primer A, and subsequently amplified using rTth DNA polymerase and primers C and D, and then purified with agarose gel electrophoresis. The resulting cDNA consisted of 2045 bp without the poly(A) tail (Fig. 1). An open reading frame was seen to encode 245 amino acids in which the MSH-B, MSH-A and β-END sequences were located in turn from the N-terminus, but the ACTH sequence was absent. Thus, this cDNA was designated as proopiomelanotropin (POM) cDNA. A signal peptide of 39 amino acids was estimated by the method of von Heijine (10). Although the β-END has not yet been isolated, MSH-A and MSH-B are flanked by basic amino acids and apparently processed further by

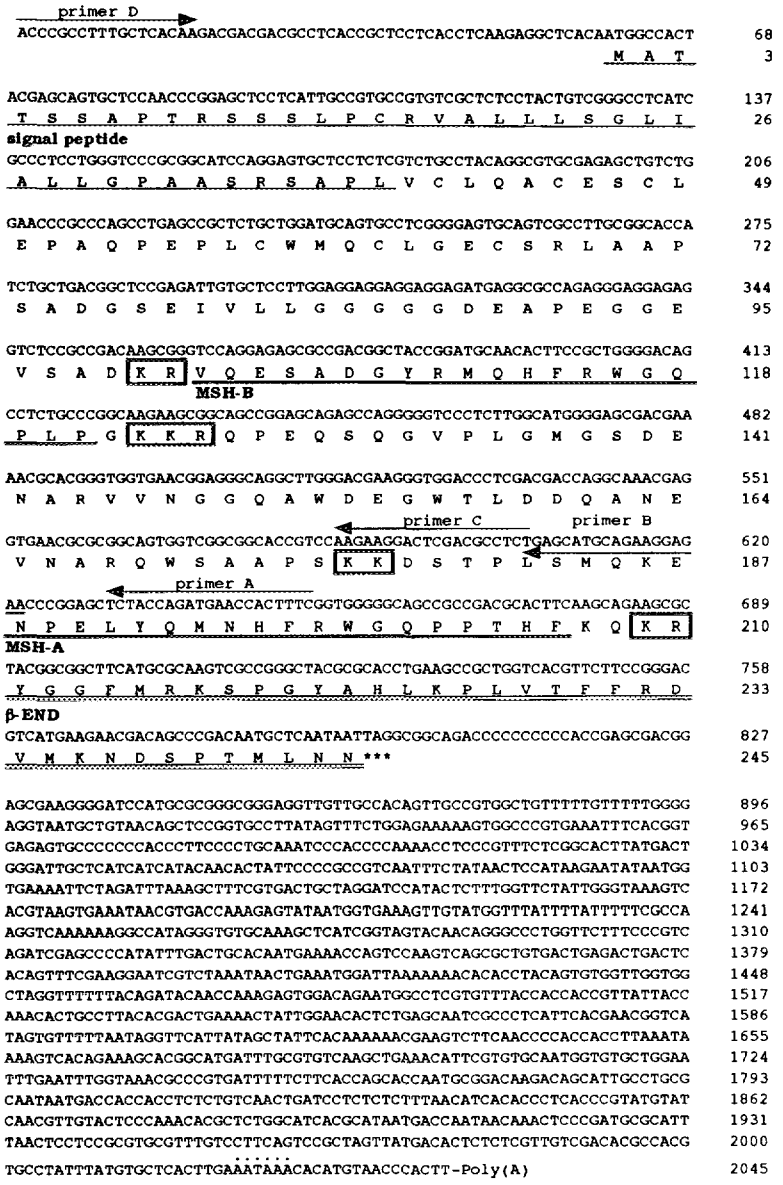

Figure 1. Nucleotide sequence and deduced amino acid sequence of POM cDNA. Number of nucleotide and amino acid sequences are indicated at both sides of line. Predicted amino acid sequence of preprocessed form of POM is numbered by designating first Met as 1. Amino acid sequences corresponding to MSH-A and MSH-B are underlined (bold). Sequence of potentially encoded β-END is indicated by double underline. Possible signal peptide is indicated by underline (thin). Possible cleavage sites are boxed. Polyadenylation signal is indicated by dots. ***: Stop codon.

proteolytic cleavage at the N-terminus of MSH-A and by amidation at the C-terminus of MSH-B.

Three clones, each ACTH-positive and MSH-B-negative, were obtained from the library and showed a nucleotide sequence of 1025 bp without a poly(A)

tail (Fig. 2). The cDNA contained an open reading frame encoding 278 amino acid residues, which was identical to the cDNA characterized by Heinig *et al.* (8). Since the open reading frame contained NHF, ACTH and a different β-END in turn from the N-terminus, but lacked the MSH-B and MSH-A sequences, this cDNA was designated as proopiocortin (POC) cDNA. NHF is a glycopeptide consisting of 121 amino acids (9) and ACTH was found to be a mixture of phosphorylated and non-phosphorylated peptides consisting of both 60 amino acids and 59 amino acids, truncated by one residue at the C-terminus (unpublished). However, peptides corresponding to ACTH (1-22) and β-END have not been isolated from pituitary extract.

Northern blot analysis detected a 2.2 kb POM mRNA in the PI, while a 1.2 kb POC mRNA was found in the PD (Fig. 3). These results, together with

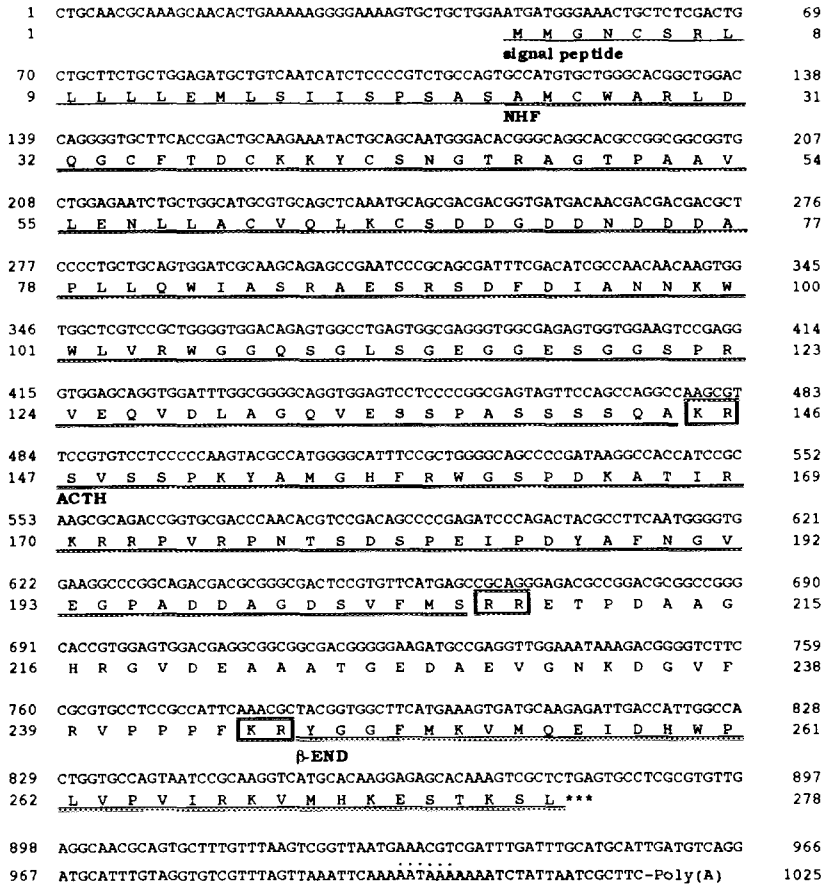

Figure 2. Nucleotide sequence and deduced amino acid sequence of POC cDNA. Amino acid sequences corresponding to NHF and ACTH are underlined (bold). Formats are equal to those in Fig. 1.

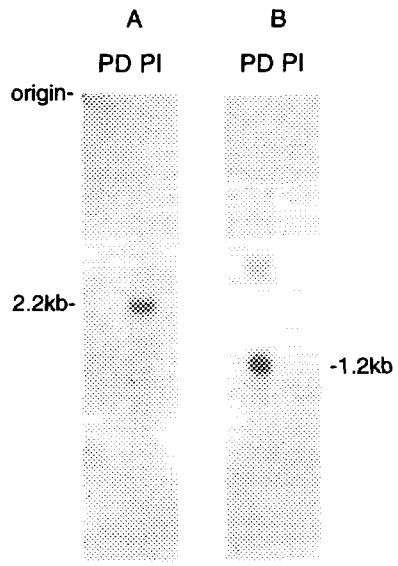

Figure 3. Northern hybridization. Total RNA (10 μg) of each tissue was applied per lane and hybridized with (A) cDNA fragment of POM cDNA (244-840) and (B) cDNA fragment of POC cDNA (224-949). PD: pars distalis. PI: pars intermedia.

the topographical distributions of ACTH- and MSH-immunoreactivity (7), confirm that POM and POC genes are expressed exclusively in the PI and PD, respectively.

DISCUSSION

The present study provides important information on the molecular evolution and functional divergence of the POMC gene. We found that in a modern representative of the earliest evolved extant vertebrate, the lamprey, MSHs and ACTH are encoded on distinct genes, POM and POC, which are expressed in the PI and PD of the adult sea lamprey, respectively. Immunocytochemical analysis showed that topographic distributions of MSH and ACTH in the adult sea lamprey pituitary are similar to those in other vertebrates; MSH is produced in the PI and ACTH in the PD (7). It is therefore highly probable that these lamprey hormones are produced by proteolytic cleavage of tissue specific prohormones from two genes. In contrast to the lamprey, these hormones are produced in other vertebrates by tissue-specific proteolytic cleavage of the same prohormone from a single gene (1-5).

Amino acid sequence of POM shows 32% sequence identity with that of POC as shown in Fig. 4. A striking difference between POM and POC is that

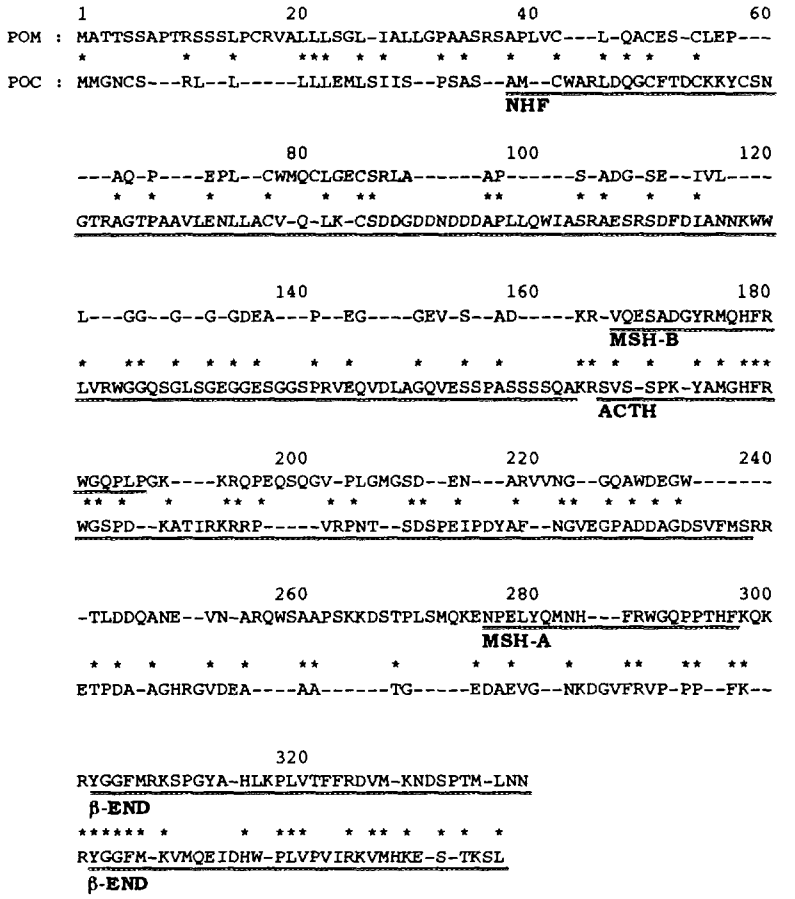

Figure 4. Amino acid sequence comparison between POM and POC. *: Identical amino acid found in two preprohormones. -: gap.

the former has two repeats of the MSH sequence, MSH-B and MSH-A, while the latter contains a single MSH sequence at the N-terminal part of ACTH. Although the N-terminal portion of POM shows little sequence identity with NHF, the locations of β-END sequences in the preprohormones are well conserved with 45% identity and MSH-B can be aligned with ACTH. It is therefore plausible that POM and POC originated from a common ancestral gene by duplication and subsequent divergence under powerful selective pressure for specialization of tissue function. In this context, the conserved β-END in both genes may fulfill a different physiological function in the lamprey.

POM and POC amino acid sequences can be schematically compared with those of bovine POMC (1) and trout POMC (11) (Fig. 5). These preprohormones share common structural features; the Cys-rich segment at

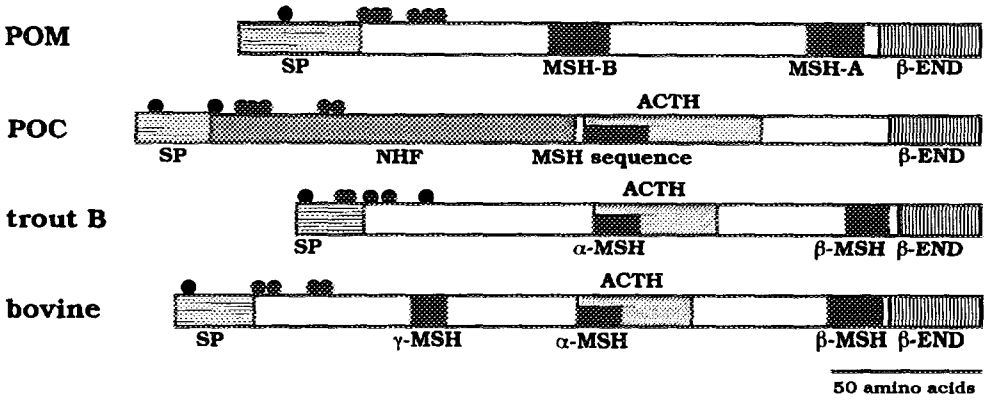

Figure 5. Schematic diagram of POMC family. Closed circles show Cys residues. SP: signal peptide. POMCs of bovine and trout-B are taken from references 1 and 11, respectively.

the N-terminus; the ACTH-MSH middle segment; the β-END segment at the C-terminus. Although γ-MSH is present only in the N-terminal segment of tetrapod POMCs, and the MSH-A counterpart in the middle segment is absent in POC, as described above, the location of MSH-B in POM or ACTH in POC corresponds to that of ACTH in other vertebrate POMCs. Consequently, MSH-A in POM appears to be homologous to β-MSH. It can therefore be assumed that the molecular architecture of at least two MSH repeats (MSH-A and MSH-B) and one β-END might be inherited from a common ancestor, and that the MSH-A homologue in lamprey POC disappeared following gene duplication during the course of lamprey evolution. To obtain a better insight into the evolution of the POMC gene, it is necessary to characterize these genes from the immediate evolutionary antecedents of the vertebrates, such as amphioxus and ascidians.

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