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Promoter activity of sea lamprey *proopiocortin* and *proopiomelanotropin* genes in AtT-20/D16v cells

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Abstract

Adrenocorticotropic hormone (ACTH) and melanophore-stimulating hormone (MSH) are produced in the pars distalis and pars intermedia, respectively, throughout vertebrates. These hormones together with β-endorphin are encoded on a single gene *proopiomelanocortin* (*POMC*) in gnathostomes, but in the sea lamprey, an agnathan, ACTH and MSH are encoded on two separate genes, *proopiocortin* (*POC*) and *proopiomelanotropin* (*POM*), respectively. Moreover, the nucleotide sequences of 5′-flanking regions of the *POC* and *POM* genes are significantly different from each other. To investigate the potential promoter activities of the *POC* and *POM* genes, we constructed promoter reporter plasmids by fusing the 5′ flanking sequences (nucleotides −1151 to +31 and −2510 to +51, respectively) to a firefly luciferase gene. Transient transfection studies in AtT-20/D16v cells, which derived from a mouse pituitary tumor cell line, revealed that the 5′-flanking sequence of the *POC* gene did not exhibit promoter activity, whereas that of the *POM* gene showed the activity at high levels nearly equivalent to SV40 promoter. Analysis of a series of the 5′-deleted reporter for the *POM* gene in the AtT-20/D16v cells demonstrated that the 422 bp 5′-flanking sequence was sufficient for promoter activity, while the sequence from −853 to −574 may contain negatively acting regulatory elements. Because the *POC* and *POM* genes are supposed to have differentiated from a common ancestor, during evolution, the *POC* gene may lack essential element(s) for expression in the AtT-20/D16v cells.

Keywords: Adrenocorticotropic hormone; Agnathan; AtT-20/D16v cell; Melanophore-stimulating hormone; Promoter activity; Proopiocortin; Proopiomelanocortin; Proopiomelanotropin; Sea lamprey

1. Introduction

Proopiomelanocortin (POMC) is the precursor of adrenocorticotropic hormone (ACTH), melanophore-stimulating hormones (MSHs), β-endorphin (β-END), and other biologically active peptides. The *POMC* gene is expressed primarily in both the pars distalis (PD) and the pars intermedia (PI) of the pituitary gland in gnathostomes (Takahashi and Kawauchi, 2005). The expression of the POMC gene in the PD and PI is differentially regulated by a hypothalamic peptide and glucocorticoid (Drouin et al., 1987; Gagner and Drouin, 1987). Subsequently, the translated POMC undergoes tissue-specific posttranslational processing in which different final

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products are generated in the PD and PI; ACTH, γ-lipotropin, pro-γ-MSH, and β-END in the PD, and α-MSH, β-MSH, γ-MSH, and N-acetylated-β-END in the PI (Castro and Morrison, 1997; Smith and Funder, 1988).

Cephalaspidomorphi (lampreys) is one of the two representatives of the oldest lineage of vertebrates, the Agnatha (Nelson, 1994). The pituitary of the sea lamprey has dual genes in the POMC family, *proopiocortin* (*POC*) and *proopiomelanotropin* (*POM*) expressed in the PD and PI, respectively (Heinig et al., 1995; Nozaki et al., 1995; Takahashi et al., 1995). This duality differs from gnathostomes in which a common *POMC* gene is expressed in both pituitary lobes (Castro and Morrison, 1997; Smith and Funder, 1988). The *POC* and *POM* genes are similar to each other in that they have two introns and that exon 3 of both genes encodes all melanocortins and β-END segments (Takahashi et al., 2005). These characteristics are also the same as those in gnathostome *POMC* genes (Cochet et al., 1982; Deen et al., 1992; Drouin et al., 1985; Gonzalez-Nunez et al., 2003; Hansen et al., 2003; Nakanishi et al., 1981; Notake et al., 1983; Takeuchi et al., 1999). However, the *POC* and *POM* genes differ from each other in that they encode different hormones; the former encodes ACTH and β-END, whereas the latter encodes two MSHs, MSH-A, and MSH-B, and β-END with a different sequence (Heinig et al., 1995; Takahashi et al., 1995). Thus, the *POC* and *POM* genes are thought to have evolved from a common ancestor in concert with the functional differentiation of the pituitary into the PD and PI during evolution in lampreys (Takahashi and Kawauchi, 2005; Takahashi et al., 2001).

Transcription of the *POMC* gene depends on the synergistic interaction of several regulatory elements which bind different nuclear proteins (Lamolet et al., 2001; Lamonerie et al., 1996; Therrien and Drouin, 1991). Recently, in sea lamprey, we determined and compared the nucleotide sequence of 5′-flanking regions of the *POC* and *POM* genes where some putative regulatory elements were present (Takahashi et al., 2005). The *POC* gene contained a TATA box, a CCAAT box, E boxes, signal transducer and activator of transcription response element (STAT), retinoic acid-inducible element (RAIE), and binding sites for pituitary homeobox 1 (Ptx1), pituitary cell-restricted T box factor (Tpit), and pituitary transcription factor-1 (Pit-1). The *POM* gene contained four TATA boxes, E boxes, STAT3, RAIE, cyclic AMP response element-like element (CRE)-like sequence and a binding site for Pit-1. However, virtually no similarity was observed in the distribution of the elements between the two genes, indicating that transcription of these genes is regulated in a distinct manner. Indeed, *POC* and *POM* are differentially expressed during the lamprey life cycle (Ficele et al., 1998; Heinig et al., 1999). The present study investigated the promoter activity of the *POC* and *POM* genes, using promoter–reporter plasmid constructs prepared by fusing the promoter regions of these genes to a plasmid encoding firefly luciferase. Because the *POC* and *POM* genes are members of the POMC family, the constructs were transiently transfected into AtT-20/D16v cells derived from a mouse pituitary tumor cell line preferentially producing POMC-related peptides.

2. Materials and methods

2.1. Promoter–reporter plasmid constructs

The genomic clone POC-5 encoding the *POC* gene −1153 to +481 was prepared as described previously (Takahashi et al., 2005), from which ten DNA fragments were generated using primer sets as follows: [clone POC-181: 5′-GGGGTACCTATCACTTTCGGAAATTGAGT-3′ (primer CF-181) and 5′-GGGCTAGCTCAGTGTTGCTTTGCGTTGCA-3′ (primer CR31); clone POC-277: 5′-GGGGTACCCAGTTACAACAGCTACGCTAC-3′ (primer CF-277) and primer CR31; clone POC-401: 5′-GGGGTACCGGAGGTGCAGCTCTGGTGGCT-3′ (primer CF-401) and primer CR31; clone POC-553: 5′-GGGGTACCTAATTGTCGAAGTTTGGTTCA-3′ (primer CF-553) and primer CR31; clone POC-705: 5′-GGGGTACCCAATGGCTGAATTTTCGATGC-3′ (primer CF-705) and primer CR31; clone POC-793: 5′-GGGGTACCTGTCATGAGCCTCTAACCTTG-3′ (primer CF-793) and primer CR31; clone POC-953: 5′-GGGGTACCGTTGCAGACAACTTACCAGGT-3′ (primer CF-953) and primer CR31; clone POC-1053: 5′-GGGGTACCTGACGTGAAAGCAGAAGTTAC-3′ (primer CF-1053) and primer CR31; and clone POC-1153a: 5′-GGGGTACCAAGCTTTGGGTAGCAGTCTAG-3′ (primer CF-1153) and primer CR31; and clone POC-1153b: primer CF-1153 and 5′-GGGCTAGCTGACTGCATTCGACAATCCGT-3′ (primer CR-604)].

The genomic clone POM-5 encoding *POM* gene −2760 to +51 was prepared as described previously (Takahashi et al., 2005), from which 11 DNA fragments were generated using primer sets as follows: [clone POM-141: 5′-GGGGTACCCGTGATGACGTGGAACAACTC-3′ (primer MF-141) and 5′-GGGCTAGCTCTTGAGGTGAGGAGCGGTGA-3′ (primer MR51); clone POM-288: 5′-GGGGTACCTGAGACACGGCCGCCTCGGAG-3′ (primer MF-288) and primer MR51; clone POM-422: 5′-GGGGTACCGCAACTCGCGTGTTCATAGTC-3′ (primer MF-422) and primer MR51; clone POM-573: 5′-GGGGTACCAGTCCATGGTGGTCGTTTCAT-3′ (primer MF-573) and primer MR51; clone POM-853: 5′-GGGGTACCGAAACACGGATCACATCGATA-3′ (primer MF-853) and primer MR51; clone POM-1002: 5′-GGGGTACCCACGGTCCAATTCACATTATC-3′ (primer MF-1002)

and primer MR51; clone POM-1162: 5′-GGGGTACCC GAATATCAGTGACTGTCATC-3′ (primer MF-1162) and primer MR51; clone POM-1290: 5′-GGGGTAC CAGCATCTTCATCACCGACGAG-3′ (primer MF-1290) and primer MR51; clone POM-1482: 5′-GGGGT ACCTATAGCCCTTGTGTGAGCCCT-3′ (primer MF-1482) and primer MR51; clone POM-2004: 5′-GGGG TACCGCGATGGATGTATGTTGAGCT-3′ (primer MF-2004) and primer MR51; and clone POM-2510: 5′-GGGGTACCCAATCGTGCAATAACGTGTTG-3′ (primer MF-2510) and primer MR51].

PCR was done using LA *Taq* with GC buffer (Takara, Tokyo, Japan) with heating of the reaction mixture at 94 °C for 2 min, and then 35 cycles of denaturation (30 s at 96 °C)–annealing (1 min at 55 °C)–extension (1.5 min at 72 °C), followed by a final extension at 72 °C for 5 min. The amplified DNAs were subcloned into *Kpn*I and *Nhe*I sites of a promoterless luciferase expression vector, pGL3-Basic vector (Promega, Madison, WI), to prepare pPOC-181/luc, pPOC-277/luc, pPOC-401/luc, pPOC-553 /luc, pPOC-705/luc, pPOC-793/luc, pPOC-953/luc, pPO C-1053/luc, pPOC-1153a/luci, pPOM-141/luci, pPOM-288/luci, pPOM-422/luci, pPOM-573/luci, pPOM-853/ luci, pPOM-1002/luci, pPOM-1162/luci, pPOM-1290/ luci, pPOM-1482/luci, pPOM-2004/luci, and pPOM-2510/luci (Fig. 1). A clone POC-1153b was inserted into the pGL3-Promoter vector to prepare pPOC-1153b/P-luci. All plasmids were confirmed by sequence analyses using the Dye Terminator Cycle Sequencing Ready Reaction Kit and 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA).

Primers described above were extended with *Kpn*I, GGTACC, or *Nhe*I, GCTAGC, sites at the 5′ end as indicated by the underlines. The numbers indicate 5′ ends of primers excluding restriction sites, which correspond to positions on the *POC* and *POM* genes.

2.2. DNA transfection and luciferase assay

AtT-20/D16v cells were maintained in Dulbecco's modified Eagle's medium with high glucose containing 10% fetal bovine serum supplemented with penicillin (15.5 μg/ml) at 37 °C with 5% CO_2. The cells were plated at 1.0×10^5 cells per well of a 24-well plate, and after 24 h

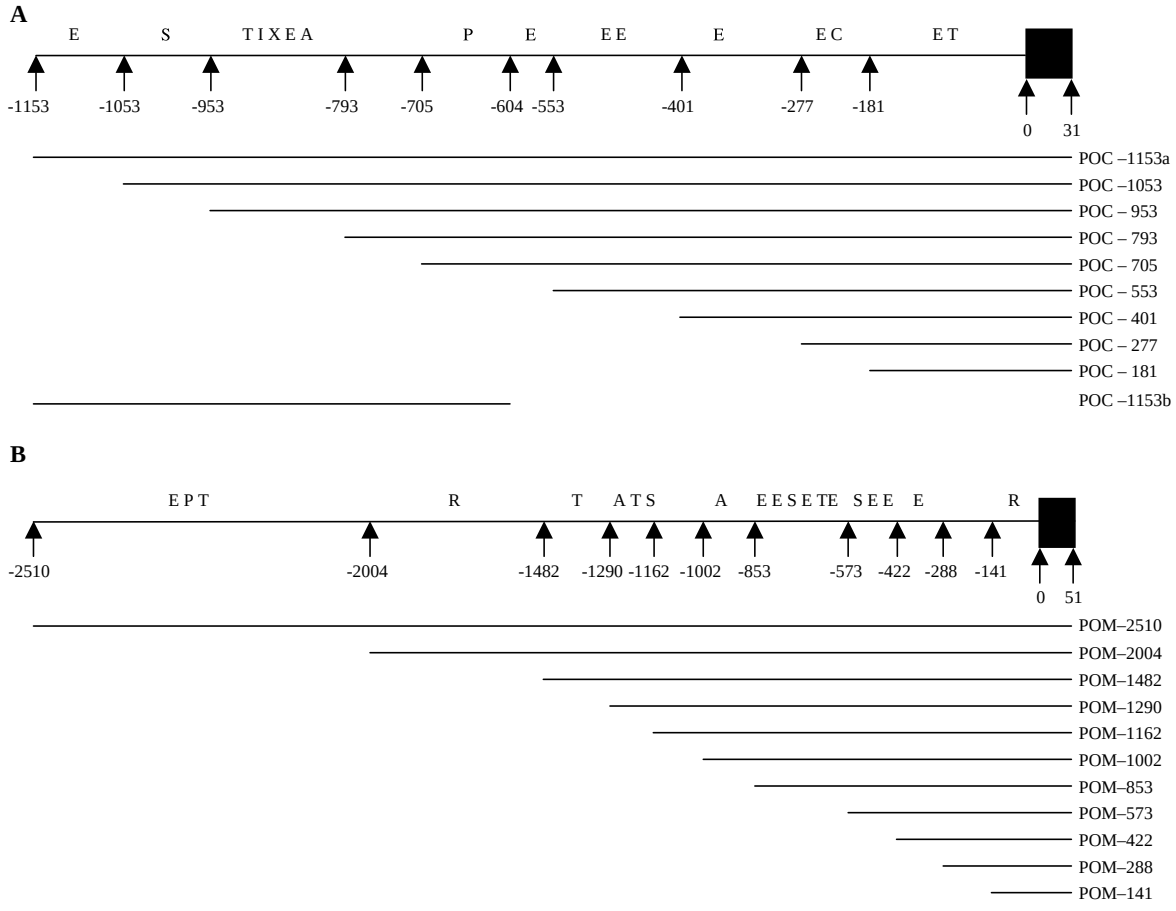

Fig. 1. Schematic diagrams for promoter regions of the *POC* gene (A) and *POM* gene (B) together with relative positions of putative regulatory elements and of DNA clones inserted into the pGL3-Basic vector excluding the clone POC-1153b, which was inserted into a pGL3-Promoter vector. Arrows show positions on the *POC* or *POM* gene corresponding to 5′ or 3′ends of clones excluding "0". C: CAAT box, E: E box, I: Tpit, A: RAIE, P: Pit1, R: CRE-like, S: STAT, T: TATA box, and X: Ptx1.

cells were transfected with 1.14 μg of a firefly luciferase promoter–reporter plasmid and 60 ng of a *Renilla* luciferase internal control plasmid, pRL-TK (Promega), using a combination of opti-MEM (Invitrogen, Carlsbad, CA) and FuGENE6 (Roche Diagnostics, Indianapolis, IN). After 48 h, the luciferase activity of triplicate samples was measured with a Luminescensor-PSN-R (Atto, Tokyo, Japan) after treatment of the cells with the Dual-Luciferase Reporter Assay System (Promega).

3. Results

No promoter activity was exhibited by the plasmid-reporter constructs prepared for the *POC* gene, pPOC-181/luc, pPOC-277/luc, pPOC-401/luc, pPOC-553/luc, pPOC-705/luc, pPOC-793/luc, pPOC-953/luc, pPOC-1053/luc, and pPOC-1153a/luci, in AtT-20 cells (Fig. 2A). pPOC-1153b/P-luci containing *POC* (−1153 to −604) was also examined to exclude possible negative effects in the vicinity of the transcription initiation site. As a result, no increases in activity were observed compared to the control, pGL3-Promoter vector (Fig. 2B).

In contrast to the *POC* gene, all plasmid-reporter constructs prepared for the *POM* gene, pPOM-141/luci, pPOM-288/luci, pPOM-422/luci, pPOM-573/luci, pPOM-853/luci, pPOM-1002/luci, pPOM-1162/luci, pPOM-1290/luci, pPOM-1482/luci, pPOM-2004/luci, and pPOM-2510/luci, showed higher levels of promoter activity than the promoterless control, pGL3-Basic vector (Fig. 2C). The strongest activity was observed for pPOM-422/luci. pPOM-141/luci and pPOM-853/luci showed less activity than the promoter-containing control, pGL3-Promoter vector.

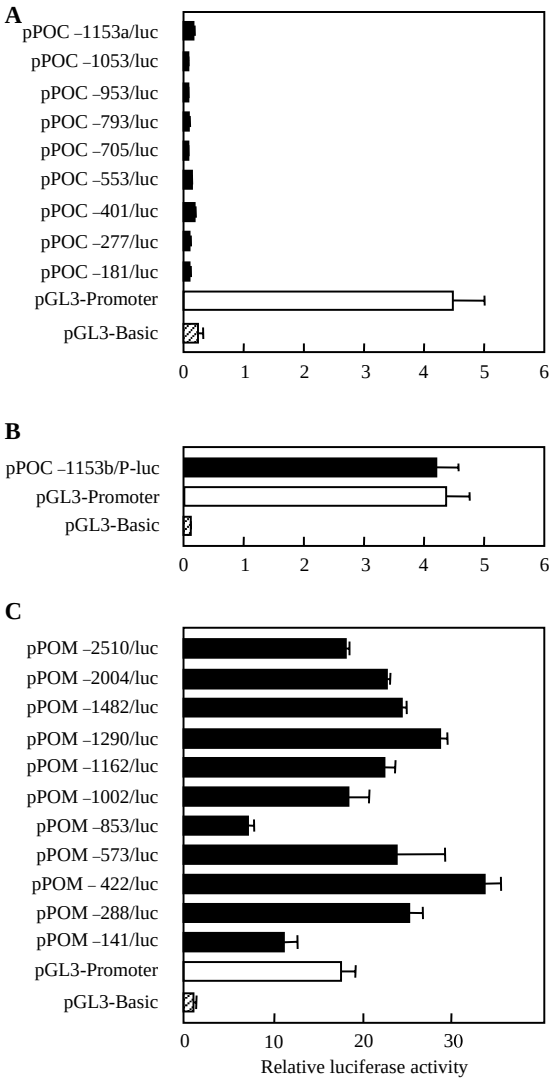

Fig. 2. Promoter activity of the *POC* and *POM* genes in AtT-20/D16v cells. (A) Activity of the *POC* gene inserted into pGL3-Basic vector. (B) Activity of the *POC* gene inserted into pGL3-Promotor vector. (C) Activity of the *POM* gene inserted into pGL3-Basic vector. Data represent the means ± SEM of triplicate assays.

4. Discussion

A remarkable difference between the *POC* and *POM* genes in the AtT-20/D16 cells was that the reporter-plasmid constructs for the *POC* gene exhibited virtually no promoter activity, whereas those for the *POM* gene exhibited substantial activity. The absence of activity in the construct for the *POC* gene suggests the presence of negatively active elements in exon 1 or a proximal region of the transcription initiation site. This working hypothesis led us to examine the activity using a plasmid-reporter construct without *POC* (−603 to 51). However, the construct, pPOC-1153b/P-luci, showed no increase in promoter activity compared to the pGL3-Promoter vector. These results reveal at least two essential properties of the *POC* gene promoter. First, the transcription of the sea lamprey *POC* gene is not initiated by machinery in the AtT-20/D16v cells. Second, regulatory elements in the *POC* gene promoter are not associated with the regulation of promoter activity of the pGL3-Promoter vector in the AtT-20/D16v cells. These properties may result from weak interaction between regulatory elements in the *POC* promoter region and transcription factors in the AtT-20/D16v cells.

Ptx1 and Tpit have been shown to regulate the transcription of the *POMC* gene in a cell-specific manner in mammals (Lamolet et al., 2001; Lamonerie et al., 1996). We recently found several putative regulatory elements including binding sites for Ptx1 and Tpit together with a TATA box proximate to the transcription initiation site in the promoter region of the *POC* gene (Takahashi et al., 2005). In contrast, the promoter region of the *POM* gene has neither Ptx1, Tpit nor a proximal TATA box. Based on these characteristics, the promoter activity of the *POC* gene was expected to be greater than that of the *POM* gene in the AtT-20/D16

cells. However, our results provided the opposite result. The *POC* and *POM* genes are supposed to have appeared from a common ancestor by gene duplication (Takahashi and Kawauchi, 2005; Takahashi et al., 2001). It is plausible that some elements in the promoter region of the ancestral gene may have been deleted with the functional differentiation of the two genes after the duplication event. The absence of promoter activity in the *POC* gene suggests that the *POC* gene may lack some essential element(s) for expression in the AtT-20/D16v cells.

The promoter activity of pPOM-141/luci was less than half of that of pPOM-288/luci, pPOM-422/luci, or pPOM-573/luci, suggesting the presence of the core promoter within *POM* (−141 to 51). The activity of the putative core promoter may be positively regulated by the elements within *POM* (−573 to −142), suggesting the contribution of STAT and E box. pPOM-853/luci showed the lowest level of activity among the eleven constructs for the *POM* gene, suggesting that STAT and the E box within *POM* (−853 to −574) are associated with the negative regulation. The promoter activity of pPOM-1002/luci was two-fold greater than that of pPOM-853/luci, suggesting the participation of RAIE within *POM* (−1002 to −854) in enhancing the activity. Other reporter-plasmid constructs, pPOM-1162/luci, pPOM-1290/luci, pPOM-1482/luci, pPOM-2004/luci, and pPOM-2510, showed comparable activity to pPOM-1002/luci, suggesting that STAT, the TATA box, the CRE-like sequence, Pit-1, and the E box within *POM* (−2510 to −1003) are responsible for positive regulation. The variation in promoter activity and the distribution of several kinds of elements suggest that the expression of the *POM* gene is regulated synergistically by the interactions of multiple transcription factors as in the case of mammals (Therrien and Drouin, 1991).

In conclusion, we evaluated the promoter activity of sea lamprey *POC* and *POM* genes in AtT-20/D16v cells. No promoter activity for the 5′-flanking region of *POC* observed in the present study suggests that the gene may lack essential elements to function in the AtT-20/D16v cells during lamprey evolution. The promoter activity of the *POM* gene is thought to be regulated by several transcription factors.

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