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Analyzing the radiation of the melanocortins in amphibians: Cloning of POMC cDNAs from the pituitary of the urodele amphibians, *Amphiuma means* and *Necturus maculosus*

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Abstract

Proopiomelanocortin (POMC) cDNAs were cloned and sequenced from brain extracts of two species of urodele amphibians: *Amphiuma means* and *Necturus maculosus*. Although the two species of urodele amphibians belong to separate families, and do not share a direct common ancestor, the level of primary sequence identity for the open reading of the POMC cDNAs was 90% at the amino acid level and 79% at the nucleotide level. It appears that the POMC gene in these urodele amphibians has been accumulating mutations at the amino acid level at a slower rate than the POMC gene in other sarcopterygian orders.
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1. Introduction

A unifying feature of the organization of the proopiomelanocortin (POMC) gene among mammals is the presence of the melanocortin core motif, His-Phe-Arg-Trp, in the sequences of ACTH/α-MSH, β-MSH, and γ-MSH; for review see [21]. Of the four melanocortins derived from mammalian POMC, the posttranslational processing of γ-MSH can result in the generation of two end products that differ in amino acid length. In all mammalian POMC genes that have been analyzed to date, the residues, Arg-Lys, located at the N-terminal of γ-MSH serve as a functional monobasic cleavage site. C-terminal to this melanocortin sequence there is a paired basic amino acid proteolytic cleavage site. Posttranslational processing at these sites yields $Lys^1\gamma_3$-MSH as an end-product [9,10]. In some species, these cleavage events would generate a 25-amino acid polypeptide as the $Lys^1\gamma_3$-MSH end product as seen, for example, in the intermediate pituitary of the mouse or rat [9,10]. However, within the primary sequences of bovine and human $Lys^1\gamma_3$-MSH, there is an internal paired basic amino acid proteolytic cleavage site that upon posttranslational processing can yield an amidated 12-amino acid polypeptide ($Lys^1\gamma_1$-MSH-NH_2) end-product [12,31].

Although γ-MSH and the other melanocortins are assumed to be present in all sarcopterygian POMC sequences, the γ_3-MSH sequence in lungfish POMC [5,17,28] is considerably shorter in amino acid length than the γ_3-MSH sequence present in either anuran amphibian POMC sequences [2,26,28,30,32], or mammalian POMC sequences [21]. This observation raises the possibility that the lungfish γ-MSH sequence may represent the ancestral condition for the Sarcopterygii. Given the proximity of lungfishes and amphibians on the vertebrate phylogenetic tree, the examination of an extant amphibian group other than the anuran amphibians may reveal the transition to the longer form of γ-MSH found in the tetrapod POMC sequences.

Amphibians first appeared in the fossil record approximately 380 million years ago during the upper Devonian

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period and evolved from lobe finned fish (rhipidistian) ancestors [11]. The extant orders of amphibians (i.e., Anura, Urodela, and Gymbophiona) trace their origins to the Jurassic period some 180 million years later [11]. Order Anura includes frogs and toads, order Urodela includes salamanders and newts, and order Gymbophiona is represented by the limbless extant amphibians [22]. There is consensus that the three orders of amphibians had a monophyletic origin and it is generally assumed that urodele amphibians occupy a phylogenetic position between the anuran amphibians and the extant lobe-finned fish represented today by the lungfishes and the coelacanth [11,22].

Previous studies of anuran POMC indicated that the γ-MSH sequence in these amphibians is similar in length to mammalian POMC sequences. However, there have been no studies done on the organization of POMC in urodele amphibians. The objective of this study was to clone and sequence POMC cDNAs from the pituitary of two urodele amphibians, *Amphiuma means* and *Necturus maculosus*, and to test the hypothesis that the γ-MSH sequence in these urodeles would have features more in common with the γ-MSH sequence of extant lobe-finned fish than with the γ-MSH sequence of anuran amphibians or amniote vertebrates.

2. Methods

Adult male and female *A. means* and *N. maculosus* were obtained from Carolina Biological Supply (Burlington, NC) and kept in a free-running freshwater aquarium at 25 °C. Animals were anesthetized using MS-222 (Sigma, St. Louis, MO) and sacrificed by decapitation. Brains were isolated, flash frozen in liquid nitrogen, and stored at −70 °C until needed.

Polyadenylated mRNA was isolated from brain tissue by direct capture onto oligo dT_{25} paramagnetic beads (Novagen, Madison, WI) using the procedure of Jakobsen et al. [27]. Approximately, 50 mg of brain tissue per amphibian species were used for each mRNA extraction. First-strand cDNA synthesis employed Superscript II reverse transcriptase (Gibco BRL, Grand Island, NY), which lacks RNAse H activity to ensure the synthesis of full-length transcripts. All first-strand cDNAs were primed off the poly-A tail with Anchor dT_{17} primer (5′GACTCGAGTCGGATCCATCGATTTTTTTTTTTTTTTTT3′). Lastly, a homopolymeric guanine tail was added to the 3′end of all first-strand cDNAs using terminal deoxynucleotidyl transferase (Gibco, Gaithersburg, MD).

A 3′RACE procedure [16] was used to amplify the 3′end of *A. means* and *N. maculosus* POMC cDNAs. The degenerate forward primer was the opioid primer, POMC 1024 [5′AA[A/G][A/C]GITA[C/T]GGIGGITT[C/T]ATG3′]; see [13]. In the sequence of the POMC 1024 primer, brackets contain mixed bases and the "I" represents deoxyinosine which was used at positions of four-fold degeneracy to minimize base pair mismatch associated with instability [29]. The reverse primer, Anchor (5′GACTCGAGTCGGATCCATCGAT3′) was targeted to the synthetic sequence incorporated by the dT_{17} primer used for first-strand synthesis. The thermal profile for the 3′RACE PCR reaction was: initial denaturation at 94 °C for 5 min, then 32 cycles of denaturation (94 °C for 1 min), annealing (7 cycles at 56 °C; 27 cycles at 58 °C for 1.5 min), and extension (72 °C for 2.5 min).

Following isolation and characterization of the 3′RACE products, these sequences were used as templates to design gene-specific reverse primers for the 5′RACE procedure. For the *A. means* POMC cDNA, the gene specific reverse primer was AmpPOMCR1 (5′CCTTGATGGGCCTCCTCTTCC3′). This reverse primer was used in conjunction with the forward primer, DC10 (5′GAATTCGCGGCCGCTTCAGTCCCCCCCCCC3′) which was targeted to the synthetic poly G tail added during first-strand synthesis. The thermal profile amplifying the remainder of the *A. means* POMC cDNA by 5′RACE was: initial denaturation at 94 °C for 5 min, then 32 cycles of denaturation (94 °C for 1 min), annealing (3 cycles at 62 °C; 4 cycles at 60 °C; 25 cycles at 58 °C for 1.5 min), and extension (72 °C for 2.5 min). A similar approach was used to amplify the 5′end of the *N. maculosus* POMC cDNA. The gene specific reverse primer was NecPOMC-R2 (5′GTGGTCAGGGTAACTGGATT3′) and the 5′RACE thermal profile was initial denaturation at 94 °C for 5 min, then 32 cycles of denaturation (94 °C for 1 min), annealing (55 °C for 1 min), and extension (72 °C for 3 min).

The 3′RACE and 5′RACE products for both species were separately subcloned and prepared for automated DNA sequence analysis as described previously [14,18]. Multiple subclones representing *A. means* 3′RACE POMC products (three clones) and 5′RACE POMC products (two clones), and multiple *N. maculosus* 3′RACE POMC products (three clones) and 5′RACE POMC products (six clones) were separately sequenced using a Beckman-Coulter CEQ 8000 autosequencer as described in Bagrosky et al. [9]. To minimize errors due to nucleotide misincorporation by *Taq* DNA polymerase, the full-length sequences represent a consensus of multiple overlapping clones. Sequences were analyzed for similarity to known genes using the BLAST algorithm [3].

Maximum parsimony analysis was done using PAUP 4.3 [35]. An exhaustive analysis was performed on the amino acid sequences of POMC from the following species: *A. means* (Accession no. AY141896), *N. maculosusus* (Accession no. AY141899), *Bombina orientalis* (Accession no. AY692246), *Spea multiplicatus* [28], *Bufo marinus* [2], *Rana ribidunda* [26], *Neoceratodus forsteri* [17], and *Protopterus annectens* [5,28]. The Port Jackson shark, *Heterodontus portusjacksoni* POMC sequence [19] was used as the outgroup sequence. Sequences were aligned as described in Dores et al. [15]. Bootstrap analysis was done for 1000 replicates.

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