# Changes of Pituitary Proopiomelanocortin mRNA Levels during Metamorphosis of the Bullfrog Larvae

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# Changes of Pituitary Proopiomelanocortin mRNA Levels during Metamorphosis of the Bullfrog Larvae

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**ABSTRACT**—Adrenal corticoids accelerate metamorphosis of amphibians by potentiating the action of thyroid hormone. Adrenal corticoid secretion is considered to be controlled mainly by adrenocorticotropic hormone generated from proopiomelanocortin (POMC) in the anterior lobe of the pituitary. In order to assess the changes in POMC mRNA levels during metamorphosis, a cDNA for POMC was isolated from a cDNA library constructed from bullfrog (*Rana catesbeiana*) pituitary polyadenylated RNA. Northern blot analysis using the POMC cDNA as a probe revealed that POMC mRNA levels in the anterior lobe were relatively low during premetamorphosis, rose during prometamorphosis, reached the maximum at the end of prometamorphosis and remained very high during climax. The POMC mRNA levels of the intermediate lobe, where α-melanophore-stimulating hormone is generated from POMC, were also determined in metamorphosing tadpoles. The POMC mRNA levels of the intermediate lobe increased as metamorphosis progressed and were maximal at mid-climax. High POMC mRNA levels were observed even in larvae that had adapted to a white background. The significance of these findings and their relationships to the hormonal requirements during metamorphosis are discussed.

## INTRODUCTION

It is well known that adrenal corticoids administered together with thyroid hormone accelerate metamorphosis of amphibian larvae (see review by Kikuyama *et al.*, 1993), presumably by increasing the conversion of thyroxine ($T_4$) to the more potent triiodothyronine ($T_3$) (Galton, 1990) and by augmenting the nuclear binding capacity for $T_3$ (Suzuki and Kikuyama, 1983; Gray and Janssens, 1990) through the elevation of thyroid hormone receptor β mRNA levels (Iwamuro and Tata, 1995). We have demonstrated that endogenous corticoids are involved in metamorphosis (Kikuyama *et al.*, 1982). According to Carstensen *et al.*, (1961) and Macchi and Phillip (1966), corticosterone and aldosterone are the major corticoids secreted by the interrenals of amphibians. In fact, both these corticoids are potent stimulators of thyroid hormone-induced tail segment resorption *in vitro* (Kikuyama *et al.*, 1983). The plasma levels of corticosterone and aldosterone are known to be elevated markedly during metamorphic climax (Jaffe, 1981; Kikuyama *et al.*, 1986) and this elevation synchronizes well with that of thyroid hormone levels (Miyauchi *et al.*, 1977; Mondou and Kaltenbach, 1979; Regard *et al.*, 1978; Suzuki and Suzuki, 1981).

As in other animals, adrenocorticotropic hormone (ACTH) is considered to be a major hormone that stimulates corticoid secretion in amphibians. The multifunctional precursor protein proopiomelanocortin (POMC) is expressed in corticotrophs of the anterior lobe of the pituitary and in melanotrophs of the intermediate lobe (Chrétien *et al.*, 1979). POMC generates several bioactive peptides, including ACTH, through tissue-specific processing. In anuran pituitaries, the presence of processing enzymes (PC1 and PC2) has recently been demonstrated immunohistochemically (Kurabuchi and Tanaka, 1997). The aim of this study was to assess the POMC mRNA levels during bullfrog (*Rana catesbeiana*) metamorphosis in order to further our understanding of the participation of the pituitary-adrenal axis in amphibian metamorphosis.

## MATERIALS AND METHODS

### Animals

Bullfrog tadpoles at various developmental stages (Taylor and Kollros, 1946) were captured in the field, kept in gray-colored containers for 5 days under laboratory conditions and were illuminated for 12 hr, from 8 am to 8 pm, each day. Premetamorphic (stage XII), prometamorphic (stages XVII and XIX) and climactic (stages XX, XXII

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and XXIV) tadpoles were sacrificed by decapitation and the anterior and neurointermediate lobes of each pituitary were frozen separately in liquid nitrogen and stored at –80°C until the RNA was extracted. One group of tadpoles was kept in a white container and another group of tadpoles was kept in a black container under constant illumination for 5 days. They were sacrificed at stage XXII and their pituitaries were treated as described above.

**Amplification of partial POMC cDNA by the reverse transcription-polymerase chain reaction (RT-PCR)**

Two 25-mer primers encoding the 5'-sense and 3'-antisense sequences corresponding to the 5'-region of bullfrog POMC cDNA (Pan and Chang, 1989) were designed: a sense primer (5'-CTCGAGAATG TTGCAGCCAGTCTGG-3') containing *Xho*I cleavage site and an antisense primer (5'-AAACTCTAGAGAGAGCTCTCTTCTC-3') containing *Sac*I cleavage site. The total RNA was isolated from the neurointermediate lobes of bullfrog pituitaries using ISOGEN, RNA extraction reagent (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (GIBCO, Gaithersburg, MD, USA) and subjected to the PCR using 50-μl reaction mixtures containing 200 μM each dNTP, 25 pmol each of the two synthetic primers described above and 1.25 U EX Taq polymerase (Takara Shuzo, Kyoto, Japan). The durations and temperatures of the PCR amplification cycle stages were 1 min at 94°C for denaturation, 1 min at 55°C for annealing and 2 min at 72°C for elongation. Partial bullfrog POMC cDNA (554-bp long) was amplified by 25 of the above cycles and then subjected to agarose gel electrophoresis.

**Construction of a cDNA library and screening of bullfrog POMC cDNA**

A cDNA library of neurointermediate lobes of adult bullfrog pituitaries was constructed using the method of Okayama and Berg (1982), as described previously (Mori *et al.*, 1991). In order to obtain full-length POMC cDNA, approximately 1000 transformants were screened with the bullfrog POMC RT-PCR fragment (554-bp long). This probe was [α-$^{32}$P]-labeled by the random priming method (Feinberg and Vogelstein 1983) using a Random Primer DNA Labeling Kit (Takara), followed by hybridization in 5×SSPE (1×SSPE comprised 150 mM NaCl, 10 mM $NaH_2PO_4$ and 1 mM $Na_2EDTA$, pH 7.4) containing 50% w/v formamide, 5×Denhardt's solution (0.1% w/v each of Ficoll, bovine serum albumin and polyvinylpyrolidone) and 0.1% w/v SDS at 42°C overnight. The filters were washed twice with 0.5× SSC-0.1% w/v SDS (1×SSC comprised 150 mM NaCl and 15 mM sodium citrate; pH 7.0) for 1hr at 68°C and the signals of the probes were detected by an imaging analyzer, BAS-2000 II (Fuji Photo Film, Tokyo, Japan). The nucleotide sequence of the POMC cDNA was determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using a Sequenase™ version 2.0 7-deaza-dGTP kit (United States Biochemical, Cleveland, OH, USA).

**Northern blot analysis of bullfrog POMC mRNA**

The POMC mRNA levels of the pituitaries were assessed by Northern blot analysis (Lehrach *et al.*, 1977). The total RNA was isolated from each sample, which consisted of 20–25 anterior or neurointermediate lobes from larvae at the same developmental stage, as described above. A 3-μg aliquot of each total RNA from anterior lobes and 1-μg aliquot of each total RNA from neurointermediate lobes thus obtained were denatured with formaldehyde, separated electrophoretically on a denaturing gel containing 1% w/v agarose-2.2 M formaldehyde, transferred to a Gene Screen Plus nylon membrane (NEN, Boston, MA, USA) and fixed to the filters by irradiation with ultraviolet light. After boiling in 1×SSC for 3 min, the filters were soaked in a hybridization solution consisting of 6×SSC, 10×Denhardt's solution and 1% w/v SDS for 2hr and hybridization was performed in this solution containing the isolated and labeled POMC cDNA described above overnight at 68°C. The filters were washed twice with 0.1×SSC-0.1% w/v SDS for 30 min each, then placed in contact with a BAS-III imaging plate (Fuji Photo Film) for 30 min and the Northern blot autoradiographs were subjected to densitometric analysis with an imaging analyzer, BAS-2000 II (Fuji Photo Film). The densitometry data for POMC mRNA were expressed as percentages of the mean value for stage XII tadpoles. Total RNAs from the anterior lobe, neurointermediate lobe, brain, kidney and liver of an adult bullfrog were subjected to Northern blot analysis, as described above, and then autoradiographed using X-OMAT film (Kodak) overnight at –80°C.

## RESULTS

**Nucleotide and deduced amino acid sequences of bullfrog POMC cDNA**

Screening of a bullfrog pituitary cDNA library, of which 40% of the transformants were recombinants, using the PCR product corresponding to part of bullfrog POMC cDNA described above showed that 1.3% of the recombinants contained a sequence related to POMC. The longest POMC cDNA contained 1180 bp and encoded the entire sequence of the POMC molecule, which consisted of 261 amino acids (Fig. 1). The amino acid sequence of bullfrog POMC deduced from the nucleotide sequence determined in this study disagreed by 5 amino acid residues from that reported by Pan and Chang (1989). These amino acid residues are included in NPP (position 38), γ-MSH (position 72), JP (position 90 and 105) and β-MSH (position 185). The amino acid sequence homologies of the POMCs of the bullfrog and a congenetic species *Rana ridibunda* (Hilario *et al.*, 1990) and of the bullfrog and the African clawed toad, *Xenopus laevis* (Martens *et al.*, 1985) were 96% and 73%, respectively.

**Northern blot analysis of POMC mRNA**

Northern blot analysis of POMC mRNAs isolated from various adult bullfrog tissues was performed using $^{32}$P-labeled POMC cDNA as a probe. A single positively hybridized band was detected at a position corresponding to about 1.4 kb (Fig. 2). Strong signals of the POMC mRNAs from the anterior and neurointermediate lobes of the pituitary (lanes 2 and 3) and faint, but definite signals of hypothalamic POMC RNA (lane 1) were detected. The kidney and liver (lanes 4 and 5) yielded no POMC mRNA signals.

**Developmental changes of POMC mRNA levels in larval pituitaries**

The POMC mRNA concentrations in the anterior and neurointermediate lobes were measured. The POMC mRNA levels of the anterior lobe increased progressively during pre- and prometamorphosis (stages XII–XIX) (Figs. 3A and 4A). At stage XIX, the maximum level, 2.5 times higher than the value for stage XII animals, was reached and during the climactic stages (XX–XXIV), the levels stayed very high. The levels of stage XXII animals kept in white, gray and black containers did not differ significantly (data not shown).

The elevations of the POMC mRNA levels of the neurointermediate lobes during pre- and prometamorphosis and early climax were more marked than those of the anterior lobes. The levels increased continuously as metamorphosis

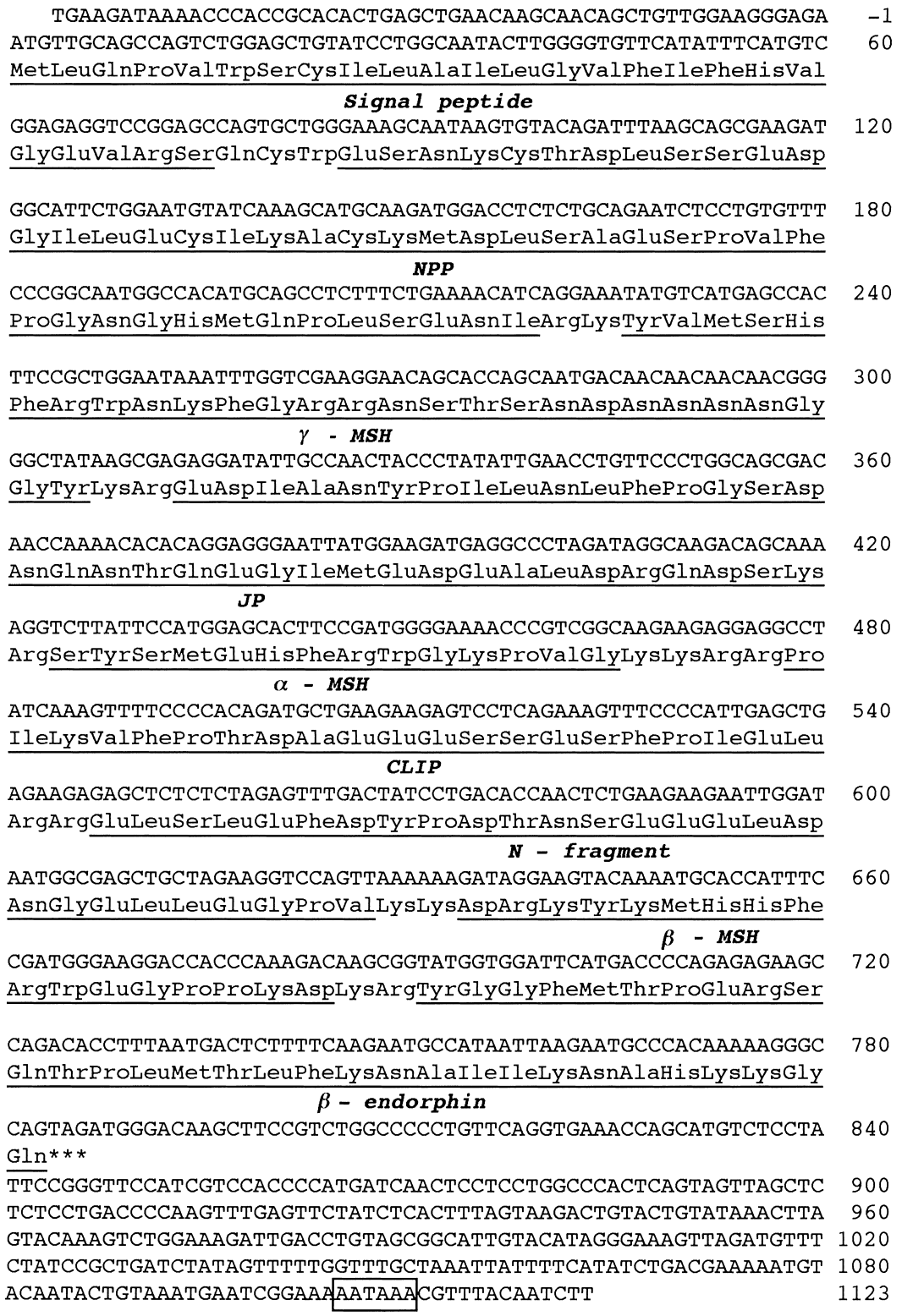


**Fig. 1.** Nucleotide and deduced amino acid sequences of *Rana catesbeiana* POMC cDNA. The numbers on the right correspond to the last nucleotide of the line and the negative numbers indicate the 5'-untranslated region. The localizations of signal peptide and POMC-derived peptides are indicated with underlines. The asterisks indicate the termination codon and the polyadenylation signal is boxed.

progressed, reaching the maximum at stage XXII, when the level was 4.5 times higher than that of stage XII animals (Figs. 3B and 4B). The mean dermal melanophore index (Hogben and Slome, 1931) of the animals kept in a gray-colored container was 3.1. The POMC mRNA levels of the neurointermediate lobes were very high, even in the stage XXII white-adapted animals, which had a mean melanophore index of 1.4 and a mean POMC mRNA level was 4 times higher than that of the stage XII group kept in a gray container. While in the stage XXII black adapted animals with a mean melano-

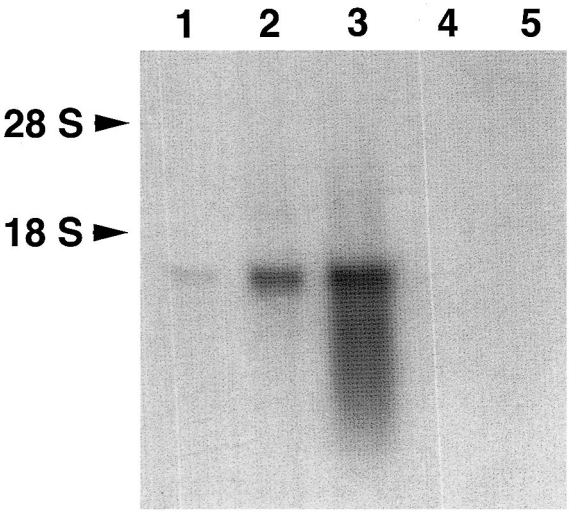


**Fig. 2.** Northern blot analysis of bullfrog POMC mRNA. Total RNAs (3 μg) prepared from adult bullfrog hypothalamus (lane 1), anterior and neurointermediate lobes (lanes 2 and 3, respectively), kidney (lane 4) and liver (lane 5) were electrophoresed on 1% w/v agarose gel containing 2.2 M formaldehyde and an autoradiogram was obtained using X-OMAT film (Kodak) overnight at –80°C. The positions of ribosomal RNAs (28S and 18S) are indicated. Bullfrog POMC mRNA was detected at positions corresponding to approximately 1.4 kb.

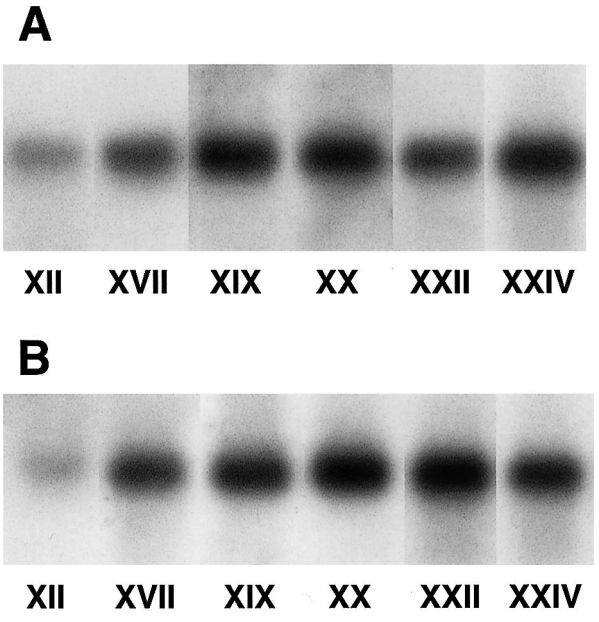


**Fig. 3.** Representative Northern blot hybridization profiles of total RNAs extracted from anterior (**A**) and neurointermediate (**B**) lobes of bullfrog tadpoles at various developmental stages as indicated. The animals were kept in gray-colored containers for 5 days before sacrifice. Each sample was prepared from 20–25 pituitaries. The amounts of total RNAs from anterior lobes and neurointermediate lobes applied were 3 and 1 μg, respectively. After electrophoresis and blotting, RNA was hybridized with the bullfrog POMC cDNA probe.

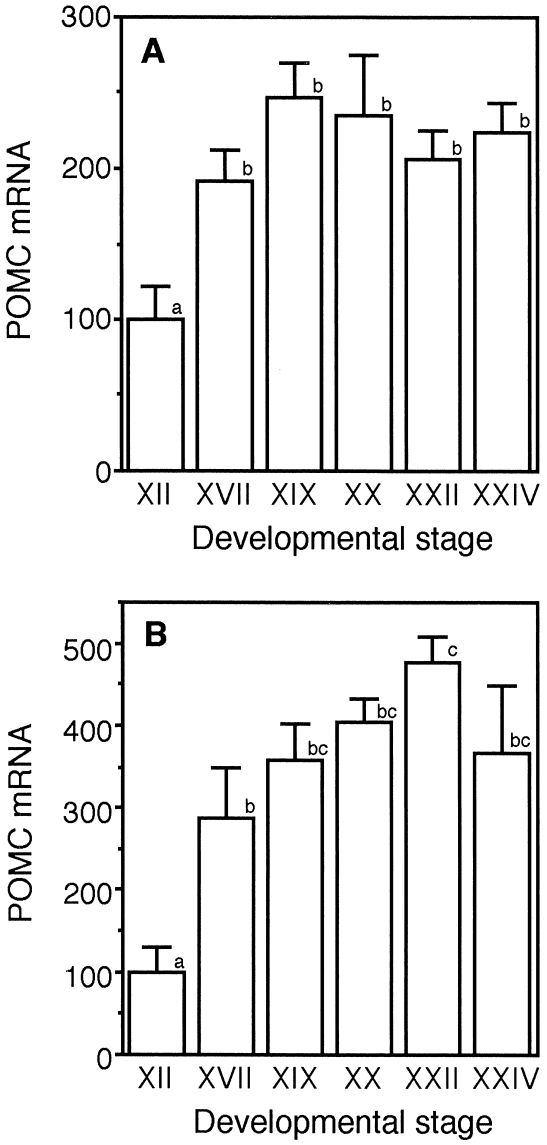


**Fig. 4.** Developmental changes in the POMC mRNA levels of the anterior (**A**) and neurointermediate (**B**) lobes of the bullfrog pituitary. Total RNAs extracted from the stage XII-XXIV tadpoles kept in gray-colored containers were subjected to Northern blot analysis of POMC mRNA. Each sample was prepared from 20–25 pituitaries. The total RNAs were quantified and equal amounts (3 μg from the anterior lobes and 1 μg from the neurointermediate lobes) were electrophoresed. The densitometry data for POMC mRNA are expressed as percentages of the mean value for stage XII tadpoles. The values are means ± SEM of 4–6 determinations and those with the same superscript do not differ significantly at the 5% level (Kruskal-Wallis and Dunn's test).

phore index of 5.0, a mean POMC mRNA level was 5.3 times higher than that of the stage XII group kept in a gray container.

## DISCUSSION

Previously, we isolated and characterized bullfrog N-terminal peptide of POMC (NPP) and joining peptide (JP) (Iwamuro *et al*., 1992), corticotropin-like intermediate lobe peptide (CLIP) and N-fragment (Kawasaki *et al*., 1991) and β-melanophore-stimulating hormone (MSH) (unpublished) and the amino acid sequences of these peptides are identical to those we deduced from the nucleic acid sequence of bullfrog POMC cDNA in this study.

The size of bullfrog POMC mRNA, determined by Northern blot analysis, was about 1.4 kb, which is in good agreement with those of other amphibian POMC mRNAs (Martens *et al*.,1985; Hillario *et al*.,1990). In addition to the pituitary, we found that the hypothalamus also expressed POMC mRNA. A similar result was obtained with *Xenopus* (Martens *et al*.,1985).

According to Jaffe (1981) and Kikuyama *et al*. (1986), the plasma corticoid levels of bullfrog larvae increase rather abruptly during the mid-climactic stages and decline during the late climactic stages. Accordingly, it has been assumed that the POMC mRNA levels of the anterior lobe will also increase around the early and/or mid-climactic stages and increase synthesis of ACTH, which stimulates corticoid release. In this study, however, we found that the maximum POMC mRNA levels of the anterior lobe were reached prior to the onset of metamorphic climax and remained high throughout the climactic period.

The obvious temporal disaccord between the POMC mRNA levels and plasma corticoid levels can be explained as follows. Firstly, elevation of POMC mRNA levels in the anterior lobe may not necessarily reflect the synthesis of POMC. Secondly, it may not be directly related to the processing of POMC molecules and the release of resulting ACTH. Further analyses of changes in the activity of prohormone convertase (PC) 1 that cleaves POMC to generates ACTH as well as the pituitary and plasma ACTH concentrations during metamorphosis are required to clarify this. It is also probable that, during metamorphosis, not only ACTH but also some other stimulants are involved in corticoid secretion by the adrenal glands. It is well known that arginine vasotosin (AVT) and its related peptides possess corticoid-releasing activity in several anurans (Iwamuro *et al*., 1989, 1991, 1992; Lacher *et al*., 1989, 1992; Kloas and Hanke, 1990). However, no information about the plasma and pituitary AVT levels and hypothalamic AVT mRNA levels of metamorphosing tadpoles is available. In addition to the neurohypophysis, the adrenal chromaffin cells of *R. ridibunda* contain AVT (Lacher *et al*., 1989). In amphibians, adrenal chromaffin cells are known to be intermingled with steroidogenic cells, suggesting that AVT is secreted in a paracrine fashion and induces neighboring steroidogenic cells to release corticoids. Finally, the responsiveness of the larval adrenal gland to ACTH should also be taken into consideration. If the responsiveness to ACTH is low, the plasma ACTH levels and, consequently, pituitary POMC mRNA levels may not reflect the plasma corticoid levels. In fact, the responsiveness of the steroidogenic cells of preclimactic tadpoles to ACTH was increased by the administration of thyroid hormone (Kikuyama *et al*., 1986), suggesting that adrenal gland responsiveness to ACTH increases as metamorphosis progresses.

Anuran larvae, with some exceptions (Kouki *et al*., 1998), respond to the background color by changing their body color by dispersing and aggregating melanin granules in the dermal melanophores. This response is known to be mediated mainly through α-MSH, one of the POMC-derived peptides. Bullfrog larvae placed in a black container released α-MSH, which led the expansion of the melanin granules in their dermal melanophores, and the release of α-MSH stopped and their melanin granules contracted when they were placed in a white container (Miyakawa *et al*., 1982). Therefore, it seems reasonable to expect that POMC mRNA levels depend primarily on the background color to which the larvae are exposed. The Northern blot analysis of POMC mRNA in the neurointermediate lobes of larval bullfrogs we performed in this study revealed that POMC mRNA levels are rather closely associated with the progress of metamorphosis. When the animals are kept in a container of the same color (gray), the POMC mRNA levels of the neurointermediate lobes increased continuously as metamorphosis proceeded. It is also noteworthy that the POMC mRNA levels of mid-climactic tadpoles kept in a white container were still very high. It is, therefore, obvious that the elevation of POMC mRNA levels we observed was not due mainly to the production of α-MSH, at least during metamorphosis. It remains to be elucidated whether the elevation of POMC mRNA levels in the intermediate lobe is related to the synthesis of POMC-derived peptides other than α-MSH and, if so, whether these peptides play any roles in the metamorphic process.

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