Cloning of a toad prolactin cDNA: expression of prolactin mRNA in larval and adult pituitaries

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ABSTRACT

A toad (Bufo japonicus) prolactin cDNA was specifically amplified from cDNAs constructed from the total RNA of adenohypophyses, employing the DNA polymerase chain reaction. Sequencing analysis revealed that the cDNA clone thus obtained was 602 bp in length, and encoded the C-terminal 134 amino acid residues of the toad prolactin molecule. The length of the toad prolactin mRNA was estimated to be about 1·0 kb by Northern blot analysis. The partial amino acid sequence deduced from the nucleotide sequence showed the following homologies between toad prolactin and the prolactins of other vertebrates: 69% with man, 80% with chicken, 81% with sea turtle, 91% with bullfrog and 38% with salmon. Using the cDNA as a probe, developmental and seasonal changes in prolactin mRNA levels in the pituitaries of toads were studied. Prolactin mRNA in the pituitary rose as metamorphosis progressed and declined at the end of metamorphosis. During the breeding season the pituitary content of prolactin mRNA was relatively high. This finding suggests that the increases in plasma and pituitary prolactin levels in larvae at metamorphic climax and in adults that remain in or migrate into water, as reported previously, accompany the increase in prolactin synthesis.

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INTRODUCTION

It has been known for some time that mammalian prolactins have antimitamorphic and growth-promoting effects in amphibian larvae (Bern et al. 1967; Etkin & Gona, 1967). However, purified amphibian prolactins have only relatively recently become available (Rana catesbeiana, Yamamoto & Kikuyama, 1981; Bufo japonicus, Yamamoto et al. 1986; Cynops pyrrhogaster, Matsuda et al. 1990; Xenopus laevis, Yamashita et al. 1993). Using these prolactins and their antisera, the biological function of prolactins in amphibians has been investigated (Yamamoto & Kikuyama, 1982a,b; Ishii & Kikuyama, 1984; Yamamoto et al. 1986; Brown et al. 1991; Matsuda et al. 1991; Carnevali et al. 1993).

In the bullfrog, the complete amino acid sequence has recently been determined (Takahashi et al. 1990; Yasuda et al. 1991). Thus, information on the primary structures of prolactins from every vertebrate class has been obtained and evolutionary changes have been studied (Yasuda et al. 1991). At present, however, the extent of the molecular biological approach to the study of amphibian prolactins is limited (Takahashi et al. 1990).

In this study, cloning of toad prolactin cDNA was performed. From the nucleotide sequence of the cDNA obtained, a partial amino acid sequence of the toad prolactin was deduced. Levels of prolactin mRNA in the pituitary glands of metamorphosing larvae and male and female adults in the aquatic and terrestrial phases were also determined using the cDNA as a probe.

MATERIALS AND METHODS

cDNA library construction and amplification of toad prolactin cDNA

Total RNA (about 10 μg) was extracted from the adenohypophyses of three toads (Bufo japonicus) according to the method described by Chirgwin et al. (1979), and cDNAs complementary to the mRNAs were specifically synthesized using an oligo(dT) primer with reverse transcriptase (Takara Syuzo Co. Ltd, Kyoto, Japan).

Toad prolactin cDNA was specifically amplified from the mRNA–cDNA complexes employing the polymerase chain reaction (PCR) method (Saiki et
al. 1988), using oligo(dT) and a specific sequence shared with vertebrate prolactin cDNAs as antisense and sense primers respectively. The reaction was performed in PCR buffer (10 mM Tris–HCl, pH 8·5, 5 mM MgCl₂, 0·5 mM dithiothreitol, 50 mM KCl) containing 0·25 mM dNTPs, 1·5 mmol each of the oligo(dT) and prolactin cDNA-specific (5′-CCNGA[AG]GA[CG]AA[CG]GA[AG]CA[AG]GC-3′) primers and 0·5 units Taq polymerase per 100 μl reaction solution. The amplified toad prolactin cDNA was blunt-ended and subcloned into the Smal site of the Bluescript plasmid vector KS M13⁺ (Stratagene, San Diego, CA, U.S.A.).

Nucleotide sequence analysis
The nucleotide sequence of the cDNA was determined by a chain-termination method (Sanger et al. 1977), using [α-32P]dCTP (Amersham International plc, Amersham, Bucks, U.K.) for the alkaline-denatured double-stranded DNA (Hattori & Sakaki, 1986) with Sequenase (United States Biochemical Co., Cleveland, OH, U.S.A.).

Northern blot analysis of toad prolactin mRNA
Prolactin mRNA was assessed and its concentrations were measured by Northern blot analysis (Lehrach et al. 1977). Total RNA, prepared as described above, was denatured with formamide and formaldehyde, electrophoresed on a denaturing gel containing 1% agarose–2·2 M formaldehyde and transferred onto a Gene Screen Plus nylon membrane (NEN, Boston, MA, U.S.A.). The filter was irradiated with u.v. light for 15 min to fix the RNA, under conditions described previously (Kato & Hirai, 1989). Double-stranded cDNA was labelled by the random-priming method (Feinberg & Vogelstein, 1983) using an oligolabelling kit (Takara Syuuzo Co. Ltd) according to the manufacturer’s instructions.

Quantitation of prolactin mRNA was performed for RNAs from the anterior pituitaries of tadpoles, juvenile toads and adult animals, captured in April and September. Tissue was removed from the animals, then frozen rapidly with liquid nitrogen and stored at −80 °C until extraction of the RNA. Each sample, consisting of 25 pituitaries of larvae at stage 38 (Limbaugh & Volpe, 1957) (hind-limb length per body length = 0·25), stage 43 (tail reduced to half) or stage 46 (tail completely gone) or of individual pituitaries of male and female adults (about 200 g body weight), was homogenized in TNE buffer (10 mM Tris–HCl, pH 7·5, 100 mM NaCl, 1 mM EDTA). Total RNA was extracted by removing the protein and genomic DNA with phenol, and subsequently precipitated in 70% ethanol. Total RNA was quantified and equal amounts of RNA were electrophoresed and blotted onto membranes followed by u.v. fixation.

After boiling in 1 x SSC (1 x SSC is 0·15 M NaCl and 0·015 M sodium citrate, pH 7·5) for 3 min, the filters were soaked in a hybridization solution consisting of 6 x SSC, 0·02% (w/v) bovine serum albumin, 0·02% (w/v) Ficoll 400, 0·02% (w/v) polyvinylpyrrolidone and 1% (w/v) sodium dodecyl

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sulphate (SDS) for 3 h at 50 °C. Hybridization was performed in the solution containing labelled cDNA overnight at 65°C. The filters were washed twice in 0.1 x SSC containing 1% SDS for 30 min at 42 °C and placed in contact with X-ray film (Eastman Kodak Co., Rochester, NY, U.S.A.) with a Quanta III intensifying screen (DuPont, Wilmington, DE, U.S.A.) for 1 or 3 days at −80°C.

Densitometry was performed on the Northern blot autoradiographs with a CS-9000 Chromatoc scanner (Shimadzu, Kyoto, Japan). The densitometry data for prolactin mRNA are expressed as contents (per pituitary) or concentrations (per total RNA) relative to the value for stage 38 animals or terrestrial male toads in April. Results are presented as means ± s.e.m. Statistical analysis was performed using Duncan's new multiple range test. P values of less than 5% were taken to be significant.

RESULTS

Specific amplification and nucleotide sequence of toad prolactin cDNA

An amplified clone was obtained from reverse transcripts of total RNA from toad anterior...
pituitaries by PCR. A single band was specifically amplified at a position corresponding to 600 bp (Fig. 1). Sequencing analysis revealed that the clone contained 602 nucleotides (Fig. 2). The deduced amino acid sequence (indicated below the nucleotide sequence in Fig. 2) showed that this clone encodes part of the sequence of the prolactin molecule consisting of 134 amino acids. When the partial amino acid sequence of toad prolactin was compared with comparable portions of other vertebrate prolactins, the levels of homology were found to be as follows: 69% for man, 69% for pig, 80% for chicken, 81% for sea turtle, 91% for bullfrog and 38% for salmon. Toad prolactin was found to be closer to reptilian, avian and mammalian prolactins than to fish prolactin.

**Northern blot analysis of prolactin mRNA**

Northern blot analysis of toad prolactin mRNA was performed using a $^{32}$P-labelled toad prolactin cDNA insert. A single band showing positive hybridization was detected at a position corresponding to about 1.0 kb (Fig. 3).

**Hybridization experiments with adenohypophyseal RNA from toad tadpoles and adults**

The prolactin mRNA levels in the adenohypophyses of prometamorphic (stage 38) and climax (stage 43) tadpoles, post-climax (stage 46) animals and adult toads were measured. Figure 3 shows that there was a linear relationship between the amount of RNA and the density measured. As shown in Fig. 4, the prolactin mRNA content of larval pituitary was relatively low in prometamorphic tadpoles, increasing slightly as metamorphosis progressed and decreasing at the end of metamorphosis. On the other hand, prolactin mRNA concentrations rose towards the end of metamorphosis. However, none of these changes were statistically significant. In the adult male pituitary, both the content and concentrations of prolactin mRNA were maximum when the animals remained in water in April. In female pituitary, the prolactin mRNA content was significantly high when the toads were migrating towards the pond in April and low when they were feeding in the bush in September. Prolactin mRNA concentrations paralleled the values for mRNA content (Fig. 5).

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DISCUSSION

Nicoll et al. (1986) have analysed the structure–function relationships of prolactin and growth hormone using mammalian and chicken hormones. When the primary sequences of prolactins from all vertebrate classes were discovered, Yasuda et al. (1991) suggested that they contained six conserved domains (PDs 1–6). Among these, PD 5 is unique in that this region is significantly conserved within tetrapod prolactins. Since the amino acid sequence of toad prolactin determined in this experiment is not complete, it contains only the portions comparable to PDs 3–6. These portions are also conserved in toad prolactin (Fig. 6). The toad prolactin mRNA was estimated to be 1.0 kb in length. This matches the size of bullfrog prolactin mRNA reported previously (Takahashi et al. 1990).

In the toad larvae, prolactin synthesis (as measured by incorporation of labelled leucine into pituitary prolactin in vitro) shows a tendency to rise as metamorphosis progresses and to decline at the end of metamorphosis (Niinuma et al. 1991). The present study revealed that developmental changes in prolactin mRNA content coincide with those in prolactin-synthesizing activity. It is known that plasma prolactin levels gradually rise in metamorphosing toad larvae. Accordingly, both the synthesis and release of prolactin in toad larvae seem to be enhanced moderately as metamorphosis progresses. In bullfrog larvae, we have also observed the rise in plasma prolactin concentrations (Yamamoto & Kikuyama, 1982a) as well as pituitary prolactin mRNA levels during climax (Takahashi et al. 1990). However, the magnitude of the increase is more pronounced in bullfrog larvae than in toad larvae. It is generally believed that, in amphibians, prolactin plays an osmoregulatory role similar to that found in teleosts when they are exposed to an hypotonic environment (Loretz & Bern, 1982; Mazzi & Vellano, 1987). It is of interest that the toad larvae migrate to the land at mid-climax, while bullfrog tadpoles at the same stage remain in an aquatic environment. This may be one explanation for the difference in prolactin cell function during climax between toad larvae and bullfrog larvae.

Monthly surveys of plasma prolactin levels in adult toads have revealed that they are highest
during the breeding month in early spring. During the breeding period, the plasma prolactin concentrations of male toads show a peak when they are swimming in a pond and those of female toads are maximum when they are moving around a pond (Ishii et al. 1989; Yamamoto et al. 1989). Changes in plasma prolactin levels during the breeding season show a good correlation with those in pituitary prolactin mRNA contents, as revealed in the present experiment, suggesting that an increase in plasma prolactin levels accompanies prolactin synthesis. In the animals which had just completed metamorphosis, pituitary prolactin mRNA contents did not parallel the prolactin mRNA concentrations. This is because the amount of total mRNA in the pituitaries of these animals is low when compared with that in the pituitaries of the metamorphosing tadpoles.

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