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Extrapituitary Expression of the Prolactin Gene in the Goldfish, African Clawed Frog and Mouse

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ABSTRACT—In mammals, the pituitary hormone prolactin is also produced in various extrapituitary tissues and may act in an auto/paracrine fashion. To explore the comparative aspects of extrapituitary prolactin, the distributions of prolactin transcripts were investigated in extrapituitary organs of the goldfish, African clawed frog and mouse by reverse transcription-polymerase chain reaction (RT-PCR). For comparison, the amount of the transcript in mouse tissues was also estimated using competitive RT-PCR. In the goldfish, the transcript was detected in the ovary, testis, liver, kidney, spleen, gill, muscle and brain in slightly lower abundance than in the pituitary, but not detected in the intestine. In the frog, the transcript was detected in the following organs with an order of abundance: pituitary >> brain > testis and ovary. In the mouse, the transcript was detected in the pituitary, brain, testis, and ovary, and its copy number per μg of total RNA was estimated at $\sim 10^{10}$ in the male pituitary, $\sim 10^{4}$ in the placenta, hypothalamus and testis, $\sim 10^{3}$ in the thymus and experimentally induced deciduoma, and $\sim 10^{2}$ in the ovary. These results suggest that the origin of extrapituitary prolactin goes back to the common ancestor of fish and tetrapods, but that distinct evolution has occurred in each lineage. The significance of extrapituitary PRL in non-mammalian species is also suggested.

INTRODUCTION

In the vertebrate body, the pituitary hormone prolactin (PRL) provokes diverse actions which can be categorized into osmoregulatory, developmental, metabolic, reproductive, behavioral, and immunoregulatory functions (Nicoll, 1980; Bole-Feysot *et al.*, 1998). In mammals, extrapituitary organs produce PRL, among them: the decidua of rats, humans and rhesus monkeys; human fibroblasts and myometrium; mammary glands of rats, sheep, goats and humans; lymphoid organs and brains of humans, rats and mice; and rat lacrimal glands (for review, see Ben-Jonathan *et al.*, 1996; Prigent-Tessier *et al.*, 1999). Other potential sites of PRL production include: murine placenta (Harigaya *et al.*, 1997); human (Untergasser *et al.*, 1997) and murine (Imaoka *et al.*, 1998) testes; human (Schwarzler *et al.*, 1997), murine (Imaoka *et al.*, 1998), bovine (Sirotkin *et al.*, 1994), and porcine ovaries (Einspanier *et al.*, 1986); and human prostate (Nevalainen *et al.*, 1997). Since these organs also possess PRL receptor (PRL-R), it is proposed that PRL is a local signal mediator. Indeed, antibodies against PRL inhibit the growth of GH_3 cells (Krown *et al.*, 1992), lymphocytes (Hartmann *et al.*, 1989), breast cancer cells (Clevenger *et al.*, 1995; Mershon *et al.*, 1995), and endothelial cells (Clapp *et al.*, 1998) *in vitro*, suggesting an autocrine effect of PRL.

Such a cytokine-like action of PRL was initially proposed for mammals. To determine the evolutionary origin of the extrapituitary PRL actions, it is apt to investigate the distribution of PRL and PRL-R gene expression in the organs of non-mammalian species. Although the distribution of PRL-R in those species has been investigated extensively (e.g. Sandra *et al.*, 1995; Zhou *et al.*, 1996; Exbrayat *et al.*, 1997; Ohkubo *et al.*, 1998), little information is available so far on the distribution of PRL (for sea bream, Santos *et al.*, 1999; for rainbow trout, Yang *et al.*, 1999).

The small quantities of the extrapituitary PRLs render the reverse transcription-polymerase chain reaction (RT-PCR) a useful tool for their investigation. Moreover, reported information on PRL mRNA sequences can be used for the analysis of PRL expression in various non-mammalian vertebrates. Therefore, in this study, the PRL gene expressions were investigated in various tissues of the goldfish and African clawed frog by RT-PCR. The expression levels in the tissues of the goldfish, African clawed frog and mouse were also compared.

MATERIALS AND METHODS

Animals

Adult male and female goldfish (*Carassius auratus*) and African clawed frogs (*Xenopus laevis*) were obtained from commercial sources. Animals were anesthetized by chilling in ice-cold water before autopsy. BALB/c mice (BALB/c/jcl; Clea Japan, Tokyo, Japan)

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were used as described by Imaoka *et al.* (1998). Dissected tissues were frozen in liquid nitrogen and kept at –85°C until use. Mouse deciduoma was induced experimentally as follows: Ovariectomized female mice aged 70 days were treated with 100 ng of estradiol-17β (E2; Sigma, St. Louis, MO, USA) for 3 days, and 10 ng of E2 plus 1 mg of progesterone (P4; Sigma) for subsequent 3 days, and then traumatized on the uterine horns with a thread for surgical operation, followed by treatment with 1 mg of P4 for 3 days.

RNA isolation, RT-PCR and Southern analysis

Procedures of RNA isolation and RT-PCR followed Imaoka *et al.* (1998). Briefly, total RNA was extracted by the acid-guanidinium phenol chloroform method (Chomczynski and Sacchi, 1987) and reverse-transcribed with SuperScript™ RNase H^- reverse transcriptase (GIBCO BRL, Gaithersburg, MA, USA) using random hexamers. The cDNA sample was subjected to PCR with Ex Taq polymerase (Takara Shuzo, Tokyo, Japan). PCR primers (Fig. 1) for goldfish, African clawed frog and mouse PRLs (gfPRL, xPRL and mPRL, respectively) were designed on the basis of previously reported sequence data (Chan *et al.*, 1996; Buckbinder and Brown, 1993; GenBank L07620). The primer sequences and the expected sizes of the amplified fragments were as follows: gfPRL-s1 (5'-AATGATTTACTGGAGCGAGCC-3') and gfPRL-a1 (5'-CAATTTTGTGAGAGTCCCTGC-3') were designed for a 498-bp gfPRL cDNA; xPRL-s1 (5'-GTGTGACTTCCCTACCAATATG-3') and xPRL-a1 (5'-TATGGGAATCCCTGCGAAGGC-3') were for a 554-bp xPRL cDNA; mPRL-s2 (5'-CTGCCAATCTGTTCCGCTGGTGAC-3') and mPRL-a3P (5'-CCTACTGCAGTTATTAGTTGAAAC-3') were for a 302-bp mPRL cDNA (Fig. 1); 5'-CTGACAGACTACCTCATGAAGATCC-3' and 5'-AGTAATCTCCTTCTGCATCCTGTC-3' were for 404-bp goldfish and frog β-actin cDNAs; and 5'-TCTAGACTTCGAGCAGGAGATGGCC-3' and 5'-GTGGATCCACCAGACAACAC-3' were for a 254-bp mouse β-actin cDNA. The PCR program was 95°C for 5 min, followed by 30–40 cycles of 95°C for 30 sec, 57°C for 1 min, and 72°C for 30 sec, except for the program for mPRL, in which 62°C instead of 57°C was adopted. The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Amplified PCR products from pituitary samples were extracted from the gel using glass powder (Easytrap; Takara Shuzo), subcloned into the pT7Blue2-T vector (Novagen, Madison, WI, USA), sequenced using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, Norwalk, CT, USA), and used as gfPRL and xPRL cDNAs. Preparation of pituitary mPRL cDNA was described previously (Imaoka *et al.*, 1998). For Southern analysis of PCR products, probes were prepared from pituitary PRL cDNAs from which primer sequences had been eliminated by restriction with Mbo II plus Hae III for gfPRL, Hinc II plus Hae III for xPRL, and EcoR I plus Xho I for mPRL (Fig. 1). From these templates, probes were random primer labeled with digoxigenin-11-dUTP (DNA Labeling and Detection Kit, non-radioactive; Boehringer Mannheim Co., Indianapolis, IN, USA). PCR products resolved by 2% agarose gel electrophoresis were transferred by capillary action to nylon membranes (Hybond N+, Amersham Pharmacia Biotech AB, Uppsala, Sweden), hybridized to 20 ng/ml of probes at 65°C, and detected by immunostaining of digoxigenin according to the instructions of the manufacturers.

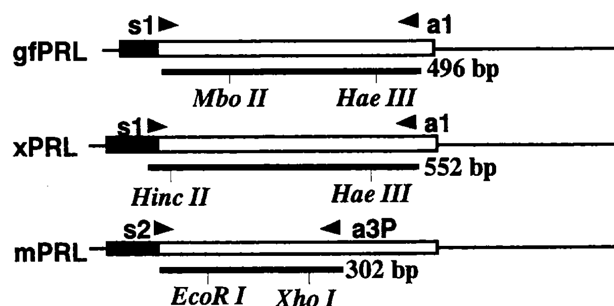
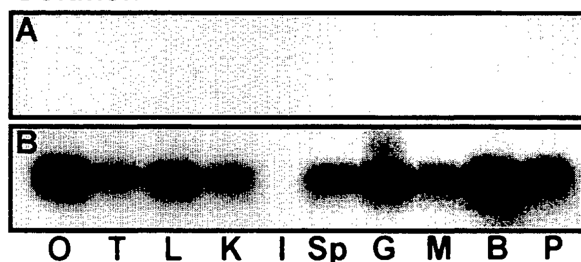
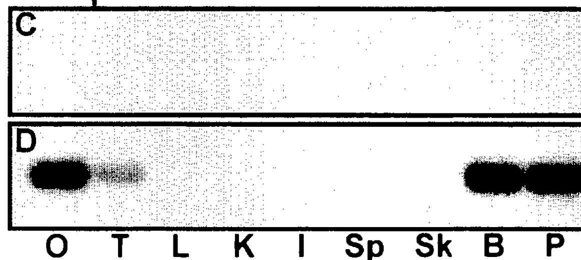


Fig. 1. Diagrams representing goldfish, African clawed frog and mouse PRL mRNAs, primers and probes. Primers (*arrowheads*) were designed within the PRL mRNAs (*lines and boxes*) of goldfish (gfPRL), African clawed frog (xPRL) and mouse (mPRL) to amplify cDNA fragments (*bold lines*) consisting of the indicated number of base pairs. The filled box, open box and flanking lines represent the sequence coding the signal peptide, the sequence coding the mature PRL peptide and the non-coding regions, respectively. Amplified fragments from the pituitary were subcloned, sequenced, and used for probe synthesis after they were rid of primer sequences using the restriction enzymes indicated.

Competitive RT-PCR and kinetic analysis of PCR

For estimating the copy number of mouse PRL transcripts per μg of total RNA, an established competitive RT-PCR system was used (Imaoka *et al.*, 1998). Briefly, tissue total RNA, together with synthetic mouse PRL RNA with 47-base truncation as an internal control, was subjected to RT-PCR with [α–32P]dCTP, and the copy num-





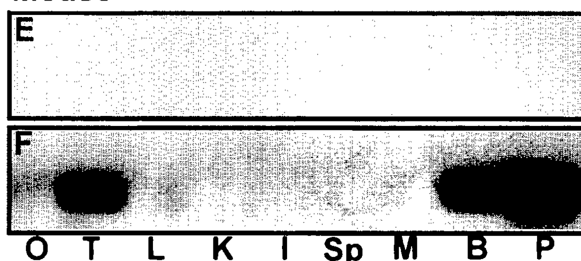


Fig. 2. RT-PCR analysis of goldfish, African clawed frog and mouse PRLs in various organs. Total RNA was extracted from the organ, reverse-transcribed, and subjected to PCR. For analysis of goldfish (A and B), African clawed frog (C and D) and mouse (E and F) PRLs, PCR products after 40, 35, 40 cycles, respectively, were electrophoresed, and Southern blots were hybridized to probes generated from pituitary PRL cDNAs. No product was detected even after 45 cycles in the samples where no PRL transcript was detected with the above number of cycles (not shown). Analyses of RNA without reverse transcription (A, C and E) and cDNA (B, D and F) are shown. Every cDNA sample yielded the expected DNA band after 30 cycles of PCR for β-actin (not shown). Representative results of three experiments were indicated. O, ovary; T, testis; L, liver; K, kidney; I, intestine; Sp, spleen; G, gill; M, skeletal muscle; B, whole brain; P, pituitary; Sk, abdominal skin.

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ber of PRL transcripts per μg of total RNA was deduced from the radioactivity of the amplified fragments from tissue PRL RNA and the internal control. For rough comparison of the expression levels of PRL in various organs, analysis of the kinetics of PCR amplification was adopted (for example, Nakabayashi *et al.*, 1992). Aliquots were removed from the PCR reaction mixtures for PRL every 5 cycles, electrophoresed, and visualized as described above. PCR amplifications for β-actin were also analyzed for standardization.

RESULTS

Extrapituitary PRL gene expression in the goldfish and African clawed frog

To explore evolutionary aspects of extrapituitary PRL production, the presence of PRL transcripts were examined in various organs of the goldfish, African clawed frog and mouse by RT-PCR followed by Southern analysis (Fig. 2). In the goldfish, a 498-bp product was detected by hybridization with the gfPRL probe in the ovary, testis, liver, kidney, spleen, gill, muscle, brain and pituitary, but not in the intestine (Fig. 2B). In the frog, a 554-bp product was detected by hybridization with the xPRL probe in the ovary, testis, brain and pituitary, but not in the liver, kidney, intestine, spleen or skin (Fig. 2D). In the mouse, a 302-bp product was detected by hybridization with the mPRL probe in the ovary, testis, brain and pituitary, but not in the liver, kidney, intestine, spleen or muscle (Fig. 2F). PCR with non-reverse-transcribed RNA samples did not yield these bands, excluding artifactual products resulting from contamination by genomic DNA (Fig. 2A, C, and E). DNA of the expected size was amplified by PCR for β-actin in every sample, indicating successful cDNA synthesis (not shown).

Comparison of expression levels of PRL between organs

The amount of PCR product is known to increase in earlier cycles of the reaction when the initial amount of the template is larger (for example, Nakabayashi *et al.*, 1992). Note that the absolute amount of the product in the late phase of PCR does not reflect the initial amount. Levels of PRL expression in various organs were compared by analyzing the kinetics of PCR (Fig. 3). For standardization, cDNA samples were diluted so that the kinetics of PCR for β-actin cDNA were identical among samples from individual organs (Fig. 3, *right*). Typical results for PRL are shown (Fig. 3, *left*). In the goldfish (Fig. 3A), PCR products from the extrapituitary organs were visible after 35–40 cycles, while that from the pituitary was visible after 30 cycles. This indicates that the levels of PRL expression were similar in the extrapituitary organs and slightly higher in the pituitary. In the African clawed frog (Fig. 3B), PCR products from the gonads, brain and pituitary were visible after 45, 40 and 30 cycles, respectively. This indicates that the pituitary was rich in PRL transcripts, that a smaller amount existed in the brain, and that still smaller existed in the testis and ovary. In the mouse (Fig. 3C), PCR products from the testis and pituitary were visible after 40 and 20 cycles, respectively, indicating that the amount of PRL transcript in the pituitary was far larger than that in the testis. For individual mouse organs, an established competitive RT-PCR system (Imaoka *et al.*, 1998) can be used to estimate the copy number of PRL transcripts per μ g of total RNA. By this method, we analyzed the organs in which the PRL transcript was detected in the above experiment (Fig. 2C). The thymus, de-

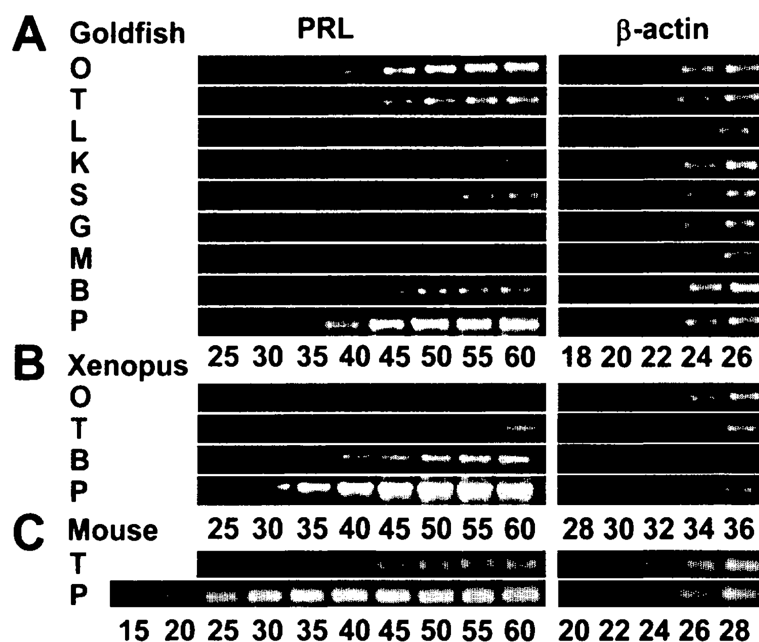

Fig. 3. Kinetics of PCR for PRL and β-actin in PRL-expressing organs in the goldfish, African clawed frog and mouse. Total RNA was extracted from the organ, reverse-transcribed, and subjected to PCR. Aliquots were removed from PCR reaction mixtures for goldfish (A), African clawed frog (B) and mouse (C) PRLs every 2 or 5 cycles (indicated below each panel) and electrophoresed. The samples that generated visible bands after fewer cycles were considered to contain more transcripts. The absolute amount of the product in the late phase of PCR does not reflect the initial amount. Reverse-transcribed samples were diluted so that the kinetics of PCR for β-actin were identical among organs (*right panels*). These samples were then subjected to PCR for PRL (*left* panels). Representative results of three experiments were indicated. O, ovary; T, testis; L, liver; K, kidney; I, intestine; S, spleen; G, gill; M, skeletal muscle; B, whole brain; P, pituitary.

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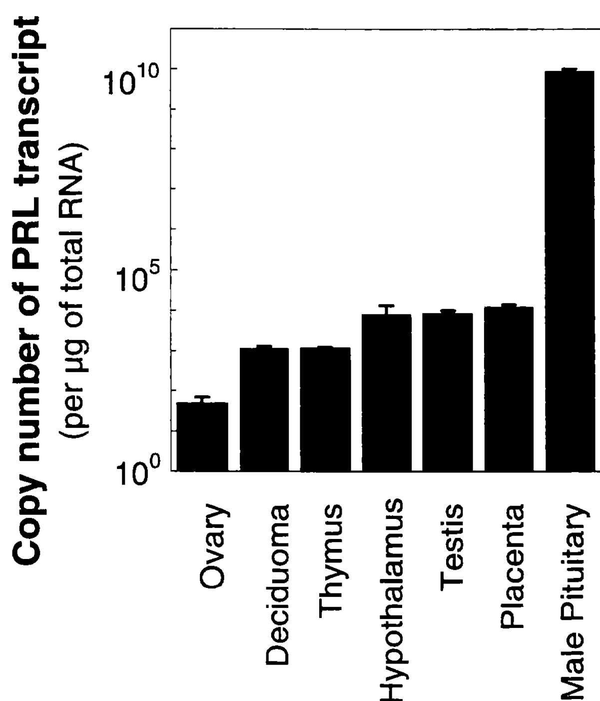

Fig. 4. Copy numbers of PRL transcripts per µg of total RNA in the mouse PRL-expressing organs. Previously established competitive RT-PCR (Imaoka *et al.*, 1998) was used to compare the amount of PRL transcripts. Briefly, isolated total RNA was diluted and mixed with 10^4 copies of truncated competitor PRL RNA. The RNA mixture was subjected to RT-PCR in the presence of [α–32P]dCTP, the reaction mixture was electrophoresed, and the copy number of PRL transcripts per µg of total RNA was deduced from the radioactivity of the amplified fragments from tissue RNA and the competitor RNA. Means and SE of 2–8 measurements are indicated.

cidua and placenta, that are known to produce PRL (Ben-Jonathan *et al*, 1996; Harigaya *et al.* 1997), were also analyzed. We used experimentally induced deciduoma, because it is a widely used model for the analysis of decidua. As a result, the copy number of PRL transcripts per µg of total RNA was estimated at about 10^{10} in the male pituitary, 10^4 in the placenta, hypothalamus and testis, 10^3 in the thymus and experimentally induced deciduoma, and 10^2 in the ovary (Fig. 4).

DISCUSSION

In mammals, PRL is produced in various extrapituitary tissues and is supposed to act as an auto/paracrine factor. As mentioned in Introduction, PRL is reported to be produced in the decidua, myometrium, breast, lymphoid organs, brain, testis, ovary, and prostate of mammals. However, hitherto the extrapituitary PRL synthesis in non-mammalian species was reported only for sea bream (Santos *et al.*, 1999) and rainbow trout (Yang *et al.*, 1999). In the present study, the tissue distribution of PRL expression was investigated using the goldfish, African clawed frog and mouse.

Evolutionary changes in the tissue distribution of PRL

The present RT-PCR results (Fig. 2) indicated that the tissue distributions of PRL transcript in the African clawed frog and mammals were similar, but that that of the goldfish was dissimilar with them. In detail, the pituitary, brain, testis and ovary expressed PRL commonly in the goldfish, African clawed frog and mammals; the liver, kidney and spleen expressed PRL only in the goldfish; and the intestine was the common non-PRL-expressing organ. This suggests that PRL expression outside the pituitary already existed in the common ancestor of those species. It is also suggested that the PRL expressions in the liver, kidney and spleen appeared only in the goldfish lineage, or that the expressions in these organs were primitive and diminished only in the tetrapod lineage. To distinguish between these possibilities, the distributions of PRL expression in more primitive species must be investigated. PRL is expressed in the liver but not in the kidney of the sea bream (Santos *et al*, 1999), whereas it is detected neither in the liver, kidney nor spleen of the rainbow trout (Yang *et al.*, 1999). Together with these data, the present result suggests that the tissue distribution of PRL diverged in the teleosts. Unlike the results previously reported for humans (Wu *et al.*, 1996) and rats (Shaw-Bruha *et al.*, 1998), PRL was not detected in the spleen of the mouse in this study. This discrepancy may be due to differences in experimental conditions, such as the immunological status of the animals or the number of PCR cycles. Since the goldfish appears to have a duplicated genome and, in agreement with this, two goldfish PRL cDNAs have been identified (Chan *et al.*, 1996), it is also possible that one of the two genes is specialized for extrapituitary expression.

Expression levels of PRL in various organs

The competitive RT-PCR used here has been shown to be sensitive and quantitative (Imaoka *et al.*, 1998). However, semi-quantification with analysis of PCR kinetics cannot always be easily interpreted, because it is possible that, for instance, non-visible PCR products are amplified simultaneously. Moreover, standardization by β-actin is not necessarily adequate because the amount of β-actin transcript can vary from one tissue to another. Nonetheless, an approximate pattern can be determined from the data obtained by this method.

In the present results of the analysis of the PCR kinetics (Fig. 3), the extrapituitary PRL expressions were similar and only slightly lower than the pituitary PRL expression in the goldfish. In the African clawed frog, PRL expressions in the gonads were lower than that in the brain and much lower than that in the pituitary. In the mouse, PRL expression in the testis was far lower than that in the pituitary, and this difference was quantified as ~10^6 fold (Fig. 4). The numbers of PRL transcripts in the mouse extrapituitary organs were nearly equal to or less than that in the testis (Fig. 4). In this way, extrapituitary PRL expressions were lower than the pituitary expression, suggesting that extrapituitary PRL works as an auto/paracrine factor. The data also showed that the relative abundance of the extrapituitary PRL to the pituitary PRL varied in those species in the following descending order: the goldfish, African clawed frog, and mouse. The relative significance of the extrapituitary PRL to the pituitary PRL might be also higher in

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the goldfish, lower in the African clawed frog, and much lower in the mouse.

The transcriptional mechanism of extrapituitary PRL expression in the goldfish, frog and mouse is currently unclear. DiMattia *et al.* (1990) have reported that the human PRL gene has a distal promoter that directs decidual and lymphocyte expression, and Shaw-Bruha *et al.* (1998) reported that the sequences in the rat PRL gene homologous to the human distal promoter may not be functional. It remains unknown whether the fish, frog and mouse PRL genes have distal promoters. PRL transcripts in mouse extrapituitary organs were unable to detect by Northern hybridization (data not shown), in contrast to the human decidual and thymocyte PRL transcripts (Gellersen *et al.*, 1989; Montgomery *et al.*, 1992). This discrepancy may reflect differences in the distal promoter activities of humans and rodents.

Possible biological role of extrapituitary PRL

In the non-mammalian vertebrates, PRL is involved in various physiological processes. For example, it regulates ion transport in the gill and kidney, the activity of immune cells, gonadal steroidogenesis, and behavior in fish; and lymphocyte proliferation, ovarian lipid metabolism, spermatogenesis, and behavior in amphibians (*see* Bole-Feysot *et al*, 1998). In the present study on the goldfish and African clawed frog, PRL transcripts were detected in the gill, kidney, gonads and brain (Fig. 2). As has been suggested for mammals, the above PRL actions may be provoked, at least in part, by locally produced PRL in those species. This is likely also because PRL is thought to have evolved from cytokines. To prove the cytokine-like functions of goldfish and frog PRLs, tissue-specific disruption of PRL expression is necessary (Ben-Jonathan *et al.*, 1996). At least, it will be necessary to examine the complete organization of the transcript, successful translation and processing of the protein, and secretion at a physiologically significant concentration. Further studies such as the Northern and Western analyses will be required to examine the organization of PRL transcript and the existence of PRL protein, respectively. Alternatively, since the human growth hormone gene, which is structurally related to PRL, also encodes a novel transcription factor (Labarriere *et al.*, 1995) and intranuclear PRL appears necessary for interleukin-2-driven proliferation of lymphocytes (Clevenger *et al.*, 1991), extrapituitary PRL might work intracellularly. Although the physiological significance of extrapituitary PRL remains unproven, the present findings suggest that it may be common among all the vertebrates and support the idea that it has many important functions.

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