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≪Research Note≫

Expression of Prolactin Receptor mRNA in Lactotrophs and Somatotrophs of the Chicken Anterior Pituitary Gland

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Prolactin (PRL) is a hormone mainly secreted by the anterior pituitary gland. In birds, PRL exerts a variety of physiological functions in target tissues expressing the PRL receptor (PRLR). In chicken, the *PRLR* mRNA is abundant in the anterior pituitary gland, but its regional and cellular localization are unknown. In the present study, we investigated the expression of the *PRLR* mRNA in cephalic and caudal lobes of the chicken anterior pituitary gland. Real-time polymerase chain reaction (PCR) revealed high levels of *PRLR* mRNA in both cephalic and caudal lobes. *In situ* hybridization revealed that the *PRLR* mRNA was distributed in a wide area of both lobes, and co-localized with the *PRL* and growth hormone (*GH*) mRNAs in the cephalic and caudal lobes, respectively. These results suggest that PRL exerts autocrine/paracrine effects through PRLR on PRL-producing lactotrophs and GH-producing somatotrophs in the chicken anterior pituitary gland.

Key words: chicken anterior pituitary gland, growth hormone, mRNA expression, prolactin, prolactin receptor

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Introduction

Prolactin (PRL) is a hormone secreted by the anterior pituitary gland that participates in a variety of physiological functions in vertebrates (Bole-Feysot *et al*., 1998). In birds, prolactin is involved in osmoregulation (Harvey *et al*., 1984; Murphy *et al*., 1986), crop milk production (Scanes *et al*., 1975), brood patch formation (Hutchinson *et al*., 1967), and incubation behavior (Saeki and Tanabe, 1955; March *et al*., 1994). These functions of PRL are mediated through the PRL receptor (PRLR) in target cells.

Studies showed that the *PRLR* mRNA is abundantly expressed in the anterior pituitary of laying White Leghorn and Bantam hens (Ohkubo *et al*., 1998a). The chicken anterior pituitary gland is composed of two lobes, namely, the cephalic and caudal lobes. Six types of endocrine cells are variably distributed in these two lobes, such as the PRL-producing lactotrophs, growth hormone (GH)-secreting somatotrophs, thyroid-stimulating hormone (TSH)-producing thyrotrophs, adrenocorticotropic hormone (ACTH)-producing corticotrophs, and follicle-stimulating hormone (FSH) or luteinizing hormone (LH)-secreting gonadotrophs (Kansaku *et al*., 1995; Proudman *et al*., 1999; Chowdhury *et al*., 2003). However, the regional and cellular localization of the *PRLR* mRNA in chicken anterior pituitary gland has not been reported. Therefore, the objective of this study is to determine the expression of the *PRLR* mRNA in lactotrophs and somatotrophs of chicken anterior pituitary gland.

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Materials and Methods

Experimental Birds and Tissue Collection

Laying White Leghorn hens (10-months-old) were obtained from Santoku Farm Matsuo Corp. (Chiba, Japan). The hens were housed under 16.5 h light and 7.5 h dark conditions. Food and water were provided *ad libitum*. Tissues were isolated after decapitation. For RNA preparation, tissues were immediately frozen in liquid nitrogen. For *in situ* hybridization, the anterior pituitary glands were frozen in dry ice powders. The frozen tissues were stored at −80℃ until further use. All procedures were conducted in accordance with the provision for animal welfare of the Nippon Veterinary and Life Science University (Approval number: 27K-44).

RNA Preparation and cDNA Synthesis

Total RNA was extracted from the tissue using the TRIzol reagent (Life Technologies, Tokyo, Japan) and was reverse-transcribed at 50℃ for 60 min with Superscript III transcriptase (Life Technologies). The resulting cDNA was amplified by PCR using LA *Taq* polymerase with GC buffer (Takara, Tokyo, Japan). PCR was performed for 33 cycles of 94℃ for 30 s, 60℃ for 30 s, and 72℃ for 2 min using

Table 1. **Primers used for PCR**

cDNA		Sequence (5′-3′)	Size (bp)	Accession No.
*PRLR**	Forward	AGGAAACATTTACCTGTTGGT	371	NM_204854.1
	Reverse	AAGCCATCCAGATCTGACATC		
PRLR †	Forward	AGGAAACATTTACCTGTTGGT	628	NM_204854.1
	Reverse	CATATGAGAGATGATAAGACACCC		
PRL †	Forward	GATGAACGTTATGCTCAGGGTCGGGGTTT	477	NM_205466.2
	Reverse	CAATTGCTATCATGGATTAGGCGGCACTT		
GH †	Forward	ATGGCTCCAGGCTCGTGGTT	651	NM_204359.2
	Reverse	TCAGATGGTGCAGTTGCTCT		

* Applied for real-time PCR. † Applied for RNA probe synthesis

forward and reverse primers specific for each cDNA. The sequences of the primers are shown in Table 1. The amplified cDNA fragments were ligated to pGEM-T Easy plasmid vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli*. The plasmid DNAs were extracted from the bacteria and purified. Sequencing of the cDNA was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies) with an ABI PRISM 310 genetic analyzer (Life Technologies).

Real-Time PCR

The plasmid DNA containing *PRLR* cDNA was used as a standard DNA for real-time PCR with forward and reverse primers. The primer sequences are shown in Table 1. Real-time PCR was conducted in accordance with the manufacturer's instructions for the real-time PCR system 7500 (Life Technologies). The thermal parameters for the PCR were 95℃ for 15 s and 60℃ for 35 s in 25 μL of buffer containing 1× Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies) and 0.2 μM each of forward and reverse primers. Quantitative measurements were performed after establishing a linear amplification curve with the serial dilutions of the *PRLR* cDNA.

In Situ Hybridization (ISH)

RNA probes for ISH were synthesized using SP6 or T7 RNA polymerases (Takara, Tokyo, Japan) and plasmid cDNAs as the template. The RNA probe for *PRLR* mRNA was labeled with digoxigenin-11-uridine-5′-triphosphate and detected using an HNPP fluorescent detection set (Roche Diagnostics, Tokyo, Japan). The RNA probes for *PRL* and *GH* mRNAs were labeled with fluorescein-12-uridine-5′-triphosphate (Roche Diagnostics). The synthesis and labeling reactions were performed in accordance with the manufacturer's instructions. These labeled probes were hybridized with frozen sections (18 μm) of the anterior pituitary gland prepared using a Leica CM1850 cryostat (Leica Biosystems, Nussloch, Germany). The slide-mounted sections were fixed in 4% paraformaldehyde (PFA) for 10 min, rinsed thrice in phosphate-buffered saline (PBS), and treated with proteinase K (Wako Pure Chemical Osaka Japan) (1 μg/m*l*) in PBS at 25℃ for 5 min and with 2 mg/mL glycine in PBS at 25℃ for 10 min. After post-fixing in 4% PFA for 10 min and rinsing thrice in PBS, the sections were acetylated with acetic anhydride (0.125%) in 100 mM triethanolamine (pH 8.0) at 25℃ for 20 min and prehybridized at 60℃ for 60 min with 200 μg/mL yeast tRNA in hybridization buffer containing 50% formamide, 5× standard saline citrate (SSC), and 0.5 μg/mL heparin in a humidity chamber. Both digoxigenin-labeled and fluorescein-labeled probes (300 ng/mL each) were added to the hybridization buffer and incubated at 60℃ for 16 hr. After washing with 2× SSC/formamide and 0.1× SSC at 60℃, the sections were incubated in a blocking buffer containing casein (5 mg/mL) and sheep serum (5 μL/mL) at 25℃ for 30 min. Subsequently, the sections were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics) diluted 1:2,000 in the blocking buffer at 4℃ for 16 hr in the humidity chamber. After washing with buffer containing 100 mM Tris-HCl (pH7.5), 150 mM NaCl, and 0.05% Tween 20, and then with detection buffer containing 100 mM Tris-HCl (pH8.0), 100 mM NaCl, and 10 mM $MgCl_2$ at 25℃, 300 μL of HNPP/Fast Red solution (Roche Diagnostics) was applied to the sections and incubated at 25℃ for 30 min. After washing with the washing buffer at 25℃, the sections were mounted with ProLong Diamond antifade mountant with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technology) and air-dried. Red fluorescence of the digoxigenin-labeled probes and green fluorescence of fluorescein-labeled probes were detected using a fluorescence microscope (Axiovert 200M; Carl Zeiss Microscopy, Tokyo, Japan).

Statistical Analysis

All data were analyzed using one-way analysis of variance, and differences in values were confirmed using the Tukey multiple comparison test. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

Comparison of the PRLR mRNA Levels in the Anterior Pituitary Gland and Other Tissues

The *PRLR* mRNA levels in the anterior pituitary gland, liver, kidney, duodenum, jejunum, ileum, ovary, and hypothalamus were determined using real-time PCR. Markedly high levels of *PRLR* mRNA were observed in both the cephalic and caudal lobes of the anterior pituitary gland than in other tissues. The levels in the cephalic and caudal lobes were almost equivalent and were more than threefold higher

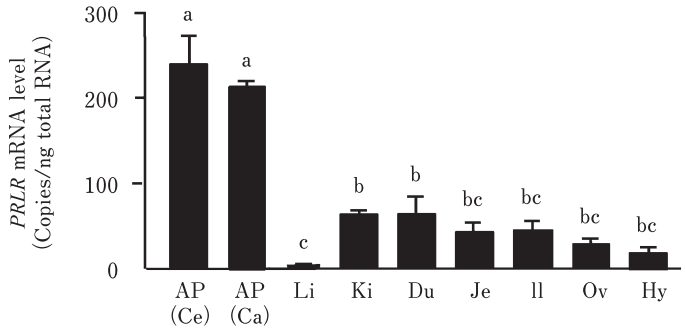

Fig. 1. **The *PRLR* mRNA levels in chicken tissues.** The *PRLR* mRNA levels were measured using real-time PCR. AP, anterior pituitary gland; Ce, cephalic lobe; Ca, caudal lobe; Li, liver; Ki, kidney; Du, duodenum; Je, jejunum; Il, ileum; Ov, ovary; Hy, hypothalamus. Each bar represents the mean±SEM (n=4). Bars with different letters are significantly different ($P<0.05$).

than those of other tissues (Fig. 1).

Regional and Cellular Distribution of PRLR, PRL, and GH mRNAs in the Anterior Pituitary Gland

First, the distribution of *PRLR*, *PRL*, and *GH* mRNAs in the anterior pituitary gland was determined using ISH. As shown in Fig. 2A, fluorescence signals in the entire anterior pituitary gland sections were detected with antisense probes, and not with sense probes, for each mRNA, indicating that the fluorescence signals were derived from *PRLR*, *PRL*, and *GH* mRNAs. The fluorescent signal of the *PRLR* mRNA was distributed in both cephalic and caudal lobes, whereas signals of *PRL* and *GH* mRNAs were predominantly detected in the cephalic and caudal lobes, respectively. Figure 2B shows magnified (20-fold) fluorescent images with *PRLR* and *PRL* antisense probes in an arbitrary area in the cephalic lobe and with *PRLR* and *GH* antisense probes in an arbitrary area in the caudal lobe. The merged fluorescent images of *PRLR* and *PRL* or *GH* mRNAs indicated that the *PRLR* mRNA was expressed in some of the *PRL* mRNA-expressing cells in the cephalic lobe and in most *GH* mRNA-expressing cells in the caudal lobe.

Discussion

Quantitative real-time PCR analyses revealed abundant expression of the *PRLR* mRNA in both cephalic and caudal lobes of the chicken anterior pituitary gland. In agreement with these results, ISH analyses revealed extensive expression of the *PRLR* mRNA in both cephalic and caudal lobes. ISH also showed that the *PRL* and *GH* mRNAs were predominantly expressed in the cephalic and caudal lobes, respectively. In the cephalic lobe, the *PRLR* mRNA was expressed in some of the *PRL* mRNA-expressing cells, highlighting expression of the *PRLR* mRNA in other endocrine cells producing TSH, ACTH, FSH, or LH. In support of this observation, PRLR has been detected in all endocrine cells of the rat anterior pituitary gland (Morel *et al*., 1994). In mammals, PRL is known to inhibit its own synthesis and secretion and stimulate internalization of its receptor in anterior pituitary gland cells (Herbert *et al*., 1979; Melded *et al*., 1980; Divots and Boutin, 1999). Co-expression of the *PRLR* mRNA with *PRL* mRNA in the chicken anterior pituitary gland cells suggests similar autocrine/paracrine functions of PRL.

Interestingly, the *PRLR* mRNA was found in *GH* mRNA-expressing cells of the caudal lobe as well as PRL mRNA-expressing cells of the cephalic lobe. Studies showed that the levels of *PRL*, *GH*, and *PRLR* mRNAs in the chicken anterior pituitary gland increase during late embryonic development and those of the *PRL* and *PRLR* mRNAs are higher in incubating hens than in laying hens (Ohkubo *et al*., 1998b; Kansaku *et al*., 1994; Leclerc *et al*., 2007). These findings suggest unknown autocrine/paracrine functions of PRL in lactotrophs and somatotrophs during embryonic development and reproductive stages.

Recently, PRL has been shown to act as an autocrine/paracrine antiproliferative and proapoptotic factor as it inhibits the expression of PRLR in rodent anterior pituitary gland, and this action is considered to be involved in cell turnover and homeostasis of the anterior pituitary gland (Ferraris *et al*., 2012; Ferraris *et al*., 2013; Ferraris *et al*., 2014). Cell proliferation and apoptosis has been observed in the cephalic and caudal lobes of the anterior pituitary gland of turkey hen following nest-deprivation, with decrease in plasma PRL concentration (Rajesh *et al*., 2001). Similarly, in both cephalic and caudal lobes of the chicken anterior pituitary gland, cell proliferation and apoptosis were observed during inhibition and resumption of laying, which may cause rejuvenation of the anterior pituitary gland (Chowdhury and Yoshimura, 2002). These findings suggest that PRL may be involved in cell turnover in the chicken anterior pituitary gland. Expression of the *PRLR* mRNA in endocrine cells other than lactotrophs and somatotrophs remains to be investigated.

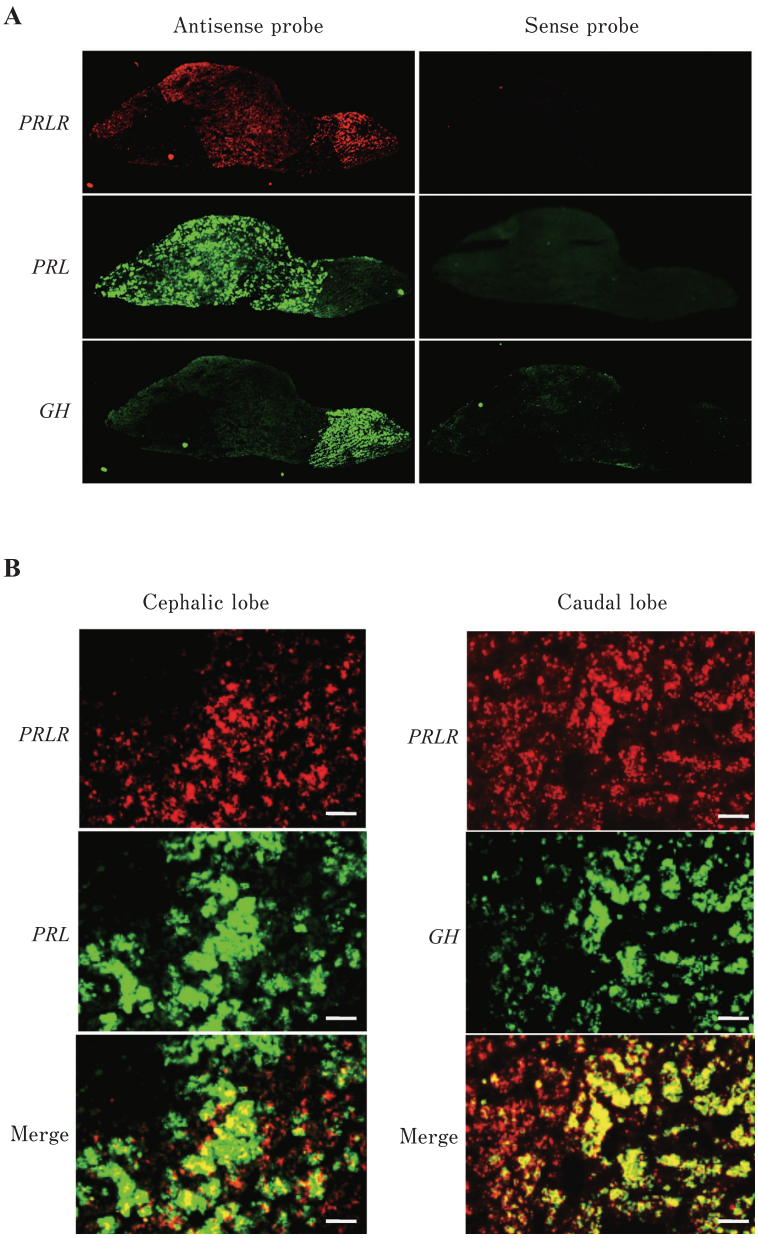

Fig. 2. **The distribution of *PRLR*, *PRL*, and *GH* mRNAs in the chicken anterior pituitary gland.** *PRLR*, *PRL*, and *GH* mRNAs were detected by ISH. Red fluorescence of digoxigenin-labeled antisense and sense probes for *PRLR* mRNA and green fluorescence of fluorescein-labeled antisense and sense probes for *PRL* or *GH* mRNAs were detected using a fluorescence microscope. A: The distribution of *PRLR*, *PRL*, and *GH* mRNAs in entire anterior pituitary gland. B: The co-expression of *PRLR* and *PRL* mRNAs in an area of the cephalic lobe and *PRLR* and *GH* mRNAs in an area of the caudal lobe. Scale bars represent 20 μm.

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