Expression of prolactin gene in incubating hens

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Summary. Steady-state levels of the 1.38-kb mRNA encoding the prolactin prohormone, measured by northern and dot blotting using a radioactive cDNA probe, rose 10-fold with concomitant increases in concentrations of prolactin in anterior pituitary and plasma from non-incubating to incubating stages in Gifujidori bantam hens. Daily injections of a low dose of thyrotrophin-releasing hormone (TRH, 0.25 µg/kg) for 4 days caused no changes in the concentrations of prolactin of the mRNA, but a high dose (2.5 µg/kg) of TRH did induce a significant increase in prolactin mRNA levels compared to saline controls. The results suggest that gene expression for prolactin increases substantially during the incubation period and TRH may contribute, at least in part, to the increase in transcription of prolactin mRNA for prolactin biosynthesis in Gifujidori bantam hens.

Keywords: gene expression; prolactin mRNA; northern hybridization; incubating hen; TRH

Introduction

Prolactin has been implicated in the regulation of broodiness in birds since Riddle *et al.* (1935) first demonstrated that injections of prolactin induce incubation behaviour. Subsequent studies with chickens and turkeys showed that the start of egg incubation is associated with a marked increase in circulating prolactin concentrations which are maintained throughout the incubation period, and, with the termination of incubation and the hatch of chicks, concentrations decline to a low value (Sharp *et al.*, 1979, 1988; Burke & Dennison, 1980; Harvey *et al.*, 1981; Proudman & Opel, 1981; Zadworny *et al.*, 1985, 1988). The increased pituitary content of prolactin during the incubation period (Saeki & Tanabe, 1955; Cherms *et al.*, 1962; Burke & Pupkoff, 1980) suggests that an increased rate of prolactin synthesis may occur which in turn sustains the high concentrations of prolactin observed in the circulating blood throughout the incubation period. However, few studies have been conducted concerning biosynthesis of prolactin or its regulation in birds with more direct indices. In mammals, it is well known that prolactin biosynthesis is stimulated via gene expression (i.e. transcriptional processing) for prolactin by thyrotrophin-releasing hormone (TRH, Murdoch *et al.*, 1985; Laveriere *et al.*, 1988) and oestradiol (Maurer, 1982; Yamamoto *et al.*, 1986; Seo, 1989). Although the transcriptional regulation for prolactin is not fully understood, TRH may be involved in gene expression via an increase in protein kinase C activity (Murdoch *et al.*, 1985) and calcium efflux (Laveriere *et al.*, 1988), while oestradiol may be involved by binding its receptor which in turn initiates transcription by binding the 5' flanking region of the prolactin gene in mammalian pituitary cells. As far as we are aware, little is known about the transcription mechanism for the prolactin gene in birds.

The mRNA encoding prolactin in the anterior pituitary of the chicken has been cloned as cDNA by Hanks *et al.* (1989) and Watahiki *et al.* (1989). Sequence determination reveals that prohormone prolactin consists of 30 amino acids as a signal peptide and 199 amino acids of mature prolactin. Although there are 3 different amino acids between sequences of the mature prolactin

deduced by the two groups, it may not be a large discrepancy unless both proteins possess different biological activities. Hence, utilization of the cDNA for prolactin as a probe to quantitate prolactin mRNA should provide a new insight to investigate the molecular basis for gene expression for prolactin biosynthesis as well as evolutionary aspects of prolactin molecules in birds. Accordingly, this study was conducted to elucidate changes in expression of the prolactin gene in relation to incubation and the effect of TRH on prolactin transcription in Gifu-jidori hens which have been reported to exhibit broody behaviour (Kono *et al.*, 1985; Zadworny *et al.*, 1989).

Materials and Methods

Animals and collections of tissue and plasma samples. Gifu-jidori hens (a strain of Japanese bantams) weighing 650–800 g were housed in deep-litter floor pens containing nest boxes under natural daylight until the experiments were initiated at Nagoya University in mid-October. Food and water were available *ad libitum*. All hens had ceased egg production but showed no visible signs of moult, i.e. they were out-of-lay (Zadworny *et al.*, 1989). The hens were then exposed to 4 h of artificial illumination in addition to natural daylight (a total of 15 h illumination; 04:00–19:00 h) until the end of the experiments.

In Exp. I, 5 hens were randomly selected and killed for collection of blood and tissue samples before addition of artificial lighting (pre-photostimulation group). At 26 days after additional illumination 5 hens were killed and blood and tissue samples were collected. The other hens were allowed to lay and accumulate clutches of eggs; those which began to incubate and had been sitting on their eggs for about 7 days were selected as incubating hens and the samples were collected.

For Exp. II, at 2 weeks after starting the 4-h artificial illumination, in addition to natural daylight, hens were randomly divided into 3 groups. Each group was assigned daily injections of saline (1.0 ml/hen) or TRH in saline (0.25 or 2.5 µg/kg, Sigma, St Louis, MO, USA) at 10:00 h for 4 days, respectively. The doses of the hormone were adopted from previous studies (Harvey *et al.*, 1978; Fehrer *et al.*, 1985). At the end of the experiment, hens were lightly anaesthetized by pentobarbital injection, and trunk blood was collected from the carotid artery into heparinized test tubes. Plasma was separated by centrifugation and stored at –20°C until required for assay. Anterior pituitary glands were removed immediately after decapitation, placed in sterile microfuge tubes, snap-frozen in liquid nitrogen, and stored at –80°C.

RNA extraction. Total RNA was prepared from individual pituitaries. Each pituitary gland was homogenized in 100 µl 0.5% Nonidet P40 (Sigma), 10 mM-Tris-HCl, 1 mM-ethyldiaminetetraacetic acid (EDTA). A portion (5 µl) of the homogenate was used for radioimmunoassay (RIA) for prolactin and the remaining homogenate for the extraction of the total RNA according to the procedures described by Chomczynski & Sacchi (1987). The amount of total RNA was estimated by spectrophotometry.

³²P-labelled chicken prolactin cDNA probe. The probe for northern and dot-blot hybridization was a purified SspI-XbaI restricted fragment (270 base pairs) of chicken prolactin cDNA (generously provided by Dr K. Nakashima, Mie University, Japan), labelled with deoxycytidine 5'-triphosphate (³²P-dCTP; 3000 Ci/mmol, New England Nuclear, Boston, MA, USA) by random primed labelling kit (Boehringer-Mannheim, Mannheim, West Germany). The radioactive probe was separated from unincorporated ³²P-dCTP by gel filtration on a Sephadex G-50 column-preequilibrated with buffer (150 mM-sodium chloride, 10 mM-Tris-HCl, and 1 mM-EDTA, pH 8.0). The specific activity of the ³²P-labelled prolactin cDNA probe was 1–5 × 10⁸ c.p.m./µg.

Northern blot hybridization procedure. Samples of total RNA (15 µg) were denatured with 4 M-glyoxal, heated at 60°C for 30 min and subjected to electrophoresis in 0.9% agarose gels and blotted on Gene Screen filters (DuPont Ltd, Southampton, UK) in 10 × SSC (1.5 M-sodium chloride, 150 mM-sodium citrate, pH 7.0) using standard techniques (Maniatis *et al.*, 1982). The filters were air dried and prehybridized in buffer (900 mM-NaCl, 5 mM-EDTA and 50 mM-sodium phosphate, pH 7.7, containing 50% formamide, 0.1% sodium dodecyl sulphate (SDS), 5 × concentrated Denhardt's solution and denatured salmon sperm DNA (200 µg/ml) at 42°C for 3 h. Radioactive cDNA probes were heated (10 min, 100°C) immediately cooled on ice, added to the prehybridization solution at a concentration of about 10 ng/ml, and then hybridized for a further 22 h at 42°C. The filters were washed in 2 × SSC and 0.1% SDS for 1 h at 65°C. Washed filters were exposed to X-ray film with intensifying screens, at –70°C.

RNA dot-blot hybridization assay. For dot blot analysis, total RNA was extracted as described above from the anterior pituitary, denatured (60°C, 15 min) in 2 × SSC containing 4 M-glyoxal, and applied to Gene Screen filters using a dot manifold according to the instruction of the manufacturer (Bethesda Research Laboratory, Gaithersburg, MD, USA). Hybridization conditions were identical to those used in the northern blot hybridization. Measurements of specific mRNA levels were determined on at least 4 dilutions of total RNA from individual pituitaries. The intensity of the ³²P-DNA-RNA hybridization was quantitated by scanning radioactivity of the Gene Screen filters using the AMBIS Radioanalytic Image System (AMBIS Radioanalytic Image System, Inc., San Diego, CA, USA) and the amount of prolactin mRNA was expressed in c.p.m./4 µg total RNA.

RIA and analyses of results. The plasma prolactin concentration and pituitary prolactin content were determined as previously described (Zadworny *et al.*, 1988). Minimum detectable concentrations of prolactin for plasma and pituitary gland are 1 ng/ml and 10 ng/mg, respectively. Samples were assayed for prolactin in a single assay to eliminate interassay variation. The intra-assay coefficient of variation for prolactin were 9.2%. Data were analysed by analysis of variance and Neuman-Keuls multiple Range Test.

Results

Detection and characterization of prolactin mRNA in anterior pituitary

A sample autoradiograph of a northern blot of chicken prolactin mRNA is shown in Fig. 1. A single, distinct species of 1.38 kb prolactin mRNA was detected in northern blots of total RNA isolated from the pituitaries of non-incubating Gifujidori bantam hens (Fig. 1, left lane). Very large amounts of 1.38 kb messages were observed in total RNA from incubating hens (Fig. 1, right lane). No prolactin mRNA was detected in total RNA from chicken liver or hypothalamus (data not shown). Although longer exposure (for 2 days) of the autoradiograph revealed the presence of larger molecular weight mRNA species (3.7 kb), it was much less abundant or negligible in comparison to the amount of 1.38 kb mRNA (data not shown).

Fig. 1. Autoradiograph of northern blot showing determination of size of prolactin mRNA of non-incubating (left) and incubating (right) bantam hens. Fifteen μ g of total RNA was applied in each lane. Numbers at left indicate molecular marker sizes in kb. Exposure was for 3 h.

Effects of photostimulation and incubation on prolactin mRNA and prolactin concentration in the anterior pituitary gland and on plasma concentration of prolactin

The out-of-lay bantam hens had regressed ovaries before photostimulation but gained body weight and ovary weight significantly increased 26 days after long-day exposure (Table 1; $P < 0.05$). Three of the 5 photostimulated hens were actively laying eggs. Two other hens showed

ovaries with atrophied follicles. About 40 days after the onset of photostimulation 3 hens laid eggs, subsequently began incubating eggs and sat on the nest for about 7 days. Body weight and ovary weight of these incubating hens significantly decreased, whereas total RNA in the pituitary gland of the incubating hens significantly increased when compared to those of the post-photostimulation group, respectively ($P < 0.05$).

Table 1. Body weight, ovary weight, pituitary weight and amount of total RNA in hens under different conditions

	Before photostimulation	After photostimulation	Incubating
Body weight (g)	698.00 $\pm$ 29.00 ^a	960.00 $\pm$ 30.0 ^b	803.00 $\pm$ 26.0 ^c
Ovary weight (g)	0.63 $\pm$ 0.09 ^a	17.43 $\pm$ 8.1 ^b	2.47 $\pm$ 0.2 ^a
Pituitary weight (mg)	4.50 $\pm$ 0.50	6.00 $\pm$ 0.9	7.80 $\pm$ 1.3
Pituitary total RNA (μ g/gland)	12.90 $\pm$ 2.40 ^a	14.90 $\pm$ 3.1 ^a	28.40 $\pm$ 5.1 ^b

Different superscripts indicate a statistically significant difference ($P < 0.05$).

Values are means $\pm$ s.e.m. for 3–5 hens.

An autoradiograph of a dot-blot hybridization showed that the hybridization signal was linear over the range of dilutions of pituitary RNA applied (1.0–8.0 μ g/dot). The lowest concentrations of prolactin mRNA (c.p.m./4 μ g total RNA) were observed in hens of the pre-photostimulation group (Fig. 2). Hens of the post-photostimulation group showed no significant increase in pituitary prolactin mRNA or pituitary prolactin and plasma prolactin concentrations (133.5 $\pm$ 41.0 c.p.m., 2.24 $\pm$ 0.49 μ g/mg, 165.3 $\pm$ 100.6 ng/ml) when compared to the pre-photostimulation group (61.6 $\pm$ 4.8 c.p.m., 0.97 $\pm$ 0.22 μ g/mg, 23.8 $\pm$ 7.4 ng/ml). In contrast, the incubating hen showed a marked increase in pituitary prolactin mRNA levels (580.8 $\pm$ 58.4 c.p.m.) and the concentrations of prolactin in the pituitary gland (20.35 $\pm$ 2.39 μ g/ml) and the plasma (945.3 $\pm$ 73.3 ng/ml). The 10-fold increase was significantly different from those of other groups ($P < 0.01$). There was a high correlation ($r = 0.81$) between pituitary prolactin mRNA and pituitary prolactin concentrations in hens used in the experiment.

Fig. 2. Amount of pituitary prolactin mRNA, pituitary and plasma prolactin concentrations in hens in different states. Values are mean $\pm$ s.e.m. for 3–5 hens. Different superscripts indicate significant difference ($P < 0.05$).

Effects of TRH on prolactin mRNA and prolactin concentrations in anterior pituitary and plasma

Body weight, ovary weight, pituitary weight and yield of total RNA from the pituitary gland after 4 days of injections of saline or TRH are shown in Table 2. All the hens in every group had

large yellow follicles and they were about to resume laying. Effects of TRH injections on quantity of prolactin mRNA and prolactin in the pituitary gland and prolactin concentrations in plasma are shown in Fig. 3. Injection of 0.25 µg TRH/kg did not result in any changes in the variables measured ($P > 0.05$), whereas injections of a larger dose TRH (2.5 µg/kg) resulted in a significant increase in pituitary prolactin mRNA (220.1 ± 42.8 c.p.m.; $P < 0.05$; Fig. 3a). At the latter dose level there were no significant differences in the concentrations of prolactin in the pituitary gland (6.34 ± 2.32 µg/mg) or plasma (74.1 ± 51.5 ng/ml) when compared to the saline group (Fig. 3b; 102.1 ± 37.2 c.p.m., 4.01 ± 0.91 µg/mg, 18.9 ± 3.34 ng/ml). There was no significant correlation between ovary weight, pituitary weight and the amount of prolactin mRNA in all the hens. Northern blot analysis revealed that only 1.38 kb RNA species characteristic of bantam hens (Fig. 1) was detected in all treatment groups (data not shown).

Table 2. Effects of TRH injections on body weight, ovary weight, and pituitary weight of photostimulated pre-laying hens

	Saline	TRH (0.25 µg/kg)	TRH (2.5 µg/kg)
Body weight (g)	958.0 ± 19.0	908.0 ± 27.0	884.0 ± 9.0
Ovary weight (g)	22.7 ± 5.3	14.4 ± 5.5	27.6 ± 1.3
Pituitary weight (mg)	6.1 ± 0.5	4.7 ± 0.4	6.0 ± 0.7
Pituitary total RNA (µg/gland)	19.8 ± 1.4	19.5 ± 1.7	22.3 ± 1.7

Values are means ± s.e.m. for 5 hens.

Fig. 3. Amount of pituitary prolactin mRNA, pituitary and plasma prolactin concentrations in out-of-lay hens treated with TRH. Values are mean ± s.e.m. for 5 hens/group. Different superscripts indicate significant difference ($P < 0.05$).

Discussion

The present study not only confirms the presence of prolactin mRNA in the pituitary gland of bantam hens (Hanks *et al.*, 1989) but also demonstrates conspicuous changes in prolactin gene expression in relation to prolactin production in the anterior pituitary of hens in different physiological states. Although the previous study reported that 0.88 kb prolactin mRNA is the major molecular weight species of mRNA (1.7–6.2 kb nuclear intermediary transcripts), the present study showed that a larger size of prolactin mRNA (1.38 kb) is the major molecular weight species.

Although the autoradiograph of a northern blot (Fig. 1, right lane) shows a large amount of prolactin mRNA ranging from 1.5 to 0.9 kb in the incubating hens, the scanning analysis of the radioactivity displayed only a single molecular species at 1.38 kb. This mRNA may be the final transcript rather than an intermediary transcript processed to the 0.88 kb mRNA in the anterior pituitary of the Gifujidori bantam hens. At present, the reason for this discrepancy remains unknown but it may not be attributable to different amounts and species of RNAs (4 µg of pituitary poly(A) RNA in the previous study and 15 µg of total RNA in this study) or different lengths of the cDNA probes used (850 bp in the previous study and 270 bp in this study). In support of this possibility, using the same procedure as described here, we have detected a 0.88 kb prolactin mRNA as the major molecular weight species in the pituitary gland of single-comb White Leghorn hens actively laying but rarely showing maternal behaviour of broodiness. The various sizes of mRNA detected in the different breeds of chickens may reflect different lengths of untranslated regions at the 5' or 3' ends of the transcript (Hanks *et al.*, 1989; Watahiki *et al.*, 1989) or may arise from differences in use of alternative polyadenylation sites (Karatzas *et al.*, 1990). It is unlikely that the coding regions are substantially different in size since prolactin cDNA from broiler chickens (Watahiki *et al.*, 1989) and from bantam chickens (Hanks *et al.*, 1989) were the same as has been reported for other avian species (turkey: Karatzas *et al.*, 1990). Direct evidence for either possibility awaits nucleotide information from different mRNAs. Monomeric turkey prolactin exists in 3 isohormone forms, one of molecular weight 22 000 and the other two about 26 500 (Proudman & Corcoran, 1981; J. A. Proudman, personal communication). We have demonstrated that mRNA isolated from the anterior pituitary glands of crossbred broiler chicken synthesizes proteins *in vitro* of molecular weight 27 000 and 27 500 which are immunoprecipitated with turkey prolactin antibody but only the smaller protein M_r 27 000 is immunodisplaceable (Shimada *et al.*, 1989). It is unclear whether the chicken pituitary gland synthesizes different forms of prolactin as does the turkey.

Although previous reports have shown a larger amount of prolactin in the pituitary gland of incubating chicken (Saeki & Tanabe, 1955) and turkey (Cherms *et al.*, 1962; Burke & Papkoff, 1980) hens when compared to non-incubating hens, the relationship between prolactin content and prolactin mRNA levels in the pituitary gland has not been evaluated. Previous studies demonstrated that pituitary prolactin content is 3 times higher in incubating than in non-incubating hens whereas the present study showed a more than 10-fold increase in pituitary prolactin content in incubating hens. Different degrees of the increase in pituitary prolactin content may be due to different sensitivities of the assays and species or breed differences. However, the >10-fold increases in prolactin mRNA and the high correlates between the levels of prolactin mRNA and of prolactin in the pituitary gland found in this study may indicate that not only accumulation of prolactin mRNA but also progressive transcription take place for prolactin biosynthesis when hens display incubation behaviour.

The mechanisms of regulation of prolactin gene expression remain unknown in birds. TRH can induce prolactin release in the chicken anterior pituitary *in vitro* (Hall *et al.*, 1975; Harvey *et al.*, 1978), whereas in-vivo administration of TRH failed to induce a release in the young chicken (Harvey *et al.*, 1978), as was observed in the present study. The latter results do not necessarily mean little effect of TRH on protein synthesis. In fact, the present study suggests that TRH may contribute to the transcriptional regulation of prolactin mRNA when it is injected at a high dose but that not all mRNA is being efficiently translated into the protein after TRH treatment. It has been claimed that the rat fetus is not capable of producing a large amount of prolactin, probably due to insufficient production of ribosomal RNA, although a large amount of prolactin mRNA is produced (Frawley & Miller, 1989). Whether possible cessation of translational process leading to prolactin synthesis in the chicken is attributable to less availability of ribosomal RNA or other factors needs further investigation. To study the relative contributions of transcriptional control on the observed stimulation of prolactin gene expression by TRH in the chicken, the transcriptional rate of the prolactin gene, as measured by nuclear run-off assays, should be compared with the effects on prolactin mRNA levels. These studies have not yet been undertaken.

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