II-3. RECOMBINANT DERIVED CHICKEN PROLACTIN: EXPRESSION, PURIFICATION AND BIOLOGICAL FUNCTION

II-3.1. Summary

Chicken PRL (cPRL) was produced in *E. coli* by manipulating the PRL cDNA cloned from White Leghorn and an expression vector pKK223-3 to confirm whether PRL protein which may be translated and released in White Leghorn is biologically active or not. The recombinant gene derived PRL exhibited a molecular mass of 23 kDa, which was in close agreement with the predicted molecular mass from the cPRL cDNA sequence. The recombinant protein showed equivalent binding kinetics to an antiserum raised against turkey PRL. Therefore, it was suggested that recombinant White Leghorn PRL has successfully product produced in *E. coli*. By injection of the recombinant PRL into pigeon crop sac as a bioassay, the recombinant White Leghorn PRL increased the weight of pigeon crop sac mucosa to a degree comparable to that induced by authentic turkey PRL derived from anterior pituitary gland. This result strongly suggests that White Leghorn PRL is biological active, and it is possible that the PRL in White Leghorn is possible to bind to PRLR in target tissues to mediated its actions.

II-3.2. Introduction

Prolactin exerts multiple functions in birds as well as in mammals (See general introduction), and numerous studies on the PRL functions have been demonstrated in domestic birds by injecting PRL (Riddle *et al.*, 1935, Bikle *et al.*, 1980, Spencer *et al.*, 1981, Kühn *et al.*, 1983, Decuypere and Kühn, 1985). However, the physiological roles of PRL including incubation behavior are still poorly understood by homologous system, because these studies were based on the injections of mammalian PRL.

Chicken PRL (cPRL) cDNAs have been cloned from White Leghorn (Watahiki *et al.*, 1989) and bantam (Hanks *et al.*, 1989a). Both groups demonstrated that the each cPRL has a 30 amino acid signal peptide followed by a 199 amino acid mature protein. However, there are 3 inconsistent amino acids at positions 141, 150 and 175 in the mature cPRL. Turkey PRL cDNA also has been cloned (Wang *et al.*, 1991) and amino acids at positions 141, 150 and 176 in turkey were the same with those in bantam. Hanks *et al.* (1989b) expressed cPRL in *E. coli* using their-cloned cPRL cDNA and found that the product possesses both immunological and biological properties. And also radioimmunoassay for recombinant cPRL has been established by using the bantam PRL cDNA and the system was suitable for measurement of PRL in the other avian species (Talbot and Sharp, 1994).

As described in Chapters II-1 and II-2, I have revealed that PRLR mRNA were highly expressed in the hypothalamus and pituitary gland in White Leghorn and bantam hens, and gross differences on the structure and transcript of PRLR were not observed in both breeds. These results indicate that breed differences on the display of incubation behavior can't be explained by the differences of PRLR expressed in tissues. As for PRL, although Shimada *et al.* (1991) have demonstrated that cDNA cloned by Watahiki *et al.* (1989) is suitable for quantification of cPRL mRNA in brooding and non-brooding chickens, it is to be considered whether the protein would be translated from the White Leghorn PRL mRNA is functional.

Moreover, the 3 different amino acids identified in the PRL cDNA of White Leghorn might be account for the lack of broodiness in White Leghorn by changing the interaction between PRL and the receptor.

Here, I describe the expression of recombinant White Leghorn PRL in *Escherichia coli* and analyses of biological and immunological properties of the protein to establish whether Leghorn PRL possesses equivalent physiological activity with authentic avian PRL or recombinant PRL produced from broody breed of chicken.

II-3.3. Materials and Methods

II-3.3.1. Plasmids and bacterial strains

The pKK223-3 plasmid (Pharmacia Sweden) was used as the expression vector to produce recombinant cPRL. This vector contains a *tac* promoter, which is induced by the addition of isopropylthio-β-galactoside (IPTG). A plasmid (pcPRL-1) contains 953 bases of entire cPRL cDNA cloned by Watahiki *et al.* (1989) was used for the construction of expression vector. *E. coli* strain JM103 was used as a host for expression of the protein.

II-3.3.2. Oligonucleotides

Two pairs of oligonucleotides were synthesized by the phosphite triester method using phosphoramidite chemistry in 381A DNA synthesizer (Applied Biosystems, Foster City, CA, USA). The oligonucleotides were purified by a Pharmacia FPLC system.

II-3.3.3. Construction of cPRL expression vectors

The pcPRL-1 plasmid was digested with *Sty* I and *Eco* RI to obtain a 757 bp fragment, which encodes all but the first 15 amino acids of mature cPRL. This fragment and synthetic DNAs, replacing the sequences for the first 15 amino acids of cPRL with AT-rich codons and providing a pair of Shine -Dalgarno (SD) sequences (Fig. II-3-1), were subcloned into a pKK223-3 plasmid.

II-3.3.4. Detection of recombinant vector derived cPRL by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried out for the detection of the recombinant cPRL protein. After the gel

electrophoresis, the gel was stained with Commassie Brilliant Blue to visualized the protein bands. To analyze the folding efficiency of the purified recombinant cPRL, the protein was treated with or without 2-mercaptoethanol before the gel electrophoresis.

II-3.3.5 Purification of recombinant cPRL from *E. coli*

The cPRL expressing cells were grown in a L-broth (50 ml) medium, containing 100 µg ampicillin/ml and 2 mM IPTG at 37°C for 24 h with vigorous shaking, and the culture was then transferred into a 500 ml medium of the same composition and grown for additional 40 hr under same conditions. The cells were precipitated from the culture medium by centrifugation, the pellets were sonicated in a buffer consisting of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.2 M Phenyl methyl sulfonyl fluoride (PMSF) and 1 mg/ml lysozyme for 10 minutes at 0°C. After the centrifugation, the pellets were suspended in a buffer consisting of 5 M urea, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 20% methanol and 0.005% Tween 80, and were followed by sonication step as described above. After the sonication, cells were precipitated by centrifugation to obtain cPRL proteins as inclusion bodies. The inclusion bodies were mixed and stirred to dissolve in 5 ml of 0.1 M Tris-HCl (pH 9.0) containing 7 M guanidine hydrochloride, and 10 mM dithiothreitol (DTT) for 1 hour at room temperature. The dissolved solution was slowly added into a 0.1 M Tris-HCl (pH 9.0) solution containing 2 M guanidine hydrochloride, 10 mM reduced glutathione, and 1 mM oxidized glutathione to achieve a final protein concentration of 0.05 mg/ml. The mixture was stirred gently for 14-18 hr at room temperature and was followed by dialysis against 10 mM Tris-HCl buffer (pH 9.0) for 24 hr. The solution containing folded cPRL was applied on 10 mm x 15 mm Sepharose-Q columns (Pharmacia, Sweden) at 4°C and eluted with a gradient solution composed of 50 mM to 1 M NaCl in 10 mM Tris-HCl buffer (pH 9.0) at a flow rate of 1

ml/min, monitoring by UV absorbance at 280 nm to collect peak fractions. The peaked fractions were reapplied on the column and purified proteins were then dialyzed against distilled water and freeze-dried. After the proteins were reconstituted in saline, protein concentration was determined and stored at –20°C until use for radioimmunoassay and pigeon crop sac assay.

II-3.3.6. Radioimmunoassay

Binding affinity of recombinant cPRL to turkey PRL (tPRL) antibodies was determined by the degree of competition with tPRL. Turkey PRL was labeled with ^{125}I by the method of Proudman and Opel (1981). The recombinant cPRL or tPRL, at levels ranging from 0.10 to 1.56 ng, was incubated with anti-tPRL antibody and the radiolabeled turkey PRL for 2 days at 4 °C, and after the addition of anti-rabbit IgG the mixtures were then incubated for an additional 2 days at 4°C. After centrifugation, the pellets were counted by an Autowell γ-counter (Aloka Co. Ltd., Tokyo, Japan) to calculate specific binding.

II-3.3.7. Stimulation of pigeon crop sac by recombinant cPRL.

The crop sac stimulation assay was carried out according the method of Nicoll (1967). Either tPRL or recombinant cPRL (5 μg) was injected to the one side of pigeon crop sac of one of 2 pigeon groups for 5 days. As a control, saline was also injected to the opposite side of the crop sac in the same bird. Twenty-four hours after the last injection, pigeons were killed and crop sacs were removed to analyze the response to the PRLs.

The statistical significance between the test and control preparations was determined by Student's *t*-test.

II-3.4. Results

II-3.4.1. Expression of cPRL in *E. coli*

Four out of 12 positive transformant clones produced an extra 23 kDa protein detected by SDS-PAGE analysis as shown in Fig. 3-2. Densitometric scanning of the gel indicated that approximately 10% of synthesized by *E. coli* was 23 kDa (Fig. II-3-2, lanes 1, 3 and 5)

II-3.4.2. Purification of recombinant cPRL from *E. coli*

After the purification steps described in Materials and Methods, the 23 kDa proteins expressed in *E. coli* were detected as a single band by SDS-PAGE analysis with Commassie stain. By SDS-PAGE analysis in the presence or absence of 2-mercaptoethanol, the cPRL proteins were shown to be successfully folded by forming disulfide bridges in the molecule (Fig. II-3-3). The yield of the protein was about 6 μg/ml culture.

II-3.4.3. Immunological characterization of recombinant cPRL

The recombinant cPRL was specifically crossreacted with anti-tPRL antibody, and the crossreactivities in the range from 0.1 and 1.56 ng of the recombinant cPRL were identical to those of tPRL (Fig. II-3-4).

II-3.4.4. Biological activities of recombinant cPRL

Five micrograms of cPRL and tPRL yielded significant ($P<0.01$ and $P<0.05$, respectively) increases in the mucosal dry weight of pigeon crop sac by injection to a hemicrop over saline controls, and the cPRL and tPRL preparations showed equivalent effects on the pigeon crop sac growth stimulation (Fig. II-3-5).

II-3.5. Discussion

It has been reported that the distance between the SD sequence and the initiation codon ATG is very important in expressing proteins in *E. coli* cells (Shepard *et al.*, 1982). Moreover the two-cistron expression system has been employed to enhance further the efficient and frequent initiation of translation (Schoner *et al.*, 1984, 1986). In this study, the synthetic DNA linker containing two cistrons was placed with an appropriate space between the promoter and the initiation codon. Consequently, a high level expression, approximately 10% of *E. coli* protein, of recombinant cPRL was achieved. The expression rate in this study was higher than that of previously reported (approximately 1.5% of total protein was recombinant protein) by Hanks *et al.* (1989).

As shown in Fig. II-3-3, the expressed protein in this study was purified by ion exchange chromatography to a discrete band of 23 kDa on SDS-PAGE, that was in close agreement with the predicted molecular mass from the cPRL cDNA sequence (Watahiki *et al.*, 1989). Though, tPRL cDNA encodes 199 amino acids as a mature protein and the molecular mass of the protein is 23 kDa (Karatzas *et al.*, 1990) as well as cPRL cDNA. However, it is reported that the molecular mass of tPRL estimated by SDS-PAGE by Burke and Papkoff (1980) was 26 kDa, whereas Proudman and Corcoran (1981) demonstrated three different forms; one 22 kDa and two 26 kDa forms. More recently, it has been described that 24 kDa of non-glycosylated PRL and 27 kDa of glycosylated PRL are released from the turkey anterior pituitary gland *in vitro* (Bedecarrats *et al.*, 1999a). The glycosylated PRL is also observed in the pituitary gland in mammalians (Lewis *et al.*, 1989, Young *et al.*, 1990, Bollengier *et al.*, 1993) and in reptile (Noso *et al.*, 1992). These results indicate that posttranslational modification such as glycosylation is naturally occurred in the pituitary gland in vertebrates.

The recombinant cPRL obtained in this study which may be higher homogeneity than that of pituitary-derived PRL possessed equivalent biological activities to those of tPRL in stimulating the mitogenesis in the crop sac (Fig. II-3-5). In addition, this protein crossreacted with anti-tPRL antibody to an equal extent as the native form of tPRL purified from the turkey pituitary gland (Fig. II-3-4). Hence, post-translational modification (e.g. glycosylation) may not be a prerequisite for biological activity. There is evidence that glycosylated PRL showed less biological activity in stimulating mitogenesis in the pigeon crop sac (Lewis *et al.*, 1989) and in the Nb2 cells (Markoff *et al.*, 1988, Sinha *et al.*, 1991), but glycosylated PRL showed greater lactogenetic activity than non-glycosylated PRL (Young *et al.*, 1990). Furthermore, higher percentages of glycosylated PRL were associated with increasing levels of total PRL in the pituitary gland in turkey embryo (Bedecarrats *et al.*, 1999b) The PRL variants may modulate physiologically diverse effects of PRL on target tissues.

It is confirmed in this study that the recombinant technique-derived chicken PRL, being produced with White Leghorn PRL cDNA, exhibited substantial biological and immunological activities. It is reported that another recombinant cPRL produced with PRL cDNA obtained from bantam, which is one breed of domestic chicken, also showed both biological and immunological activities (Hanks *et al.*, 1989b). These facts indicated that these two preparations possess the equivalent potency in both biological and immunological activities as PRL, although one breed, White Leghorn, never display incubation behavior and another breed, bantam manifests the behavior.

Therefore, it is concluded that differences of PRL cDNA observed between White Leghorn and bantam may not influence on biological activity of PRL and the structural difference of PRL in two breeds may not responsible for different manifestation of incubation behavior.

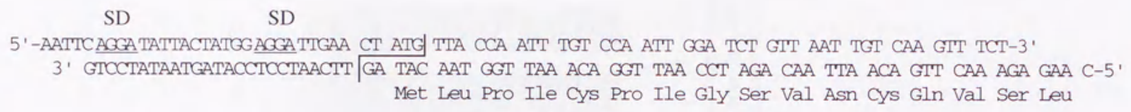

Fig. II-3-1 Sequece of synthetic DNAs encoding the *N*-terminus of cPRL and linker region of the two-cistron translation system. Four single strand oligonucleotides were synthesized independently and annealed. The sequence of duplex was chosen to be rich in AT without changing the amino acid sequence of the protein. Two Shine-Dalgarno sequences were underlined and marked as SD above the sequence.

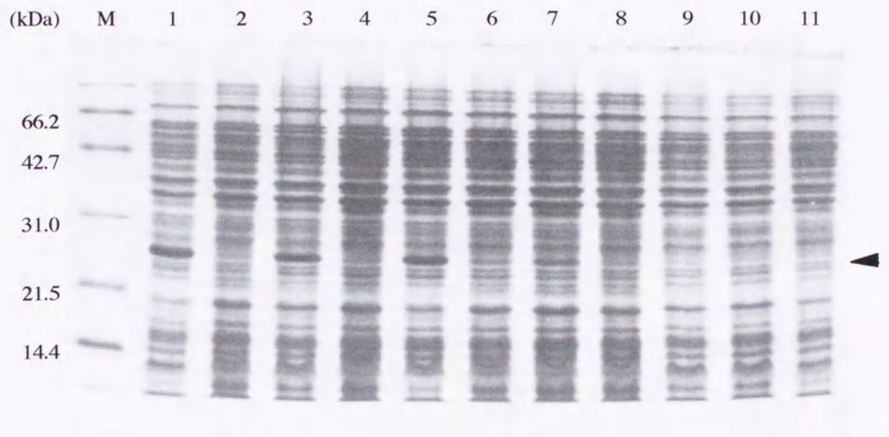

Fig. II-3-2 SDS-PAGE analysis of putative recombinant cPRL expressed in E. coli. Samples of cell lysates of 0.5 ml-cultures grown to the stationary phase in L-broth containing 2 mM IPTG were electrophoresed in 13.5 % polyacrylamide. Lanes 1, 3, 5 and were positive clones expressing cPRL.

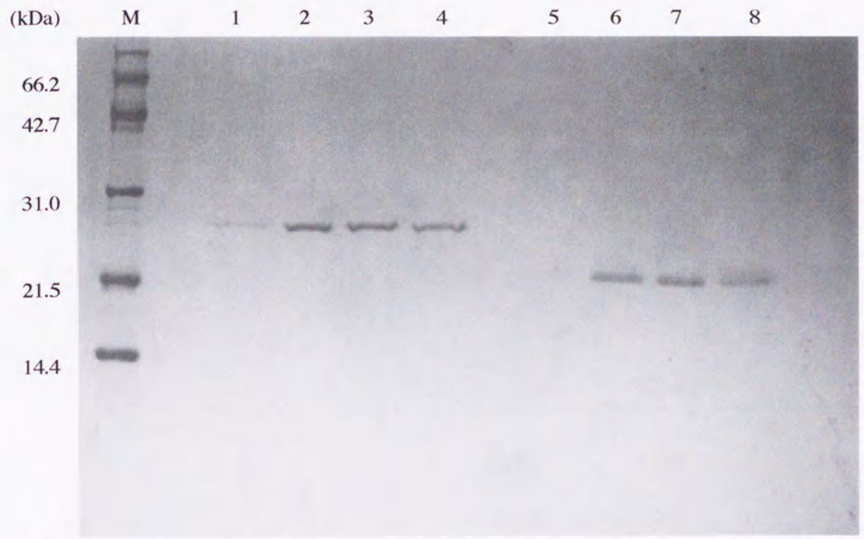

Fig. II-3-3 SDS-PAGE analysis of recombinant cPRL purified by ion exchange chromatography. Lanes 1-4 and lanes 5-8 indicate different fractions of major peak portion after chromatography under reduced and non-reduced conditions, respectively.

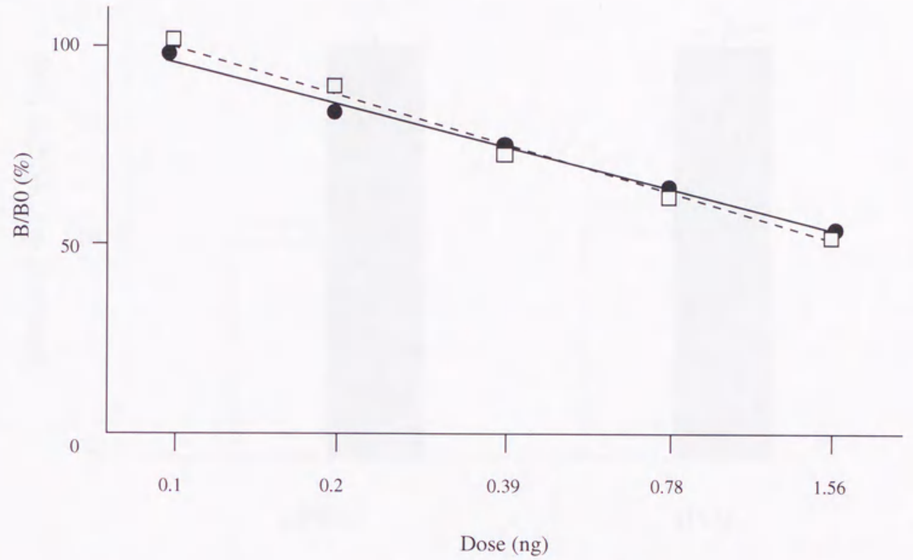

Fig. II-3-4 Binding activity of recombinant cPRL and tPRL to tPRL antibody.

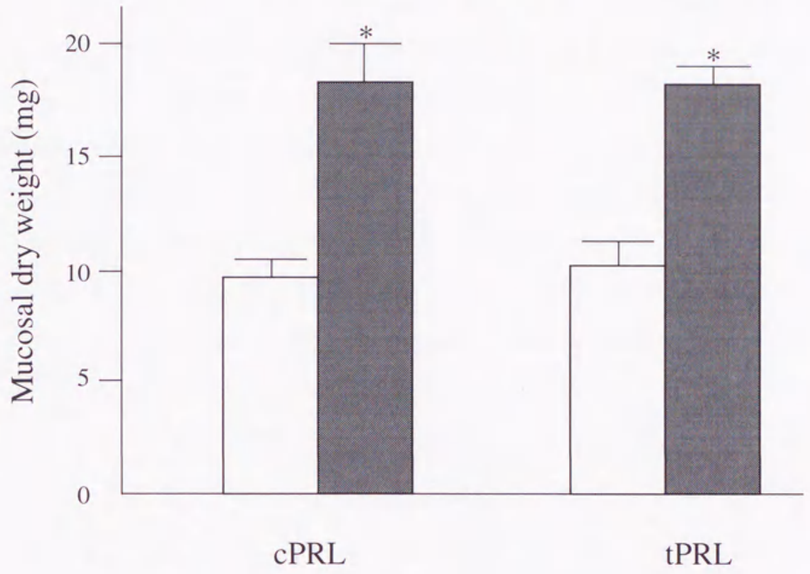

Fig. II-3-5 Pigeon crop mucosal weight after the injection of 5 μg cPRL and 5 μg tPRL (hatched bar), with the corresponding saline control (open bar). Values are mean ± SEM (n=5). Asterisk indicates significant differences between the control and the treatment ($P<0.01$)

II-4. MOLECULAR CLONING AND POSSIBLE COOPERATIVE ACTIVATION OF THE CHICKEN PROLACTIN GENE BY Pit-1 AND cAMP-INDUCED FACTOR, CBP

II-4.1. Summary

Transcription of the prolactin (PRL) gene has been reported to be activated by a nuclear factor, Pit-1. However, the precise molecular mechanisms of the Pit-1 mediated PRL gene activation are still unclear. We have cloned the chicken PRL (cPRL) gene and its 5'-flanking region to analyze their structure and transcription initiating mechanism. In luciferase assay, forskolin activated the proximal promoter region between -248 and -76 to transcribe the cPRL gene in pituitary cells, but not in non-pituitary cells, although the pituitary Pit-1 mRNA expression was not enhanced by the cAMP stimulation. In gel mobility shift assay, two DNA fragments, -133/-103 and -104/-76, each containing putative Pit-1 binding site, were bound by nuclear factors from the pituitary cells. Pretreating the nuclear extracts with either anti-Pit-1 or anti-CBP antibodies prevented the binding between the fragments and the factors. These results indicate that Pit-1 requires CBP as a cooperative activator to bind to the two proximal promoter sites to activate the cPRL gene expression.

4.2. Introduction

Prolactin (PRL) is a member of the family of related hormones including growth hormone, placental lactogen, proliferin and somatolactin which was purified and identified from the pituitary glands of teleost (Ono *et al.*, 1990). It is believed that they have evolved from a common ancestral gene by means of gene duplication (Nial *et al.*, 1971, Millar and Eberhardt, 1983, Nicoll *et al.*, 1986) and these hormones have highly homologous primary structures as well as similar biological properties.

A lot of studies have been demonstrated to identify transcription factors for PRL gene expression and to understand regulatory mechanism of PRL gene expression in mammals. The transcription factor Pit-1/GHF-1 (Pit-1) has been shown to play an important role in the regulation of the gene expression for PRL, growth hormone (GH) and β chain of thyroid-stimulating hormone (TSH) in mammals. For transcriptional activation of mammalian PRL genes, Pit-1 protein interacts with its conserved binding site localized in the promoter regions of the genes, and this Pit-1 dependent gene expression of the PRL is enhanced by cAMP (Mangalam *et al.*, 1989, Iverson *et al.*, 1990, Howard and Maurer, 1995). Several hormones and peptides including dopamine (Maurer, 1981), estrogen (Maurer, 1982), vasoactive intestinal peptide (VIP) (Carrillo *et al.*, 1985, Escalada *et al.*, 1996), epidermal growth factor (Supowit *et al.*, 1984, Stanley, 1988) and thyrotropin releasing hormone (Murdoch *et al.*, 1983, 1985) have also been reported to regulate the PRL gene expression. Furthermore, it is described that the regulation of PRL gene expression by dopamine or VIP are modulated via the cAMP pathway (Maurer, 1981, Carrillo *et al.*, 1985). There is also evidence that VIP also increases cellular cAMP concentration in the perifused chicken anterior gland as well as in mammals (Shimada and Kansaku, 1997).

Recently, the turkey PRL gene has been cloned and nucleotide sequence including 5'-flanking region was revealed (Kurima *et al.*, 1995). By comparison of the turkey PRL gene with those of mammalians, it has been revealed that the exon/intron arrangement of turkey PRL gene is similar to mammalian PRL genes, and predicted Pit-1 binding sites are localized in the promoter region of the gene (Kurima *et al.*, 1995). On the other hand, multiple forms of Pit-1 cDNA have been cloned in turkey (Wong *et al.*, 1992 Kurima *et al.*, 1998) and chicken (Tanaka *et al.*, 1999), suggesting the existence of Pit-1 factors to regulate PRL gene expression in the avian pituitary.

During incubation behavior, PRL mRNA in the pituitary gland are up-regulated in chicken (Talbot *et al.*, 1991, Shimada *et al.*, 1991) and turkey (Wong *et al.*, 1991). And VIP plays an important role in controlling PRL synthesis in the anterior pituitary gland in chicken (Sharp *et al.*, 1989, Talbot *et al.*, 1991, 1995, Kansaku *et al.*, 1995) and turkey (Youngren *et al.*, 1994, El Halawani *et al.*, 1995).

In Chapter II-2, it has been described that VIP is effective on PRL release from the anterior pituitary gland of White Leghorn and bantam. Moreover, in Chapter II-3, it has been suggested that PRL proteins produced in the both breeds are biologically active. However, the regulatory pathways by Pit-1 an/or other factors are not full understood in birds. Therefore, it is very important to reveal how PRL is transcriptionally regulated by transcription factors to understand the mechanism of broody behavior. And it might be possible that transcriptional regulation of PRL gene is different between non-broody breed and broody breed.

In this study, I have initially cloned chicken (White Leghorn) PRL gene to compare its structure with that of turkey PRL gene. And furthermore transcriptional regulation has been analyzed to clarify the promoting mechanism of PRL gene expression by Pit-1 and cAMP in birds.

II-4.3. Materials and Methods

II-4.3.1. Cloning and sequencing of cPRL gene

Chicken genomic DNA was extracted from the liver and partially digested with *Sau*3AI, and fractionated by sucrose density gradient centrifugation (Sambrook *et al.*, 1989). DNA fragments of 10 to 20 kb were cloned into λEMBL3 vector and packaged *in vitro*. The genomic library was screened by ^{32}P-labeled cPRL cDNA (Watahiki *et al.*, 1989). The clone containing the cPRL gene was digested with several restriction enzymes and appropriate restriction fragments were subcloned into pUC118 and sequenced by dideoxy chain termination methods (Sanger *et al.*, 1977). Some restriction fragments were deleted on their 5' and/or 3' ends by T4 exonuclease and Mung bean nuclease before subcloning.

II-4.3.2. Reporter gene construction and transfection

A 1.4 kb fragment spanning from -1380 to +33 in the cPRL promoter region, harboring a *Hin*dIII site at 3'-end, was amplified by PCR using appropriate primers, and then the amplified fragment was subcloned into pCR™II plasmid vector (Invitrogen, Carlsbad, CA) and sequenced. The DNA fragment digested with *Sac*I and *Hin*dIII was subcloned into *Sac*I and *Hin*dIII sites of the pGL-2 vector (Promega, Madison, WI) to generated reporter plasmid (-1336 luc). The -1366del luc was generated by removal of *Xba*I fragments from -1336 luc. DNA fragments for other reporter constructs were prepared by 5' deletion and subcloned into pGL-2 vector and used.

The GH3 cells derived from rat pituitary tumor were supplied from Japan Cancer Research Resources Bank. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2.5% fetal bovine serum and 15% horse serum in 5% CO_2/95 % air at

37°C. The cells were transfected with reporter genes by CellPhect transfection kit (Amersham Pharmacia, Tokyo, Japan) according to the manufacture's instruction. Twenty four hours after transfection, the cells were treated with 10 μM forskolin (Sigma, St. Louis, MO) or with PBS(-) in serum-free DMEM for additional 24 h, and then the cells were harvested. Luciferase activity was determined as described previously (De Wet *et al.*, 1987).

II-4.3.3. Luciferase assay and β-galactosidase assay

Both luciferase and β-galactosidase activities were determined using Luciferase assay System (Promega) and β-Galactosidase Assay System (Promega), respectively. The protein concentration of each extract was determined with a Bio-Rad protein assay kit (Bio-Lad Laboratories, Hercules, CA). The luciferase activity obtained from cell extracts was normalized to the β-galactosidase activity to correct for differences in transfection efficiency.

II-4.3.4. Northern blot hybridization

Total RNA was extracted from GH3 cells at the time of forskolin stimulation and 2, 4, 8 and 12 h after forskolin treatment using a commercial kit (Nippon Gene, Toyama, Japan), and separated by electrophoresis on a 1% agarose gel containing 2.2M formaldehyde. The separated RNAs were blotted onto a Gene Screen membrane (NEN life Science, Boston, MA) which was hybridized to the ^{32}P-labeled 271 bp rat Pit-1 cDNA probe encoding from 264 to 534. After hybridization, the membrane was washed sequentially in 2xSSC containing 0.1% SDS at room temperature, 1xSSC containing 0.1% SDS at 65°C for 1 h, and 0.1xSSC containing 0.1% SDS at 65°C for 1 h. It was then exposed to X-ray film (Fuji photo film, Tokyo, Japan) with an intensifying screen at -70°C for 3 days to generate an autoradiogram.

II-4.3.5. DNA gel mobility shift analysis

Both strands of oligonucleotide probes corresponding to a region between -1301 and -1280 (DF), between -104 and -76 (PF1) and between -133 and -103 (PF2) were synthesized. The probes were annealed and radiolabelled with [α-^{32}P] dCTP by Klenow fragment. The radiolabelled probes, DF or PF1 and PF2 (each 1.0×10^4 cpm), were then incubated with 5 μg of nuclear extracts prepared from chicken pituitary gland and COS-7 cells transfected with chicken Pit-1 (cPit-1) expression vector or nuclear proteins extracted from chicken pituitary gland and GH3 cells, respectively. The reactions were made at 30°C for 30 min in a binding buffer containing 20 mM HEPES-KOH (pH 7.9), 0.1M KCl, 20% glycerol, 0.5mM dithiothreitol, 1mM $MgCl_2$, 12.5mM spermidine and 1μg of poly (dI-dC)·poly (dI-dC). The reaction mixtures were applied to 4% polyacrylamide gel electrophoresis as described previously (Singh *et al.*, 1986). For supershift analysis, nuclear extracts prepared from GH3 cells were preincubated with anti-Pit-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-CBP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 6 h under the conditions described above, and then radiolabelled PF2 probe was added to analyze in 4% polyacrylamide gel by electrophoresis.

II-4.4. Results

II-4.4.1. Structural analysis of cPRL gene

The cPRL gene was cloned and sequenced. It comprised five exons and four introns. The nucleotide sequences of the exons and the donor and acceptor regions of the introns, together with the deduced amino acid sequence, are shown in Fig. II-4-1. The exon/intron arrangement of cPRL gene is similar to that of turkey PRL gene (Kurima *et al.*, 1995), and all the exon-intron junctions conform to the GT-AG rule (Breathnach and Cambon, 1981). The length of cPRL gene is about 6.8 kb, which is close to that of turkey PRL gene (Kurima *et al.*, 1995). At the 3'-end, two putative polyadenylation signals were located at 209 and 276 nucleotides downstream from the termination codon.

The 5'-flanking region of the cPRL gene is shown in Fig. II-4-2. A typical TATA-box was localized 24 bp upstream of the transcription start point deduced from the 5'-end of cPRL cDNA (Watahiki *et al.*, 1989). Three avian and teleost type (Ohkubo *et al.*, 1996) and one mammalian type (Bodner *et al.*, 1988, Ingraham *et al.*, 1988) Pit-1 binding sites were localized in the distal 5'-flanking region of the cPRL gene between -1314 and -1128. The proximal region from -128 to -67 in the cPRL promoter showed higher similarities with the P2 and P3 regions of the rat PRL promoter region where mammalian type Pit-1 binding sites reside (Mangalam *et al.*, 1989). In the cPRL proximal promoter region, no canonical Pit-1 consensus sequence, neither avian and teleost type nor mammalian type was observed, but two putative Pit-1 binding site-like sequences were localized at -123 and -98 regions. On the other hand, no candidate for cAMP-responsive element (CRE) was found either in the proximal region or in more upstream region up to -1366.

II-4.4.2. Promoter activities of the 5'-flanking region of cPRL gene in GH3 and HeLa cells

The transcription-initiating activities of various 5'-regions of the cPRL gene were analyzed by luciferase assay. A set of fragments from the 5'-flanking region of the cPRL gene were fused to the luciferase reporter gene and transfected into GH3 cells, to determine whether forskolin induced increases in intracellular cAMP concentration induces the cPRL promoter activity in GH3 cells (Fig. II-4-3). The luciferase activities in -76 luc and -41 luc even after the forskolin addition were equivalent to the background activity. But in other constructs, luciferase activities were markedly increased by forskolin treatment. Furthermore, luciferase activities after forskolin treatment in the reporter genes from -1117 luc to -248 luc were higher than those of -1366 luc and -1366del luc. In contrast to the results in GH3 cells, forskolin didn't enhance the luciferase activities in HeLa cells and the activities were equivalent to that of a promoter-less control (Table II-4-1).

II-4.4.3. Pit-1 mRNA expression in GH3 cells following forskolin stimulation

Northern blot hybridization was carried out to determine the effect of forskolin on the Pit-1 gene expression in GH3 cells (Fig. II-4-4). Pit-1 mRNA expression was significantly decreased 2 h after the forskolin stimulation and the expression was recovered to the level before forskolin stimulation at 8 h after the treatment.

II-4.4.4. Gel mobility shift analysis of distal Pit-1 binding site in cPRL gene 5'-flanking region

To determine the transcription factor binding sites in the cPRL promoter region, gel mobility shift analysis was performed. A specifically shifted band was observed when a DNA fragment from -1301 to -1280 (DF) was incubated with the nuclear extracts prepared from the chicken pituitary gland or COS-7 cells transfected with chicken Pit-1 expression

vector. The shifted band was competitively reduced by the addition of excess amounts of the non-radioactive probe (Fig. II-4-5).

II-4.4.5. Nuclear factors binding to proximal promoter region of cPRL gene

To determine the transcription factors promoting cPRL gene expression, nuclear extracts from the chicken pituitary or GH3 cells were incubated with two DNA fragments, PF1 from -104 to -76 and PF2 from -133 to -103, of the proximal promoter region of the cPRL gene, and competition has been made by the addition of non-radioactive PF1, PF2 or a DNA fragment from -1301 to –1280 (DF) which region contains a putative Pit-1 binding consensus sequence. With PF1, specifically shifted band was observed when it was incubated with nuclear proteins from chicken pituitary and the shifted band of PF1 was much reduced by the addition of non-radioactive PF1. Furthermore, its binding was blocked partially by the addition of PF2 or DF (Fig. II-4-6B). In the same assay by using PF1 probe and GH3 nuclear extracts, specifically shifted band was also observed and the binding was suppressed by the addition of non-radiolabeled PF1 probe. However, the interaction between the PF1 probe and nuclear proteins that prepared from GH3 cells was not reduced by the addition of non-radioactive DF and PF2 probes (Fig. II-4-6C). On the other hand, specific interaction between PF2 probe and chicken pituitary or GH3 cells nuclear extracts was not observed (Figs. II-4-6B and II-4-6C).

By supershift assay using antibodies against CBP or Pit-1, the band signals showing the interaction between the nuclear factors and the DNA probe was suppressed by treating the nuclear factors with antibodies against CBP or Pit-1, and the signal appeared at the origins on the gel. Densitometric analysis showed that the signals of the shifted bands were reduced by 14%, 21% and 34% by the antibodies against CBP, Pit-1 and both, respectively (Fig. II-4-7).

II-4.5.Discussion

The structure of the 5'-flanking region cPRL gene showed approximately 80 % similarity with that of turkey (Kurima *et al.*, 1995), indicating that the regulatory mechanism of the gene may be very similar in these 2 birds. Furthermore, comparison of the regulatory sequences between chicken and mammalian PRL genes revealed that highly homologous sequences were conserved in their proximal promoter regions. The cPRL promoter has two proximal Pit-1 binding regions corresponding to the P2 and P3 regions of rat PRL promoter (Mangalam *et al.*, 1989), but it has no corresponding sequence to the P1 region of the rat promoter which is also considered to be a putative Pit-1 binding site in mammals (Mangalam *et al.*, 1989). The turkey PRL gene promoter has similar structural features to that of the cPRL promoter (Kurima *et al.*, 1995).

The present study demonstrated that the proximal region up to -248 is sufficient to promote the cAMP-dependent PRL gene expression in chicken, and the region from -76 to the transcription start point has no activity to initiate the cAMP-dependent gene expression (Fig. II-4-3). This result indicates that the sequence between –248 and –76 contain the key elements to promote the cAMP-dependent cPRL gene expression. In the rat, Pit-1 has been reported to interact with the P2 and P3 regions in the proximal promoter region to stimulate the PRL gene expression (Mangalam *et al.*, 1989). Furthermore, the Pit-1 dependent gene expression is enhanced by cAMP (Peers *et al.*, 1991, Liang *et al.*, 1992, Gaiddon *et al.*, 1995) and other factors (Howard and Maurer, 1995) interacting with the proximal promoter region. Moreover, it has been confirmed in mammals that the cAMP-dependent stimulation of the PRL gene expression is occurred only in the pituitary cells, but not in heterologous cell lines lacking Pit-1 protein production (Mangalam *et al.*, 1989, Peers *et al.*, 1991). It has been also confirmed in this study that cAMP-induced expression of the PRL gene was observed in GH3 cells but not in HeLa cells (Table 4-1). In addition, in the chicken, forskolin induced the

PRL gene expression significantly in the anterior pituitary gland (Kansaku *et al.*, 1995, Shimada and Kansaku, 1997). Therefore, it can be hypothesized that both Pit-1 and a cAMP-responsive factor(s) play important roles in the promotion of the PRL gene expression in birds and mammals by interacting with the proximal Pit-1 binding domains. To support this hypothesis, following experiments have been carried out.

Northern analysis was performed to verify whether forskolin can directly induce Pit-1 mRNA expression to stimulate PRL gene expression. Forskolin treatment did not stimulate Pit-1 gene expression in GH3 cells, but it rather decreased the gene expression in 2 h (Fig. II-4-4) while the promoter activities were elevated by the forskolin treatment (Fig. II-4-3). This result suggests that an increase in Pit-1 level is not necessary to stimulate PRL gene expression in the pituitary gland, consistent with the observation that the PRL mRNA in the pituitary gland is increased during the incubation period without an increase in Pit-1 mRNA expression in turkey (Wong *et al.*, 1992). In addition, DNA probes prepared from the promoter regions highly conserved between chicken and mammalian PRL genes, PF1 and PF2, which correspond to P2 and P3 in mammalian PRL genes, respectively, were tested to identify *cis*-acting element(s) of the cPRL gene by DNA gel mobility shift assay (Fig. II-4-6). The PF1 probe specifically bound with nuclear factors derived from chicken pituitary gland and GH3 rat pituitary tumor cells (Figs. II-4-6B and II-4-6C). With PF2 probe that is also a conserved promoter region between chicken and mammalian PRL genes, but similarity between PF2 and P3 is less than that between PF1 and P2 region, specific interaction was not visualized between nuclear factors prepared from both chicken pituitary gland and GH3 cells. This result suggests that PF1 region between –76 and –103 may mediate cAMP-dependent activation of cPRL gene.

It has been widely believed that the cAMP-dependent gene expressions are mediated by CRE binding protein (CREB), which binds to the specific motif, TGACGTCA so called CRE

(Bokar *et al.*, 1988, Deutsch *et al.*, 1988). Several studies have predicted that PRL gene expression is also regulated by the same manner on CRE, because CRE-like element was found in the proximal promoter region in rat PRL gene, showing transcriptional responses to cAMP treatments (Iverson *et al.*, 1990, Liang *et al.*, 1992). However, it has also been observed that CRE motif can not competitively reduce specific binding between the proximal promoter region of human PRL gene and nuclear extracts derived from pituitary tumor cells (Peers *et al.*, 1991). Furthermore, such CRE-like site observed in mammalian PRL gene was not conserved in the chicken PRL promoter region. Considering these results, it is unlikely that both Pit-1 and CREB bind directly to the *cis*-elements in the PRL promoter region to control its cAMP dependent expression. On the other hand, it has been reported that CREB binding protein (CBP) (Chrivia *et al.*, 1993) and p/CAF (Yang *et al.*, 1996) form a co-activator complex, which interacts with nuclear coactivator to mediate the nuclear receptor function (Torchia *et al.*, 1997). Recent studies show that the activity of Pit-1 is also regulated by the balance between the coactivator complex and a co-repressor complex (Heinzel *et al.*, 1997, Alland *et al.*, 1997). CBP is stimulated by cAMP (Xu *et al.*, 1998) and directly binds to the POU-specific domain of Pit-1 to mediate the Pit-1-dependent activation of the PRL gene (Xu *et al.*, 1998, Tolón *et al.*, 1998). Present result that Pit-1 and cAMP-induced CBP mediated the PRL gene expression without existence of CRE is consistent with this mechanism, and therefore, it is possible that the Pit-1 alone may not directly induce the PRL gene expression, but may enhance the PRL gene expression after interaction with cAMP-stimulated CBP. Then, we have tested that protein binding to specific DNA sequence (PF1) by supershift assay using GH3 nuclear extracts and antibodies to CBP or Pit-1. Unexpectedly, we have not observed distinct supershift band, but the interaction between protein(s) and DNA probe was much reduced by co-incubation with nuclear factors and these antibodies (Fig. II-4-7). This result suggests that the formation of a protein complex between

Pit-1 and CBP is important for binding to the *cis*-element of the cPRL gene and that the Pit-1/CBP complex can induce the PRL gene expression.

It has been also revealed in this study that existence of distal promoter region between -1366 and -1255 repressing the forskolin-stimulated cPRL gene transcription (Fig. II-4-3). There is evidence that mutations in the corresponding region increase plasma PRL levels in turkey (personal communication). Therefore the distal promoter region might contain functional repressor element(s). This region, however, contained three canonical Pit-1 binding sites conserved for Aves and teleosts (Ohkubo *et al.*, 1996), being specifically bound by Pit-1 protein and chicken pituitary nuclear proteins (Fig. II-4-5). Furthermore, this promoter region contains a CANNTG promoter element (E-box) that has been suggested to bind transcription factors such as zinc finger-homeodomain proteins and helix-loop-helix proteins to repress transcription of various genes in mammals (Genetta *et al.*, 1994, Barabletz *et al.*, 1999, Gregoire and Romeo, 1999, Lu et al., 1999). Either competition between these distal Pit-1 binding sites and the proximal promoter domains to bind to Pit-1 factors or binding of E-box to transcriptional repressors to reduce the gene expression might be occurring. The mechanism(s) of the negative regulation through the distal promoter region should be elucidated further.

Although, it should be confirmed that Pit-1 protein is occurred in the lactotroph in chicken pituitary gland, Pit-1 protein is detected in turkey pituitary gland by Western blot analysis (Kurima *et al.*, 1998). In addition, there is evidence that forskolin induces remarkably the PRL gene expression in the anterior pituitary gland in chicken (Kansaku *et al.*, 1995, Shimada and Kansaku, 1997). Therefore, it is concluded that the PRL gene expression is cooperatively promoted by Pit-1 and cAMP-induced CBP, probably forming a complex, by binding to the proximal Pit-1 binding sites upstream of the TATA-box. In

addition, distal promoter region around −1300 may contain functional repressor that negatively regulates PRL gene expression.

Table II-4-1 Luciferase assay of cPRL promoter activity in HeLa cells by forskolin

vector	treatment	luciferase activity (light units)
-248 luc	control	117.6 ± 7.0
	forskolin	126.2 ± 6.2
pGL-2 basic	control	109.8 ± 5.5
	forskolin	110.6 ± 4.9

Data represent the mean ± SEM of results with five independent dishes.

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CAGGTATTGAGATTTCTTTCTGGTAGAGCAAGTCATCACACAGAATCCCTACCATGAGCAACAGAGGGGCTTCATTGAAAGgtaaggctt      90
                                                    M  S  N  R  G  A  S  L  K                    9
ttgccattcactgtctgataacttctgtttttaagttttgattgaattaagaagaagctgaaggttaacaactctaga

                                .........Intron 1 (1.5 Kb).........

gtgcttcactctgtttatttcaagagtcagcacatcgggtactctgagccatgtgctgagcaaagccaacatccctgcattctgacactg      90
cctctgacagctatttccaaatgcatgcttgtgtcaatatgttcccattttggttttgttttcaaactgaacagGTTTGTTTCTGGCGGT     180
                                                                  G  L  F  L  A  V               15
TCTTCTGGTGTCCAACACACTTCTGACCAAGGAAGGAGTGACCTCCCTGCCAATCTGCCCCATTGGATCAGTCAACTGCCAAGTTTCCCT     270
  L  L  V  S  N  T  L  L  T  K  E  G  V  T  S  L  P  I  C  P  I  G  S  V  N  C  Q  V  S  L    45
TGGGGAACTTTTTGATCGGGCAGTTAAACTTTCACACTACATACACTACCTCTCTTCAGAAATATTCAATGAATTTgtaagtactttctc     360
  G  E  L  F  D  R  A  V  K  L  S  H  Y  I  H  Y  L  S  S  E  I  F  N  E  F                    70
ttttcctgggggtgtgatcacgcaaaatccagttcagtatttttgagcagaaacatgttaatctatgaatttaatttgt                  440

                                .........Intron 2 (1.0 Kb).........

ataacctctccttgctagtctgcagGATGAACGTTATGCTCAGGGTCGGGGTTTCATTACAAAAGCTGTTAATGGCTGCCACACTTCCTC      90
                                D  E  R  Y  A  Q  G  R  G  F  I  T  K  A  V  N  G  C  H  T  S  S    92
CTTAACCACTCCTGAAGATAAGGAGCAAGCTCAGCAGATTCATgtaagtcctacatctaatgccaatgactgtgagatagagatgtgggg     180
  L  T  T  P  E  D  K  E  Q  A  Q  Q  I  H                                                      106
gtaagaaagagggaggagaaagacgtagagaggggcagacagagacagaggaaatcagatct                                  244

                                .........Intron 3 (1.4 Kb).........

gtgactgtgcaatattgcacacaattaccaagacaatatcagaaattgtatagagagatatctaagatagggctgtagggagcagtcagt      90
gaattcccaaatttctgcacttgagaacattgctttaattgcctcctaatgctgccaacatatagccttcttattttaactacctttctc     180
taatgtggaccagCATGAAGACCTACTGAATTTAGTAGTGGGAGTGCTGCGTTCCTGGAATGATCCCCTGATCCATCTGGCCTCTGAAGT     270
              H  E  D  L  L  N  L  V  V  G  V  L  R  S  W  N  D  P  L  I  H  L  A  S  E  V    132
GCAAAGAATCAAAGAAGCTCCAGATACCATTCTCTGGAAGGCTGTAGAGATTGAGGAGCAAAACAAGAGGCTTCTAGAAGGAATGGAGAA     360
  Q  R  I  K  E  A  P  D  T  I  L  W  K  A  V  E  I  E  E  Q  N  K  R  L  L  E  G  M  E  K    162
AATAGTTGGGCGGgtaagtattaggtccttacagtcttccagcctattggtgcaaagaagccatgtaagggact                      434
  I  V  G  R                                                                                    166

                                .........Intron 4 (1.7 Kb).........

gcttagaaattttaaggaaataaactgaaatatctggtggaaattcattcaaaatctgtttgatggtctaaagaactctttgcagaacaa      90
aagggagacataacaggaaacactttacaagctgtaccactactgactagtgttcctcaaggtcaatatttcttaattctctgttctaca     180
cccagacagattgactatcatctcttactgtatgattatgtctctctagGTTCATTCTGGTGATGCTGGAAATGAAATTTACTCTCACTG     270
                                                    V  H  S  G  D  A  G  N  E  I  Y  S  H  W    180
GGACGGCCTTCCATCCCTGCAACTCGCTGATGAGGACTCCAGACTCTTTGCTTTTTATAACCTGCTGCATTGCCTCCGCAGAGATTCCCA     360
  D  G  L  P  S  L  Q  L  A  D  E  D  S  R  L  F  A  F  Y  N  L  L  H  C  L  R  R  D  S  H    210
CAAAATTGACAACTATCTTAAAGTTTTGAAGTGCCGCCTAATCCATGATAGCAATTGCTAAGTACCTGTGGGCTGCATTACTCACTGAAA     450
  K  I  D  N  Y  L  K  V  L  K  C  R  L  I  H  D  S  N  C  *                                   229
CCATTCATCATGGTGTTCTTGTTGCTTTGCCACTTTTCACTGCGAACTTTAGCAAAAACCACATTGTACAGCACCAGGATCATCAGTAAC     540
TTTCAGGCATGCTTGTGTGAATTCTGGCTGCAACTATTAGTGCCATTTATCTTGGTGTTTAAATGCTGTAAATCACTTGTGTAACAAGAA     630
TAAACCTTTCTGTCTAATTTCCTCACTTATTAGCATTTCATTGATAAGTAGATCATGTAGATACAAATAAAAATATTATTAAATTCAAAA     720
CCACGTAACAACAAGTGGTATCATGAACAAAAATAATAGCTGGGATGGCTTTCTACCTTTAGCAAAACTTTTTCTTTTGGATCTCTTCAT     810
TTATGGCCTGGCAAGCCTAGCTGTGGTGGTTTCAAATAC                                                         849
```

Fig. II-4-1 Structure and sequence of cPRL gene. Exons are shown in upper-case letters, introns and flanking regions in lower-case letters. The number 1 of nucleotide in exon 1 corresponds to the 5' end of cPRL cDNA (Watahiki et al., 1989) and the first amino acid of cPRL is designated as 1. Potential polyadenylation signals are under lined.

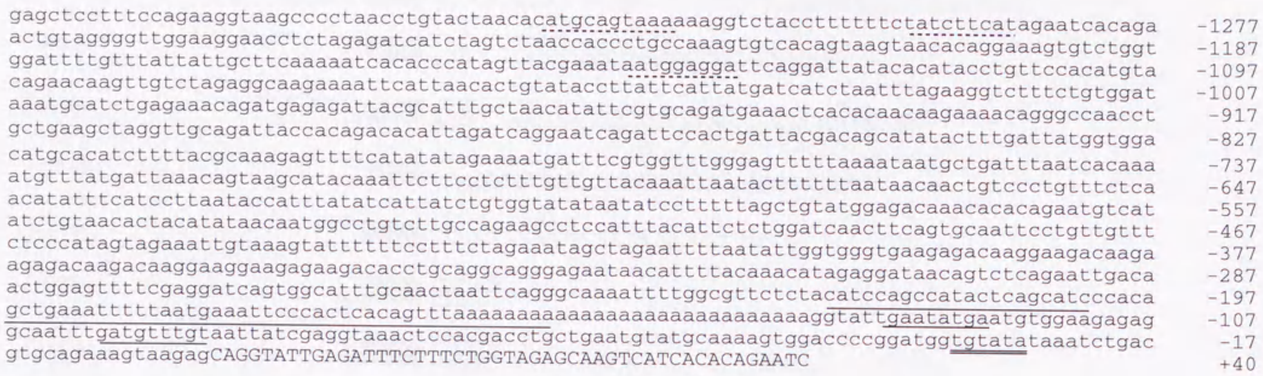

Fig. II-4-2 Sequence of cPRL promoter region. Potential TATA-box is double underlined. The canonical distal Pit-1 binding sites are underlined by a dashed line, and putative proximal Pit-1 binding domains are underlined. Highly conserved promoter sequence between the chicken and mammalian PRL genes is overlined.

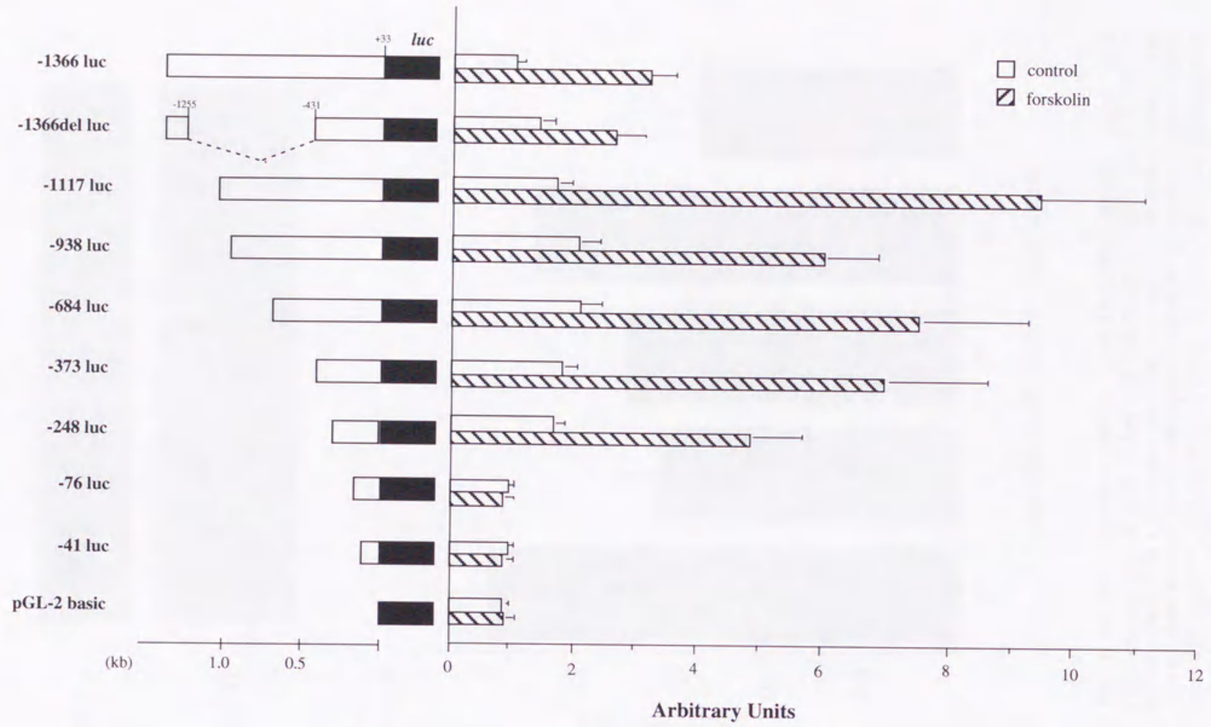

Fig. II-4-3 Promoter activity of cPRL gene in GH3 rat pituitary tumor cells in the presence and absence of forskolin. Various deletions of the 5'-flanking region of cPRL gene were fused to the luciferase reporter gene, and transiently expressed in GH3 cells. The left panel represents reporter genes containing various portion of cPRL gene. The luciferase activity in each transformant was described as mean ± SEM of five independent transfections (right panel).

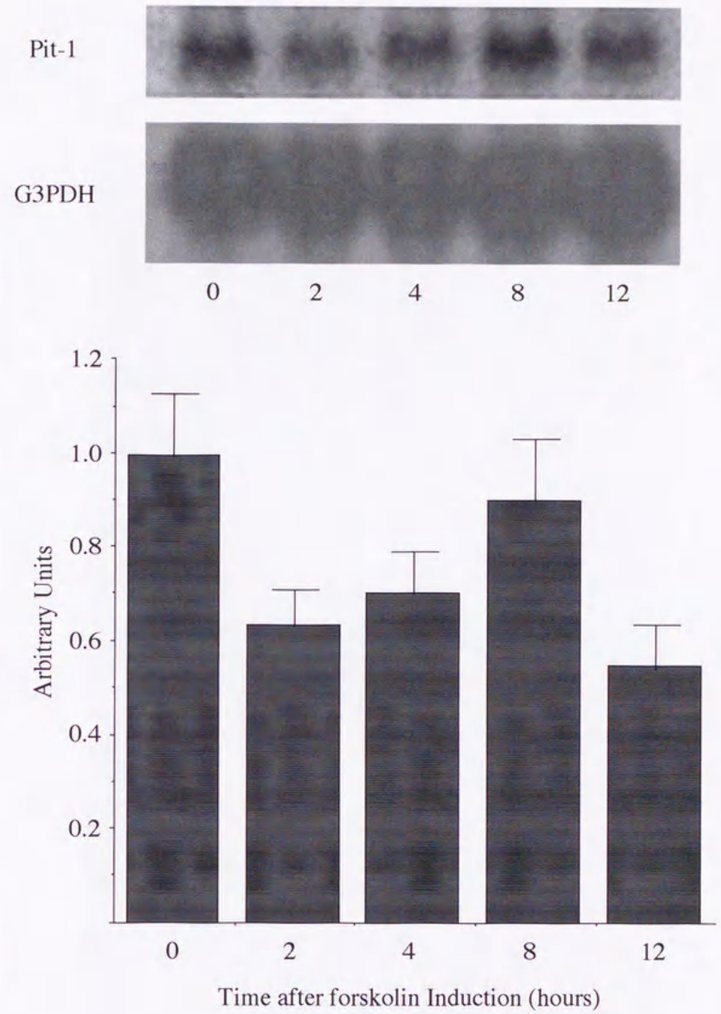

Fig. II-4-4 Pit-1 mRNA expression after forskolin induction. Upper panel represents an autoradiogram probed with radiolabelled rat Pit-1 cDNA or G3PDH cDNA, which is as a corresponding control to verify the amount of loaded RNA. Lower panel shows a densitometricquantification of the autoradiograms. Values were calculated as a multiple of value for the time zero. Values are given as mean ± SEM for four independent experiments.

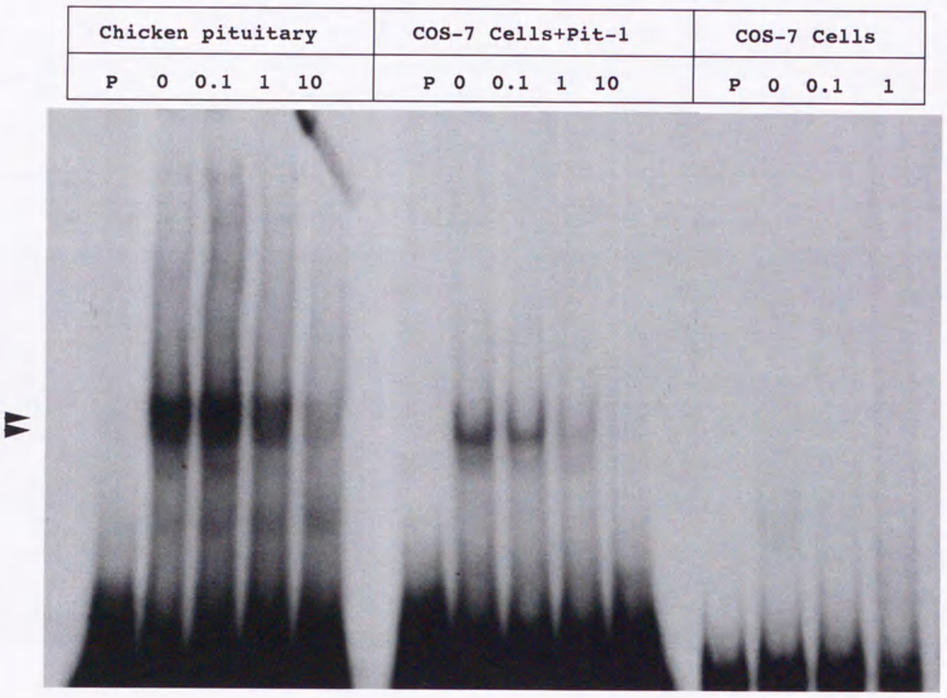

Fig. II-4-5 DNA gel mobility shift analysis of the Pit-1 binding sequences in the distal cPRL promoter region. Synthetic double-stranded oligonucleotides representing -1301 to -1280 region of the cPRL gene was incubated with either nuclear extracts from the chicken pituitary or COS-7 cells transfected with chicken Pit-1 expression vector. The arrows indicate the distinct bands determined by the nuclear extracts.

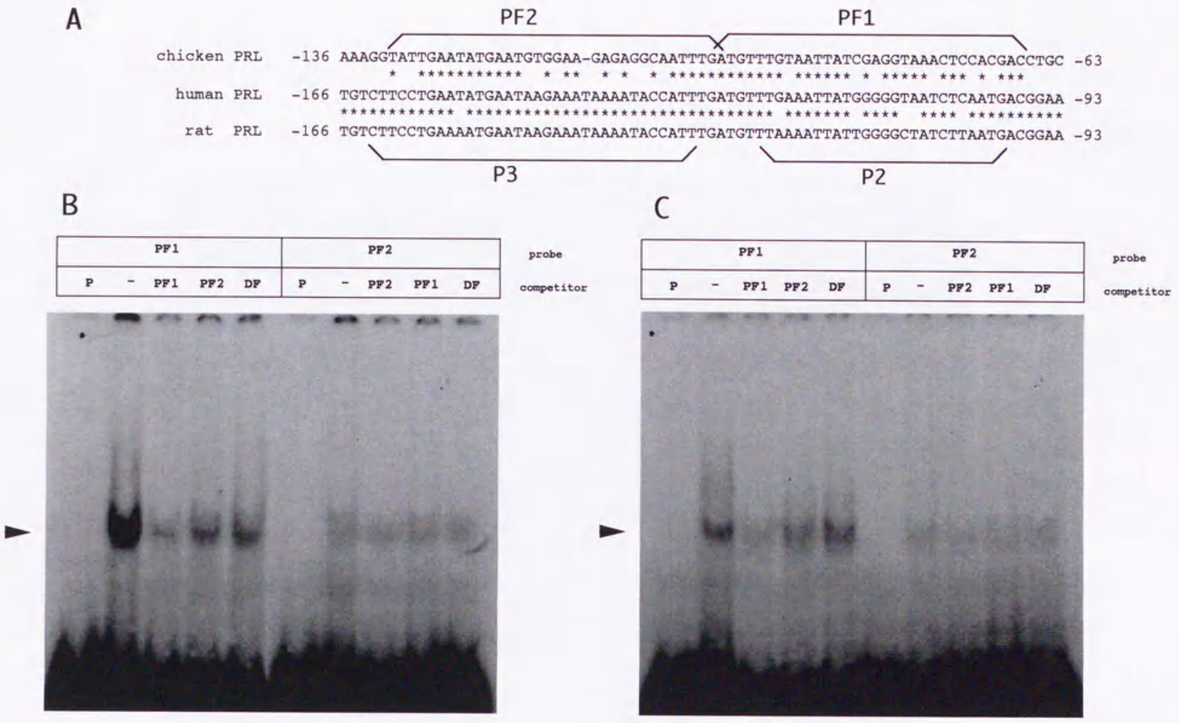

Fig. II-4-6 Interaction between the proximal cPRL promoter domain and nuclear extracts from chicken pituitary gland or GH3 cells. A, alignment of conserved promoter sequence among chicken, human and rat PRL genes. P2 and P3 indicate proposed Pit-1 binding sites in mammals and PF1 and PF2 indicate the fragments used for DNA gel mobility shift assay. B, interaction between chicken pituitary nuclear extracts and the PF1 or PF2 fragments. Gel shift assay was performed as described in Materials and Methods. The radiolabelled DNA fragments are indicated in the top of the panel and competitors are shown in the bottom. The competition was made in the presence of 10 mM of unlabeled probe. Distinct shifted bands are indicated by arrowheads. C, interaction between GH3 cell nuclear extracts and PF1 or PF2 fragments.

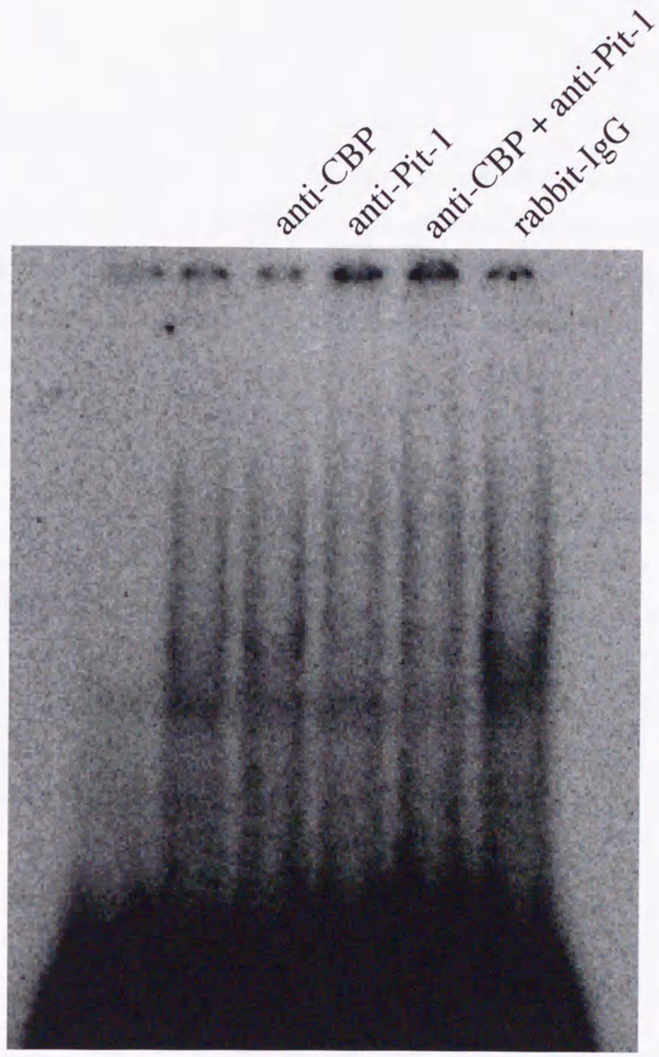

Fig. II-4-7 Supershift analysis of proximal promoter regions in the cPRL gene. Supershift assay was performed as described in Materials and Methods. 32P-labeled PF2 probe was used for the assay. Nuclear extracts prepared from GH3 cells were incubated with antibodies to CBP (lane 3), antibodies to Pit-1 (lane 4) and antibodies to CBP and Pit-1 (lane 5). Nuclear extracts preincubated without antibodies (lane 2) and with rabbit normal IgG (lane 6) were used as the controls.

III GENERAL DISCUSSION

Since Riddle *et al.* (1935) first reported that PRL can induce incubation behavior in domestic chicken (Riddle *et al.*, 1935), numerous researchers have focused on the relationship between plasma PRL level and the behavior in birds, and a lot of findings have been reported. From the findings, the induction pathway of incubation behavior by PRL can be summarized as follows. PRL released from the anterior pituitary gland is transported into the brain via the choroid plexus and binds to the receptor localized in the hypothalamus to induce the incubation behavior. On the other hand, circulating PRL acts, directly or indirectly, on the peripheral tissues and exerts its functions such as brood patch formation, ovarian regression and so on. However, the mechanism for PRL to initiate and maintain the incubation behavior is not yet known. In addition, one breed of domestic chicken, the White Leghorn, never becomes broody after PRL injections (Saeki and Inoue, 1979), while other breeds show the behavior. However, why this breed of domestic chicken (White Leghorn) cannot be induced the behavior by PRL is still uncertain. This thesis has focused on to clarify how PRL modulates incubation behavior and why White Leghorn cannot display incubation behavior by molecular biological point of view.

In Chapters II-1 and II-2, quantitative analyses have demonstrated PRLR mRNA expression in the central and peripheral tissues of domestic chickens. The widespread distribution of PRLR mRNA in the peripheral tissues has been found in chickens (Fig. II-2-3). PRL is known to participate in variety of physiological functions in birds, and therefore PRLR is widely distributed in central and peripheral tissues. It has been revealed that PRLR mRNA is highly expressed in the pituitary gland and hypothalamic regions in chickens (Figs II-1-2, II-1-3, II-1-4, II-1-5, II-1-6 and II-2-2). It is reported that PRLR-immunoreactivity was detected in hypothalamus (Roky *et al.*, 1996, Pi and Grattan, 1998) and pituitary gland (Morel et al., 1994) in mammals. In ring dove, PRL binding sites are concentrated in the

hypothalamic areas (Buntin *et al.*, 1993) and PRLR antibody inhibited the specific binding of PRL in hypothalamus (Li et al., 1995). Consequently, it is possible that PRLR mRNAs expressed in these areas in chicken are translated into protein to mediate PRL actions. It is expected that parental care, including incubation behavior, is mediated via the hypothalamic region in the brain. This view is supported by the observations that lesions in this area in the turkey block the development of incubation behavior (Youngren *et al.*, 1989) and in the ring dove disrupt PRL-induced parental feeding behavior (Slawski and Buntin, 1995). Therefore PRLR in the hypothalamus has a critical role in controlling incubation behavior in chicken. In addition, it has also been revealed that the concentration of PRLR mRNA in the basal hypothalamus was lowered in incubating than in laying or out-of-lay bantam hens. These similar results are observed in the turkey hypothalamus (Zhou *et al.*, 1996). They suggest that increased plasma PRL down-regulates hypothalamic PRLR mRNA. However, such inverse relationship between plasma PRL levels and the concentration of PRLR mRNA in the basal hypothalamus was not observed in the preoptic hypothalamus in the same comparison (Fig. II-1-6). Furthermore, in comparison between the PRLR mRNA expression and PRL levels in different physiological states such as sex, photoperiod and the stage of broody cycle, it is difficult to conclude that the expression levels of PRLR in central tissues would correlate with the PRL levels in plasma in chicken. In addition, high abundance of PRLR mRNA expression in the hypothalamus has been archived before sexual maturation (Fig. II-1-3) and was maintained by sexual maturation and following reproductive stages. This result may indicate that once an adequate number of PRLR is established in the hypothalamus, steady-level of PRLR mRNA expression may be sufficient to maintain the receptor protein to mediate the PRL actions including incubation behavior in the hypothalamus. Moreover, it is unlikely that circulating PRL directly regulate PRLR mRNA gene expression in the chicken neuroendocrine tissues.

In Chapter II-4, I have cloned chicken PRL gene and its 5'-flanking to investigate the transcription initiating mechanism. In the experiments of luciferase assay and gel shift assay, the PRL gene expression in chicken is absolutely dependent on the pituitary specific transcription factor, Pit-1 as was observed in mammals and the Pit-1-dependent expression of the PRL gene is mediated via *cis*-acting element in the proximal promoter region between -76 and -133 of the gene. Furthermore, I have demonstrated that Pit-1 requires CBP as a cooperative activator to bind to the proximal promoter region to activate the chicken PRL gene expression. CBP is stimulated by cAMP (Xu *et al.*, 1998) and directly binds to the POU-specific domain of Pit-1 to induce the Pit-1-dependent activation of the PRL gene in mammalian system (Xu *et al.*, 1998, Tolón *et al.*, 1998). Avian Pit-1 shares common structural features with mammalian one, and also has highly conserved POU-specific domain (Wong *et al.*, 1992, Kurima *et al.*, 1998, Tanaka *et al.*, 1999). It is suggested that in birds, Pit-1 interacts with CBP to activate PRL gene expression by the same manner observed in mammals. Moreover VIP increases cellular cAMP concentration in the perfused chicken anterior gland (Shimada and Kansaku, 1997) and accumulation of intracellular cAMP induces PRL gene expression in the chicken anterior pituitary gland (Kansaku *et al.*, 1995). cAMP/protein kinase A (PKA) pathway might involve VIP-dependent-PRL gene expression in the chicken anterior pituitary gland. Recent study also revealed that VIP receptor expresses predominantly in the cepharic lobe of anterior pituitary gland which condenses PRL producing cells in the chicken (Shimada and Kansaku, 1997). It is likely that VIP affects both PRL release and gene expression via VIP receptor localized in the PRL producing cells. Furthermore, it has been reported in turkey that PRL mRNA in the pituitary gland is increased during incubation period without the increase in Pit-1 mRNA expression (Wong *et al.*, 1992). This observation suggests that induction of Pit-1 gene expression is not necessary

to activate PRL gene expression, but activation of CBP by cAMP/PKA pathway is important to interact with Pit-1 protein to activate PRL gene expression.

Consequently, the molecular mechanism for PRL to initiate and maintain the incubation behavior in birds as follows (Figs. III-1 and III-2).

1. Transcriptional initiation of avian PRL gene: First, VIP released from hypothalamus binds to the VIP receptor in the lactotrophs in the anterior pituitary gland in birds. Second, the VIP and receptor complex stimulates adenylate cyclase to increase intracellular cAMP. Third, the cAMP activates PKA to activate CBP by phosphorylation. Finally, the activated-CBP binds to Pit-1 and the complex induces PRL gene expression via the proximal promoter region between –133 and –76 in the chicken PRL gene. In the mean time, VIP stimulates PRL producing cells to release the hormone into circulating blood (Fig III-1).

2. Modulation of incubation behavior by PRL:

Existence of eggs in the nest triggers to activate VIP neurons to stimulate PRL release and gene expression (Fig. III-1). In peripheral tissues, increased circulating PRL binds to the target organs to show the action such as brood patch formation and ovarian regression. In central tissues, circulating PRL is transported into brain via choroid plexus. Transported PRL binds to the receptor localized in the hypothalamic regions to induce and maintain the incubation behavior. In basal hypothalamus, PRL may exert a negative feedback control over its secretion by associating with VIP neurons. In preoptic hypothalamus, PRL may contribute to maintain motivation for the parental behavior. However, these regulations may not be associated, at least in the preoptic hypothalamus, with the number of PRL binding sites in the target tissues, and is dependent on circulating PRL levels in plasma.

It was hypothesized that functional defect of PRL or PRLR might be responsible for the lack of incubation behavior in White Leghorn. However, breeds difference on the display of

incubation behavior is not interpreted by the quantitative and qualitative differences of PRL and its receptor.

In Chapter II-2, comparison of the PRLR mRNA expression in the brain in White Leghorns, non-broody breed, and bantams, broody breed revealed that PRLR mRNA concentration in the brain did not differ significantly between laying White Leghorns and bantams. In addition, the expression levels of PRLR mRNA in the central nervous system are similar to the levels obtained in the experiments in the Chapter II-1. Equivalent levels of PRLR gene expression were seen in the peripheral tissues in both breeds. Furthermore, Southern and Northern blot analyses showed that the gross structures of the PRLR gene and the PRLR mRNA were not different between these two strains. Possibility of the point mutation in the PRLR gene and PRLR mRNA cannot be excluded from the results of Southern or Northern analyses. However, if point mutation(s) responsible for the defect of broodiness occur in White leghorn, other physiological disorder might be observed, since PRL modulates a considerable number of physiological functions in vertebrates. Indeed, the null mutation of the PRLR gene in mice causes multiple reproductive defects, such as infertility in males, reduced mammary gland development, irregular estrus cycles, defective preimplantation embryonic development and lack of psuedopregnancy in females (Ormandy *et al.*, 1997). Therefore, it is unlikely that PRLR gene of White Leghorn contains defective point mutation(s).

In Chapter II-3, I have produced recombinant cPRL by using PRL cDNA cloned from White Leghorn and analyzed its biological and immunological activities to elucidate whether PRL protein in White Leghorn is functional or not. The recombinant cPRL showed equivalent biological activity with pituitary-derived turkey PRL on the development of the crop sac in pigeon and also crossreacted with anti tPRL antibody. In addition, the recombinant PRL influenced thyroid hormone metabolism in the chicken embryo (Kühn *et*

al., 1996). These results may indicate that functional PRL protein is generated from the PRL gene of White Leghorn in the anterior pituitary gland. Furthermore, another recombinant cPRL produced by using PRL cDNA from bantam possesses both biological and immunological activities (Hanks *et al.*, 1989b). Therefore, it is likely that PRL proteins produced either in non-broody breed (White Leghorn) or in broody-breed (bantam) shows equivalent activity to mediate PRL action in chickens.

In Chapter II-2, the experiment of intravenous injection of VIP in White Leghorn revealed that VIP acts as PRL releasing factor in this breed. Moreover, in Chapter II-4, indirect evidence suggests that VIP can induce White Leghorn PRL gene expression. Therefore, the absence of broodiness in White Leghorns is not due to a lack of a PRL response to the avian PRL releasing and synthesizing hormone, VIP.

There is also evidence that no differences in the PRLR mRNA levels were observed in the preoptic and basal hypothalamus in control hens and nest-deprived hens for 24 hours or 72 hours (Fig. III-3). However, these birds showed different profiles on the plasma PRL levels and on the behavior. Control birds showed the incubation behavior through out the experiment, and plasma PRL level remained high. The nest-deprived hens for 24 and 72 hours showed low plasma PRL levels after hens being moved from the nest. When the 24 or 72 of nest-deprived birds were transferred again to the nest, all 24 hours of nest deprived hens have been back to the nest within 30 minutes to incubate eggs, while 72 hours of nest-deprived birds have never gone back to the nest. Hence, it is strongly suggested that either the numbers of PRLR in the hypothalamus or high levels of PRL in plasma are not directly associated with maintenance of broodiness.

Since breeds difference on the display of incubation behavior can not be interpreted by the differences of PRL and its receptor, it is hypothesized that other factor(s) induced by PRL through PRLR may play an essential role in displaying the behavior in the brain. This

hypothesis came from observation that mouse target-disrupted PRLR gene can't display the maternal behavior (Ormandy *et al.*, 1997). This result suggests that the PRLR-dependent signal is important to develop the maternal behavior in mammals. There is also good evidence to resolve the effects of breed difference on the expression of incubation behavior. In support of the latter, it is notable that the fosB knockout mice showed normal reproductive functions, except that maternal care of the pups was ablated (Brown *et al.*, 1996). Furthermore, fosB expression is induced in the preoptic area in female rats after exposure to pups (Brown *et al.*, 1996). The fosB protein belongs to the fos family of transcription factors and these genes are activated during a widely variety of adaptive neuronal responses. It is reported that fos or fos–like proteins are highly expressed in brain regions including the preoptic hypothalamus during parental behavior (Calamandei and Keverne, 1994, Fleming and Walsh, 1994, Fleming *et al.*, 1994, Luckman, 1995, Numan and Numan, 1995). These views are consistent with the observation in the ring dove that fos-like immunoreactivity was increased in the preoptic hypothalamus after egg laying and 12-14 days of incubation (Sharp *et al.*, 1996). In addition, plasma PRL concentration is correlated with fos-immunoreactivity in the rat hypothalamus during the estrus cycle (Rowe and Erskine, 1993). Consequently, fos protein may be activated by PRL through PRLR in the central nervous system. Further, the fos family of proteins induced by the input of PRL signal may activate the expression of other gene(s) which directly regulate the broody behavior in birds as is suggested in fosB knockout mice (Brown *et al.*, 1996). Therefore, breeds difference on the display of incubation behavior in domestic chicken might be explained as follows (Fig. III-4)

Prolactin transported into brain binds to the receptor localized in the hypothalamus and the signal is mediated to nuclei to stimulate immediately early genes including fos family of transcription factors. The transcription factors activate following gene expressions to initiate incubation behavior and reduce reproductive functions in the central nervous system. This

mechanism may involve stimulation of VIP synthesis and release. In White Leghorn, fos-related protein would be mutated in the signaling pathway from PRL-PRLR which resulting the lack of broody behavior. This view is supported by the observation that PRL injection can't induce incubation behavior in White Leghorn hens, while other breeds show the behavior by the injection of PRL (Riddle *et al.*, 1935).

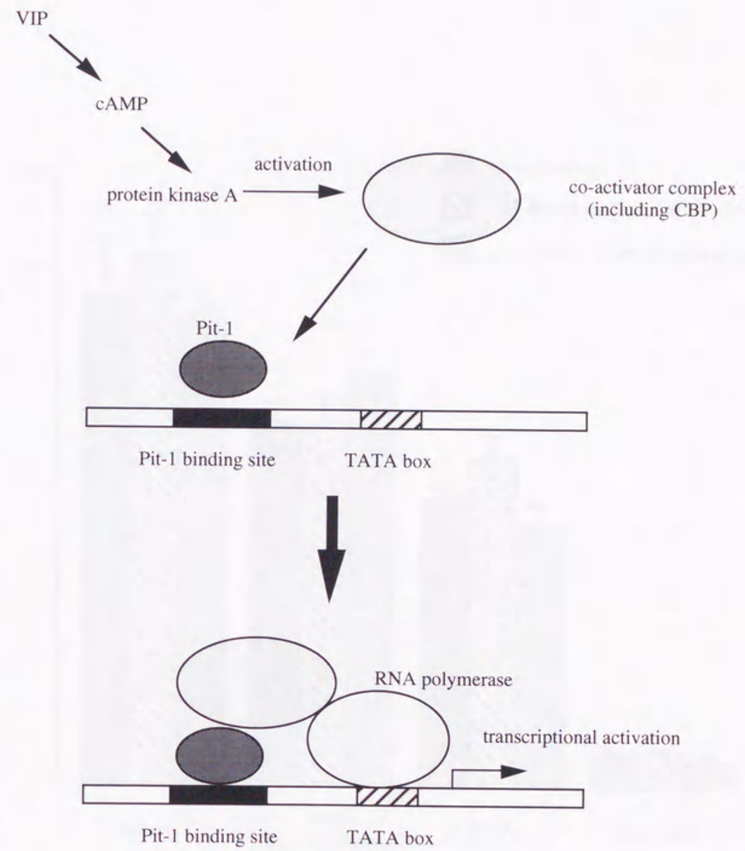

Fig. III-1 A proposed model of PRL transcription by Pit-1 and CBP. VIP increases cAMP and activates subsequent protein kinase A (PKA) in the pituitary gland. The PKA activates CBP by phosphorylation, and the activated CBP forms complex with Pit-1 to initiate PRL gene expression.

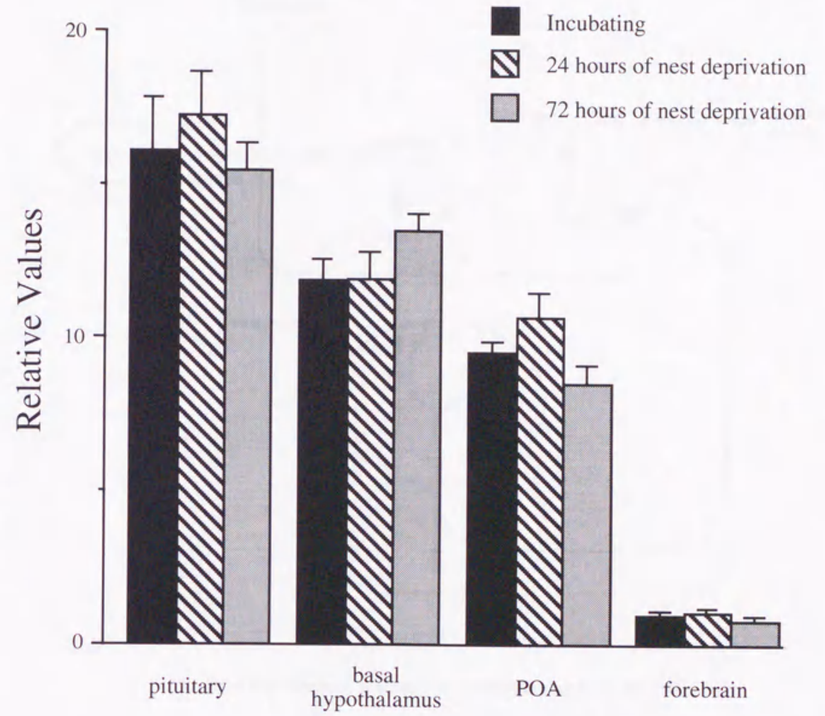

Fig. III-2 Comparison of the relative amount of PRLR mRNA in the brain in incubating and nest-deprived bantam hens

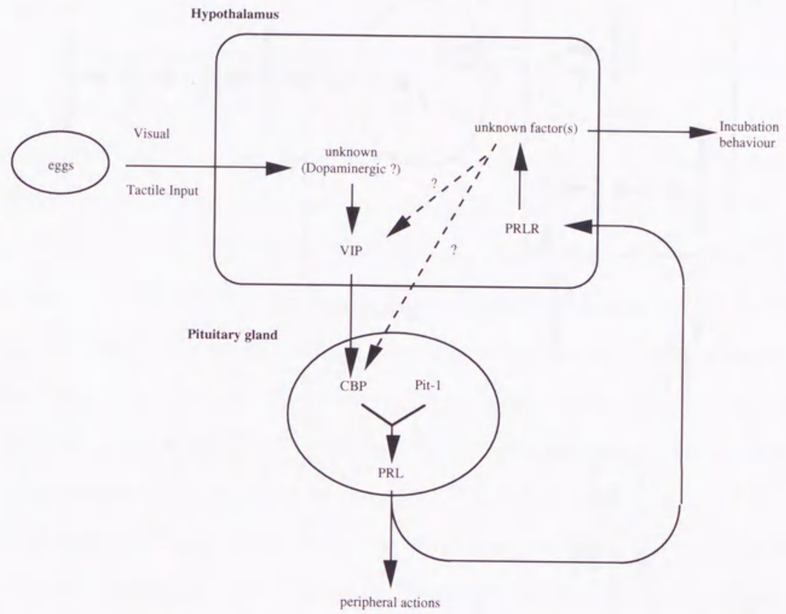

Fig. III-3 Hypothetical pathway of incubation behavior by PRL

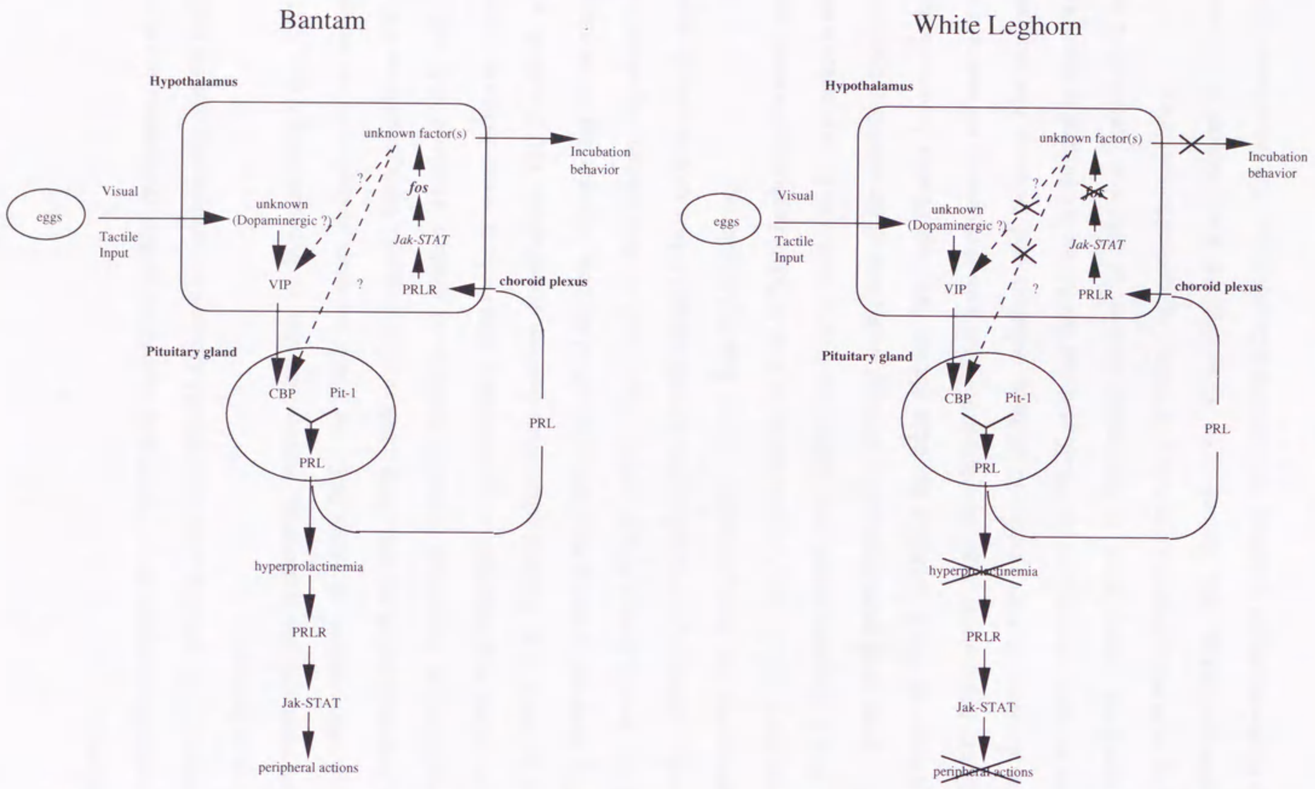

Fig. III-4 Hypothetical model of incubation behavior by PRL in domestic chickens

IV SUMMARY

The regulatory mechanisms of PRLR gene expression in the hypothalamus in chicken and functions of PRL and PRL gene from White Leghorn were investigated, and the results obtained are as follows.

I have examined the relationship between amounts of hypothalamic/pituitary PRLR mRNA and concentrations of plasma PRL. The PRLR expresses abundantly in the preoptic and basal hypothalamus in bantams. This result strongly suggests that hypothalamic PRLR has a critical role in controlling incubation behavior in chicken. Further PRLR mRNA expressions in the hypothalamus in the incubating bantam hens were decreased during incubation behavior. The inverted relationship between hypothalamic PRLR mRNA and plasma PRL observed in laying, incubating and out-of-lay hens suggests that the increased plasma PRL down-regulates PRLR mRNA expression in the chicken hypothalamus. However, this relationship is not sustained in the physiological states such as juvenile males and females in short day, adult cockerels or hens in long day or short day.

Comparison of PRLR mRNA expression in the brain in White Leghorn and bantam hens has been made to examine whether breed differences is associated with the expression levels of the PRLR gene which might account for the breed differences on the incubation behavior. The concentration of PRLR mRNA in the brain did not differ significantly between laying White Leghorns and bantams. Equivalent levels of PRLR gene expression were also seen in the peripheral tissues in both breeds. In addition, Southern and Northern blot analyses showed that the gross structures of the PRLR gene and mRNA are not differed in these two strains, respectively. Furthermore, an intravenous injection of VIP was confirmed to be equally effective in stimulating prolactin release in White Leghorn and bantam hens.

Recombinant chicken PRL derived from White Leghorn was produced in *E. coli*, purified and characterized its biological and immunological potencies. The recombinant PRL

possesses the molecular mass of 23 kDa that is consistent with the molecular weight calculated from the amino acid sequence encoded in the cDNA. This protein crossreacted with turkey PRL antibody and showed equivalent biological activity with pituitary-derived turkey PRL by pigeon crop sac assay.

Chicken PRL gene was cloned and the transcriptional regulation of the gene was extensively analyzed by luciferase assay and gel mobility shift assay. The transcription of the gene is critically controlled by pituitary specific transcription factor, Pit-1 via the proximal promoter region between –76 and –133. The Pit-1 dependent transcription requires the formation of a complex with coactivator protein named CBP and this cascade is activated by cAMP/PKA pathway.

Based on these results, it is concluded that high level of PRL in plasma during incubation behavior is transcriptionally regulated via proximal promoter region between –133 and –76 by interacting Pit-1 and CBP, and the PRL binds to the receptor localized in the hypothalamus to display the behavior in chicken. However, lack of the broodiness in White Leghorn cannot be explained by the dysfunction of PRL and the PRLR, since all parameters in the VIP-PRL-PRLR axis in White Leghorn hens was the same with those in the bantam hens. Therefore, it is hypothesized that breed differences in the expression of incubation behavior in these broody non-broody breeds are due to the factor(s) existing in the PRLR-mediated signaling cascade in the hypothalamus (Fig. III-4).

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VI REFERENCES

Ali, S., Edery, M., Pellegrini, I., Lesueur, L., Paly, J., Djiane, J., and Kelly, P. A. (1992) The Nb2 form of prolactin receptor is able to activate a milk protein gene promoter. *Mol. Endocrinol.* **6**, 1242-1248.

Alland, L., Muhle, R., Hou, H. Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* **387**, 49-55.

Andersen, B., and Rosenfeld., M. G. (1994) Pit-1 determines cell types during development of the anterior pituitary gland. *J. Biol. Chem.* **269**, 29335-29338.

Arámburo, C., Montiel, J. L., Proudman, J. A., Berghman, L. R., and Scanes C. G. (1992) Phosphorylation of prolactin and growth hormone. *J. Mol. Endocrinol.* **8**, 183-191.

Armstrong, D. G., and Hogg, C. O. (1992) The expression of a putative insulin-like growth factor-I receptor gene in the liver of the developing chick. *J. Mol. Endocrinol.* **8**, 193-201.

Baily, D. J., and Herbert, J. (1982) Impaired copulatory behaviour of male rats with hyperprolactinemia induced by domperidone or pituitary grafts. *Neuroendocrinology* **35**, 193-201.

Balthazart, J., Foidart, A., and Harada, N. (1990) Immunocytochemical localization of aromatase in the brain. *Brain Res.* **514**, 327-333.

Bazan J. F. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain. *Biochem. Biophys. Res. Commun.* **164**, 788-795

Bazan J. F. (1990) Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6934-6938.

Bedecarrats, G., Guémené, D., Morvan, C., Crisostomo-Pinto, S., Kuhnlein, U., and Zadworny, D. (1999a) In vitro release of isoforms of prolactin from pituitary glands of turkey hens at different physiological stages. *Gen. Comp. Endocrinol.* **113**, 105-111.

Bedecarrats, G., Guémené, D., Morvan, C., Kuhnlein, U., and Zadworny, D. (1999b) Quantification of prolactin messenger ribonucleic acid, pituitary content and plasma levels of prolactin, and detection of immunoreactive isoforms of prolactin in pituitaries from turkey embryos during ontogeny. *Biol. Reprod.* **61**, 757-763.

Ben-Jonathan, N., Arbgast, L. A., and Hyde, J. F. (1989) Neuroendocrine regulation of prolactin release. *Prog. Neurobiol.* **33**, 399-447.

Bikle, D. D., Spencer, E. M., Burke, W. H., and Post, C. R. (1980) Prolactin but not growth hormone stimulates 1, 25 dehydroxyvitamin D_3 production by chick renal preparations in vitro. *Endocrinology* **107**, 81-84.

Bodner, M., Castrillo J. -L., Theill, L. E., Deerinck, T., Ellisman, M., and Karin, M. (1988) The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. *Cell* **55**, 505-518.

Bokar, J. A., Roesler, W. J., Vandenbark, G. R., Katezel, D. M., Hanson, R. W., and Nilson, J. H. (1988) Characterization of the cAMP responsive elements from the genes for α-subunit of glycoprotein hormones and phosphoenolpyruvate carboxykinase (GTP). Conserved features of nuclear protein binding between tissues and species. *J. Biol. Chem.* **25**, 19740-19747.

Bole-Feysot, C., Goffin, V., Edery, M, Binart, N., and Kelly, P. A. (1998) Prolactin (PRL) and its receptor: action, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Rev.* **19**, 225-268.

Bollengier, F., Geerts, A., Matton, A., Mahler, A., Velkeniers, B., Hooghe-Peters, E., and Vanhaeist, L. (1993) Identification and localization of 23,000 and glycosylated rat prolactin in subcellular fractions of rat anterior pituitary and purified secretory granules. *J. Neuroendocrinol.* **5** 669-676.

Brabletz, T., Jung, A., Hlubek, F., Lohberg, C., Meiler, J., Suchy, U., and Kirchner, T. (1999) Negative regulation of CD4 expression in t cells by transcriptional repressor ZEB. *Int. Immunol.* **11**, 1701-1708.

Bridges, R. S., Numan, M., Ronsheim, P. M., Mann, P. E., and Lupini, C. E. (1990) Central prolactin infusions stimulate maternal behavior in steroid-treated nulliparous female rats. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8003-8007.

Brown, J. R., Ye, H., Bronson, R.T., Dikkes, P., and Greenberg, M. E. (1996) A defect in nurturing in mice lacking the immediate early gene *fos*B. *Cell* **86**, 297-309.

Buntin J. D., and Ruzycki, E. (1987) Characteristics of prolactin binding sites in the brain of the ring dove (*Streptopelia risoria*). *Gen. Comp. Endocrinol.* **65**, 243-2553.

Buntin, J. D., Lea, R. W., and Figge, G. R. (1988) Reductions in plasma LH concentration and testicular weight in ring doves following intracranial injection of prolactin and growth hormone. *J. Endocrinol.* **118**, 33-40.

Buntin, J. D., Ruzycki, E., and Witebsky, J. (1993) Prolactin receptors in dove brain: Autoradiographic analysis of binding characteristic in discrete brain regions and accessibility to blood-borne prolactin. *Neuroendocrinology* **57**, 738-750.

Buntin, J. D. (1996) Neural and hormonal control of parental behavior in birds. *Advan. Stud. Behav.* **25**, 161-213.

Burke, W. H., and Papkoff, W. (1980) Purification of turkey prolactin and the development of a homologous radioimmunoassay for its measurement. *Gen. Comp. Endocrinol.* **40**, 297-307.

Calamandrei, G., and Keverne, E. B. (1994) Differential expression of Fos protein in the brain of female mice dependent on pup sensory cues and maternal experience. *Behav. Neurosci.* **108**, 113-120.

Carrillo, A. J., Pool, T. B., and Sharp, Z. D., (1985) Vasoactive intestinal peptide increases prolactin messenger ribonucleic acid content in GH3 cells. *Endocrinology* **116**, 202-206.

Casia, R. V., Scanes, C. G., and Malamed, S. (1987) Polyhormonal regulation of avian and mammalian corticosteroidogenesis *in vitro*. *Comp. Biochem. Physiol.* **88A**, 131-140.

Chen, X. J., and Horseman, N. D. (1994) Cloning, expression and mutational analysis of the pigeon prolactin receptor. *Endocrinology* **135**, 269-276.

Chiu, S., Koos, R. D., and Wise, P. M. (1992) Detection of prolactin receptor (PRL-R) mRNA in the rat hypothalamus and pituitary gland. *Endocrinology* **130**, 1747-1752.

Chiu, S., and Wise, P. M. (1994) Prolactin receptor mRNA localization in the hypothalamus by in situ hybridization. *J. Neuroendocrinol.* **6**, 191-199.

Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodmam, R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* **365**, 855-859.

Clarke, L. A., Edery, M., Loudon, A. S. I., Randall, V. A., Postal-Vinay, M. -C., Kelly, P. A., and Jabbour, H. N. (1995) Expression of the prolactin receptor gene during the breeding and non-breeding seasons in red deer *Cervus elaphus*: Evidence for the expression of two forms in the testis. *J. Endocrinol.* **146**, 313-321.

Clevenger, C. V., and Medaglia, M. V. (1994) The protein tyrosine kinase p59fyn is associated with prolactin (PRL) receptor and is activated by PRL stimulation of T-lymphocytes. *Mol. Endocrinol.* **8**, 674-681.

Cohen, L. E., Wondisford, F. E., and Radovic, S. (1997) Role of Pit-1 in the gene expression of growth hormone, prolactin, and thyrotropin. *Endocrinol. Metab.* **25**, 523-540.

Corcoran, D. H., and Proudman J. A. (1991) Isoforms of turkey prolactin: evidence for differences in glycosylation and in tryptic peptide mapping. *Comp. Biochem. Physiol.* **99B**, 563-570.

Crumeyrolle-Arias, M., Latouche, J., Jammes, H., Djaine, J., Kelly, P. A., Reymond, M. J., and Haour, F. (1993) Prolactin receptors in the rat hypothalamus: autoradiographic localization and characterization. *Neuroendocrinology* **57**, 457-466.

DaSilva, L., Howard, Z. O. M., Rui, H., Kirken, R. A., and Farrar, W. L. (1994) Growth signaling and JAK2 association mediated by membrane-proximal cytoplasmic regions of prolactin receptor. *J. Biol. Chem.* **269**, 18267-18270.

DaSilva, L., Rui, H., Erwin, R. A., Howard, O. M., Kirken, R. A., Malabarba, M. G., Hackett, R. H., Larner, A. C., and Farrar, W. L. (1996) Prolactin recruits STAT1, STAT3 and STAT5 independent of conserved receptor tyrosines TYR402, TYR479, TYR515 and TYR580. *Mol. Cell. Endocrinol.* **117**, 131-140.

Decuypere, E., and Kühn, E. R. (1985) Effect of a single injection of prolactin on the serum concentrations of thyroid hormones and corticosterone and liver monodeiodinase in the domestic fowl before and after hatching. *J. Endocrinol.* **104**, 363-366.

Deutsch, P. J., Hoeffler, J. P., Jameson, J. L., Lin, J. C., and Habener, J. F. (1988) Structural determinants for transcriptional activation by cAMP-responsive DNA elements. *J. Biol. Chem.* **263**, 18466-18472.

Di Carlo, R., Meli, R., Florio, S., Mattace Raso, G., and Pagnini, G. (1996) The effect of age and sex on the expression of prolactin binding activity in the chicken bursa of Fabricius. *Life Sci.* **59**, 1803-1808.

Drago, F., Canonico, P. L., Bitetti, R., and Scapagnini, U. (1980) Systemic and intraventricular prolactin induces excessive grooming. *Eur. J. Pharmacol.* **65**, 451-458.

Edery, M., Jolicoeur, C., Levi-Meyrueis, C., Dusanter-Fourt, I., Pétridou, B., Boutin J. -M., Lesueur, L., Kelly, P. A., and Djiane, J. (1989) Identification and sequence analysis of a second form of prolactin receptor by molecular cloning of complementary DNA from rabbit mammary gland. *Proc. Acad. Natl. Sci. U.S.A.* **86**, 2112-2116.

El Halawani, M. E., Silsby, J. L., Behnke, E. J., and Fehrer, S. C. (1986) Hormonal induction of incubation in ovariectomized female turkey (*Meleagris gallopavo*) *Biol. Reprod.* **35**, 59-67.

El Halawani, M. E., Silsby, J. L., Rosenboim, I., Pitts, G. R. (1995) Increased egg production by active immunization against vasoactive intestinal peptide in the turkey (*Meleagris gallopavo*). *Biol. Reprod.* **52**, 179-183.

Erpel, T. and Courtneidge, S. A. (1995) Src family protein kinases and cellular signal transduction pathways. *Curr. Opin. Cell Biol.* **7**, 176-182.

Escalada, J., Cacicedo, L., Ortego, J., Melian, E., and Sánchez-Franco, F. (1996) Prolactin gene expression and secretion during pregnancy and lactation in the rat: role of dopamine and vasoactive intestinal peptide. *Endocrinology* **137**, 631-637.

Etches, R. J., Williams, J. B., and Rzasa, J. (1984) Effects of corticosterone and dietary changes in the hen on ovarian function, plasma LH and steroids and the response to exogenous LHRH. *J. Reprod. Fertil.* **70**, 121-130.

Fechner, J. H. Jr., and Buntin, J. D. (1989) Localization of prolactin binding sites in ring dove brain by quantitative autoradiography. *Brain Res.* **487**, 245-254.

Finidori, J., and Kelly, P. A. (1995) Cytokine receptor signaling through two novel families of transducer molecules: Janus kinases, and signal transducers and activators of transcription. *J. Endocrinol.* **147**, 11-23.
Fleming, A. S., and Walsh, C. (1994) Neuropsychology of maternal behavior in the rat: *c-fos* expression during mother-litter interactions. *Psyconeuroendocrinol.* **19**, 429-443.
Fleming, A. S., Suh, E. J., Korsmit, M., and Rusak, B. (1994) Activation of Fos-like immunoreactivity in the medial preoptic area and limbic structures by maternal and social interactions in rats. *Behav. Neurosci.* **108**, 724-734.
Gaiddon, C., Mercken, L., Bancroft, C, and Loeffler, J. P. (1995) Transcriptional effects in GH3 cells of Gs alpha mutants associated with human pituitary tumors: stimulation of adenosine 3', 5'-monophosphate response element-binding protein-mediated transcription and of prolactin and growth hormone promoter activity via protein kinase A. *Endocrinology* **136**, 4331-4338.
Gao, J., Hughes, J. P., Auperin, B., Buteau, H., Edery, M., Zhuang, H., Wojchowski, D. M., and Horseman, N. D. (1996) Interactions among JANUS kinases and the prolactin (PRL) receptor in the regulation of a PRL response element. *Mol. Endocrinol.* **10**, 847-856.
Genetta, T., Ruezinsky, D., and Kadesch, T. (1994) Displacement of an E-box-binding repressor by basic helix-loop-helix proteins: implications for B-cell specificity of the immunoglobulin heavy-chain enhancer. *Mol. Cell. Biol.* **14**, 6153-6163.
Georgiou, G. G., Sharp, P. J., and Rea, R. W. (1995) [^{14}C]-2 deoxyglucose uptake in the brain of Ring Dove (*Streptopelia risoria*). II. Differential uptake at the onset of incubation. *Brain Res.* **700**, 137-141.
Goujon, L., Allevato, G., Simonin, G., Paquereau, L., Le Cam, A., Clark, J., Nielsen, J. H., Djiane, J., Postel-Vinay, M. -C., Edery, M., and Kelly, P.A. (1994) Cytoplasmic sequences of the growth hormone receptor necessary for signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* **91**,957-61.
Gregoire, J. M., and Romeo, P. H. (1999) T-cell expression of the human GATA-3 gene is regulated by a non-lineage-specific silencer. *J. Biol. Chem.* **274**, 6567-6578.
Hanks, M. C., Alonzi, J. A., Sharp, P. J., and Sang, H. M. (1989a) Molecular cloning and sequence analysis of putative chicken prolactin cDNA. *J. Mol. Endocrinol.* **2**, 21-30.
Hanks, M. C., Talbot, R. T., and Sang, H.M. (1989b) Expression of biologically active recombinant-derived chicken prolactin in *Escherichia coli*. *J. Mol. Endocrinol.* **3**, 15-21.
Harlan, R. E., Shivers, B. D., and Pfaff, D. W. (1983) Midbrain microinfusions of prolactin increase the estrogen dependent behaviour, lordosis. *Science* **219**, 1451-1453.
Harpur, A. G., Andres, A. -C., Ziemiecki, A., Aston, R. R., and Wilks, A. F. (1992) Jak 2, a third member of the Jak family of tyrosine kinases. *Ondogene* **7**, 1347-1353.
Harvey, S., Hall, T. R., and Chadwick, A. (1984) Growth hormone and prolactin secretion in water-deprived chickens. *Gen. Comp. Endocrinol.* **54**, 46-50.
He, X., Treacy, M. G., Simmons, D. M., Swanson, L. W., and Rosenfeld, M. G. (1989) Expression of a large family of POU-domain regulatory genes in mammalian brain development. *Nature* **340**, 35-42.
Heinzel, T., Lavinsky, R.M., Mullen, T. -M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenmann R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**, 43-48.
Herr, W., Sturm, R. A., Clerc, R. G., Corcoran, L. M., Baltimore, D., Sharp, P. A., Ingraham, H. A., Rosenfeld, M. G., Finney, M., and Ruvkin, G. (1988) The POU

domain: A large conserved region in the mammalian pit-1, oct-1, oct-2 and *C. elegans* unc-86 gene product. *Genes Dev.* **2**, 1513-1516.
Hinuma, S., Habata, Y., Fujii, R., Kawamata, Y., Hosoya, M., Fukushimi, S., Kitada, C., Masuo, Y., Asano, T., Matsumoto, H., Sekiguchi, M., Kurokawa, T., Nishimura, O., Onda, H., and Fujino, M. (1998) A prolactin releasing peptide in brain. *Nature* **394**, 302
Hnasko, R. M., and Buntin, J. D. (1993) Functional mapping of neural sites mediating prolactin-induced hyperphage in doves. *Brain Res.* **623**, 257-266.
Hou, X. S., Melnick, M. B., and Perrimon, N. (1996) Marelle acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATs. *Cell* **84**, 411-419
Howard, P. W., and Maurer, R. A. (1995) A composite Ets/Pit-1 binding site in the prolactin gene can mediate transcriptional responses to multiple signal transduction pathway. *J. Biol. Chem.* **270**, 20930-20936.
Hu, Z., Zhuang, L., and Dufau, M. L. (1996) Multiple and tissue-specific promoter control of gonadal and non-gonadal prolactin receptor gene expression. *J. Biol. Chem.* **271**, 10242-10246.
Hutchison, R. E., Hind, R. A., and Steel, E. (1967) The effects of oestrogen, progesterone and prolactin on brood patch formation in overiectomized canaries. *J. Endocrinol.* **39**, 379-385.
Ihle, J. N. (1996) STATs: signal transducers and activators of transcription. *Cell* **84**,331-334.
Ingraham, H. A., Chen, R., Mangalam, H. J., Elsholtz, H. P., Flynn, S. E., Lin, C. R., Simmons, D. M., Swanson, L., and Rosenfeld, M. G. (1988) A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* **55**, 519-529.
Itoh, N., Yonehara, S., Schreurs, J., Gorman, D. M., Maruyama, K., Ishii, A., Yahara, I., Arai, K., and Miyajima, A. (1990) Cloning of an interleukin-3 receptor gene: a member of a distinct receptor gene family. *Science* **247**, 324-327.
Iverson, R. A., Day, K. H., d'Emden, M., Day, R. N., and Maurer, R. A. (1990) Clustered point mutation analysis of the rat prolactin promoter. *Mol. Endocrinol.* **4**, 1564-1571.
Jahn, G. A., Edery, M., Belair, L., Kelly, P. A., and Djiane, J. (1991) Prolactin receptor gene expression in rat mammary gland and liver during pregnancy and lactation. *Endocrinology* **128**, 2976-2984.
Janik, D. S., and Buntin, J. D. (1985) Behavioural and physiological effects of prolactin in incubating ring doves. *J Endocrinol* **105**, 201-209.
Juhn, M., and Harris, P. (1958) Moult of capon feathering with prolactin. *Proc. Soc. Exp. Biol. Med.* **98**, 662-672.
Kansaku, N., Shimada, K., and Saito, N. (1995) Regionalized gene expression of prolactin and growth hormone in the chicken anterior pituitary gland. *Gen. Comp. Endocrinol.* **99**, 60-68.
Karatzas, C. N., Zadworny, D., and Kuhnlein, U. (1990) Nucleotides sequence of turkey prolactin. *Nuc. Acids Res.* **18**, 3071.
Karatzas, C. N., Guémené, D., Zadworny, D., and Kuhnlein, U. (1992) The ratio of prolactin isoforms in the pituitary gland: changes during the reproductive cycle of the turkey. *Proceedings of the Fifth International Symposium on Avian Endocrinology.* Edinburgh: AFRC Roslin Institute, Abstract 13.
Kühn, E. R., Decuypere, E., Hemschoote, K., Berghman, L., and Paulussen, J. (1983) Antagonism of serum tri-iodothyronine changes after injections of prolactin in the domestic fowl before and after hatching. *J. Endocrinol.* **99**, 401-407.

Kühn, E. R., Shimada, K., Ohkubo, T., Vleurick, L. M., Berghman, L. R., and Darras, V. M. (1996) Influence of recombinant chicken prolactin on thyroid hormone metabolism in the chick embryo. *Gen. Comp. Endocrinol.* **103**, 349-358.

Kurima, K., Proudman, J. A., El Halawani, M. E., and Wang, E. A. (1995) The turkey prolactin-encoding gene and its regulatory region. *Gene* **156**, 309-310.

Kurima, K., Weatherly, K. L., Sharova, L., and Wong, E. A. (1998) Synthesis of turkey Pit-1 mRNA variants by alternative splicing and transcription initiation *DNA Cell Biol.* **17**, 93-103.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lal, P., Sharp, P.J., Dunn I. C., and Talbot, R. T. (1990) Absence of an effect of naloxone, on opioid antagonist, on luteinising hormone release in vivo and luteinising hormone-releasing hormone I release in vitro in castrated, and food restricted cockerels. *Gen. Comp. Endocrinol.* **77**, 239-245.

Lea, R. W., Talbot, R. T., and Sharp, P. J. (1991) Passive immunization against chicken vasoactive intestinal polypeptide suppresses plasma prolactin and crop sac development in incubating ring doves. *Horm. Behav.* **25**, 287-294.

Lebrun, J.J., Ali, S., Sofer, L., Ullrich, A., and Kelly, P. A. (1994) Prolactin induced proliferation of Nb2 cells involves tyrosine phosphorylation of the prolactin receptor and its associated tyrosine kinase. *J. Biol. Chem.* **269**, 14021-14026.

Lebrun, J.J., Ali, S., Goffin, V., Ullrich, A., and Kelly, P. A. (1995a) A single phosphotyrosine residue of the prolactin receptor is responsible for activation of gene transcription. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4031-4035.

Lebrun, J. -J., Ali, S., Ullrich, A., and Kelly, P. A. (1995b) Proline-rich sequence-mediated Jak2 association to the prolactin receptor is required but not sufficient for signal transduction. *J. Biol. Chem.* **270**, 10664-10670.

Lewis, U. J., Singh, R. N., and Lewis, L. J. (1989) Two forms of glycosylated human prolactin have different pigeon crop sac-stimulating activities. *Endocrinology* **124**, 1558-1563.

Li, C., Kelly, P. A., and Buntin, J. D. (1995) Inhibitory effects of anti-prolactin receptor antibodies on prolactin binding in the brain and prolactin-induced feeding behavior in ring doves. *Neuroendocrinology* **61**, 125-135.

Liang, J., Kim, K. E., Schoderbek, W. E., and Maurer, R. A. (1992) Characterization of a nontissue-specific, 3', 5'-cyclic adenosine monophosphate-responsive element in the proximal region of the rat prolactin gene. *Mol. Endocrinol.* **6**, 885-892.

Lu, J., Webb, R., Richardson, J. A., and Olson, E. N. (1999) MyoR: a muscle-restricted basic helix-loop-helix transcription that antagonizes the actions of MyoD. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 552-557.

Luckman, S. M. (1995) Fos expression within regions of the preoptic area, hypothalamus and brainstem during pregnancy and parturition. *Brain Res.* **669**, 115-124.

Majumdar, S., Irwin, D. M., and Elsholtz, H. P. (1996) Selective constraints on the activation domain of transcription factor Pit-1. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10256-10261.

Mangalam, H. J., Albert, V. R., Ingraham, H. A., Kapiloff, M., Wilson, L., Nelson, C., Elsholtz H., and Rosenfeld M. (1989) A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. *Genes Dev.* **3**, 946-958.

March, J. B., Sharp, P. J., Wilson, P. W., and Sang, H. M. (1994) Effect of active immunization against recombinant-derived chicken prolactin fusion protein on the onset of

broodiness and photoinduced egg laying in bantam hens. *J. Reprod. Fertil.* **101**, 227-233.
Markoff, E., Sigel, M. B., Lacour, N., Seavey, B. K., Friesen, H. G., and Lewis, U. J. (1988) Glycosylation selectively alters the biological activity of prolactin *Endocrinology* **123**, 1303-1306.
Maruyama, M., Matsumoto, H., Fujiwara, K., Kitada, C., Hinuma, S., Onda, H., Fujino, M., and Inoue, K. (1999) Immunocytochemical localization of prolactin-releasing peptide in the rat brain. *Endocrinology* **140**, 2326-2333.
Maurer, R. A. (1981) Transcriptional regulation of the prolactin gene by ergocrypyine and cyclic AMP. *Nature* **294**, 94-97.
Maurer, R. A. (1982) Estradiol regulates the transcription of the prolactin gene. *J. Biol. Chem.* **257**, 2133-2136.
Miller, W. L., and Eberhardt, N. L. (1983) Structure and evolution of the growth hormone gene family. *Endocrine Rev.* **4**, 97-130.
Moore, B. J., Gerardo-Gettens, T., Horowitzs, B. A., and Stern, J. S. (1986) Hyperprolactinemia stimulates food intake in the female rat. *Brain Res. Bull.* **17**, 563-569.
Morel, G., Ouhtit, A., and Kelly, P. A. (1994) Prolactin receptor immunoreactivity in rat anterior pituitary. *Neuroendocrinology* **59**, 78-84.
Muccioli, G., Ghè, C., and Di Carlo, R. (1991) Distribution and characterization of prolactin binding sites in male and female rat brain: effects of hypophysectomy and ovariectomy. *Neuroendocrinology* **53**, 47-53.
Muccioli, G., and Di Carlo, R. (1994) Modulation of prolactin receptors in the rat hypothalamus in response to changes in serum concentrations of endogenous prolactin or to ovine prolactin administration. *Brain Res.* **663**, 244-250.
Murdoch, G. H., Franco, R., Evans, R. M., and Rosenfeld, M. G. (1983) Polypeptide hormone regulation of gene expression. Thyrotropin releasing hormone rapidly stimulates both transcription of the prolactin gene and the phosphorylation of a specific nuclear protein. *J. Biol. Chem.* **258**, 15329-15335.
Murdoch, G. H., Waterman, M., Evans, R. M., and Rosenfeld, M. G. (1985) Molecular mechanisms of phorbol ester, thyrotropin-releasing hormone and growth factor stimulation of prolactin gene expression. *J. Biol. Chem.* **260**, 11852-11858.
Murphy, M. J., Brown, P. S., and Brown, S. C. (1986) Osmoregulatory effects of prolactin and growth hormone in embryonic chicks. *Gen. Comp. Endocrinol.* **62**, 485-492.
Mustafa, A., Nyberg, F., Bogdanovic, N., Islam, A., Suliman, I., Lindgren, U., Roos, P., and Adem, A. (1995) Prolactin binding sites in the rat brain and liver: effects of long-term ovariectomy and ovarian steroids. *Neurosci. Lett.* **200**, 179-182.
Nagano, M., and Kelly, P. A. (1994) Tissue distribution and regulation of rat prolactin receptor gene expression. *J. Biol. Chem.* **269**, 13337-13345.
Nelson, C., Albert, V. R., Elsholtz, H. P., Lu, L. E. -W., and Rosenfeld, M. G. (1988) Activation of cell-specific expression of rat growth hormone and prolactin gene by a common transcription factor. *Science* **239**, 1400-1405.
Niall, H. D., Hogan, M. L., Sauer, R., Rosenblum, I. Y., and Greenwood, F. C. (1971) Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene duplication. *Proc. Natl. Acad. Sci. U.S.A.* **68**, 866-869.
Nicoll, C. (1967) Bioassay of prolactin. Analysis of the pigeon crop sac response to local prolactin injection by an objective and quantitative method. *Endocrinology* **80**, 641-655.

Nicoll, C. S., Meyer, G. L., and Russel, S. M. (1986) Structural features of prolactins and growth hormones that can be related to their biological properties. *Endocrine Rev.* **7**, 169-203.

Noso, T., Swanson, P., Lance, V. A., and Kawauchi, H. (1992) Isolation and characterization of glycosylated and non-glycosylated prolactins from alligator and crocodile. *Int. J. Peptide Protein Res.* **39**, 250-257.

Numan, M., and Numan, M. J. (1995) Importance of pup-related sensory inputs and maternal performance for the expression of Fos-like immunoreactivity in the preoptic area and ventral bed nucleus of the stria terminalis of postpartum rats. *Behav. Neurosci.* **109**, 135-149.

Ohkubo, T., Araki, M., Tanaka, M., Sudo, S., and Nakashima, K. (1996) Molecular cloning and characterization of the yellowtail GH gene and its promoter: a consensus sequence for teleost and avian Pit-1/GHF-1 binding sites. *J. Mol. Endocrinol.* **16**, 63-72.

Ohkubo, T., Tsukada, A., Tanaka, M., and Nakashima, K. (1998) Cloning and expression of pigeon growth hormone receptor cDNA in COS-7 monkey kidney cells. *Comp. Biochem. Physiol.* **120B**, 449-455.

Ono, M., Takayama, Y., Rand-Weaver, M., Sakata, S., Yasunaga, T., Nose, T., and Kawauchi, H. (1990) cDNA cloning of somatolactin, a pituitary protein related to growth hormone and prolactin. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4330-4334.

Ono, M, and Takayama, Y. (1992) Structures of cDNAs encoding chum salmon pituitary-specific transcription factor, Pit-1/GHF-1. *Gene* **226**, 275-279.

Opel, H. (1971) Induction of incubation behavior in the hen by brain implants of prolactin. *Poult. Sci.* **50**, 1613.

Ormandy, C. J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N., and Kelly, P. A. (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev.* **11**, 167-78.

Peers, B., Monget, P., Nalda, M. A., Voz, M. L., Berwaer, M., Belayew, A., and Martial, J. A. (1991) Transcriptional induction of the human prolactin gene by cAMP requires two cis-acting elements and at least the pituitary-specific factor Pit-1. *J. Biol. Chem.* **266**, 18127-18134.

Pezet, A., Buteau, H., Kelly, P. A., and Edery, M. (1997) The last proline of Box 1 is essential for association with JAK2 and functional activation of the prolactin receptor. *Mol. Cell. Endocrinol.* **129**, 199-208.

Pi, X. J., and Grattan, D.R. (1998) Distribution of prolactin receptor immunoreactivity in the brain of estrogen-treated ovariectomized rats. *J. Comp. Neurol.* **394**, 462-474.

Proudman J. A., and Corcoran, D. H. (1981) Turkey prolactin: purification by isotachophoresis and partial characterization. *Biol. Reprod.* **25**, 375-384.

Proudman, J. A., and Opel, H. (1981) Turkey prolactin: Validation of a radioimmunoassay and a measurement of change associated with broodiness. *Biol. Reprod.* **25**, 573-580.

Quelle, D. E., Quelle, F. W., and Wojchowski, D. M. (1992) Mutations in the WSAWSE and cytosolic domains of the erythropoietin receptor affect signal transduction and ligand binding and internalization. *Mol. Cell. Biol.* **12**, 4553-4561.

Ramesh, R., Kuenzel, W. J., Buntin, J. D., and Proudman, J. A. (2000) Identification of growth-hormone- and prolactin-containing neurons within the avian brain. *Cell Tissue Res.* **299**, 371-383.

Riddle, O., Bates, R. W., and Lahr, E. L. (1935) Prolactin induces broodiness in fowl. *Am. J. Physiol.* **111**, 352-360.

Roky, R., Valatx, J. L., Paut-Pango, L., and Jouvet, M. (1994) Hypothalamic injection of prolactin or its antibody alters the rat sleep-wake cycle. *Physiol. Behav.* **55**, 1015-1019.
Roky, R., Paut-Pango, L. P., Goffin, V., Kitahama, K., Valatx, J. -L., Kelly, P. A., and Jouvet, M. (1996) Distribution of prolactin receptors in the rat forebrain. *Neuroendocrinology* **63**, 422-429.
Rowe, D. W., and Erskine, M. S. (1993) c-Fos proto-oncogene activity induced by mating in the preoptic area, hypothalamus and amygdala in the female rat: role of afferent input via the pelvic nerve. *Brain Res.* **621**, 25-34.
Rozakis-Adcock, M., and Kelly, P. A. (1991) Mutational analysis of the ligand-binding domain of the prolactin receptor. *J. Biol. Chem.* **266**, 16472-16477.
Rozakis-Adcock, M., and Kelly, P. A. (1992) Identification of ligand binding determinants of the prolactin receptor. *J. Biol. Chem.* **267**, 7428-7433.
Saeki, Y., and Inoue, Y. (1979) Body growth, egg production, broodiness, age and first egg size in Red Jungle fowls, and an attempt at their genetic analysis by reciprocal crossing with White Leghorns. *Jpn. Poult. Sci.* **15**, 121-126.
Saldanha, C. J., and Silver, R. (1995) Intraventricular prolactin inhibits hypothalamic vasoactive intestinal polypeptide expression in doves. *J. Neuroendocrinol.* **11**, 881-887.
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, 2nd Ed., Cold Spring Harbor, NY.
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467.
Sarkar, D., and Yen, S. S. C. (1985) Hyperprolactinemia decreases the luteinizing hormone releasing hormone concentration in pituitary portal plasma: A possible role for beta endorphin as a mediator. *Endocrinology* **116**, 2080-2084.
Schlinger, B. A., and Arnold, A. P. (1991) Brain is the major site of estrogen synthesis in a male songbird. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4191-4194.
Schoner, B. E., Hsiung, H. M., Belagaje, R. M., Mayne, N. G., and Schoner, R. G. (1984) Role of mRNA translational efficiency in bovine growth hormone expression in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5403-5407.
Schoner, B. E., Belagaje, R. M., and Schoner, R. G. (1986) Translational two-cistron mRNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8506-8510.
Shamgochian, M. D., Avakian, C., Truong, N. H., Stone, S., Tang, K. -T., and De Vito, W. J. (1995) Regulation of prolactin receptor expression in the female rat brain. *Neuro Report* **6**, 2537-2541.
Sharp, P. J., Macnamee, M. C., Sterling, R. J., Lea, R. W., and Pedersen, H. C. (1988) Relationships between prolactin, LH and broody behaviour in broody bantam hens. *J. Endocrinol.* **118**, 279-286.
Sharp, P. J., Sterling, R. J., Talbot, R. T., and Huskisson, N. S. (1989) The role of vasoactive intestinal polypeptide in the maintenance of prolactin secretion in incubating bantam hens: observations using passive immunization, radioimmunoassay and immunohistochemistry. *J. Endocrinol.* **122**, 5-13.
Sharp, P. J., Li, Q., Georgiou, G., Lea, R. W. (1996) Expression of *fos*-like immunoreactivity in the hypothalamus of the ring dove (*Streptopelia risoria*) at the onset of incubation. *J. Neuroendocrinol.* **8**, 291-298.
Shepard, H. M., Yelverton, E., and Goeddel, D. V. (1982) Increased synthesis in *E. coli* of fibroblast and leukocyto interferons through alteration in ribosome binding sites. *DNA* **1**, 125-131.
Shimada, K., Ishida, H., Seo, H., Sato, K., and Matsui, N. (1991) Expression of prolactin gene in incubating hens. *J. Reprod. Fertil.* **91**, 147-154.

Shimada, K., and Kansaku, N. (1997) VIP receptor for prolactin gene expression. In *Advances in Comparative Endocrinology*, Monduzzi Editore, Bologna, 957-961.
Singh, H., Sen, R., Baltimore, D., and Sharp, P. A. (1986) A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunogloblin gene. *Nature* **319**, 154-158.
Sinha, Y. N. (1992) Prolactin variants. *Trends in Endocrinol. Metab.* **3**, 100-106.
Sinha, Y. N., DePaolo, L. V., Haro, L. S., Singh, R. N., Jacobsen, B. P., Scott, L. E., and Lewis, U. J. (1991) Isolation and biochemical properties of four isoforms glycosylated porcine prolactin. *Mol. Cell. Endocrinol.* **80**, 203-213.
Skwarlo-Soñta, K., Rosolowska-Huszcz, D., Sotowska-Brochocka, J., and Gajewska, A. (1986) Daily variation in response of certain immunity indices to prolactin in White Leghorn chickens. *Exp. Clin. Endocrinol.* **87**, 195-200.
Skwārlo-Soñta, K. (1992) Prolactin as an immunoregulatory hormone in mammals and birds. *Immunol. Lett.* **33**, 105-122.
Slawski, B. A., and Buntin, J. D. (1995) Preoptic area lesions disrupt prolactin-induced parental feeding behavior in ring doves. *Horm. Behav.* **29**, 248-266.
Sreekumar, K. P., and Sharp, P. J. (1998a) Ontogeny of the photoperiodic control of prolactin and luteinizing hormone secretion in male and female bantams (*Gallus domesticus*). *Gen. Comp. Endocrinol.* **109**, 69-74.
Sreekumar, K. P., and Sharp, P. J. (1998b) Effect of photostimulation on concentrations of plasma prolactin in castrated bantams (*Gulls domesticus*). *Gen. Comp. Endocrinol.* **110**, 147-154.
Stanley, F. (1988) Stimulation of prolactin gene expression by insulin. *J. Biol. Chem.* **263**, 13444-13448.
Sugiyama, T., Minoura, H., Kawabe, N., Tanaka, M., and Nakashima, K. (1994) Preferential expression of long form prolactin receptor mRNA in the rat brain during the oestrous cycle, pregnancy and lactation: Hormones involved in its gene expression. *J. Endocrinol.* **141**, 325-333.
Supowit, S. C., Potter, E., Evans R. M., and Rosenfeld, M. G. (1984) Polypeptide hormone regulation of gene transcription: specific 5' genomic sequences are required for epidermal growth factor and phorbol ester regulation of prolactin gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2975-2979.
Talbot, R. T., Hanks, M. C., Sterling, R. J., Sang, H. M., and Sharp, P. J. (1991) Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: effects of manipulating plasma levels of vasoactive intestinal polypeptide. *Endocrinology* **129**, 496-502.
Talbot, R. T., Burke, W. H., Scougall, R. K., and Sharp, P. J. (1992) Multiple forms of prolactin in the chicken pituitary in different reproductive states. *Proceedings of the Fifth International Symposium on Avian Endocrinology*. Edinburgh: AFRC Roslin Institute, Abstract P5.
Talbot, R. T., and Sharp, P. J. (1994) A radioimmunoassay for recombinant-derived chicken prolactin suitable for the measurement of prolactin in other avian species. *Gen. Comp. Endocrinol.* **96**, 361-369.
Talbot, R. T., Dunn, I. C., Wilson, P. W., Sang, H. M., and Sharp, P. J. (1995) Evidence for alternative splicing of the chicken vasoactive intestinal polypeptide gene transcript. *J. Endocrinol.* **15**, 81-91.
Tanaka, M., Maeda, K., Okubo, T., and Nakashima, K. (1992a) Double antenna structure of chicken prolactin receptor deduced from the cDNA sequence. *Biochem. Biophys. Res. Commun.* **188**, 490-496.

Tanaka, M., Hosokawa, Y., Watahiki, M, and Nakashima, K. (1992b) Structure of the chicken growth hormone-encoding gene and its promoter region. *Gene* **112**, 235-239.
Tanaka, M., Yamamoto, I., Ohkubo, T., Wakita, M., Hoshino, S., and Nakashima, K (1999) cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. *Gen. Comp. Endocrinol.* **114**, 441-448.
Theill, L. E., and Karin, M. (1993) Transcriptional control of GH expression and anterior pituitary development. *Endocrine Rev.* **14**, 670-689.
Tolón, R. M., Castillo, A. I., and Aranda, A. (1998) Activation of the prolactin gene by peroxisome proliferator-activated receptor-alpha appears to be DNA binding-independent. *J. Biol. Chem.* **273**, 26652-26661.
Torchia, J., Rose, D. W., Inostroza, J., Kamel, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387**, 677-684.
Tortonese, D. J., Brooks, J., Ingleton, P. M., and McNeilly, A. S. (1998) Detection of prolactin receptor gene expression in the sheep pituitary gland and visualization of the specific translation of the signal in gonadotrophs. *Endocrinology* **139**, 5215-5223.
Voss, J. W., and Rosenfeld, M. G. (1992) Anterior pituitary development: short tails from dwarf mice. *Cell* **70**, 527-530.
Walsh, R. J., Mangurian, L. P., and Posner, B. I. (1990) The distribution of lactogen receptors in the mammalian hypothalamus: an in vitro autoradiographic analysis of the rabbit and rat. *Brain Res.* **530**, 1-11.
Watahiki, M., Tanaka, M., Masuda, N., Sugisaki, K., Yamamoto, M., Yamakawa, M., Nagai, J., and Nakashima, K. (1989) Primary structure of chicken prolactin deduced from the cDNA sequence: conserved and specific amino acid residues in the domains of the prolactin. *J. Biol. Chem.* **264**, 5535-5539.
Wilk, A. F., Harpur, A. G., Kurban, R. R., Ralph, S. J., Zürcher, G., and Ziemiecki, A. (1991) Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. *Mol. Cell. Biol.* **11**, 2057-2065.
Wold, F. (1981) In vivo chemical modification of proteins. (Posttranslational Modification). *Annu. Rev. Biochem.* **50**, 783-814.
Wong, E. A., Ferrin, N. H., Silsby, J. L., and El Halawani, M. E. (1991) Cloning of a turkey prolactin cDNA: expression of prolactin mRNA throughout the reproductive cycle of the domestic turkey (*Meleagris gallopavo*). *Gen. Comp. Endocrinol.* **83**, 18-26.
Wong, E. A., Silsby, J. L., and El Halawani, M. E. (1992) Complementary DNA cloning and expression of Pit-1/GHF-1 from domestic turkey. *DNA Cell Biol.* **11**, 651-660.
Xu, L., Lavinsky, R. M., Dasen, J. S., Flynn, S. E., Mclnerney, E. M., Mullen, T. -M., Heinzel, T., Szeto, D., Korzus, E., Kurokawa, R., Aggarwal, A. K., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1998) Signal-specific co-activator domain requirements for Pit-1 activation. *Nature* **395**, 301-306.
Yamada, S., Hata, J. -I., and Yamashita, S. (1993) Molecular cloning of fish Pit-1 cDNA and its functional binding to promoter of gene expressed in the pituitary. *J. Biol. Chem.* **268**, 24361-24366.
Yan, R., Small, S., Desplan, C., Dearolf, C. R., and Darnell, J. E. Jr. (1996) Identification of a Stat gene that functions in Drosophila development. *Cell* **84**, 421-430.
Yang, X. -Y., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996) p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature*, **382**, 319-324.

Yasuda, A., Yamaguchi, K., Kobayashi, T., Yamamoto, K., Kikuyama, S., and Kawauchi, H. (1991) The complete amino acid sequence of prolactin from bullfrog (*Rana catesbeiana*). *Gen. Comp. Endocrinol.* **83**, 218-226.

Yoshimura, A., Zimmers, T., Neumann, D., Longmore, G., Yoshimura, Y., and Lodish, H. F. (1992) Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. *J. Biol. Chem.* **267**, 11619-11625.

You, S., Foster, L. K., Silsby, J. L., El Halawani, M. E., and Foster, D. N. (1995) Sequence analysis of the turkey LHβ subunit and its regulation by gonadtrophin-releasing hormone and prolactin in cultured pituitary cells. *J. Mol. Endocrinol.* **14**, 117-129.

Young, K. H., Buhi, W. C., Horseman, N., Davis, J., Kraeling, R., Linzer, D., and Bazer, F. W. (1990) Biological activities of glycosylated and nonglycosylated porcine prolactin. *Mol. Cell. Endocrinol.* **18**, 155-162.

Youngren, O. M., El Halawani, M. E., Phillips, R. E., and Silsby, J. L. (1989) Effects of preoptic and hypothalamic lesions in female turkeys during a photoinduced reproductive cycle. *Biol. Reprod.* **41**, 610-617.

Youngren, O. M., El Halawani, M. E., Silsby, J. L., and Phillips, R. E. (1991) Intracranial prolactin perfusion induces incubation behavior in turkey hens. *Biol. Reprod.* **44**, 425-443.

Youngren, O. M., Silsby, J. L., Rozenboim, I., Phillips R. E., and El Halawani, M. E. (1994) Active immunization with vasoactive intestinal peptide prevents the secretion of prolactin induced by electrical stimulation of the turkey hypothalamus. *Gen. Comp. Endocrinol.* **95**, 330-336.

Zhou, J. F., Zadworny, D., Guémené, D., and Kuhnlein, U. (1996) Molecular cloning, tissue distribution, and expression of the prolactin receptor during various reproductive states in *Meleagris gallopavo*. *Biol. Reprod.* **55**, 1081-1090.

主論文（要約）の印刷公表の方法および時期

1) Ohkubo, T., Tanaka, M., Nakashima, K., Shimada, K., Saito, N., and Sato, K.
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2) Ohkubo, T., Tanaka, M., Nakashima, K., Talbot, R.T., and Sharp, P.J.
Prolactin receptor gene expression in the brain and peripheral tissues in broody and nonbroody breeds of domestic hen.
Gen. Comp. Endocrinol. 109, 60-68 (1998).

3) Ohkubo, T., Tanaka, M., Nakashima, K., and Sharp, P.J.
Relationship between prolactin receptor mRNA in the anterior pituitary gland and hypothalamus and reproductive state in male and female bantams (Gallus domesticus).
Gen. Comp. Endocrinol. 111, 167-176 (1998).

4) Ohkubo, T., Tanaka, M., and Nakashima, K.
Molecular cloning of the chicken prolactin gene and activation by Pit-1 and cAMP-induced factor in GH3 cells.
Gen. Comp. Endocrinol. 119, 208-216 (2000).

Kurima, K., Proudman, J. A., El Halawani, M. E., and Wong, E. A. (1995). The turkey prolactin-encoding gene and its regulatory region. *Gene* **156,** 309–310.

Kurima, K., Weatherly, K. L., Sharova, L., and Wong, E. A. (1998). Synthesis of turkey Pit-1 mRNA variants by alternative splicing and transcription initiation. *DNA Cell Biol.* **17,** 93–103.

Liang, J., Kim, K. E., Schoderbek, W. E., and Maurer, R. A. (1992). Characterization of a nontissue-specific, 3′,5′-cyclic adenosine monophosphate-responsive element in the proximal region of the rat prolactin gene. *Mol. Endocrinol.* **6,** 885–892.

Mangalam, H. J., Albert, V. R., Ingraham, H. A., Kapiloff, M., Wilson, L., Nelson, C., Elsholtz H., and Rosenfeld M. (1989). A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. *Genes Dev.* **3,** 946–958.

March, J. B., Sharp, P. J., Wilson, P. W., and Sang, H. M. (1994). Effect of active immunization against recombinant-derived chicken prolactin fusion protein on the onset of broodiness and photoinduced egg laying in bantam hens. *J. Reprod. Fertil.* **101,** 227–233.

Maurer, R. A. (1981). Transcriptional regulation of the prolactin gene by ergocrypvine and cyclic AMP. *Nature* **294,** 94–97.

Maurer, R. A. (1982). Estradiol regulates the transcription of the prolactin gene. *J. Biol. Chem.* **257,** 2133–2136.

Murdoch, G. H., Franco, R., Evans, R. M., and Rosenfeld, M. G. (1983). Polypeptide hormone regulation of gene expression: Thyrotropin releasing hormone rapidly stimulates both transcription of the prolactin gene and the phosphorylation of a specific nuclear protein. *J. Biol. Chem.* **258,** 15329–15335.

Murdoch, G. H., Waterman, M., Evans, R. M., and Rosenfeld, M. G. (1985). Molecular mechanisms of phorbol ester, thyrotropin-releasing hormone and growth factor stimulation of prolactin gene expression. *J. Biol. Chem.* **260,** 11852–11858.

Ohkubo, T., Araki, M., Tanaka, M., Sudo, S., and Nakashima, K. (1996). Molecular cloning and characterization of the yellowtail GH gene and its promoter: A consensus sequence for teleost and avian Pit-1/GHF-1 binding sites. *J. Mol. Endocrinol.* **16,** 63–72.

Peers, B., Monget, P., Nalda, M. A., Voz, M. L., Berwaer, M., Belayew, A., and Martial, J. A. (1991). Transcriptional induction of the human prolactin gene by cAMP requires two *cis*-acting elements and at least the pituitary-specific factor Pit-1. *J. Biol. Chem.* **266,** 18127–18134.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.

Sharp, P. J., Sterling, R. J., Talbot, R. T., and Huskisson, N. S. (1989). The role of vasoactive intestinal polypeptide in the maintenance of prolactin secretion in incubating bantam hens: Observations using passive immunization, radioimmunoassay and immunohistochemistry. *J. Endocrinol.* **122,** 5–13.

Shimada, K., and Kansaku, N. (1997). VIP receptor for prolactin gene expression. *In* "Advances in Comparative Endocrinology," pp. 957–961. Monduzzi Editore, Bologna.

Singh, H., Sen, R., Baltimore, D., and Sharp, P. A. (1986). A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin gene. *Nature* **319,** 154–158.

Stanley, F. (1988). Stimulation of prolactin gene expression by insulin. *J. Biol. Chem.* **263,** 13444–13448.

Supowit, S. C., Potter, E., Evans R. M., and Rosenfeld, M. G. (1984). Polypeptide hormone regulation of gene transcription: Specific 5′ genomic sequences are required for epidermal growth factor and phorbol ester regulation of prolactin gene expression. *Proc. Natl. Acad. Sci. USA* **81,** 2975–2979.

Talbot, R. T., Dunn, I. C., Wilson, P. W., Sang, H. M., and Sharp, P. J. (1995). Evidence for alternative splicing of the chicken vasoactive intestinal polypeptide gene transcript. *J. Endocrinol.* **15,** 81–91.

Talbot, R. T., Hanks, M. C., Sterling, R. J., Sang, H. M., and Sharp, P. J. (1991). Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: Effects of manipulating plasma levels of vasoactive intestinal polypeptide. *Endocrinology* **129,** 496–502.

Tanaka, M., Hosokawa, Y., Watahiki, M, and Nakashima, K. (1992). Structure of the chicken growth hormone-encoding gene and its promoter region. *Gene* **112,** 235–239.

Tanaka, M., Yamamoto, I., Ohkubo, T., Wakita, M., Hoshino, S., and Nakashima, K. (1999). cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary. *Gen. Comp. Endocrinol.* **114,** 441–448.

Tolón, R. M., Castillo, A. I., and Aranda, A. (1998). Activation of the prolactin gene by peroxisome proliferator-activated receptor-alpha appears to be DNA binding-independent. *J. Biol. Chem.* **273,** 26652–26661.

Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass C. K., and Rosenfeld, M. G. (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387,** 677–684.

Watahiki, M., Tanaka, M., Masuda, N., Sugisaki, K., Yamamoto, M., Yamakawa M., Nagai, J., and Nakashima, K. (1989). Primary structure of chicken prolactin deduced from cDNA sequence. *J. Biol. Chem.* **264,** 5535–5539.

Wong, E. A., Silsby, J. L., and El Halawani, M. E. (1992). Complementary DNA cloning and expression of Pit-1/GHF-1 from domestic turkey. *DNA Cell Biol.* **11,** 651–660.

Xu, L., Lavinsky, R. M., Dasen, J. S., Flynn, S. E., McInerney, E. M., Mullen, T.-M., Heinzel, T., Szeto, D., Korzus, E., Kurokawa, R., Aggarwal, A. K., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1998). Signal-specific co-activator domain requirements for Pit-1 activation. *Nature* **395,** 301–306.

Yang, X.-Y., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. (1996). p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382,** 319–324.

Youngren, O. M., El Halawani, M. E., Silsby, J. L., and Phillips, R. E. (1991). Intracranial prolactin perfusion induces incubation behavior in turkey hens. *Biol. Reprod.* **44,** 425–443.

Youngren, O. M., Silsby, J. L., Rozenboim, I., Phillips R. E., and El Halawani, M. E. (1994). Active immunization with vasoactive intestinal peptide prevents the secretion of prolactin induced by electrical stimulation of the turkey hypothalamus. *Gen. Comp. Endocrinol.* **95,** 330–336.

参考論文の印刷公表の方法および時期

1) Kuwayama, T., Shimada, K., Saito, N., Ohkubo, T., Sato, K., Wada, M., and Ichinoe, K.
Effects of removal of chicks form hens on concentrations of prolactin, lutenizing hormone and oestradiol in plasma of brooding Gifujidori hens.
J. Reprod. Fert. 95, 617-622 (1992).

2) Tanaka, M., Maeda, K., Okubo, T., and Nakashima, K.
Double antenna structure of chicken prolactin receptor deduced from the cDNA Sequence.
Biochem. Biophys. Res. Commun. 188, 490-496 (1992).

3) Shimada, K., Ohkubo, T., Saito, N., Talbo,t R. T., and Sharp, P.J.
The molecular biology of prolactin.
In: Avian Endocrinology (Ed. P. J. Sharp)
Journal of Endocrinology, Ltd, Bristol, 135-148 (1993).

4) Sakaguchi, K., Ohkubo, T., Sugiyama, T., Tanaka, M., Ushiro, T., and Nakashima, K.
Differential regulation of prolactin receptor mRNA expression in rat liver and kidney by testosterone and oestradiol.
J. Endocrinol. 143, 383-392 (1994).

5) Tanaka, M., Toma, Y., Ohkubo, T., Sudo, S., and Nakashima, K.
Sequence of the flounder (*Paralichthys olivaceus*) growth hormone encoding gene and its promoter region.
Gene 165, 321-322 (1995).

6) Tanaka, M., Hayashida, Y., Sakaguchi, K., Ohkubo, T., Wakita, M., Hoshino, S., and Nakashima, K.
Growth hormone-independent expression of insulin-Like growth factor I messenger ribonucleic acid in extrahepatic tissues of the chicken. Endocrinology 137, 30-34 (1996).

参考論文の印刷公表の方法および時期

7) Ohkubo, T., Araki, M., Tanaka, M., Sudo, S., and Nakashima, K.
Molecular cloning and characterization of yellowtail growth hormone gene and its promoter: a consensus sequence for teleost and avian Pit-1/GHF-1 binding sites.
J. Mol. Endocrinol. 16, 63-72 (1996).

8) Sakaguchi, K., Tanaka, M., Ohkubo, T., Doh-ura, K., Fujikawa, T., Sudo, S., and Nakashima, K.
Induction of brain prolactin receptor long-form mRNA expression and maternal behavior in the pup-contacted male rats: promotion by prolactin administration and suppression by female contact.
Neuroendocrinology, 63, 559-568 (1996).

9) Kühn, E.R., Shimada, K., Ohkubo, T., Vleurick, L.M., Berghman, L.R., and Darras, V.M.
Influence of recombinant chicken prolactin on thyroid hormone metabolism in the chick embryo.
Gen. Comp. Endocrinol. 103, 349-358 (1996).

10) Sudo, S., Fujikawa, T., Nagakura, T., Ohkubo, T., Sakaguchi, K., Tanaka, M., Nakashima, K., and Takahashi, T.
Structures of mollusc shell framework proteins.
Nature, 387, 563-564 (1997).

11) Tanaka, M., Taniguchi, T, Ohkubo, T., and Nakashima, K.
cDNA cloning and characterization of neuron-specific enolase from chicken.
Biochim Biophys Acta. 1395, 28-33 (1998).

12) Ohkubo, T., Tsukada, A., Tanaka, M., and Nakashima, K.
Cloning and expression of pigeon growth hormone receptor cDNA in COS-7 monkey kidney cells.
Comp. Biochem. Physiol. B Biochem. Mol. Biol., 120, 449-455 (1998).

参考論文の印刷公表の方法および時期

13) Tsukada, A., Ohkubo, T., Sakaguchi, K., Tanaka, M., Nakashima, K., Hayashida, Y., Wakita, M., and Hoshino, S.
Thyroid hormones are involved in insulin-like growth factor-I (IGF-I) production by stimulating hepatic growth hormone receptor (GHR) gene expression in the chicken.
Growth Horm. IGF Res., 8, 235-242 (1998).

14) Sakaguchi, K., Tanaka, M., Ohkubo, T., Yoshizato, H., Hanai Y., Fujikawa, T., Kaneko, H., and Nakashima, K.
Tissue-specific regulation of growth hormone receptor and growth hormone binding protein gene expression during pregnancy and lactation in the rat. Endocrine J., 45 (Suppl), S105-S107 (1998).

15) Tanaka, M., Taniguchi, T., Yamamoto, I., Sakaguchi, K, Yoshizato, H., Ohkubo, T., and Nakashima, K.
Gene and cDNA structures of flounder insulin-like growth factor-I (IGF-I): Multiple mRNA species encode a single short mature IGF-I.
DNA Cell Biol., 17, 859-868 (1998).

16) Tanaka, M., Yamamoto, I., Ohkubo, T., Wakita, M., Hoshino, S., and Nakashima, K.
cDNA cloning and developmental alterations in gene expression of the two Pit-1/GHF-1 transcription factors in the chicken pituitary.
Gen. Comp. Endocrinol. 114, 441-448 (1999).

17) Tsukada, A., Ohkubo, T., Tanaka, M., Nakashima, K., Wakita, M., and Hoshino, S.
Effects of thyroid hormone on serum insulin like growth factor binding proteins (IGFBPs) and IGFBP-2 mRNA expression in chickens.
Jpn. Poult. Sci. 36, 155-166 (1999).

18) Ohkubo, T., Tanaka, M., and Nakashima, K.
Structure and tissue distribution of chicken leptin receptor (cOb-R) mRNA.
Biochim. Biophys. Acta 1491, 303-308 (2000).

参考論文の印刷公表の方法および時期

19) Tanaka, M., Yamamoto, I., Hayashida, Y., Nakao, N., Ohkubo, T., Wakita, M., and Nakashima, K.
Two novel first exons in the prolactin receptor gene are transcribed in a tissue-specific and sexual maturation-dependent manner to encode multiple 5'-truncated transcripts in the testis of the chicken.
Biochim. Biophys. Acta 1491, 279-284 (2000).