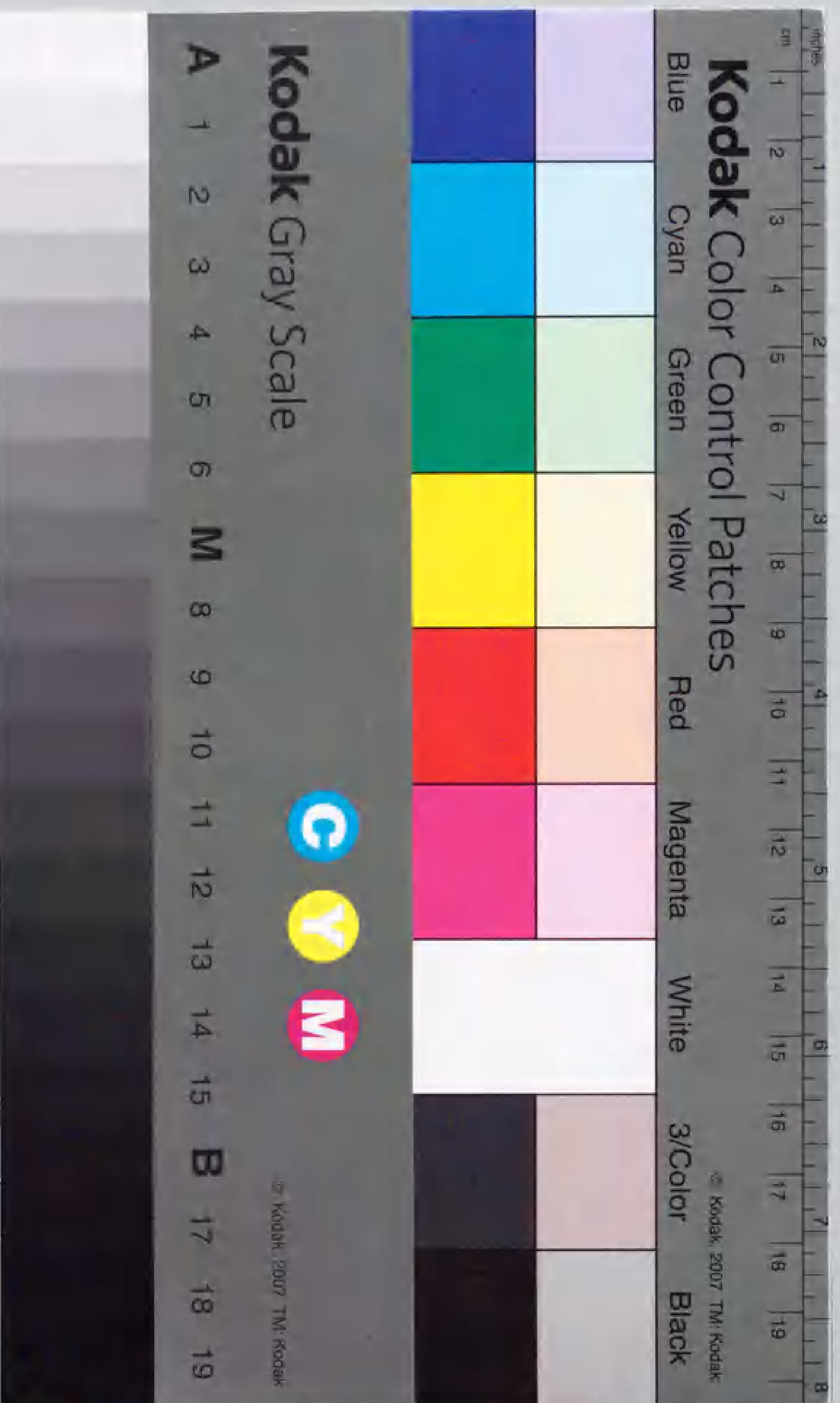
Kodak Color Control Patches
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
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Kodak Gray Scale
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19
C Y M
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報告番号 乙第 5864 号

STUDIES ON GENE EXPRESSION MECHANISMS OF PROLACTIN AND PROLACTIN RECEPTOR IN THE CHICKEN

(ニワトリプロラクチン及びプロラクチン受容体遺伝子の発現機構に関する研究)

BY

TAKESHI OHKUBO

CENTER FOR MOLECULAR BIOLOGY AND GENETICS

MIE UNIVERSITY

THESIS SUBMITTED TO THE GURADUATE SCHOOL OF BIOAGRICULTURAL SCIENCES, NAGOYA UNIVERSITY FOR THE DEGREE OF DOCTOR OF AGRICULUTURAL SCIENCES

2000 December

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I. GENERAL INTRODUCTION

Prolactin (PRL) exhibits a variety of physiological functions in vertebrates. In birds, its functions are diverse and it participates in functions such as Columbiform crop sac development (Nicoll, 1967), brood patch formation (Hutchison *et al.*, 1967) and incubation behavior (Janik and Buntin, 1985, Sharp *et al.*, 1988, Youngren *et al.*, 1991). Especially, the incubation behavior is an essential and unique behavior in birds to conserve the species. Since the report by Riddle *et al.* (1935) that injection of ovine PRL causes incubation behavior in domestic chicken, numerous studies have demonstrated the relationship between PRL and the behavior in the turkey and chicken. In 1989, the cloning of chicken PRL cDNA was demonstrated by two groups and the nucleotide sequences were revealed (Watahiki *et al.*, 1989, Hanks *et al.*, 1989a). After these findings, further advances of the biology of avian PRL have been made by the application of molecular biological techniques. In 1992, chicken PRL receptor cDNA was cloned (Tanaka *et al.*, 1992) and this finding made it possible to understand the functions of PRL via its receptor in birds. However, the regulation mechanism of PRL functions including incubation behavior is not fully understood.

I-1. PHYSIOLOGICAL FUNCTIONS OF PROLACTIN

Prolactin has a variety of physiological actions in vertebrates including birds. In a recent review, these actions were subdivided into 6 broad categories: 1) water and electrolyte balance, 2) growth and development, 3) endocrinology and metabolism, 4) brain and behavior, 5) reproduction, and 6) immunoregulation and protection (Bole-Feysot *et al.*, 1998). In birds, numbers of studies have demonstrated the functions of PRL and these functions are also divided into these categories (Table I-1). Among these functions, behavioral and reproductive actions are well characterized in birds.

I-1.1. Parental behavior

Parental behavior in birds typically includes nest building, incubating the eggs, brooding the newly hatched chicks, and taking care of the young until they are ready to live independently. Birds show enormous diversity in parental behavior. For example, female cuckoos and cowbirds deposit their eggs secretly into the nest of other birds and never engage in parental behavior. In other avian species, such as chicken and duck, only the females provide parental behavior, and whereas only males tend nests and incubate their eggs in jacana. Biparental care, in which both males and females equally contribute to parental care, is the most common pattern of parental behavior among birds.

The pattern of avian parental behavior on which most endocrine investigations have focused is pigeons and doves that provide food to the young. Unique parental behavior in pigeons and doves is that both parents produce crop milk to feed their young, which is sufficiently similar to mammal's parental behavior, suggesting that a common underlying physiological mechanism may be involved.

I-1.2. Incubation behavior

Incubation behavior is one of the parental behavioral phases in birds and this behavior can refer to sitting on eggs in a nest and hens stop laying eggs before starting incubation behavior. Therefore, studies by a large number of poultry scientists have been aimed at preventing incubation behavior in order to increase egg production.

It has been suggested that the onset of incubation behavior is initiated by increasing PRL in the blood (El Halawani *et al.*, 1986, Youngren *et al.*, 1991, March *et al.*, 1994), and PRL is maintained at high levels throughout the incubation phase in both pituitary gland and circulating blood. Moreover, the levels of PRL mRNA in the pituitary gland show a similar trend in chicken (Talbot *et al.*, 1991, Shimada *et al.*, 1991) and turkey (Wong *et al.*, 1991). It

has been shown that active immunization against recombinant-derived PRL reduced the incidence, delayed the development, or prevented the occurrence of incubation behavior in birds (March *et al.*, 1994). Furthermore, more direct evidence comes from the observation that incubation behavior can be induced by systemic or intracranial administration of ovine PRL (Riddle *et al.*, 1935). These results strongly suggest that PRL regulates the onset and/or maintenance of incubation behavior in galliformes.

It is expected that parental care, including incubation behavior, is mediated via the hypothalamic region in the brain. In support of this view, autoradiographic studies in the ring dove using ^{125}I- labeled prolactin reveal high concentrations of PRL binding sites in the hypothalamus (Buntin and Ruzycki 1987, Buntin *et al.*, 1993) and PRLR mRNA is expressed in the turkey hypothalamus (Zhou *et al.*, 1996). Furthermore, this view is supported by the observations that lesions in this area block the development of incubation behavior in the turkey (Youngren *et al.*, 1989), and disrupt prolactin-induced parental feeding behavior in the ring dove (Slawski and Buntin, 1995).

I-2. MOLECULAR FORM OF PROLACTIN

I-2.1. Avian PRL cDNA

Nucleotide sequences of chicken PRL cDNAs (Watahiki *et al.*, 1989, Hanks *et al.*, 1989a) and turkey PRL cDNAs (Karatzas *et al.*, 1990, Wong *et al.*, 1991) were reported. Both chicken and turkey PRL cDNAs contain a signal peptide of 30 amino acid residues followed by a mature PRL of 199 residues (Fig. I-1). The mature PRL has a calculated molecular weight of 23 kDa. The six cysteine residues at positions 4, 11, 58, 174, 191 and 199 are conserved in the same positions as in prolactins of other species. They are responsible for the secondary structure of the molecule forming disulfide loops by linking residues 4-11, 58-174, and 191-199.

Analyses of sequence similarity between chicken PRL and other species demonstrate the phylogenetic relationship between birds and other vertebrate classes. The highest homology is with reptiles (alligator, 92 %; sea turtle, 86 %), high with amphibian (bull frog, 72 %) and the lowest with teleosts (salmon, 28 %) (Yasuda *et al.*, 1991, Noso *et al.*, 1992). Amongst mammalian PRLs, similarities with chicken PRL range between 79 % for pig and 54 % for mouse (Hanks *et al.*, 1989a).

I-2.2. PRL variants

The diverse biological actions of PRL may be mediated by PRL variants. These variants could be generated by alternative splicing of mRNA transcript or by post-translational modifications including glycosylation, phosphorylation, deamidation, sulphation or the formation of high molecular weight polymers of PRL (Sinha, 1992). In birds, multiple forms of PRL are observed in turkey (Proudman and Corcoran, 1981, Corcoran and Proudman, 1991, Arámburo *et al*, 1992, Karatzas *et al.*, 1992) and chicken (Arámburo *et al*, 1992, Talbot *et al.*, 1992). The possibility that some forms may be phosphorylated has been demonstrated in incubation of chicken and turkey prolactin *in vitro* with the catalytic subunit of protein kinase A and [γ-^{32}P] ATP (Arámburo *et al*, 1992). Glycosylated variant forms of avian PRL have also been reported. In the glycosylated forms of PRL in whale, horse, human, pig and sheep, the carbohydrate moiety is attached to the asparagine sequence (Asn-X-Ser) for *N*-linked glycosylation (Sinha, 1992). Since this sequence does not occur at this position in either chicken or turkey, it has been proposed that *N*-linked glycosylation may occur in avian PRL at Asn-X-Cys sites (Proudman and Corcoran, 1981). The biological significance of glycosylated forms of PRL is not fully understood, but turkey (Proudman and Corcoran, 1981) and mammalian forms are less biologically active than their non-gycosylated

variants (Sinha, 1992). At least one form of turkey PRL, which has a molecular size of 25 kDa and may be glycosylated, is present in reduced amounts in the pituitary gland of the incubating turkey (Karatzas *et al.*, 1992).

I-3. REGULATION OF PROLACTIN RELEASE AND GENE EXPRESSION

I-3.1. Prolactin releasing factors

A hypothalamic peptide that stimulates PRL release has recently been found as a ligand of an orphan receptor in mammals and named PRL-releasing peptide (PrRP) (Hinuma *et al.*, 1998). The PrRP is mainly detected in hypothalamus by immunocytochemistry and its receptor is observed in pituitary gland in mammals (Maruyama *et al.*, 1999). In avian, PrRP and its receptor have not been identified yet. However, a peptide hormone released from the hypothalamus, which is named as vasoactive intestinal polypeptide (VIP), stimulates PRL release from the avian pituitary gland.

I-3.2. Vasoactive intestinal polypeptide (VIP)

Vasoactive intestinal polypeptide (VIP), a well characterized PRL releasing factor in birds, exerts a stimulatory effect on PRL release in chicken (Sharp *et al.* 1989, Talbot *et al.* 1991, Talbot *et al.*, 1995) and turkey (Youngren *et al.*, 1994, El Halawani *et al.*, 1995). Passive immunization of incubating bantam hens with anti-chicken VIP results in an immediate decrease in concentration of plasma PRL to below levels of detection (Sharp *et al.*, 1989) and passive immunization of incubating ring doves with the same antiserum prevents the development of the crop sac (Lea *et al.*, 1991). There is also evidence that in addition to stimulating PRL release, VIP also stimulates the PRL gene expression *in vivo* (Sharp *et al.*, 1989, Talbot *et al.*, 1991) and *in vitro* (Kansaku *et al.*, 1995).

I-3.3. GHF-1/Pit-1

GHF-1/Pit-1 (Pit-1) is a pituitary-specific transcription factor containing two conserved regions designated as POU-specific domain and POU homeodomain (Herr *et al.*, 1988, He *et al.*, 1989, Cohen *et al.*, 1997). In addition to its role in pituitary organogenesis (Voss and Rosenfeld, 1992, Andersen and Rosenfeld, 1994), Pit-1 directs the gene expression for growth hormone (GH), PRL, thyroid-stimulating hormone-β subunit (TSHβ) in pituitary somatotrophs, lactotrophs and thyrotrophs, respectively (Bodner *et al.*, 1988, Ingraham *et al.*, 1988, Nelson *et al.*, 1988, Mangalam *et al.*, 1989, Voss and Rosenfeld, 1992, Theill and Karin, 1993, Cohen *et al.*, 1997).

In birds, two and three Pit-1 cDNAs, which are generated by alternative splicing of premature mRNA, have been cloned from chicken (Tanaka *et al.*, 1999) and turkey (Wong *et al.*, 1992, Kurima *et al.*, 1998), respectively (Fig.I-2). The chicken Pit-1α and turkey Pit-1* are structurally comparable to the mammalian Pit-1α, and turkey Pit-1β*, which was not detected in chicken even by RT-PCR (Tanaka *et al.*, 1999), is comparable to the mammalian Pit-1β. On the other hand, chicken Pit-1γ and turkey Pit-1w* contain a unique sequence of 11 amino acids at their N-terminus. Noteworthy, all avian Pit-1 isoforms contain 38 amino acid insertions in the transactivation domain of the protein as compared to mammalian Pit-1 proteins. Similar amino acid insertions of 33 residues are observed at the same site in fish Pit-1 proteins (Ono and Takayama, 1992, Yamada *et al.*, 1993, Majumdar *et al.*, 1996), and this insertion has been demonstrated to be essential for the activation of salmon PRL gene but not for rat PRL gene (Majumdar *et al.*, 1996), suggesting that the insertion of 38 amino acid conserved in avian Pit-1s may also be very important for the activation of the target genes in the pituitary gland in birds. Moreover, a consensus Pit-1 binding site conserved in the avian

and teleost PRL/GH gene family has been proposed (Ohkubo *et al.*, 1996) (Fig.I-3). This consensus Pit-1 binding sequence for aves and teleosts slightly differs from that conserved in mammals, and the site is found in chicken GH gene (Tanaka *et al.*, 1992). However, the transcriptional regulation of pituitary hormones by Pit-1 is not fully understood in birds.

I-4. PROLACTIN RECEPTOR

Avian prolactin receptor (PRLR) cDNA has been reported in chicken (Tanaka *et al.*, 1992a), pigeon (Chen and Horseman, 1994) and turkey (Zhou *et al.*, 1996). PRLR belongs to the cytokine receptor superfamily (class 1 cytokine receptor), which possesses common structural features including two pairs of cystine residues and a Trp-Ser-X-Trp-Ser (WSXWS) motif in the extracellular region (Bazan, 1989, 1990). Further, the avian PRLRs have a unique structure compared to mammalian PRLRs in which the extracellular domain of the cDNAs contains two tandemly repeated similar units. This means that avian PRLRs contain two putative ligand binding units as a double antenna structure. A similar repeated structure has been reported for mouse interleukin-3 receptor, though its amino-terminal antenna unit does not contain a complete WSXWS motif (Itoh *et al.*, 1990). Another common structure in the receptor superfamily is a proline rich region, so called box 1, which is observed in the cytoplasmic region. This region is proposed to interact with janus kinase (JAK) mediating signals of PRL (Lebrun *et al.*, 1995a) and growth hormone (Goujon *et al.*, 1994) to the signal transducers and activators of transcription (STAT) family (Finidori and Kelly, 1995, Ihle, 1996). The sequence of the box 1 is highly conserved in the avian and mammalian PRLRs and also in the avian PRLRs and growth hormone receptors (Fig.I-4).

I-5. SIGNAL TRANSDUCING PATHWAY VIA PROLACTIN RECEPTOR

Prolactin actions result from the interaction of PRL with its receptor in various target cells, which leads to the activation of a cascade of intracellular events. However, the signal transduction pathway of avian PRL through the receptor is not understood in avian species. As described in section II-1, avian PRLRs share common structural features with mammalian ones. Therefore, it is possible that the actions of avian PRL may be mediated by the same manner observed in mammals. Here, the signal transduction pathways proposed in mammals are described in detail.

I-5.1. The JAK-STAT pathway

Janus kinases (JAKs) are newly identified kinase that mediates the signals through cytokine receptor, and four members, Tyk2, JAK1, JAK2 and JAK3 form the JAK family. The structures of the four members of the JAK family are very similar (Fig. I-5). They are 120-135 kDa cytosolic kinases, devoid of Src-homology 2 (SH2) and Src-homology 3 (SH3) regulatory domain. Seven highly conserved domains have been described from the C-terminus towards the N-terminus and named JH1 to JH7 (Harpur *et al.*, 1992). The C-terminus kinase domain (JH1) has all the characteristics of protein kinase and was shown to have a kinase activity when expressed as a bacterial fusion protein (Wilk *et al.*, 1991). The second domain (JH2) is also very similar to known kinases, but the conserved residues essential for ATP-binding site are missing. Therefore, this region has no kinase activity, but it may have a regulatory function.

Signal transducers and activators of transcription (STAT) were identified as a family of DNA binding proteins that are involved in cytokine receptor signaling. The STAT gene family contains eight members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6 and dSTAT which is a homologue of STAT5 found in *Drosophila* (Hou *et al.*, 1996,

Yan *et al.*, 1996). STAT contains five common structural features (Fig. I-6): a DNA binding domain, an SH3-like domain, an SH2 domain, a ubiquitous tyrosine, and a C-terminal transacivation domain (from the N- to C-terminus, respectively).

JAK2 is the major PRLR-associated Janus kinase. JAK 2 is constitutively associated with PRLR (Lebrun *et al.*, 1994) and the interaction between PRLR and JAK 2 involves the membrane proximal region of the PRLR cytoplasmic domain (box 1, which is highly enriched in proline residues). This interaction is very important to mediate the signals to the downstream. It is now believed that the signaling of cytokines including PRL is modeled as following. First, the cytokine-bound receptor results in tyrosine phosphorylation by the associated JAK. Second, phosphorylated tyrosine interacts with the SH2 domain of a STAT, making the receptor-JAK complex. Third, the receptor-bound STAT is phosphorylated by the JAK belonging to the complex. Finally, phosphorylated STAT dissociates from the receptor, and that forms homo- or hetero-dimer to translocate to the nucleus where it activates specific DNA elements in the promoter of the target genes. For the activation of PRLR, JAK2 and three members of STAT proteins, STAT1, STAT3, mainly STAT5, are involved. There is evidence that pigeon PRLR interacts with JAK2 and also JAK3 to stimulate rat β-casein gene expression in vitro, and this activation is STAT5-dependent (Gao *et al.*, 1996).

I-5.2 Other signaling pathways

Although the JAK-STAT cascade is presumably the most important signaling pathway, other transducing pathways are also involved in signal transduction by PRL. It is reported that the MAP kinase pathway is activated in different cells under PRL stimulation. Fyn, a member of the Src kinase family (Erpel and Courtneidge, 1995), is associated with PRLR and is activated by PRL in the Nb2 rat T lymphoma cell line (Clevenger and Medaglia, 1994).

Recent observations suggest that phospholipase-C and protein kinase C are also involved in PRL signaling. However, these pathways are to be elucidated.

It is essential to clarify the regulatory mechanism of incubation behavior for the understanding of the reproduction of avian species and for the improvement of egg and meat productions. Therefore, this thesis focuses on the molecular mechanisms for the regulation of PRL and PRL receptor gene expressions with relation to incubation behavior of chickens, especially to know how the incubation behavior is controlled and why one of domestic chickens (White Leghorn) can't show incubation behavior.

Table I-1 Physiological functions of PRL in birds

Categories / Organ	Effect
Water and electrolyte balance	
Kidney	Plasma urate
Nasal salt gland	Elimination of excess salt
Growth and development	
Body	Weight gain
Feather	Growth
	Molting
Skin	Broodpatch defeathering
	Broodpatch epidermal growth
Hair follicle	Hair growth
Crop sac	Proliferation of epithelium
Intestine	Proliferation of Intestinal mucosa
Gonads	Weight gain
Endocrinology and metabolism	
Adipocyte	Increase lipoprotein lipase activity
Brain and behavior	
Brain	Migration
	Nesting behavior
	Nest attendance
	Incubation behavior
	Feeding behavior
	Hyperphagia
	Regurgitation feeding
Reproduction	
Crop sac	Growth
	Mitosis of germinal layer
	Thickening of epithelium
	Annexin Icp 35
	Crop milk polypeptide 58 and 50.5
	Lipoprotein lipase
	Ornithine decarboxylase
	25 kDa protein
Ovary	Decrease estradiol and induce ovarian regression
Immunoregulation and protection	
Lymphocytes	T cell engrafment

Modified from Bole-Feysot *et al.*, 1998

```
LeuProIleCysProIleGlySerValAsnCysGlnValSerLeuGlyGluLeuPheAsp     20
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  -  - SerSer -  -  -  -  -  -  -  -  -  -  -  -  -  -

ArgAlaValLysLeuSerHisTyrIleHisTyrLeuSerSerGluIlePheAsnGluPhe     40
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  - Arg -  -  -  -  -  - Phe -  -  -  -  -  -  -  -  -

AspGluArgTyrAlaGlnGlyArgGlyPheIleThrLysAlaValAsnGlyCysHisThr     60
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

SerSerLeuThrThrProGluAspLysGluGlnAlaGlnGlnIleHisHisGluAspLeu     80
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  -  -  -  -  -  -  -  -  - Thr -  -  -  -  -  - Glu -

LeuAsnLeuValValGlyValLeuArgSerTrpAsnAspProLeuIleHisLeuAlaSer    100
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  - IleLeu -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

GluValGlnArgIleLysGluAlaProAspThrIleLeuTrpLysAlaValGluIleGlu    120
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

GluGlnAsnLysArgLeuLeuGluGlyMetGluLysIleValGlyArgValHisSerGly    140
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  -  -  - Arg -  -  -  -  -  -  -  -  -  - Ile -  -  -

HisAlaGlyAsnGluIleTyrSerHisSerAspGlyLeuProSerLeuGlnLeuAlaAsp    160
Asp -  -  -  -  -  -  -  - Trp -  -  -  -  -  -  -  -  -  -
Asp -  -  -  - ValPhe - GlnTrp -  -  -  -  -  -  -  -  -  -

GluAspSerArgLeuPheAlaPheTyrAsnLeuLeuHisCysHisArgArgAspSerHis    180
 -  -  -  -  -  -  -  -  -  -  -  -  -  - Leu -  -  -  -  -
 -  -  -  -  -  -  -  -  -  -  -  -  -  - Leu -  -  -  -  -

LysIleAspAsnTyrLeuLysValLeuLysCysArgLeuIleHisAspSerAsnCys       199
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -
 -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  - Asn -  -
```

Fig. I-1 A comparison of the amino acid sequences of mature chicken and turkey prolactins as deduced from their cDNA nucleotide sequences. The two chicken cDNAs were cloned by Watahiki *et al.*, 1989 (top lane) and Hanks *et al.*, 1989a (second lane) and the turkey cDNA by Wong *et al.*, 1991 (bottom lane).

α

MetThrCysGlnAlaPheAlaSerSerAspAsnPhe

ValProLeuAsnSerAspSerSerProSerLeuProLeuIleMetHisHisSerAlaAlaGluCysLeuProValSerAsnHisAlaThrAsnValValSerThr
(Ser)

γ

MetTyrLeuGlu

SerSerCysIlePheLeuPro[ValProSerValLeuSerLeuIleGlnThrProLysCysSerHisLeuHisPheAlaMetMetThrSerGlyAsnValSerAla]
(Phe)

Common

GlyLeuHisTyrSerValProSerCysHisTyrGlyAsnGlnAlaSerThrTyrGlyValMetAlaGlyIleLysProAlaThrProGluMetLeuSerAlaSer
(Thr) (Thr)

LeuSerGlnSerArgIleLeuGlnThrCysSerMetProHisProAsnValValAsnGlyValSerThrLeuGlnSerSerLeuThrProCysLeuTyrLysPhe

ProGluHisAlaLeuSerAlaSerSerCysAlaLeuGlyHisSerPheThrProMetHisGlnThrLeuLeuSerAspAspProThrAlaSerAspPheLysGln
(Ala)

GluPheArgArgLysSerLysSerValGluGluProValAspMetAspSerProGluIleArgGluLeuGluLysPheAlaAsnGluPheLysLeuArgArgIle

LysLeuGlyTyrThrGlnThrAsnValGlyGluAlaLeuAlaAlaValHisGlySerGluPheSerGlnThrThrIleCysArgPheGluAsnLeuGlnLeuSer
(Ser)

PheLysAsnAlaCysLysLeuLysSerIleLeuSerLysTrpLeuGluGluAlaGluGlnValGlyAlaLeuTyrAsnGluLysValGlyValAsnGluArgLys

ArgLysArgArgThrThrIleSerIleSerAlaLysGluAlaLeuGluArgHisPheGlyGluGlnSerLysProSerSerGlnGluIleMetArgMetAlaGlu
(Ala)

GlyLeuAsnLeuGluLysGluValValArgValTrpPheCysAsnArgArgGlnArgGluLysArgValLysThrSerLeuHisGlnAsnAlaPheSerSerIle
(Phe)

IleLysGluHisHisGluCysArg

Fig. I-2 A comparison of the amino acid sequences of chicken and turkey Pit-1. Amino acid residues differing from the turkey Pit-1* and Pit-1w* are shown in parentheses under the corresponding residues of chicken Pit-1 α and Pit-1γ, respectively. The turkey Pit-1β* specific region are boxed, and which is inserted between Pit-1 α specific residues and common region. The chicken and turkey Pit-1 cDNAs were cloned by Tanaka *et al.*, 1999 and by Kurima *et al.*, 1998 respectively.

Mammalian consensus	**T ATATNCAT**	
yellowtail	ACCTCCAT	-105/-112
rainbow trout G4c	TTCTCCAT	-38/-31
Chicken	ATCTGCAT	-112/-105
Teleost & Avian consensus	**T ANCTNCAT**	

Fig. I-3 A Comparison of the Pit-1/GHF-1 binding sequences in the promoter regions from yellowtail, rainbow trout and chicken. The mammalian consensus sequence and a novel consensus sequence proposed for teleost and avian Pit-1/GHF-1 binding sites are also shown. (From Ohkubo *et al*., 1996)

```
pGHR    LILLSKQSRL KMLIFPPVPV PKIKGIDPDL LKKGKLDEVN SILA  260/303
cGHR    LILLSKQPRL KMLIFPPVPV PKIKGIDPDL LKKGKLDEVN SILA  257/300
hGHR    VFLFSKQQRI KMLILPPVPV PKIKGIDPDL LKEGKLEEVN TILA  284/327
rGHR    VVIFSKQQRI KMLILPPVPV PKIKGIDPDL LKEGKLEEVN TILG  285/328
mGHR    RIRHPKQQRI KMLILPPVPV PKIKGIDPDL LKEGKLEEVN TILG  320/363
                        ****  *******  * *  **  *

pPRLR   WTMVLKGYRM IAFILPPVPG PKIKGIDTHL LETGKSEELL SALG  459/502
cPRLR   WTMVLKGYRM ITFMLPPVPG PKIKGIDTHL LETGKSEELL SALG  458/501
```

Fig. I-4. Alignment of amino acid sequences of the proline-rich box 1 region in GHRs and PRLRs. The boxed area is conserved among GHRs from different species, and asterisks indicate the identical amino acid residues among GHR and avian PRLRs. hGHR, rGHR, mGHR, cGHR, cPRLR and pPRLR indicate GHRs for human, rat, mouse, chicken, and PRLRs for chicken and pigeon, respectively. Each nucleotide number was indicated in the right margin.
(From Ohkubo *et al*., 1998)

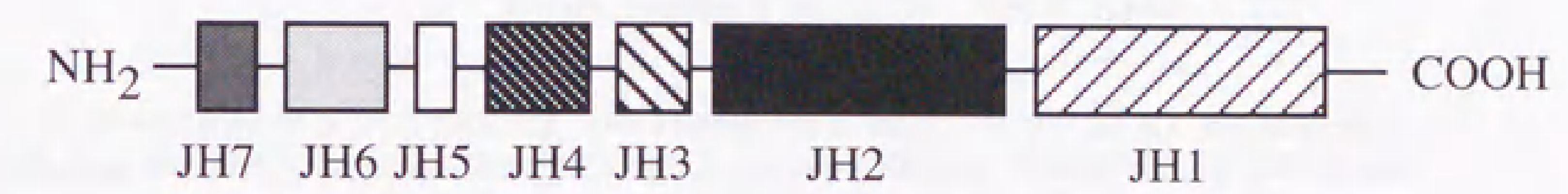

Fig. I-5 Structure of the JAK family . The overall organization of the family members is described. The homologous regions are indicated as JH1 to JH7 as described by Harpur et al., 1992. The JH1 and JH2 are the catalytic domain of tyrosine kinase and the kinase-like domain, respectively.

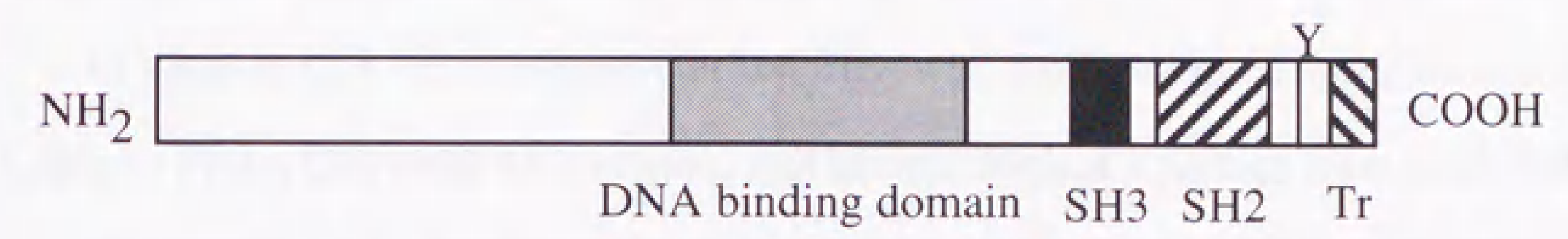

Fig. I-6 Structure of STAT proteins. The functional domains of the STAT are indicated, including the DNA binding domain, a Src homology domain 3-like region (SH3), the highly conserved Src homology domain 2 (SH2), the critical site of tyrosine phosphorylation (Y), and C-terminal transcriptional activation domain (Tr).

II. MAIN CHAPTERS

The MAIN CHAPTERS consist of four chapters. Chapter II-1 describes the regulatory mechanism of PRL receptor gene expression in neuroendocrine tissues at different physiological states, investigating whether PRL may up-regulate or down-regulate the PRL receptor gene expression.

In Chapter II-2, the mRNA expression of PRL receptor has been compared in the brain areas between broody (bantam) and non-broody (White Leghorn) breeds respectively.

In Chapter II-3, recombinant-chicken PRL was produced in *Escherichia coli* using White Leghorn PRL cDNA and its biological and immunological activities were analyzed.

In Chapter II-4, cloning and sequence analysis of chicken PRL gene have been carried out, and the regulatory elements of the gene transcription have been identified.

II-1. RELATIONSHIP BETWEEN PROLACTIN RECEPTOR mRNA IN THE ANTERIOR PITUITARY GLAND AND HYPOTHALAMUS, AND REPRODUCTIVE STATE IN MALE AND FEMALE BANTAMS (*Gallus domestics*)

II-1.1. Summary

The aim of this study is to clarify the relationship between the concentrations of plasma PRL by age, sex or reproductive status of the chicken and amounts of PRLR mRNA in the hypothalamus and pituitary. Comparisons were made on concentrations of PRLR mRNA in the anterior pituitary gland and basal and preoptic hypothalamus in adult males and females on long days; in 3-week-old juvenile males and females on short days; in 8-week-old on short days; and females held on short days; in adult cockerels exposed to long or short days; in adult hens exposed to long or short days; and in adult laying, incubating and out-of-lay females. There was a sex difference in anterior pituitary and basal hypothalamic PRLR mRNA with lower values in both tissues in females than in males. In adult birds of either sexes held on long or short days, there was no difference in pituitary PRLR mRNA levels while PRLR mRNA in the basal hypothalamus was lower on short days. As compared to the laying and out-of-lay hens, the incubating hens showed increased and decreased PRLR mRNA concentrations in the anterior pituitary and basal hypothalamus, respectively. Sex, reproductive state or photoperiod did not affect the PRLR mRNA level in the preoptic hypothalamus. It is concluded that there is no consistent relationship between the plasma PRL levels within the physiological range and the expression levels of PRLR mRNA in the anterior pituitary gland, basal hypothalamus or in preoptic hypothalamus.

II-1.2. Introduction

Prolactin (PRL) has been reported to exert multiple effects on the brain of vertebrates, being involved in the control of parental behaviors (Bridges *et al.*, 1990, Youngren *et al.*, 1991, Buntin, 1996), sexual behavior (Bailey and Herbert, 1982, Harlan *et al.*, 1983), sleep/wake activity (Roky *et al.*, 1994), grooming (Drago *et al.*, 1980), appetite (Moore *et al.*, 1986, Li *et al.*, 1995), gonadotrophin secretion (Sarkar and Yen, 1985, Buntin *et al.*, 1988), and prolactin secretion (Ben-Jonathan *et al.*, 1989, Saldanha and Silver, 1995). PRL receptor (PRLR) or PRL binding sites are widely distributed in the brain, being abundant particularly in the hypothalamus (Muccioli *et al.*, 1991, Buntin *et al.*, 1993, Crumeyrolle-Arias *et al.*, 1993, Chiu and Wise, 1994, Nagano and Kelly, 1994, Roky *et al.*, 1996, Zhou *et al.*, 1996) and anterior pituitary gland (Chiu *et al.*, 1992, Nagano and Kelly, 1994, Zhou *et al.*, 1996). A part of the regulatory mechanisms whereby PRL acts on the brain may involve changes in the abundance of PRLR mRNA. Investigations in the rat have suggested that the concentrations of PRLR mRNA in each region of the brain are differently regulated by prolactin and/or ovarian steroids (Nagano and Kelly, 1994, Sugiyama *et al.*, 1994, Shamgochian *et al.*, 1995).

Recently, PRLR cDNAs have been cloned in the chicken (Tanaka *et al.*, 1992a), pigeon (Chen and Horseman, 1994) and turkey (Zhou *et al.*, 1996) and tissue expression of the PRLR has been investigated. The PRLR mRNA has been quantified in the turkey, where it is abundant in the hypothalamus and anterior pituitary gland. In addition, in the turkey, it has been suggested that PRL may up- and down-regulate PRLR in the anterior pituitary gland and hypothalamus, respectively. However, neither the expression levels of PRLR in the separate brain areas nor regulation of PRLR by circulating PRL have been described in chicken. The aim of present study is to establish the expression profile of PRLR mRNA in the separate brain areas in bantam, one breed of domestic chickens (*Gallus domestics*) to identify the

targets of PRL in the central nerves system in chicken. Especially, hypothalamus were dissected into two regions, basal and preoptic hypothalamus, to measure PRLR mRNA, because these brain areas are thought to mediate different control actions of PRL (Buntin 1996) and have a critical role in controlling in the incubation behavior in birds (Youngren *et al.*, 1989). Furthermore, I have tested the hypothesis made in turkey that circulating prolactin is inversely related to hypothalamic PRLR mRNA levels and directly related to anterior pituitary PRLR mRNA concentrations (Zhou *et al.*, 1996) to understand the relationship between hyperprolactinemia during incubation period and expression level of PRLR mRNA in the chicken brain. Concentrations of PRL in the plasma are dependent on age, sex (Sreekumar and Sharp, 1998a), photoperiod (Sreekumar and Sharp, 1998b) or the stage of the broody cycle (Sharp *et al.*, 1988) in the domestic chicken. Therefore comparisons were made of PRLR mRNA in neuroendocrine tissues in bantams in which concentrations of plasma PRL were different because of sex, photoperiod, or stage of the broody cycle.

II-1.3. Materials and Methods

II-1.3.1. Animals

Bantams from the Roslin Institute's flock were maintained at an ambient temperature of 20 to 23°C exposed to a lighting intensity 220-300 lux with free access to food and water. The birds were exposed to 8, 14 or 16 hours light/day depending on experimental requirements. Blood samples (0.5 ml) were taken from a brachial vein for prolactin measurements 4 to 8 hours after the lights came on. The birds were killed by cervical dislocation and brain tissues were immediately removed and frozen in liquid nitrogen before RNA extraction. The preoptic and basal hypothalami were dissected as described (Lal *et al.*, 1990), and the anterior pituitary glands and fragments of hyperstriatum accessorium (forebrain) were collected. Additional tissues were collected from the optic lobes and cerebellum if necessary.

II-1.3.2. Prolactin measurement

Plasma prolactin was measured in a single assay as described by Talbot and Sharp (1994). The intra- and interassay coefficients of variation were 8.1 and 12.5%, respectively.

II-1.3.3. Ribonuclease (RNase) protection assay for PRLR mRNA

Total RNA was extracted by using RNA Isolator (Genosys, Woodland, Texas, USA) following the manufacturer's instructions (Fig. II-1-1). pBluescript SK(-) plasmid vector containing bases 1101 to 1505 of the White Leghorn chicken PRLR cDNA (Tanaka *et al.*, 1992) was linearized using *Nco* I (Boehringer Mannheim, Lewes, East Sussex, UK) for antisense RNA synthesis. A 95 bp fragment of chicken (c)β-actin cDNA, used as a RNA

loading control and subcloned into pGEM-3Z (Armstrong and Hogg, 1992), was linearized with *Bam* HI (Boehringer Mannheim) for antisense RNA synthesis. The antisense probes for cPRLR and cβ-actin from linealized plasmids were labeled with [α-^{32}P] UTP (Amersham, Little Chalfont, Buckinghamshire, UK) using T3 and T7 RNA polymerases (Promega, Southampton, UK), respectively.

Total RNA (20μg) from each tissue to be assayed was incubated with ^{32}P-labeled cPRLR and cβ-actin cRNA probes for 16 hours at 50°C in 30 μl hybridization buffer containing 80% formamide, 400 mM NaCl, 1 mM EDTA (pH 8.0) and 40 mM PIPES-NaOH (pH 6.7, piperazine-NN'-bis-2-ethanesulphonic acid mono sodium salt). Following digestion with RNases A (10 μg/ml) and T1 (5 μg/ml, Boehringer Mannheim) for 30 min. at 30°C, the reaction mixture was treated with proteinase K (125 μg/ml, Boehringer Mannheim) for 15 min at 37°C. The samples were electrophoresed on a 6% polyacrylamide gel containing 8 M urea to separate the protected RNA fragments. The gel was dried and exposed in a Phosphor Imager (Molecular Dynamics, Sunnyvale, California, USA), for 16 hours in order to quantify the protected RNA bands.

The images of the protected RNA bands were quantified by image analysis (Image Quant, Molecular Dynamics). The concentration of β-actin mRNA in the anterior pituitary was lower than in brain tissues but the ratio between pituitary and brain tissue β-actin mRNAs were the same in each experimental group. In all experiments densitometric quantification of the β-actin mRNA-protected bands showed that there were no differences in RNA amounts loaded among the tissues. Densitometric measurements of PRLR mRNA in

the different brain areas were arbitrary values expressed as a multiple of the value for one forebrain value, which was set at one, selected at random from one experimental group in each experiment. All the densitometric values for PRLR mRNA were normalized to the β-actin mRNA values before statistical analysis. Samples from each experimental group were assayed on a single gel.

II-1.3.4. Experiment 1: Sex difference in pituitary and hypothalamic PRLR mRNA levels in adult chickens maintained on long days

The birds were 42 weeks old and had been individually caged, exposed to 14 hours light/day from 18 weeks of age. All hens were regularly laying eggs and the cockerels had fully developed testes at necropsy. Five cockerels and five hens were taken for measurements of PRLR mRNA concentration.

II-1.3.5. Experiment 2: Differences in pituitary and hypothalamic PRLR mRNA levels in juvenile male and female chicks maintained on short days

The chicks were maintained on 8 hours light/day in groups of 4-6. At 3 and 8 weeks, blood samples were taken from males and females for prolactin measurements immediately before sacrifice for the collection of brain samples.

II-1.3.6. Experiment 3: Comparison of pituitary and hypothalamic PRLR mRNA concentrations in sexually mature cockerels and hens with photoperiodically increased or decreased plasma prolactin levels

Individually caged cockerels or hens reared on 8 hours light/day were divided into two groups (n=5) at 18 weeks of age. One group was retained on 8 hours light/day and the other

was photostimulated by transfer to 14 hours light/day. At 22-24 weeks of age, blood samples and neuroendocrine tissues such as preoptic and basal hypothalami, forebrain, and anterior pituitary gland were taken for prolactin and PRLR mRNA measurements, respectively. At necropsy, both long- and short-day-exposed cockerels and hens had fully developed testes and ovaries, respectively.

II-1.3.7. Experiment 4: Comparison of pituitary and hypothalamic PRLR mRNA levels in laying, incubating and out-of-lay hens

Individually caged laying hens reared on 8 hours light/day, and stimulated into egg production by transfer to 14 hours light/day at 18 weeks, were randomly divided into three groups at 34 weeks of age. The first group was retained, individually caged, on 14 hours light/day (laying). The second group was retained in individual cages and transferred to 8 hours light/day to induce ovarian regression (out-of-lay). At the time of death, the birds taken for the experiment from this group had stopped laying for at least 2 weeks. The third group was moved to floor pens (4 m x 2 m, 4 to 5 birds/pen) with nest boxes containing artificial eggs to induce incubation behavior and exposed to 14 hours light/day (incubating). At the time of death, the birds selected for the experiment from this group had been persistently nesting and emitting characteristic broody calls for 10-14 days. Blood samples and neuroendocrine tissues were taken from laying, out-of-lay and incubating hens to determine the plasma prolactin concentration and PRLR mRNA level. At necropsy, it was confirmed by gross examination that the ovaries from the incubating and out-of-lay hens were completely regressed, and the ovaries of the laying hens were fully developed.

II-1.3.8. Statistical Analysis

The values are given as mean ± SEM. The significance of differences between the values was analyzed by unpaired Student's *t* test after performing the F test (two groups) or Barlett test (more than two groups). Plasma prolactin values were subjected to logarithmic transformation before statistical analysis. A value of $P<0.05$ was considered significant.

II-1.4. Results

II-1.4.1. Sex difference in pituitary and hypothalamic PRLR mRNA levels in adult chickens maintained on long days

The concentrations of PRLR mRNA in the anterior pituitary and basal hypothalamus in adult cockerels were greater than in laying hens (Fig. II-1-2). However no sex difference was observed in the concentrations of PRLR mRNA in the preoptic hypothalamus, optic lobe, cerebellum or forebrain. In both sexes the concentrations of PRLR mRNA in the preoptic hypothalamus were less ($P<0.05$) than in basal hypothalamus (Fig. II-1-2).

The concentration of plasma prolactin in cockerels (14.6 ±4.2 ng/ml, n=7) was significantly lower ($P<0.05$) than in hens (39.2 ± 5.2 ng/ml, n=5).

II-1.4.2. Differences in pituitary and hypothalamic PRLR mRNA level in juvenile male and female chicks maintained on short days

At 3 weeks of age, there was no sex difference in the concentrations of PRLR mRNA in the anterior pituitary gland, but at 8 weeks of age there was a difference, with higher values in the males than in females (Fig II-1-3A, B). At 3 and 8 weeks of age the concentration of PRLR mRNA in the preoptic hypothalamus was lower in females than in males but this difference was not observed in the 8 week-old birds. No sex difference was seen at 3 or 8 weeks in the forebrain PRLR mRNA concentrations.

The concentration of plasma prolactin in 3 week-old males and females were moderately high, with the values in males (46.0 ± 3.6 ng/ml) being higher ($P<0.05$) than in females (23.6 ± 2.0 ng/ml). The concentrations of plasma prolactin in 8 week-old males (0.7 ± 0.2

ng/ml) and females (0.9 ± 0.1 ng/ml) were very low and not significantly different between the sexes.

II-1.4.3. Comparison of pituitary and hypothalamic PRLR mRNA expressions in sexually mature cockerels and hens showing photoperiodically increased or decreased plasma prolactin level

The PRLR mRNA concentrations in the anterior pituitary gland were not significantly different in sexually mature cockerels held under the long- or short-day conditions (Fig. II-1-4). The concentration of basal hypothalamic PRLR mRNA in the long-day cockerels was higher than that in the short day cockerels (Fig. II-1-4). No effect of the photoperiod was observed on the PRLR mRNA levels in the preoptic hypothalamus or forebrain. The plasma prolactin concentration in the long-day cockerels (10.9 ± 3.1 ng/ml) was, however, markedly higher ($P<0.01$) than that in the short-day cockerels (0.7 ± 0.2 ng/ml).

The concentrations of anterior pituitary gland PRLR mRNA in the long- and short-day laying hens were not significantly different (Fig. II-1-5). The basal hypothalamic PRLR mRNA concentration was higher in the long-day laying hens than in the short-day ones (Fig. II-1-5). There was no effect of the photoperiod on the preoptic hypothalamus and forebrain PRLR mRNA concentrations. The plasma prolactin concentrations in the long-day hens (27.2 ± 6.7 ng/ml) was significantly higher ($P<0.001$) than that in the short day hens (0.9 ± 0.1 ng/ml).

II-1.4.4. Comparison of pituitary and hypothalamic PRLR mRNA levels in laying, incubating and out-of-lay hens

The PRLR mRNA concentration in the anterior pituitary gland in the laying hens was less than that in the incubating hens but much greater than that in the out-of-lay hens (Fig. II-1-6). In the basal hypothalamus, the concentration of PRLR mRNA in the incubating hens was lower than that in out-of-lay or laying hens (Fig. II-1-6). No significant differences were observed in concentrations of PRLR mRNA in the preoptic hypothalamus or forebrain among out-of-lay, incubating and laying hens (Fig. II-1-6).

The plasma prolactin concentration in the incubating hens (716.4 ± 43.9 ng/ml) was remarkably higher ($P<0.001$) than that in laying (29.2 ± 9.6 ng/ml) or out-of-lay (3.1 ± 0.1 ng/ml) hens. The concentration of plasma prolactin in the laying hens was greater than that in out-of-lay hens ($P<0.001$).

II-1.5. Discussion

In this study, it has been confirmed that PRLR mRNAs are present in the pituitary gland and hypothalamus at higher concentrations than those in other brain regions (Figs. II-1-1 to II-1-6). Observation that PRLR was highly expressed in the hypothalamus is consistent with studies in doves using autoradiography to localize ^{125}I-labeled PRL binding sites (Fechner and Buntin, 1989, Buntin *et al.*, 1993) and in turkeys using competitive-PCR to measure the PRLR gene expression (Zhou *et al.*, 1996) as well as in mammals (Walsh *et al.*, 1990, Muccioli *et al.*, 1991, Crumeyrolle-Arias *et al.*, 1993, Chiu and Wise, 1994, Roky *et al.*, 1996). Similar amounts of PRLR mRNA were found in the basal and preoptic hypothalamus, suggesting the presence of indispensable and maybe different functions of PRL in these two areas. Prolactin receptors in the basal hypothalamus occur in the same area as that for VIP neurons, which exert stimulatory action on PRL synthesis and release (Sharp *et al.*, 1989, Talbot *et al.*, 1991, 1995). These neurons may be the targets for PRL action, allowing circulating levels of the hormone to exert a negative feedback control over its secretion. Evidence to support this view comes from the observation that intracerebroventricular injection of PRL in incubating ring doves suppresses the activity of basal hypothalamic VIP neurons, as assessed by quantitative immunocytochemistry (Saldanha and Silver, 1995).

Prolactin receptor in the preoptic hypothalamus has been predicted to mediate the expression of parental behavior, since lesions in this area in the turkey blocks the development of incubation behavior (Youngren *et al.*, 1989), and disrupt prolactin-induced parental feeding behavior in the ring dove (Slawski and Buntin, 1995). Furthermore, in the ring dove, the development of incubation behavior is associated with increased c-*fos* expression and uptake of 2-[^{14}C] deoxyglucose in the preoptic hypothalamus (Georgiou *et al.*, 1995, Sharp *et al.*, 1996). Prolactin receptor expressed in either preoptic hypothalamus

or basal hypothalamus may also mediate the suppression of gonadotrophin secretion (Buntin *et al.*, 1988) and stimulation of the food intake (Hnasko and Buntin, 1993).

The presence of PRLR mRNA in the anterior pituitary gland also confirms previous findings in the rat (Chiu *et al.*, 1992, Nagano and Kelly, 1994) and turkey (Zhou *et al.*, 1996). One of the functions of PRLR in the chicken anterior pituitary could be to mediate the suppressive effect of PRL on LHβ subunit gene expression in chicken (Kansaku *et al.*, 1994) and turkey (You *et al.*, 1995). The LHβ mRNAs expressed in the cephalic and caudal lobes of anterior pituitary gland were inversely correlated with the expression level of PRL mRNA in bantam hens (Kansaku *et al.*, 1994). This is consistent with the observation that marked increase in circulating PRL levels during the incubation period decreased circulating LH levels in bantam (Sharp *et al.*, 1988). Furthermore, PRLR and LH were co-localized in the same cells in mammals, (Tortonese *et al.*, 1998). This result suggests that PRL may be involved in the regulation gonadotrophin secretion through a paracrine mechanism within the pituitary, even though no direct evidence that PRLR are expressed in the gonadotrophs is available in birds. Furthermore a sex difference was seen in the amount of PRLR mRNA in the anterior pituitary gland in adult and 8 week-old juvenile bantams. The sex difference in pituitary PRLR mRNA in adult and 8 week-old juvenile bantams suggests that it is related to sex differences in gonadal hormones. The absence of a sex difference in pituitary PRLR mRNA at 3 weeks may be attributed to the extreme functional immaturity of the ovary at this age (Sreekumar and Sharp, 1998a).

The concentration of PRLR mRNA in the (basal) hypothalamus in the bantam, was lower in the incubating hens than those in laying or out-of-lay hens, and inversely related to the plasma prolactin levels (Fig. II-1-6). This relationship was a part of evidence presented by Zhou *et al.* (1996) to suggest that increased level of plasma prolactin down-regulates

hypothalamic PRLR mRNA expression. It is consistent with observations in the long day adult bantam cockerels and hens whose plasma prolactin concentrations are inversely correlated with their basal hypothalamic PRLR mRNA level (Fig. II-1-2). This conclusion cannot be sustained in bantams when correlating the plasma prolactin concentration and basal hypothalamic PRLR mRNA levels in the short-day kept juvenile males and females (Fig. II-1-3), adult long-day or short-day cockerels (Fig. II-1-4) or adult long-day or short-day hens (Fig. II-1-5). There was no consistent relationship between the plasma prolactin concentration and the expression level of basal hypothalamic PRLR mRNA in these comparisons.

There are no reports on the differences in brain PRLR mRNA content in males and females of the mammals and birds, except that observations on the brain PRL binding sites quantified using ligand binding (Muccioli *et al.*, 1991) have been made in the rat. They showed that there is no sex difference in PRL binding at various brain regions except in the hypothalamus where a higher binding level was observed in females. This has suggested that the sex difference in circulating hormones level in the males and females might regulate hypothalamic PRL binding activity. Furthermore, ovariectomy markedly decreased the PRL binding to the rat hypothalamus, which was reversed by the estradiol treatment (Muccioli *et al.*, 1991, Mustafe *et al.*, 1995). However, this effect of estrogen may be secondary to the estrogen-induced increase in plasma PRL level. In support of this view, PRL has been observed to increase the specific binding of ^{125}I-labeled rat PRL to rat hypothalamic membranes (Muccioli and Di Carlo, 1994).

Recent observations in the rat suggest that, in addition to PRL, estrogen may also regulate the PRLR gene expression in the brain (Nagano and Kelly, 1994, Sugiyama *et al.*, 1994, Shamgochian *et al.*, 1995). In view of this prediction, it could be possible that differences in anterior pituitary and basal hypothalamic PRLR gene expression in bantams

under various reproductive conditions might be explained by the circulating estrogen. This possibility, however, seems to be unlikely, since it is assumed that ovarian development is directly related to the circulating concentrations of plasma estrogen (Etches *et al.*, 1984). Full ovarian development was associated with depressed anterior pituitary PRLR mRNA expression levels for the comparison between adult male and female bantams (Fig. II-1-2), whereas immature ovaries were associated with depressed anterior pituitary PRLR mRNA for the comparison between juvenile male and females (Fig. II-1-3B) or between the laying and out-of-lay hens (Fig. II-1-6). Full ovarian development was also associated with depressed basal hypothalamic PRLR mRNA for the comparison between adult males and females (Fig. II-1-2). However, this relationship is unlikely to be causal, since basal hypothalamic PRLR mRNA level was depressed in juvenile females when compared with juvenile males (Fig. II-1-3), in the out-of-lay hens when compared with the laying hens (Fig. II-1-6), and in the laying hens kept on short days when compared with the laying hens held on long days (Fig. II-1-5).

It is concluded that pituitary gland and hypothalamus are the targets for the central action of PRL, since high levels of the mRNA expression were observed in the all comparisons in this study. However, within its physiological range, circulating prolactin may not regulate the PRLR gene expression in chicken neuroendocrine tissues. In addition, indirect evidence suggests that circulating estrogen acting alone does not regulate PRLR gene expression in chicken neuroendocrine tissues. However, recent studies revealed that PRL-producing neurons are presented in the turkey brain (Ramesh *et al.*, 2000) and also enzymatic activity of cytochrome P450 aromatase, which is called estrogen synthetase, is observed in the brain of several birds (Balthazart *et al.*, 1990, Schlinger, and Arnold, 1991). Furthermore, there is evidence that it is suggested that multiple promoters regulate tissue specific gene expression of PRLR in mammal (Hu *et al.*, 1996). Supporting the observations in mammals, my

colleagues and I have found that at least three alternative first exons existed in chicken PRLR gene and one of which is used as liver-specific manner (unpublished data). This observation strongly suggests that multiple promoters also control the tissue specific gene expression of the PRLR in the chicken tissues as well as mammals. Hence these hormones might control PRLR gene expression in chicken brain by paracrine or autocrine mechanism and tissue specific promoter(s) might contribute on the differential regulation of PRLR gene expression by these hormones in chicken neuroendocrine tissues.

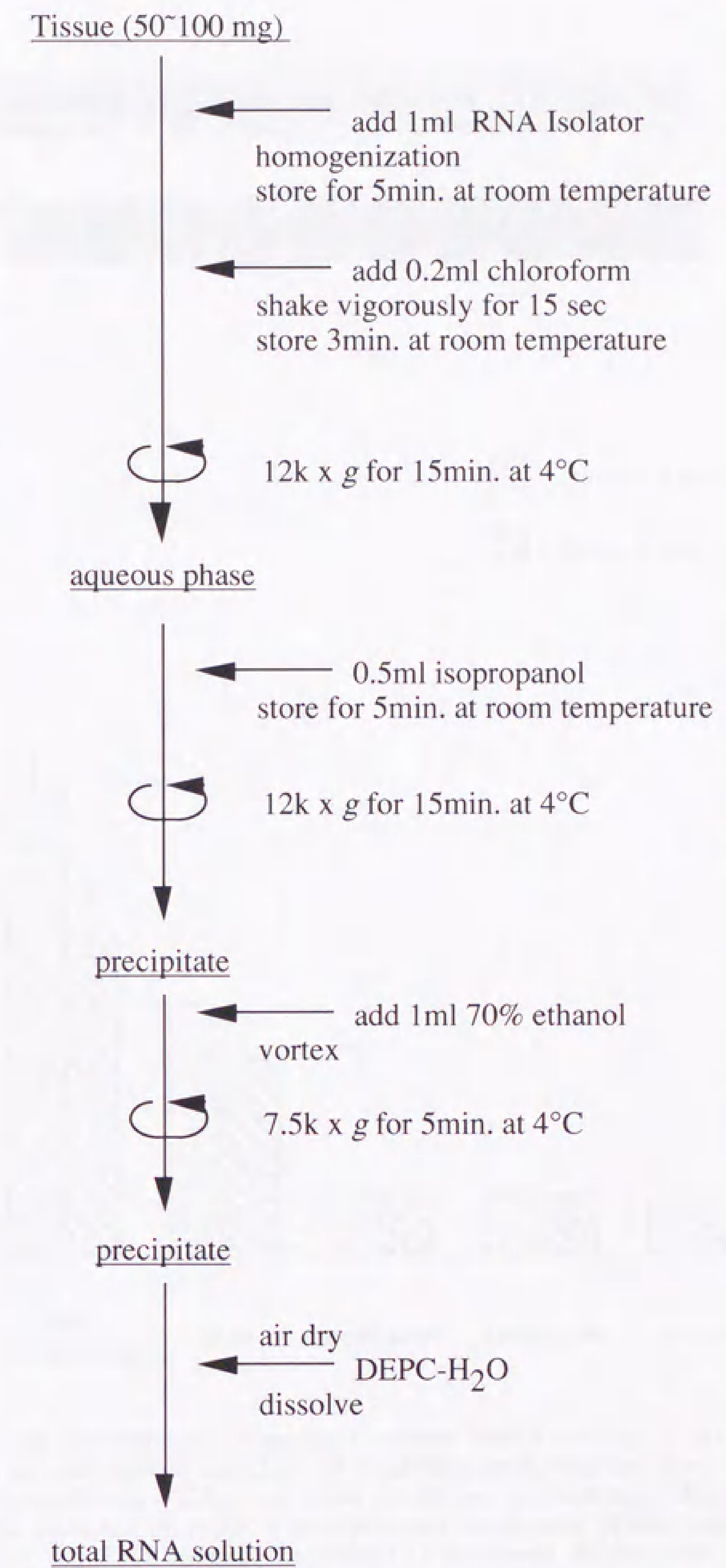

Fig. II-1-1 Instruction of total RNA isolation by RNA Isolator (Genosys)

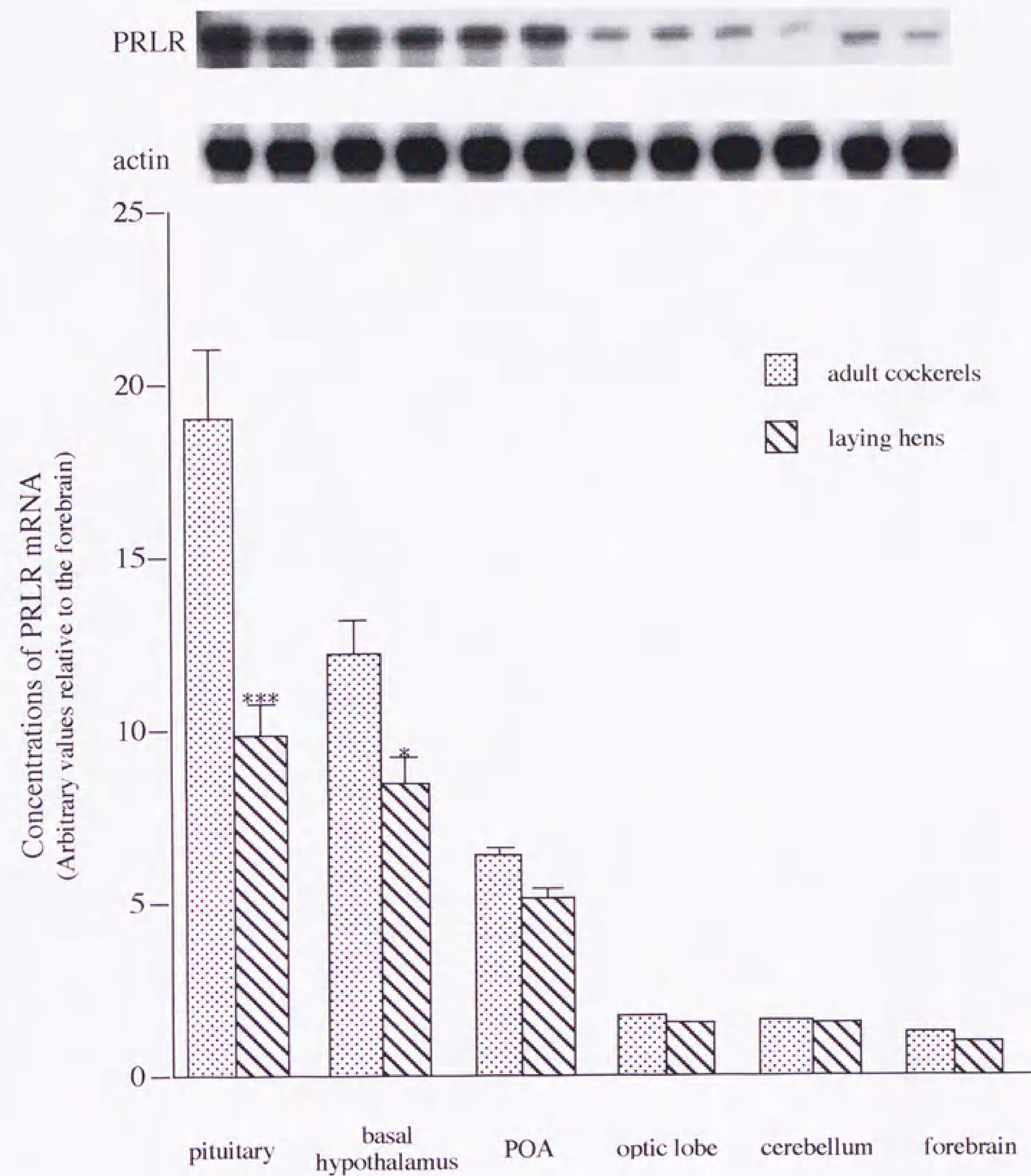

Fig. II-1-2 A comparison of concentrations of prolactin receptor (PRLR) mRNA in sexually mature bantam cockerels and hens held on long days (14 h light/day) in the pituitary gland, basal hypothalamus, preoptic hypothalamus (POA), optic lobe, cerebellum and forebrain. The upper part of the figure shows examples of PRLR-mRNA-protected bands with β-actin mRNA-protected bands to control for RNA loading. Each band is positioned directly above the corresponding densitometric quantification of the group from which it came. Values are calculated relative to that of one forebrain sample, which was assigned a value 1. ***$P < 0.001$, * $P < 0.05$ compared with cockerels. Values are means±SEM, n=5.

Fig. II-1-3 A comparison of concentrations of prolactin receptor (PRLR) mRNA in the pituitary gland, basal hypothalamus, preoptic hypothalamus (POA) and forebrain in juvenile male and female bantams exposed to short days (8h light/day) at (A) 3 weeks, when plasma prolactin is moderately high, and (B) 8 weeks of age, when plasma prolactin is low. The upper part of the figure shows examples of PRLR-mRNA-protected bands with β-actin mRNA-protected bands to control for RNA loading. Each band is positioned directly above the corresponding densitometric quantification of the group from which it came. Values are calculated relative to that of one forebrain sample, which was assigned a value 1. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ compared with males. Values are means±SEM, n=5.

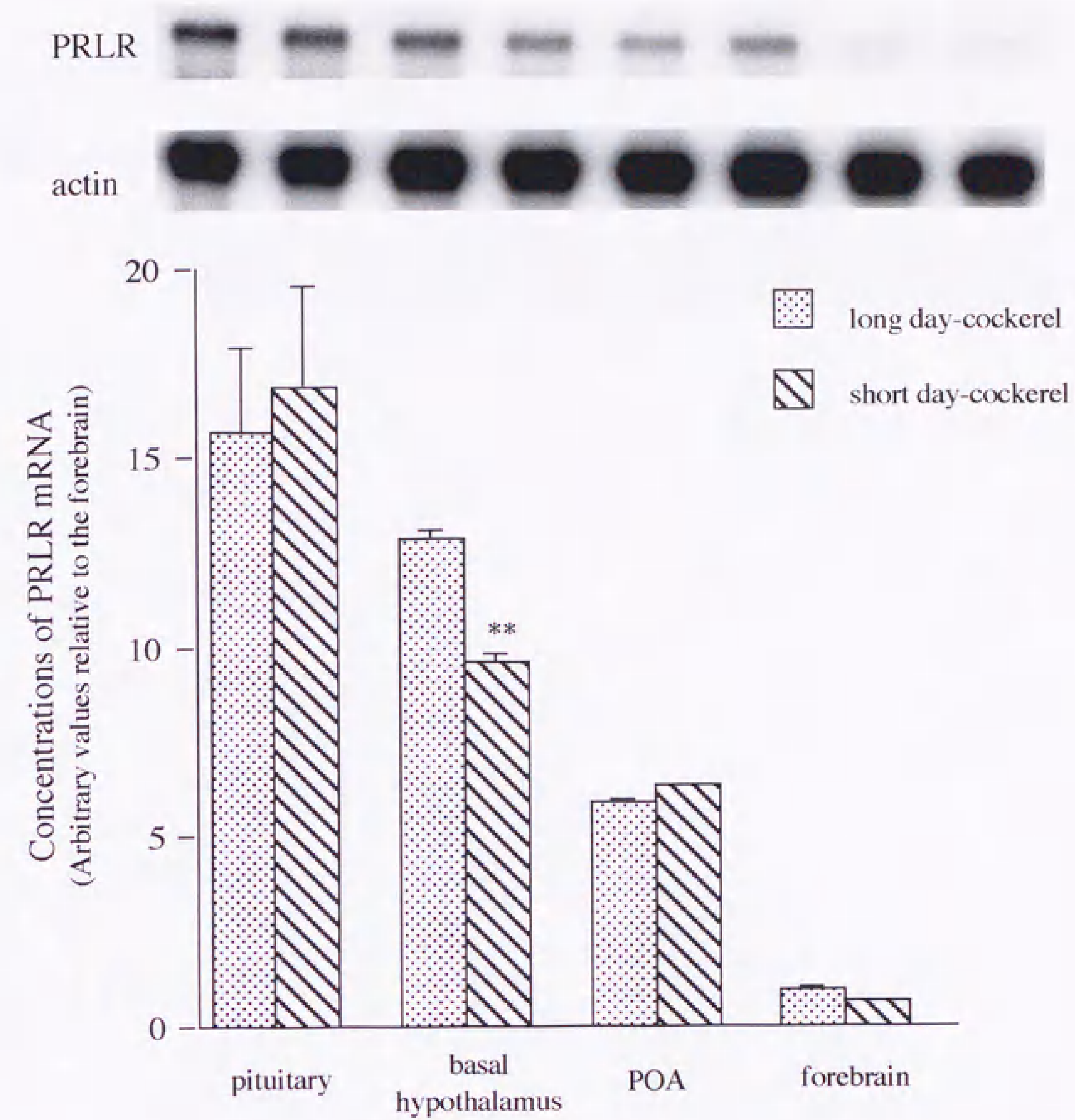

Fig. II-1-4 A comparison of concentrations of prolactin receptor (PRLR) mRNA in the pituitary gland, basal hypothalamus, preoptic hypothalamus (POA) and forebrain in sexually mature bantam cockerels. The birds were exposed to either long (14 h light/day) or short (8h light/day) days to increase or maintain low concentrations of prolactin, respectively. The upper part of the figure shows examples of PRLR-mRNA-protected bands with β-actin mRNA-protected bands to control for RNA loading. Each band is positioned directly above the corresponding densitometric quantification of the group from which it came. Values are calculated relative to that of one forebrain sample, which was assigned a value 1. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with long day value. Values are means±SEM, n=5.

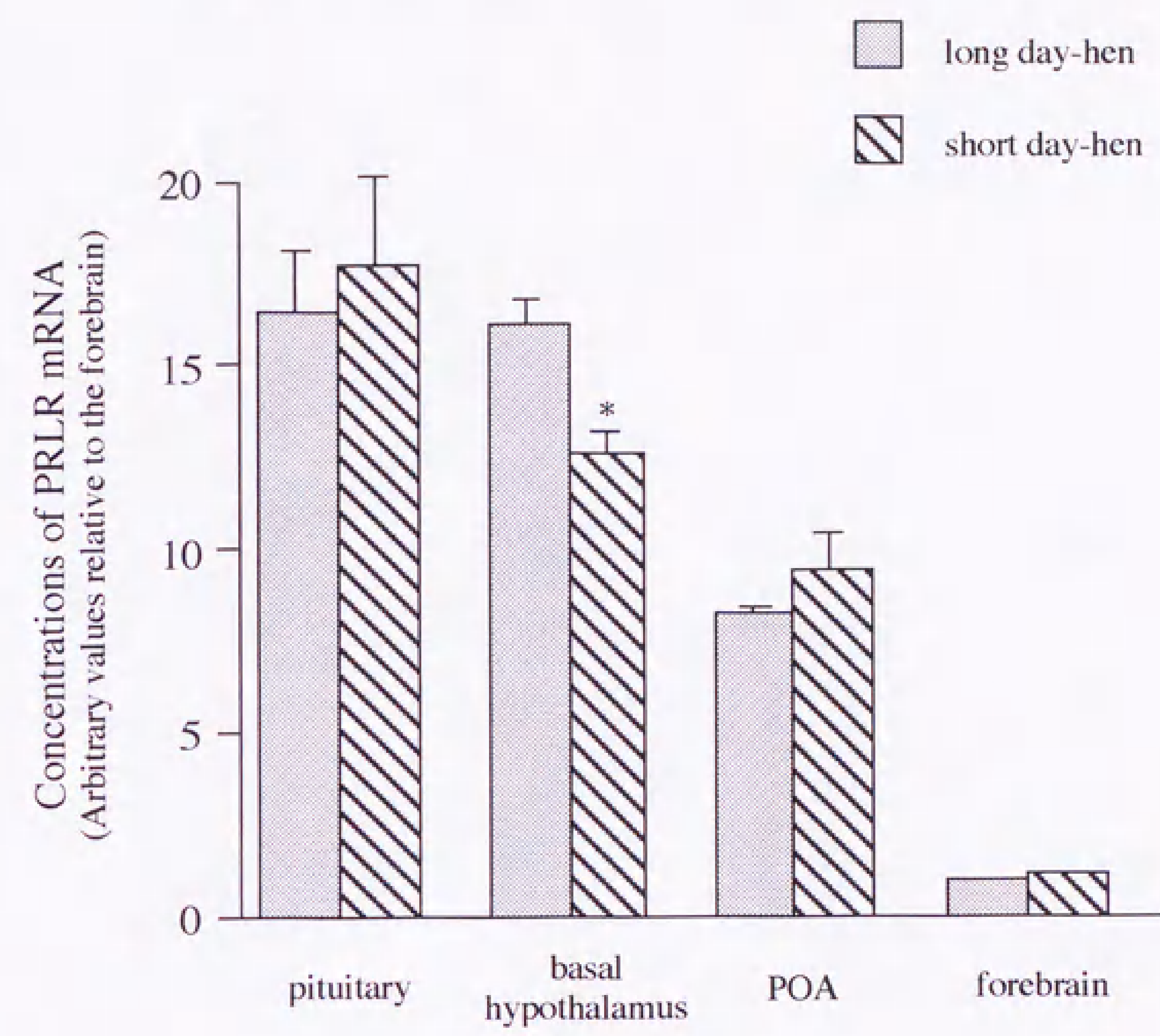

Fig. II-1-5 A comparison of concentrations of prolactin receptor (PRLR) mRNA in the pituitary gland, basal hypothalamus, preoptic hypothalamus (POA) and forebrain in laying bantam hens. The birds were exposed to either long (14 h light/day) or short (8h light/day) days to increase or maintain low concentrations of prolactin, respectively. The upper part of the figure shows examples of PRLR-mRNA-protected bands with b-actin mRNA-protected bands to control for RNA loading. Each band is positioned directly above the corresponding densitometric quantification of the group from which it came. Values are calculated relative to that of one forebrain sample, which was assigned a value 1. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with long day value. Values are means±SEM, n=5.

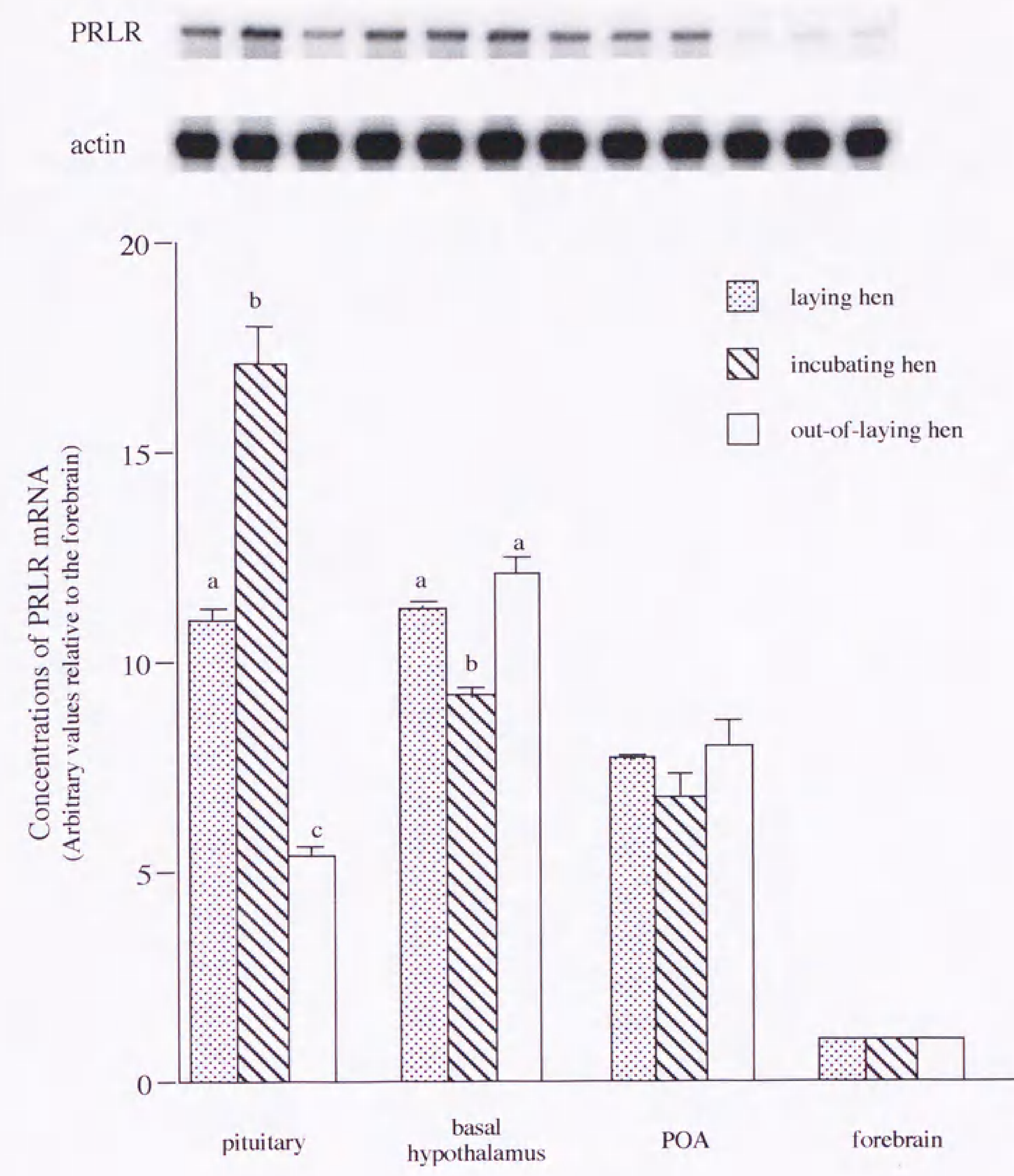

Fig. II-1-6 A comparison of concentrations of prolactin receptor (PRLR) mRNA in the pituitary gland, basal hypothalamus, preoptic hypothalamus (POA) and forebrain in out-of-lay, incubating and laying bantam hens. The upper part of the figure shows examples of PRLR-mRNA-protected bands with β-actin mRNA-protected bands to control for RNA loading. Each band is positioned directly above the corresponding densitometric quantification of the group from which it came. Values with different superscripts in the pituitary and basal hypothalamus are significantly different, $P < 0.05$. Values are means±SEM, n=5.

II-2. PROLACTIN RECEPTOR GENE EXPRESSION IN THE BRAIN AND PERIPHERAL TISSUES IN BROODY AND NONBROODY BREEDS OF DOMESTIC HEN

II-2.1 Summary

The aim of this study is to determine whether any breed-dependent difference is present or not in the structure and/or expression pattern of the PRLR gene accounting for the observation that broodiness does not occur in the White Leghorn hens but does occur in other breeds of domestic hens, including the bantam. In the brain, PRLR mRNA expression was dominantly detected in the pituitary gland and basal and preoptic hypothalami both in laying White Leghorns and bantams. However, the amounts of PRLR mRNA in the brain didn't differ significantly between laying White Leghorn and bantam hens. Southern blotting analysis using chicken PRLR cDNA revealed that the restriction patterns were identical in the PRLR genes from White Leghorn and the bantam. Northern blotting analysis also revealed that the identical two species of PRLR mRNA transcripts in hypothalami both in laying White Leghorns and bantam hens. It is thus concluded that different manifestations of the broodiness in the White Leghorn and bantam hens cannot be explained by different expression patterns of PRLR mRNA in the hypothalami or by different gross structures of the PRLR gene in these two breeds.

II-2.2. Introduction

In domestic birds, genetical segregation had been continued to improve productivity on egg and meat for long years. As the result of efforts, meat-type or egg-type has been established in chickens. The White Leghorn is one of the best known and the most important commercial egg-producing breed in the world. A good hens lay in the vicinity of 300 eggs a year and hens of this strain never become broody (Saeki and Inoue, 1979), while other breeds show the behavior.

The onset of incubation behavior in birds is initiated by an increase in plasma prolactin (PRL) (El Halawani *et al.*, 1986, Youngren *et al.*, 1991, March *et al.*, 1994), which is believed to be transported into the brain via the choroid plexus to induce the behavior (Buntin *et al.*, 1993). Autoradiographic studies in the ring dove using ^{125}I-labelled prolactin have revealed the presence of highly concentrated PRL binding sites in the hypothalamus (Buntin and Ruzycki 1987, Buntin *et al.*, 1993). I have described in Chapter II-1 that high levels of PRLR mRNA expression were detected in the pituitary gland and hypothalamic region in the bantam. This result strongly suggested that both hypothalamus and pituitary gland are the targets for initiation and/or maintenance of incubation behavior and reproductive functions. These results indicate the possibility of difference in the brain PRLR between White Leghorns and others including bantams. Chicken PRLR cDNA originally has been cloned from White Leghorn and the existence of the mRNA expression in the whole brain of White Leghorn has been demonstrated (Tanaka *et al.*, 1992a). However, comparison study of the expression of PRLR mRNA between White Leghorn and broody breeds or localization and amount of PRLR mRNA in the neuroendocrine tissues in White Leghorn has not been demonstrated. In addition, prolactin cDNAs have been cloned from both White Leghorn (Watahiki *et al.*, 1989) and bantam (Hanks *et al.*, 1989a), but it is not confirmed whether VIP could stimulate PRL release or gene expression in White Leghorn as were observed in

broody breeds of chicken (Talbot *et al.*, 1991, Kansaku *et al.*, 1995). If VIP is not effective on PRL release from anterior pituitary gland in White Leghorn, VIP might be responsible for the lacking of broody behavior in White Leghorn.

Therefore, in this chapter, I have initially analyzed the effect of VIP on PRL release from the anterior pituitary gland in White Leghorn to demonstrate that the lack of broodiness in White Leghorn is not due to the lack of pituitary responsiveness to the VIP. And furthermore, I have analyzed PRLR mRNA expression in the neuroendocrine and peripheral tissues, and the gross structures of PRLR gene in both broody and non-broody breeds of domestic chicken have been compared to establish whether the expression of incubation behavior in the bantam hens, but not in White Leghorn, can be explained by breed differences in the structure or transcription of the gene encoding PRLR.

II-2.3. Materials and Methods

II-2.3.1. Animals

Thirty to forty-weeks age of Laying bantams and White Leghorns (Ross strain) from the Roslin Institute's flocks were maintained under the long-day (16 hours light/day) condition and fed on food and water *ad libitum*. The birds that have continuously laid eggs for at least two weeks before the experiments were killed by cervical dislocation and brain and peripheral tissues were immediately removed and frozen in liquid nitrogen before RNA extraction.

II-2.3.2. Vasoactive intestinal polypeptide (VIP)-induced prolactin release

VIP (50 μg/kg body wt) or saline vehicle was injected into a wing vein and blood samples (0.5 ml) were collected by direct venipuncture from the contralateral wing vein immediately before and 2, 10, and 30 min after the injection. Plasma was separated by centrifugation, and prolactin was measured by radioimmunoassay (Talbot and Sharp, 1994) in a single assay. The intra-assay coefficient of variation was 9.8%.

II-2.3.2. RNase protection assay

RNase protection assay of PRLR mRNA was carried out as described in II-1.3.3.

II-2.3.3. Southern blot hybridization

Genomic DNA (10 μg), isolated from the White Leghorn or bantam using Nucleon I DNA isolation kit (Scotlab, Coatbridge, UK) was digested with *Bam* HI, *Eco* RI, *Xba* I or *Pst* I (Boehringer Mannhein). After electrophoresis on 0.5% agarose gel, the DNA fragments were transferred to a nylon membrane by capillary blotting. The membrane was hybridized

with the 2.2 kb chicken PRLR cDNA probe labeled with [α-^{32}P] dCTP by the random primer labeling method (Sambrook *et al.*, 1989). The chicken PRLR cDNA probe was a subclone of White Leghorn PRLR cDNA (λCPRLR-1), previously described by Tanaka *et al.* (1992a). After hybridization, the membrane was washed sequentially by 2 x SSC containing 1% SDS at 22°C for 30 min., 1 x SSC containing 1% SDS at 65°C for 1 h, and 0.1 x SSC plus 1% SDS at 65°C for 1 h before exposure to a X-ray film (Genetic Research Instrumentation, Essex, UK) with an intensifying screen at -70°C.

II-2.3.4. Northern blot hybridization

Total RNA was extracted from the kidney, hypothalamus, and hippocampus of White Leghorn and bantam chickens using a commercial kit (Genosys). RNA was separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde, and blotted onto a Hybond-N$^+$ membrane (Amersham) before hybridization to the 2.2 kb chicken cDNA probe (Tanaka *et al.*, 1992a) labeled with [α-^{32}P] dCTP as described above in II-2.3.3 Southern hybridization. The membrane was then processed to generate an autoradiogram as described for Southern hybridization.

II-2.4. Results

II-2.4.1. Comparison of VIP-induced prolactin release in laying White Leghorn and bantam hens

By intravenous injection of VIP, plasma PRL levels were increased in 2 minutes after the injection, peaked at 10 minutes after the injection and high levels of plasma PRL were observed by 30 minutes after the injection in both White Leghorn and bantam hens. Prolactin levels after the injection were higher than those of vehicle controls in both White Leghorn and bantam hens ($P<0.01$). Plasma PRL levels were not differed in both breeds and an intravenous injection of VIP was found to be equally effective in stimulating the prolactin release in White Leghorn and bantam hens (Fig. II-2-1).

II-2.4.2. Comparison of the amounts of PRLR mRNA in the brains of laying White Leghorn and bantam hens

The amounts of PRLR mRNA expressed in the pituitary gland, basal hypothalamus, and preoptic hypothalamus were greater than those in the forebrain, cerebellum, and optic lobe in both White Leghorns and bantams (Fig. II-2-2). However, the amounts of PRLR mRNA in these and other brain areas tested did not differ significantly between White Leghorn and bantam hens (Fig. II-2-2).

II-2.4.3. Comparison of the amounts of PRLR mRNA in peripheral tissues of laying White Leghorn and bantam hens

The PRLR mRNA expression was widely detected in peripheral tissues in both White Leghorn and bantam hens in the following order of abundance; kidney, leg skin, brood

patch, duodenum, intestine > thyroid gland > adrenal gland, liver, ovary >> adipose tissue > thymus, spleen > muscle > blood cells (Fig. II-2-3).

II-2.4.4. Southern blot hybridization with of the PRLR genes in White Leghorn and bantam

Digestion with *Bam* HI, *Eco* RI, *Xba* I and *Pst* I generated one, four, three, and five DNA fragments, respectively, which hybridized with the chicken PRLR cDNA probe (Fig. II-2-4). The restriction patterns of the PRLR gene were identical in White Leghorn and bantam hens (Fig. II-2-4).

II-2.4.5. Northern blot hybridization of the PRLR mRNA in White Leghorn and bantam

Two transcripts of 7.5 and 3.3 kb were detected by hybridization with the chicken PRLR cDNA probe in the kidney, hypothalamus, and hippocampus of White Leghorn and bantam hens (Fig. II-2-5). No differences were seen in the size of the PRLR mRNA between the two breeds of hens (Fig. II-2-5).

II-2.5. Discussion

Vasoactive intestinal polypeptide (VIP) is a well characterized PRL releasing factor to stimulate prolactin release from the anterior pituitary gland in chicken (Sharp *et al.* 1989, Talbot *et al.* 1991, Talbot *et al.*, 1995) and turkey (Youngren *et al.*, 1994, El Halawani *et al.*, 1995). However, it has not been established whether or not VIP can show the stimulatory effect on the PRL release in White Leghorn hens. At first, in this study, it has been confirmed that VIP can induce PRL release from the anterior pituitary gland in White Leghorn, non-broody breed *in vivo* (Fig. II-2-1). This fact strongly suggests that the absence of incubation behavior in White Leghorn hens cannot be explained by an inability of the VIP to stimulate prolactin release. In addition, Riddle *et al.* have demonstrated that incubation behavior is not induced by the injection of ovine PRL in White Leghorn hens (Riddle *et al.*, 1935). It may therefore be possible that differences in the manifestation of incubation behavior in White Leghorn and bantam hens are due to differences in the expression, or gross structure of the genes encoding PRLR.

In both the White Leghorn and bantam breeds, the expression level of the gene encoding PRLR in the brain was highest in the hypothalamus (Fig. II-2-2). And also the occurrence of PRLR mRNA expression in the anterior pituitary gland both in White Leghorn and bantam has been confirmed in this study as was observed in the laying turkey (Zhou *et al.*, 1996). However, in the laying White Leghorn and bantam hens, unlike laying turkey hens, the amounts of PRLR mRNA in the anterior pituitary gland were greater than those in the hypothalamus. These results are consistent with the observation in Chapter II-1.

The widespread distribution of PRLR mRNA in the peripheral tissues in White Leghorn and bantam hens confirms the observations in the chicken (Tanaka *et al.*, 1992a), turkey (Zhou *et al.*, 1996), and mammals (Nagano and Kelly, 1994). It indicates parts of the mechanism through which prolactin acts on variety of tissues to exert its multiple effects. In

birds, there is evidence that prolactin plays roles in osmoregulation (Harvey *et al.*, 1984, Murphy *et al.*, 1986), brood patch formation (Hutchison *et al.*, 1967), molt (Juhn and Harris, 1958), adrenocortical function (Casia *et al.*, 1987), and immunoresponsiveness (Skwarlo-Soñta, 1992). All tissues involved in these functions were found to contain PRLR mRNA in both White Leghorn and bantam hens.

Southern blot analysis using four restriction enzymes revealed identical restriction patterns of the DNA fragments in White Leghorns and bantams. This result indicates that gross differences in the structures of the genome encoding PRLR do not exist between White Leghorn and bantam, though, this observation does not eliminate the possibility that there may be point mutations in the PRLR gene in the White Leghorn breed. Any such difference may affect the JAK tyrosine kinase signal transduction pathway (DaSilva *et al.*, 1994) mediating the prolactin action to induce the incubation behavior. However, it is unlikely that such critical mutation(s) for PRL function would occur in White Leghorn. The structures of PRLR are highly conserved among chicken (White Leghorn), turkey and pigeon, and White Leghorn PRLR is shows 89.9% and 81.7% of similarity with that of turkey and pigeon, respectively (Fig. II-2-6). Several common structural features are known to mediate the PRL signal to following cascades in mammals. Two pairs of cysteines in N-terminal region are known to be critical to the maintenance of the structural and functional integrity of the PRLR (Rozakis-Adcock and Kelly, 1991, 1992). WSXWS motif located near the transmembrane domain is suggested to participate variety of processes, including ligand binding, receptor internalization and signal transduction (Quelle *et al.*, 1992, Yoshimura *et al.*, 1992). Proline-rich sequence, box 1, and tyrosine residues in the cytoplasmic region play a important role in the signal transduction of PRL (Lebrun *et al.*, 1995a, 1995b, DaSilva *et al.*, 1996, Pezet *et al.*, 1997). All these motifs are also satisfactory conserved in avian PRLRs. Because of difference of the length in avian PRLRs, chicken PRLR encodes amino acids and turkey and

pigeon PRLRs encode 830 amino acids, variety of the secondary structures was observed in the cytoplasmic region of the genes (Fig. II-2-6). However, these varieties may not be important for the signal transduction of PRL. In Nb2 cells, truncated form of PRLR which lacks a segment of 198 amino acids is able to activate lactogenic signal transduction and mitogenetic action by PRL (Ali *et al.*, 1992).

It is also unlikely that differences in the transcription of the PRLR gene in the hypothalamus account for the difference in the manifestation of incubation behavior in White Leghorns and bantams. In both breeds, 3.3 and 7.5 kb PRLR mRNAs were observed on Northern hybridization. The 3.3 kb transcript is similar in size to the PRLR mRNA species reported in the dove (Chen and Horseman, 1994) and turkey (Zhou *et al.*, 1996), and this is the size predicted for the mature transcript (Tanaka *et al.*, 1992). The 7.5 kb mRNA may correspond to the larger transcripts reported in studies of the mammalian PRLR gene expression (Edery *et al.*, 1989, Jahn *et al.*, 1991, Clarke *et al.*, 1995). One possibility is that the two PRLR mRNA transcripts derived from the different transcription start sites. However, the significance of the larger transcript is still uncertain. Recently, unique truncated PRLRs which lack extracellular and transmembrane domains have been cloned from the testis of sexually matured chicken (Mao *et al.*, 1999). The truncated transcripts were specifically expressed in the testis chicken (White Leghorn) and no transcript was observed in any other tissues including the brain (Tanaka *et al.*, 2000).

The presence of PRLR mRNA in the brain and peripheral tissues of White Leghorn and bantam hens does not directly mean the production of the correctly processed receptor protein. A failure in further processing in the PRLR gene expression in the hypothalamus of the White Leghorn hen could possibly account for the absence of incubation behavior. Therefore, further work is required to investigate this possibility using autoradiography with prolactin as the ligand or immunocytochemistry with anti-prolactin receptor antibodies in

bantam, as has been recently shown in the rat (Roky *et al.*, 1996), that hypothalamic PRLR mRNA is translated into protein. However, it is likely that functional PRLR occurs in the central and peripheral tissues in chicken, since injections of prolactin into the preoptic hypothalamus induce incubation behavior in turkeys (Opel, 1971) and an injection of prolactin into the ventromedial hypothalamus induces hyperphagia in the ring dove (Hnasko and Buntin, 1993). Furthermore, injection ovine PRL decrease the thymus and bursa of Fabricius weight (Skwarlo-Soñta *et al.*, 1986) and specific bindings with ovine PRL was observed in the chicken bursa of Fabricius (Di Carlo *et al.*, 1996).

In conclusion, the absence of incubation behavior in White Leghorn hens and its presence in bantam hens cannot be explained by the absence of PRLR gene expression at the mRNA level in the preoptic area of the hypothalamus in the White Leghorn hen or by breed differences in the transcription mechanism or gross structure of the gene.

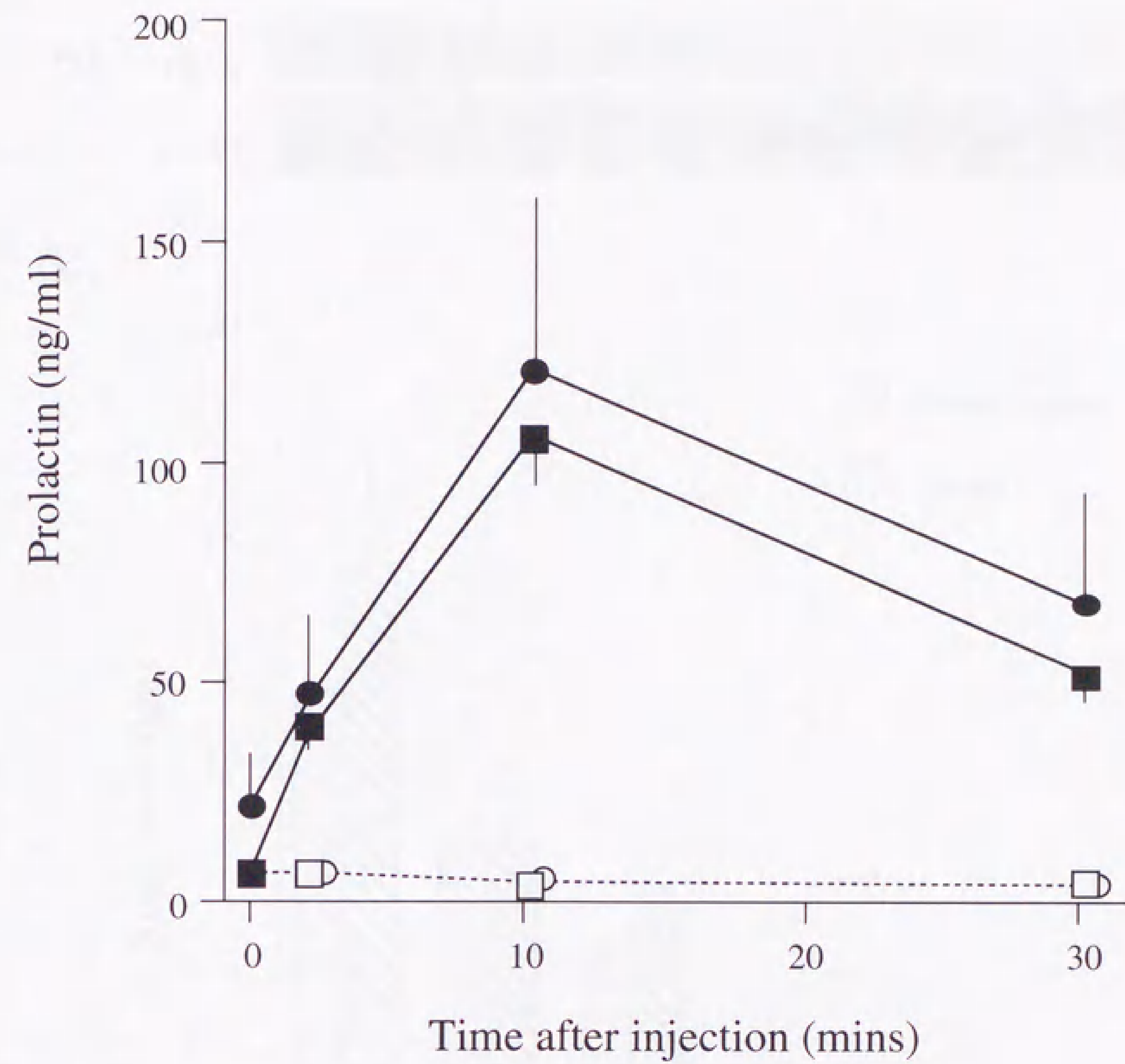

Fig. II-2-1 Changes in concentrations of plasma prolactin after an intravenous injection of vasoactive intestinal peptide (VIP) or saline vehicle in laying White Leghorn and bantam hens. Values are given as means ± SEM, n=8. Solid squares, White Leghorn with VIP; open squares, White Leghorn with saline; solid circles, bantam with VIP; open circles, bantam with saline.

(A)

(B)

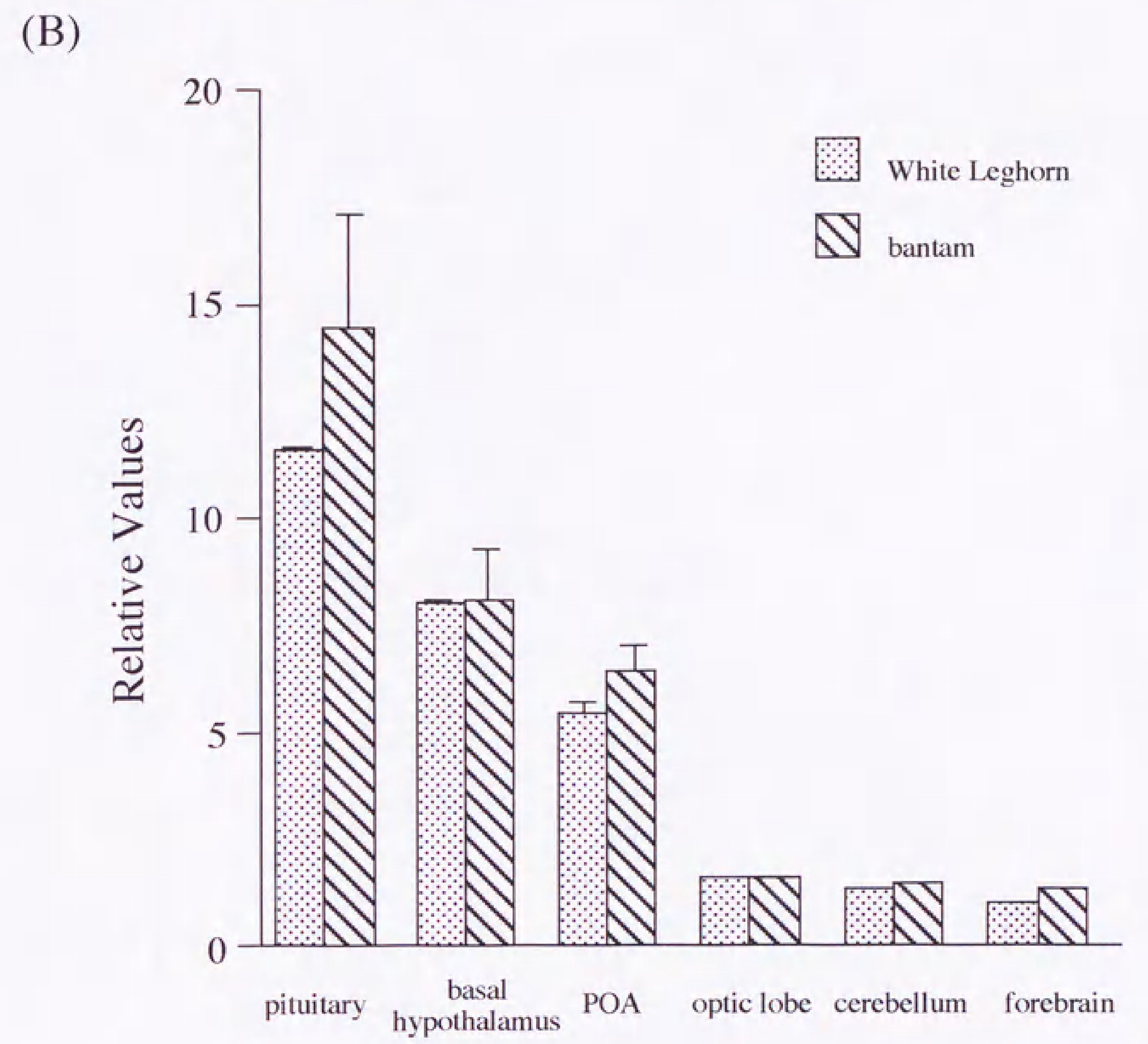

Fig. II-2-2 A comparison of the relative amount of prolactin receptor mRNA, measured using RNase protection assay, in the pituitary gland, hypothalamus, and other brain areas in laying White Leghorn and bantam hens showing (A) an autoradiogram of chicken prolactin receptor mRNA protected bands with β-actin mRNA protected bands to to control for RNA loading and (B) a densitometric quantification of the autoradiograms. Comparisons using Student's t test showed that there were no differences in the two breeds of chicken in any of the brain areas measured relative to the forebrain, which is assigned a value of one. Values are given as means ± SEM, n=5.

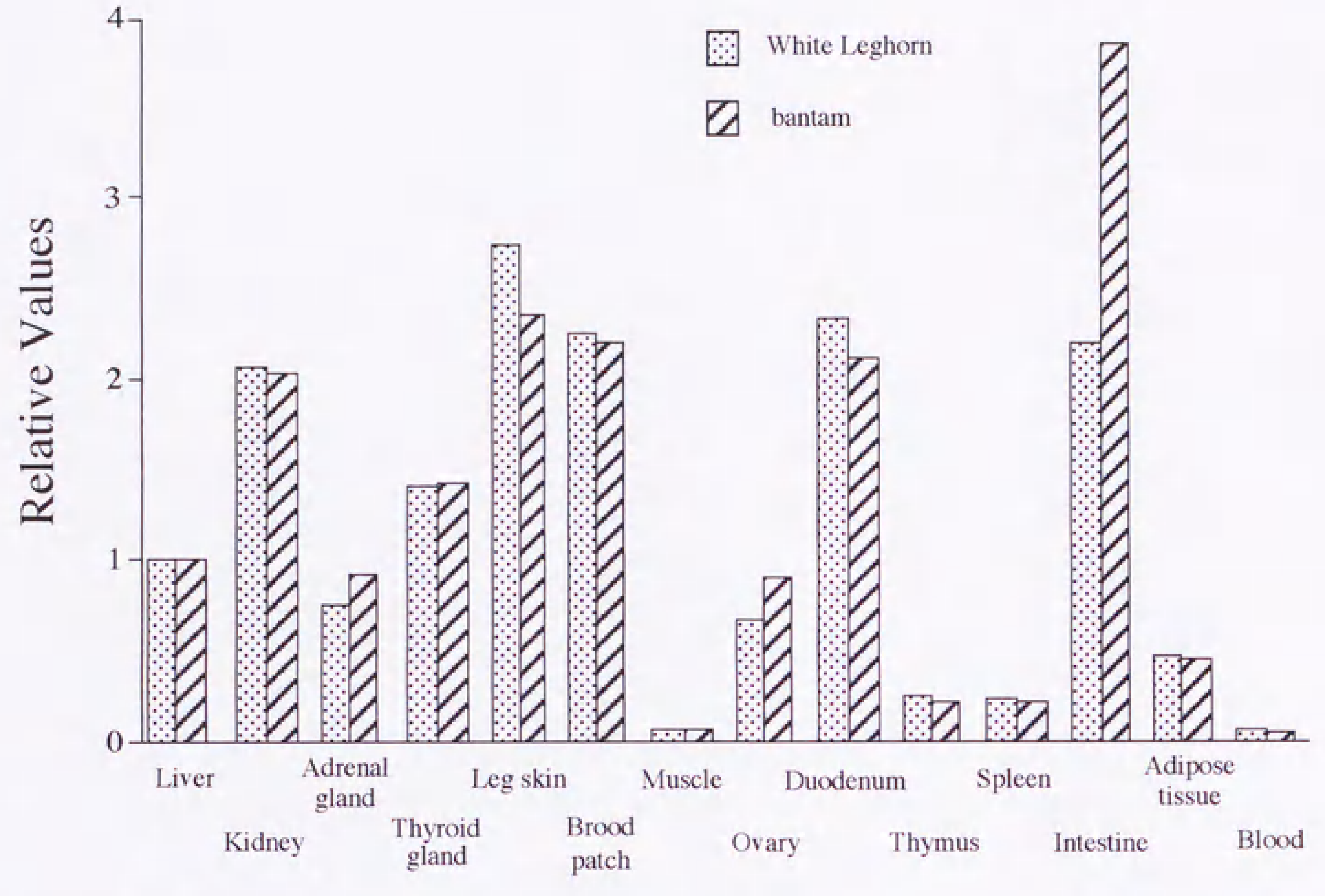

Fig. II-2-3 A comparison of the relative amount of prolactin receptor mRNA, measured using RNase protection assay, in the peripheral tissues in laying White Leghorn and bantam hens showing (A) an autoradiogram of chicken prolactin receptor mRNA protected bands with β-actin mRNA protected bands to to control for RNA loading and (B) a densitometric quantification of the autoradiograms. The values are the mean of single measurements of tissues from two laying White Leghorn hens and two bantam hens and are calculated relative to the liver, which assigned a value of one.

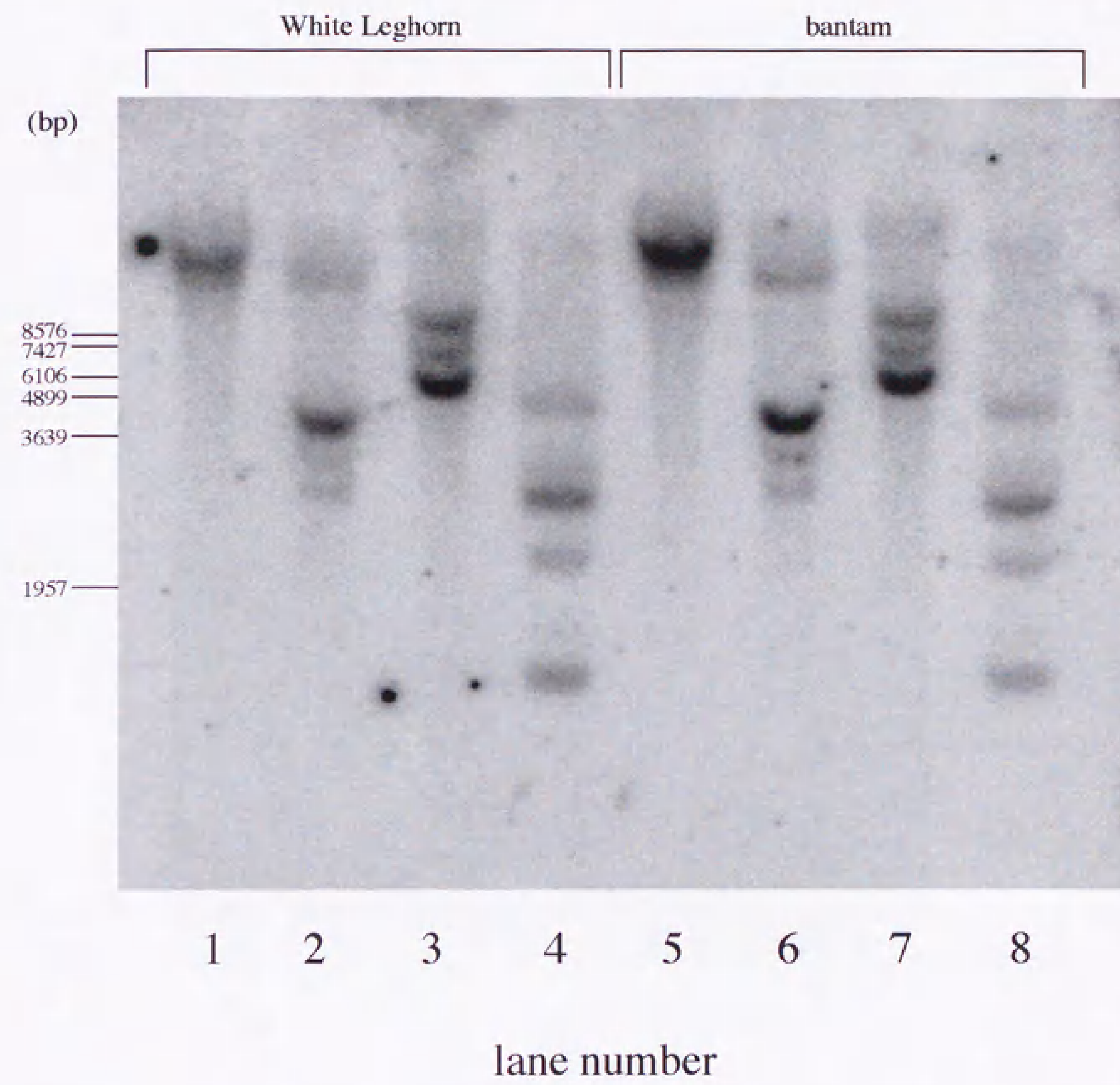

Fig. II-2-4 A comparison of the restriction patterns of the chicken prolactin receptor gene in laying White Leghorn and bantam hens after digestion with four restriction enzymes. Gemomic DNA (10 μg) was digested with *Bam* HI (lanes 1 and 5), *Eco* RI (lanes 2 and 6), *Xba* I (lanes 3 and 7), and *Pst* I (lanes 4 and 8) separated on a 0.5 % agarose gel, blotted onto a nylon membrane, and hybridized to a 2.2-kb [α-^{32}P]dCTP-labeled fragment of a chicken PRLR cDNA.

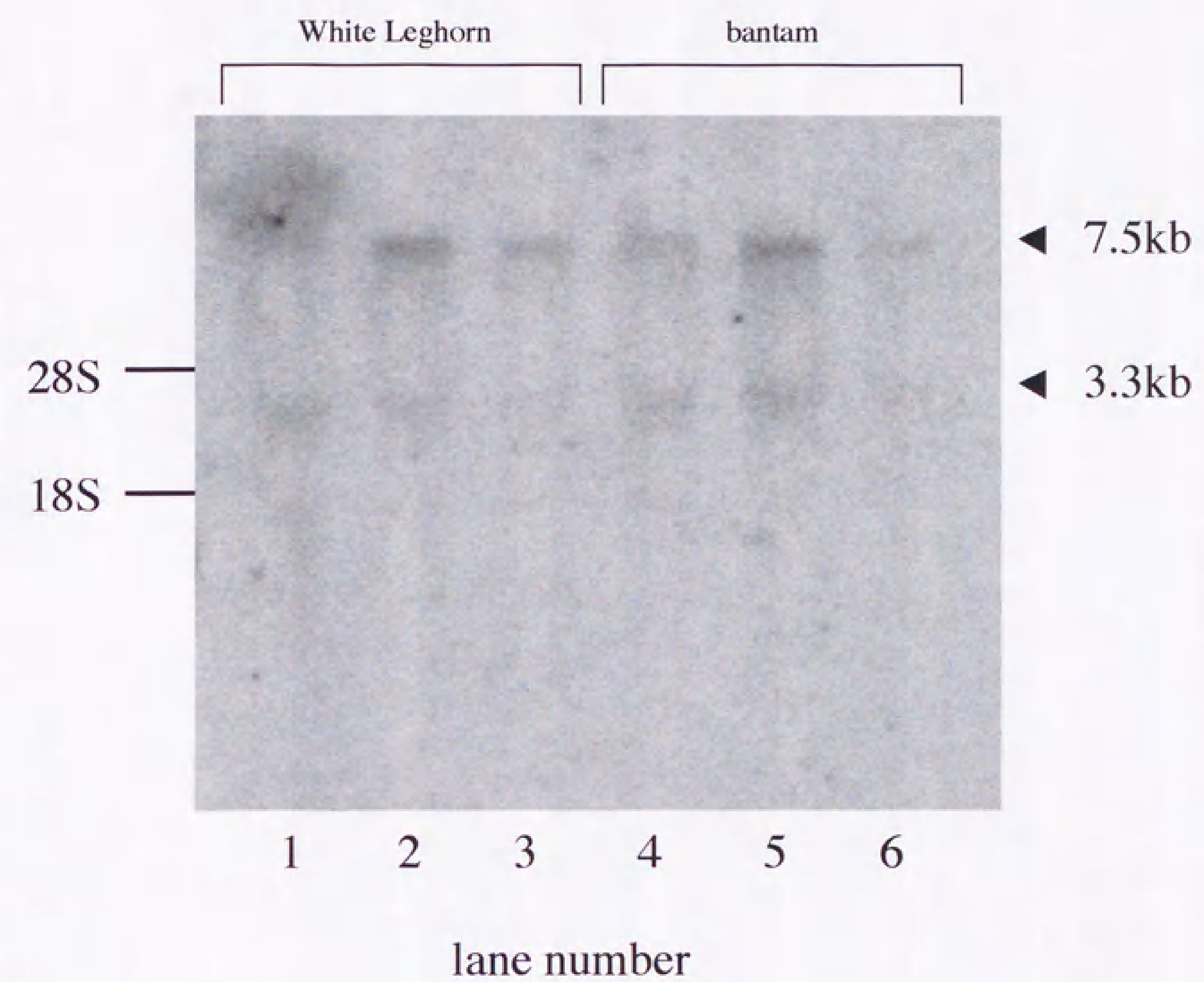

Fig. II-2-5 A comparison of the sizes of prolactin receptor mRNAs in laying White Leghorn and bantam hens. Total RNA (50 μg) from kidney (lanes 1 and 4), hypothalamus (lanes 2 and 5), and hippocampus (lanes 3 and 6) was separated on a 1 % agarose containing 2.2 M formaldehyde, blotted onto a nylon membrane,and hybridized to a 2.2-kb [α-^{32}P]dCTP-labeled fragment of a chicken PRLR cDNA.

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chicken    1 MKQDLISSVQIILFLPLTTVGLAGQSFPGKPKIIRCRSLEKETFSCWWKPGSDGGLPTNYTLFYSKDSEEEIYECPDYRTSGPNSCYFNKNHTSPWTTFN 100
turkey     1 MKQNLISSVQIILLLPLTTVGLTSQSFPGKPKIIRCRSLEKETFSCWWKPGSDGGLPTNYTLFYSKDSEEKIYECPDYRTSGPNSCYFNRNYTNSWTTYN 100
pigeon     1 MKQKLRSSVQIILLFALTAVGLTGQSYPGKPKIIRCRSLEKETFSCWWKPGSDGGLPTNYTLFYSKDSEEKIYECPDYGMSGPNSCYFDKNHTNPWTTYN 100

chicken  101 ITVTATNEIGSNSSDPQYVDVTSIVQPGSPVNLTLETKRSANIMYLWAKWSPPLLAD-ASSNHLYHYELRIKPEEKEEWETISVGVQTQCKINRLNAGMR 199
turkey   101 ITVTATNEIGSNSSDPQYVDVTSIVQPGSPVNLTLETQRYANIMYLWAKWSPPLLAD-ASSNHLYHYELRLKPEEKEEWETVPVGVQTQCKINRLNAGMR 199
pigeon   101 ITVMAMNEIGSNSSDPQYVDVTSIVQPDAPVNLSLETKTSASTTYLLAKWSPPPLADVTSNSHVYRYELRLKPEEKEEWETVSVGVQTQYKVNRLQAGVK 200

chicken  200 YVVQVRCTLDPGEWSEWSSERHILIPSGQSPPEKPTIIKCRSPEKETFTCWWKPGLDGGHPTNYTLLYSKEGEEQVYECPDYRTAGPNSCYFDKKHTSFW 299
turkey   200 YVVQVRCMLDPGEWSEWSSERRILISGGLSPPEKPTITKCRSPEKETFTCWWKPGLDGGHPTNYTLLYSKEGEEQVYECPDYRTAGPNSCYFDKKHTSFW 299
pigeon   201 YVVQVRCVLDIGEWSEWSSERHIHIPNGESPPEKPTIIKCRSPEKETFTCWWKPGSDGGHPTNYTLLYSKEGEERVYECPDYKTAGPNSCYFDKKHTSFW 300
                         WSXWS

chicken  300 TIYNITVRATNEMGSNSSDPHYVDVTYIVQPDPPVNVTLELKKPINRKPYLVLTWSPPPLADVRSGWLTLEYELRLKPEEGEEWETIFVGQQTQYKMFSL 399
turkey   300 TVYNITVKATNEMGSNSSDPHYVDVTYIVQPDPPANVTLELKKPINRKPYLMLTWSPPPLADVRSGWLTLDYELRLKPEEGEEWETVFVGQQTQYKMFSL 399
pigeon   301 TIYNITVKATNEIGSNVSDPLYVDVTYIVQTDPPVNVTLELKKTVNRKPYLVLTWSPPPLADVRSGWLTLDYELRLKPEEAEEWETIFVGQQTHYKMFSL 400

chicken  400 NPGKKYIIQIHCKPDHHGSWSEWSSENYIQIPNDFRVKDMIVWIVLGVLSSLICLIMSWTMVLKGYRMITFMLPPVPGPKIKGIDTHLLETGKSEELLSA 499
turkey   400 NPGKKYIVQIHCKPDHHGSWSEWSSENYIEIPNDFRVKDMIVWIVLGVLSSLICLIMSWTMVLKGYRMITFILPPVPGPKIKGIDTHLLETGKSEELLSA 499
pigeon   401 NPGKKYIVQIHCKPDHHGSWSEWSLEKYLQIPTDFRIKDMVVWIIVGVLSSLICLVMSWTMVLKGYRMIAFILPPVPGPKIKGIDTHLLETGKSEELLSA 500
                              WSXWS                         tmd                      box 1

chicken  500 LGCHGLPPTSDCEELLIEYLEVEDSEDQQLMPSHDNGHPSKNAKITRKETDSDSGRGSCDSPSLLSEKCRETCALPPVLQTQEVRDVQEKKAAKRSWETQ 599
turkey   500 LGCHGFPPTSDCEELLIEYLEVEDSEDHQLMPSHDSGRPSKNAKITLKETDRDSGRGSCDSPSLLSEKCRETCALPSALQIQDVRDVQAKKAGKRSWESY 599
pigeon   501 LGCHGFPPTSDCEELLIEYLEVEDSEDQQLMPSHDNGHPSKNAKMIAKETDSDSGRGSCDSPSLLSEKCRESRAILSTLQTQDIRDVQENNG-RRHWETQ 599

chicken  600 YVASERKALLSNSESAKSSTWPAVQLPNSQPPMFAYHSIVDAHKITLNTTNTNVAAVLVEDEEEHQSQCSLTETIPGEMEKQGEMENLHSKTEQTTAQVK 699
turkey   600 CVASERKALLFNNESAKSSTWPAVQLPNNQPPTFAYHSIVEANKITSTTTNMNVAAVLVENEERHQSLYSISETISGGMEKQEEMENLHSKTTQTTVQVR 699
pigeon   600 CIASEQKILLFNNESTKSPIWPAAQLPDNQPPMFAYHSTVDVHKITLCTIDVNIAPVLVENEEQHQPQYPITETVHDNMEKHREVENLYSKTDQTTVQVK 699

chicken  700 QNRSNERLPFLDAALMDYVEVHKVIRQDEEPAVLLKHKENSGKIEKYTISGASKEYTKVSTVMDHNILVLMPDSRVPHTPASQ-EPAKETSQSLQQGQVE 798
turkey   700 QNRSNEKLPFLNAALMDYVEVHK-VRQDEEPTVLLKHKEKSGKIEKYTISGASKEYTKVSTVMNHNILVLMPDSRVLHTPTSQEEPAKETSQNPQQGQVE 798
pigeon   700 QNRPNDKSPFSKPKLMDYVEVHKV-RQDEVAAVLLKHKENSGKIEKYTVPGTSKEYTKVSTVVDHNILVLMPDSRIQHIPVSQ-EPAMEMSQNLQQGQTE 797

chicken  799 KNMSYCLTAPSDCKRETGGSEYMDPSSFMPSFK 831
turkey   799 TNMSYCMTAPRDCQREPSGSEYMDPSSFMPSFK 831
pigeon   798 KNMSYCLTVPSECKRETSASEYMDPSSFIPAFK 830
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Fig. II-2-6 Multiple alignment of the amino acid sequences of avian PRLRs. Conserved residues are shaded, and identical residues in the three birds are black-boxed. The WSXWS motifs (WSXWS), transmembrane domain (tmd) and box 1 are underlined.